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Analysis of insulin-stimulated insulin receptor activation and glucose transport in cultured skeletal muscle cells from obese subjects☆

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Abstract

Obesity is associated with impaired insulin-stimulated glucose disposal in the skeletal muscle, but whether this is an intrinsic or acquired factor is unknown. In many patients with type 2 diabetes mellitus (T2D) and their nondiabetic relatives, who have a genetic predisposition for diabetes, insulin resistance is maintained in cultured muscle cells. To study the association of obesity with defects in insulin action, we investigated insulin stimulation of both insulin receptor (IR) autophosphorylation and subsequent glucose transport in primary skeletal muscle cell cultures obtained from both nonobese and obese nondiabetic subjects. In these 2 groups, there was no difference in the ability of insulin to induce autophosphorylation of the IR, phosphorylation of the downstream serine kinase Akt/PKB, or stimulation of glucose transport. Moreover, there were no major differences in cultured muscle cell content of either the IR, the IR antagonist PC-1, or GLUT 1 and GLUT 4. These data therefore indicate that the insulin resistance associated with obesity is not maintained in cultured muscle cells and suggest that this insulin resistance is an acquired feature of obesity.
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1. Introduction

Insulin resistance in the skeletal muscle precedes and contributes to the development of type 2 diabetes mellitus (T2D) and the metabolic syndrome [1-3]. Impaired responses to insulin in the skeletal muscle may result from both intrinsic genetic factors as well as acquired components related to diet, activity, and other lifestyle factors [1,4]. Obesity, for example, is associated with a reduced capacity for insulin-stimulated glucose disposal in muscle both in vivo and in vitro [5,6]. However, in obesity, whether skeletal muscle insulin resistance is an acquired or intrinsic factor has not been determined.

Primary skeletal muscle cultures have been used to directly study insulin action in human muscle [7,8]. This model system has been used both to study intrinsic defects in muscle insulin action and to characterize the effects of potential mediators of insulin resistance. Insulin resistance has consistently been reported in muscle cells from patients with T2D [7,9,10]. Whether insulin resistance also occurs in cultured muscle cells from obese individuals is unknown.

In our previous studies, we compared insulin action in skeletal muscle strips prepared from rectus abdominis muscles of both nonobese and obese nondiabetic individuals [6,11]. Insulin stimulation of glucose transport and downstream insulin receptor (IR) signaling events including IRS-1-PI3K–associated activity and Akt activation were impaired in muscle strips from obese individuals [6,11]. We have now investigated insulin action in cultured muscle cells from rectus abdominis muscles that were obtained from a similar population of individuals. When cultured muscle cells from nonobese individuals are compared with cells from obese individuals, the cells from obese subjects respond to insulin with normal activation of IR signaling and insulin stimulated glucose transport. These data suggest therefore that skeletal muscle cells from obese individuals do not retain their insulin-resistant phenotype.

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2. Materials and methods

2.1. Materials

Microtiter plates (Maxisorp Immunoplates) were from Nunc (Copenhagen, Denmark). Bovine serum albumin (BSA) was from Intergen (Purchase, NY). Crystalline porcine insulin was obtained from Eli Lilly (Indianapolis, Ind). Biotin-conjugated antiphosphotyrosine antibody was from UBI (Lake Placid, NY). Horseradish peroxidase (HRP)–conjugated streptavidin was from Pierce (Rockford, Ill). The ELAST amplification kit used was from DuPont NEN (Boston, Mass). The biotinyl tyramide (TMB) reagent kit was from Kirkegaard and Perry Laboratories (Gaithersburg, Md). Defined media components were from Clonetics (Walkersville, Md). All other reagents were from Sigma (St Louis, Mo).

2.2. Subjects

Nonobese and obese nondiabetic subjects were individuals undergoing elective abdominal surgery. Body mass, height, ethnicity, sex, age, and diabetic status were recorded as part of preoperative procedures. A fasting blood sample was obtained for subsequent analysis of insulin and glucose. Subjects were categorized into groups based on body mass index (BMI) (nonobese, <30; obese, $\geq 30\ kg/m^2$), and the presence of diabetes was exclusionary. All the nonobese subjects were women and 2 of the obese subjects were men. During surgery, a biopsy specimen of the rectus abdominis was obtained for subsequent cell culture. All procedures were approved by the East Carolina University Human Studies Committee.

