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### Research Article

Insulin resistance is a predictor of the development of noninsulin-dependent diabetes mellitus (NIDDM) in humans. It is unclear whether insulin resistance is a primary defect leading to NIDDM or the result of hyperinsulinemia and hyperglycemia. To determine if insulin resistance is the result of extrinsic factors such as hyperinsulinemia primary skeletal muscle cell cultures were established from muscle biopsies from Pima Indians with differing in vivo insulin sensitivities. These cell cultures expressed a variety of muscle-specific phenotypes including the proteins alpha-actinin and myosin, muscle-specific creatine kinase activity, and RNA encoding GLUT4, MYF5, MYOD1, and MYOGENIN. Labeled glucose was used to measure the insulin-stimulated conversion of glucose to glycogen in these cultures. The in vivo rates of insulin-stimulated glycogen production (insulin resistance) were correlated with in vitro measures of glycogen production ( $P = 0.007$ ,  $r = 0.58$ ). This defect in insulin action is stable in a uniform culture environment and is retained over time. The retention of insulin resistance in myoblast derived cell cultures is consistent with the expression of an underlying biochemical defect in insulin resistant skeletal muscle.

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# Human Primary Myoblast Cell Cultures from Non-Diabetic Insulin Resistant Subjects Retain Defects in Insulin Action

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## Abstract

Insulin resistance is a predictor of the development of non-insulin-dependent diabetes mellitus (NIDDM) in humans. It is unclear whether insulin resistance is a primary defect leading to NIDDM or the result of hyperinsulinemia and hyperglycemia. To determine if insulin resistance is the result of extrinsic factors such as hyperinsulinemia primary skeletal muscle cell cultures were established from muscle biopsies from Pima Indians with differing in vivo insulin sensitivities. These cell cultures expressed a variety of muscle-specific phenotypes including the proteins  $\alpha$ -actinin and myosin, muscle-specific creatine kinase activity, and RNA encoding *GLUT4*, *MYF5*, *MYOD1*, and *MYOGENIN*. Labeled glucose was used to measure the insulin-stimulated conversion of glucose to glycogen in these cultures. The in vivo rates of insulin-stimulated glycogen production (insulin resistance) were correlated with in vitro measures of glycogen production ( $P = 0.007$ ,  $r = 0.58$ ). This defect in insulin action is stable in a uniform culture environment and is retained over time. The retention of insulin resistance in myoblast derived cell cultures is consistent with the expression of an underlying biochemical defect in insulin resistant skeletal muscle. (*J. Clin. Invest.* 1996. 98:2346–2350.) Key words: myoblast • insulin • glycogen • cell culture • skeletal muscle

## Introduction

Insulin resistance, or a reduced rate of insulin-stimulated glucose uptake is considered a defect of skeletal muscle (1). Specifically, data from hyperinsulinemic euglycemic clamp studies in conjunction with nuclear magnetic resonance studies in insulin resistant humans indicate the reduced capacity of skeletal muscle to take up and convert glucose to glycogen (2, 3). It remains controversial, however, as to whether insulin resistance is an inherent feature of skeletal muscle cells, or an acquired defect resulting from hyperinsulinemia, or other factors extrinsic to these cells (4). This controversy stems partly from in vivo analyses, where interactions between the different organ systems responsible for glucose homeostasis can not be com-

pletely controlled experimentally. Resolution of this debate concerning the intrinsic versus extrinsic foundation of insulin resistance depends, in part, upon demonstrating reduced insulin-stimulated glucose uptake and glycogen synthesis in skeletal muscle in a uniform environment and retention of this defect over time. Evidence supporting an intrinsic biochemical defect underlying insulin resistance in skeletal muscle can be obtained with the establishment of cell cultures in vitro followed by measurements of insulin-stimulated glycogen synthesis.

Primary myoblast cultures have been used to study normal muscle development as well