2.3. Plasma analysis

Plasma was analyzed for glucose (YSI 2300 STAT Plus Glucose and Lactate Analyzer, YSI Inc, Yellow Springs, Ohio) and for insulin with a microparticle enzyme immunoassay (IMx, Abbott Laboratories, Abbott Park, Ill). Glucose and insulin concentrations were used to determine homeostasis model assessment (HOMA) values {(fasting glucose [mg/dL] $\times$ 0.05551) $\times$ fasting insulin [μU/mL]/22.1} as an index of in vivo insulin action [12].

2.4. Cell culture

The harvesting and subsequent culturing of satellite cells from rectus abdominis skeletal muscle tissue were adapted with modification from Henry et al [8]. Muscle biopsy specimen was immediately placed in ice-cold Dulbecco's Modified Eagle's Medium (DMEM) and transported to the cell culture facility. The tissue was then dissected free of adipose and connective tissue in ~3 mL DMEM + ~5 mL $Ca^{2+}Mg^{2+}$ free Hanks buffered saline (HBBS) at room temperature. The tissue was minced (2-mm pieces) and washed twice by centrifugation for 10 minutes with 20 mL of Hanks at 550 g (Beckman TJ-6). Satellite cells were isolated by subjecting the tissue to a trypsin digestion cocktail containing 0.25% (wt/vol) trypsin, 0.1% (wt/vol) type IV collagenase, and 0.1% (wt/vol) BSA for 30 minutes on a low shaker setting at room temperature. The cellular suspension was treated with 5% fetal bovine serum (FBS) (wt/vol) to terminate digestion and preplated in uncoated 25-cm^2 flasks for 1 to 3 hours at 37°C to remove fibroblasts. The residual cellular suspension was carefully transferred to collagen I–coated 25-cm^2 flasks in 3 mL SkGM (skeletal growth medium [skeletal basal medium supplemented with 10% FBS, 0.5 mg/mL BSA, 0.5 mg/mL fetuin, 20 ng/mL human epidermal growth factor, 0.39 μg/mL dexamethasone, and 50 μg/mL gentimicin/amphotericin B]) and incubated in a 5% CO_2, 37°C humidified atmosphere. The volume of SkGM was increased to 5 mL after 24 hours of incubation, speeding myoblast attachment. The SkGM was changed every 5 days. After reaching 70% confluence, myoblasts were subcultured onto 6- or 24-well type I collagen–coated plates at densities of 6.4×10^4 cells or 1.6×10^4 cells per well, respectively. Cultures used for experimental procedures were between the fourth and eighth population doubling. When cells reached 80% confluence, they were switched to low-serum media (2% FBS) to induce differentiation. At 8 to 9 days postdifferentiation and before analysis of insulin action, myoblasts were serum starved for 18 hours. For studies of insulin signaling, cells growing in 6-well plates were exposed to various concentrations of insulin for 5 minutes. Cells were then washed with phosphate-buffered saline at 4°C and then solubilized in 50 mM HEPES, pH 7.6, 150 mM NaCl, 1% Triton X-100, 2 mM Na_3VO_4, 1 mM PMSF, 2 μM leupeptin, 2 μM pepstatin A. Lysates were allowed to solubilize for 1 hour at 4°C and were then centrifuged for 20 minutes at 14 000 rpm. Supernatants were collected and stored at −70°C for subsequent study. Protein content of cellular extracts was determined by the Bradford method [13]. Differentiation states of myoblasts were determined by assaying extracts for creatine phosphokinase (CPK) activity (Sigma) [8,14]. Assessment of myoblast CPK activity confirmed that each cell line was of a muscle-specific lineage. Undifferentiated myoblast morphology was confirmed visually by the absence of fused myotubes and a relatively low CPK activity [8,14].

2.5. Glucose transport

To assess 2-deoxyglucose uptake, muscle cells were cultured in 24-well, collagen-coated plates. Myotubes were allowed to differentiate for 10 days and serum starved 16 hours before assay. Cells were then washed and incubated with transport buffer (20 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA), containing varying concentrations of insulin, for 1 hour at 37°C. Cytochalasin B (final concentration, 10 μM) was added to wells to determine nonspecific incorporation of label. 2-Deoxy-[^{3}H]glucose was added to all wells (final

concentration, 10 μM 1 μCi/mL) followed by 60 minutes of incubation at 37°C. Cell uptake of glucose was terminated by aspirating and washing (3 times) each well with ice-cold phosphate-buffered saline containing 20 mM D-glucose. The myotubes were solubilized in 500 μL 0.03% sodium dodecyl sulfate, and membranes were disrupted by sonication. A total of 200 μL lysate aliquots was counted in a scintillation counter, and the protein concentration in the remaining lysate was measured by Bicinchoninic Acid (BCA) protein assay (Pierce).

2.6. Insulin receptor enzyme-linked immunosorbent assay

Insulin receptor content of muscle cell lysates was determined by specific enzyme-linked immunosorbent assay (ELISA) as described previously [15]. Briefly, microtiter 96-well plates were coated with 2 μg/mL of a monoclonal antibody to the IR α-subunit (MA-20) for 18 hours at 4°C. After washing and blocking the plate, solubilized cellular extract containing 2 to 5 μg protein of each sample was added to each well for triplicate determination and allowed to bind overnight at 4°C. Readout of bound IR was accomplished with the sequential addition of biotinylated monoclonal anti-IR antibody CT-1, peroxidase-conjugated streptavidin (Pierce), ELAST ELISA amplification system (NEN Research Products, Boston, Mass) for signal enhancement, and TMB peroxidase substrate system (Kirkegaard and Perry) for color development. The absorption at 450 nm of each well was measured in a microtiter plate reader (DuPont-NEN). Insulin receptor content for each sample was calculated as an average of triplicate values.

2.7. Insulin receptor autophosphorylation

The ELISA for tyrosine phosphorylation of the IR was performed as described previously [15]. The volume of cell lysate containing 20 pg of IR was applied to 96-well ELISA plates coated with monoclonal antihuman IR antibody, MA-20. Insulin receptor from the samples was allowed to bind during an 18-hour incubation at 4°C. Next, the 96-well ELISA plates were washed with Tris-buffered saline with Tween (TBST: NaCl, 150 mM; Tween 20, 0.05%; Tris, 20 mM; pH 7.4). Biotin-conjugated antiphosphotyrosine antibody was added to the wells for a 2-hour incubation at 22°C. Wells were again washed and then incubated with streptavidin-HRP. After addition of a peroxidase substrate, TMB, the degree of tyrosine phosphorylation was quantified by having a plate reader read the optical density (OD) at 451 nm.

2.8. Insulin-like growth factor-1R autophosphorylation

Tyrosine phosphorylation of the insulin-like growth factor-1R resulting from insulin stimulation of cells was determined by ELISA. The protocol was identical to that for the IR phosphorylation ELISA, except that 3 to 10 μg of soluble cell protein was loaded onto a 96-well pate coated with αIR3, a monoclonal antibody recognizing the insulin-like growth factor-1R α-subunit.

2.9. PC-1 content ELISA

Cellular content of membrane glycoprotein PC-1 was determined by specific ELISA as previously described [16]. The protocol was similar to that described above for the IR ELISA, with the capture antibody to PC-1 provided by Dr I Yamashina of Kyoto University, Kyoto, Japan. The secondary antibody was a biotinylated anti–PC-1 monoclonal antibody [16].

2.10. Determination of Akt phosphorylation

Activation of Akt due to serine phosphorylation of residue 473 was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a phospho-specific antibody against Akt. A total of 30 μg of soluble muscle protein was loaded onto an 8% to 16% polyacrylamide mini gel. The samples were run through the gel at 30 mA for 1 hour. The proteins were electrophoretically transferred to a nitrocellulose membrane at 30 V for 1.6 hours. After transfer, the membranes were incubated for 30 minutes in 2% nonfat milk, TBST-blocking buffer. The membranes were then incubated at 4°C with a polyclonal antibody raised against phospho Akt (Ser473) (Cell Signaling, Beverly, Mass), diluted 1:1000 in superblock (Pierce). After an overnight incubation, the membranes were washed with TBST. Next, membranes were incubated with HRP- conjugated goat antirabbit IgG (Cell Signaling), diluted 1:10 000 in superblock for 1.5 hours at 22°C. Membranes were washed again with TBST. Phosphorylated Akt was visualized by enhanced chemiluminescence (Pierce).

2.11. Determination of GLUT1 and GLUT4 content

GLUT1 and GLUT4 measurements were determined by slot blot procedure. Cell lysates were diluted to 5 μg/mL in TBST with 0.1% BSA. A 100 μL sample was applied in triplicate to a Minifold II Slot Blot System (Schleicher & Schuell, Keene, NH) fitted with nitrocellulose filter. Vacuum was applied until samples soaked into the

Table 1
Subjects' clinical data

	Nonobese (n = 9)		Obese (n = 16)	
	Average	Range	Average	Range
Age (y)	44 ± 1	39-51	38 ± 3	19-54
BMI (kg/m^2)	25.5 ± 0.9	21-30	54 ± 4.2*	36-77
Fasting plasma glucose (mg/dL)	83 ± 6	61-98	91 ± 3	78-100
Fasting plasma Insulin (μU/mL)	3.6 ± 1.7	0.8-12.0	20.1 ± 3.2**	1.6-27.0
HOMA	0.8 ± 0.3	0.3-2.4	3.3 ± 0.6**	0.2-6.5

To measure insulin sensitivity, HOMA was calculated as described in Materials and methods.

* $P < .00001$.

** $P < .05$.

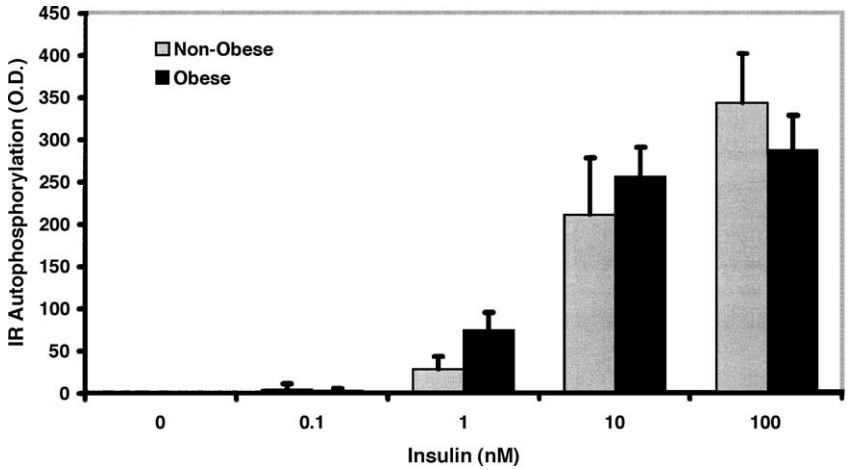

Fig. 1. Insulin dose-response curves for IR signaling in human skeletal muscle cells. Insulin receptor autophosphorylation was determined by ELISA in cells exposed to varying concentrations of insulin for 5 minutes. Values are mean ± SEM of triplicate determinations. There are no significant differences between groups at any concentration of insulin.

nitrocellulose. The wells were then washed with TBST containing 0.1% BSA. Next, the nitrocellulose blots were dried at 37°C for 30 minutes, then blocked with 25 mL TBST containing 5% milk for 1 hour. After being washed 3 times, the blots were incubated with anti-GLUT1 or anti-GLUT4 antibody (1:1500 in TBST containing 5% milk) overnight at 4°C. (Both antibodies were generous gifts from Dr Mike Mueckler, Washington University School of Medicine, St. Louis, Mo) Next, blots were washed 3 times with TBST, then incubated with HRP-conjugated sheep antirabbit IgG (Amersham, Piscataway, NJ) diluted 1:16 000 in TBST with 5% milk for 90 minutes at room temperature. After being washed, blots were incubated with SuperSignal (Pierce) and exposed to film. Values were determined by scanning densitometry.

2.12. Statistics

All data analyses were performed using MedCalc statistical software (Mariakerke, Belgium). Values are presented as mean ± SEM. Differences between obese and nonobese groups were analyzed by the Student *t* test. Significance was accepted as $P < .05$.

3. Results

3.1. Subjects

Muscle cells were cultured from 9 nonobese and 11 obese nondiabetic subjects. General characteristics of the subjects at the time of biopsy are presented in Table 1. Obesity (BMI, ≥30) was associated with impaired insulin action in these subjects as determined by HOMA.

3.2. Cell differentiation

The degree to which myoblasts were induced to differentiate into multinucleated myotubes was determined by both visual inspection and assaying CPK activity. All muscle cell cultures exhibited the expected myotube morphology. Creatine phosphokinase activity was not associated with any difference in insulin action.

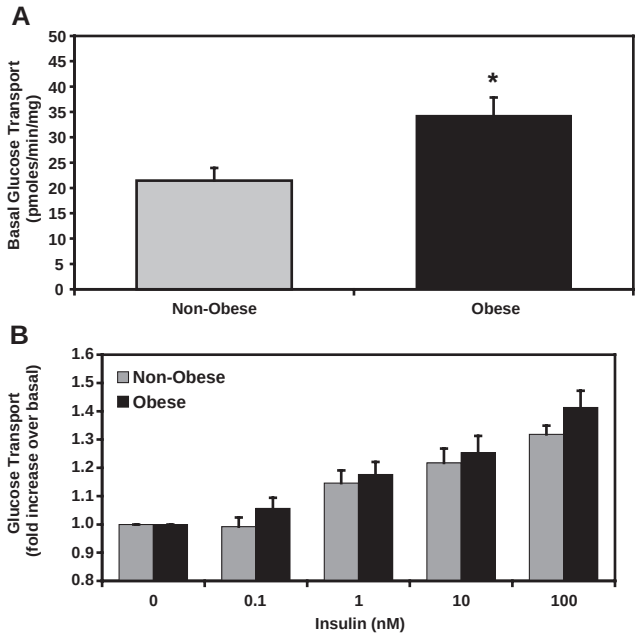

Fig. 2. Effects of obesity on glucose transport in cultured human skeletal muscle cells. Differentiated myotubes were serum starved overnight, exposed to varying concentrations of insulin for 60 minutes (or 100 nM insulin plus wortmanin), and incubated with 10 μM 2-Deoxy-[^{3}H]glucose for 30 minutes. A, Absolute rates of basal glucose transport in muscle cells from obese subjects are elevated. Values are mean rates of 2-deoxyglucose transport in the absence of insulin for muscle cell cultures from nonobese (gray bars) and obese (black bars) individuals ± SEM. Asterisk indicates absolute glucose transport rates significantly greater than those in nonobese controls ($P < .05$). B, The fold increase over basal transport rates, stimulated by insulin, for cells from nonobese (gray bars) and obese (black bars) subjects. Values are mean ± SEM of triplicate determinations. There are no significant differences between groups at any concentration of insulin.

Table 2
Biochemical data of myotubes from nonobese and obese subjects

	Nonobese	Obese
CPK activity (nmol/mg per min)	434 ± 130	260 ± 75
IR content (ng/mg)	11.7 ± 1.6	8.1 ± 1.4
GLUT1 content (OD)	161 ± 15	135 ± 14
GLUT4 content (OD)	75 ± 3	66 ± 4
PC-1 content (ng/mg)	116 ± 18	136 ± 42

Creatine phosphokinase activity, an indication of myotube differentiation, was determined by enzymatic assay, IR and PC-1 content were determined by ELISA, and GLUT1 and GLUT4 content were determined by Western blots. All values are normalized to cell lysate protein content.

3.3. Effects of obesity on the IR signaling system

Insulin dose curves of IR autophosphorylation were determined in muscle cell cultures by specific ELISA. There were no differences in insulin stimulation of IR autophosphorylation between cells from nonobese subjects and those from obese subjects at any concentration of insulin (Fig. 1). Likewise, although serine phosphorylation of Akt was elevated in the basal state in cells from nonobese subjects (68 ± 11 vs 21 ± 14 OD units, nonobese vs obese, respectively; $P < .05$), there was no difference in the ability of insulin to induce Akt activation between the nonobese and obese groups (100 nM insulin values, 240 ± 100 vs 169 ± 16 OD units, nonobese vs. obese, respectively; P = NS).

3.4. Glucose transport

Rates of glucose transport were determined in the muscle cell cultures. The basal rates of glucose transport were significantly elevated in cells from obese subjects (Fig. 2A). Differences in the absolute rates of glucose transport could not be ascribed to differences in cellular content of glucose transporters because there was no significant difference in the cellular content of either GLUT1 or GLUT4 (Table 2).

Insulin stimulation of glucose transport was determined over a range of insulin concentrations. Insulin at a concentration of 1 nM significantly increased transport ($P < .05$) and at 100 nM insulin transport was maximal (Fig. 2B). The effect of insulin was fully inhibited by the PI3-kinase inhibitor, wortmanin, which reduced insulin-stimulated glucose transport rates in the cells to 74.4% ± 5.6% of basal values. Mean stimulation of glucose transport by 100 nM insulin was 1.38 ± 0.05 fold. The fold increase in myotube glucose transport stimulated by insulin was not significantly different between nonobese and obese subjects (Fig. 2B). Further transport was not related to either BMI ($r = -0.04$; P = NS) or any other clinical measure of insulin sensitivity (data not shown).

3.5. Other parameters

There was no significant difference between the obese and nonobese groups with regard to the cellular content of any of the following: the IR, the IR antagonist PC-1, GLUT1, or GLUT4 (Table 2).

4. Discussion

In the present study, we found that the insulin resistance that characterizes the skeletal muscle from obese subjects was not maintained in cultured muscle cells. Insulin stimulation of IR autophosphorylation was not different in the obese and nonobese groups. Glucose transport (fold increase) stimulated by insulin was the same in muscle cells from both groups. However, several aspects of the insulin signaling-glucose transport pathway were altered in the cells from obese subjects compared with those from nonobese control subjects. Most notably, basal rates of muscle cell glucose transport were elevated by 50% with morbid obesity. We also observed small decrements in cellular GLUT4 levels associated with obesity, but these did not have any apparent impact on insulin action in the cells.

In vivo, muscle insulin sensitivity is regulated on a long-term basis by factors such as obesity and in a short-term basis by changes in dietary habits and physical activity [17]. Thus, the serial passage of muscle cells in tissue culture should remove them from these in vivo regulators of insulin action. Therefore, this cell model system has been used to analyze the intrinsic function, independent of acquired factors, of both the insulin signaling and glucose transport systems. We previously reported insulin signaling in myoblasts from Pima Indians, a group of native Americans for which the high rate of diabetes is related both to a high prevalence of obesity as well as to inherited insulin resistance [15]. These studies indicated that impaired IR function is an intrinsic characteristic of muscle cells from certain types of individuals with insulin resistance that is distinct from the effects of obesity.

Basal and insulin-stimulated glucose transports have been studied in various types of individuals with a genetic basis for insulin resistance. Several groups have reported that cultured muscle cells from patients with T2D are insulin resistant. Muscle cell cultures derived from these subjects typically show reduced basal and insulin-stimulated glucose transports, reduced fold stimulation by insulin, and impaired activation of downstream signaling [7-10,18]. Diverging results have been observed however in studies of cultured muscle cells from nondiabetic but insulin-resistant individuals. Jackson et al [19] reported that the basal glucose transport values were elevated in cells from insulin-resistant relatives of individuals with T2D but that the fold stimulation of glucose transport by insulin was reduced. In contrast, Krutzfeldt et al [20] observed no differences in either basal or insulin-stimulated glucose transport between muscle cells from insulin-resistant and insulin-sensitive first-degree relatives of patients with T2D. The reasons for the different results between the last 2 studies are unknown, but, in view of all the above studies, it appears likely that most insulin-resistant subjects (patients with T2D and their relatives) have acquired defects in insulin-stimulated glucose transport that are preserved in cultured muscle cells.

In obese nondiabetic subjects, there is most likely no intrinsic insulin resistance. A previous study of obese subjects with impaired glucose tolerance reported normal basal but impaired insulin-stimulated glucose transport in cultured muscle cells [21]. In the present study, however, in muscle cells from euglycemic obese subjects, we observed elevated basal glucose transport with no alteration in the ability of insulin to stimulate this parameter. The differences between that earlier study and the present one may reflect the presence of intrinsic insulin resistance factors that contributed to impaired glucose tolerance in the earlier study.

In the present study, it is unclear how obesity produced alterations in the rates of basal glucose transport in muscle cells in culture. Basal glucose transport rates were unrelated to cellular content of either GLUT1 or GLUT4. It is possible that the increased glucose transport rates could be attributable to elevations in the amount of glucose transporters in the plasma membrane in the absence of differences in the total cellular content of GLUT1 or GLUT4. Unfortunately, these data could not be collected in the present study because of the small amount of cells available from each subject.

Other aspects of the obese phenotype have been reportedly maintained in cultured muscle cells. Mott et al [18] reported that palmitate oxidation was increased in undifferentiated myoblasts from obese insulin-resistant subjects. Although the authors suggested that these results provided evidence of intrinsic abnormalities in fat oxidation in muscle, it is possible that muscle cells from obese subjects retain alterations in both glucose and lipid metabolism in culture that were acquired secondary to obesity.

In summary, we have explored insulin signaling and glucose transport in myotubes cultured from nonobese and obese nondiabetic individuals. We have observed that the insulin resistance that characterizes the obese state is lost during culture and serial passage of muscle cells. However, we also observed that muscle cells from obese insulin-resistant individuals display enhanced glucose transport rates in the absence of insulin. Thus, the obese state is associated with both reversible and imprinted effects on muscle cells.

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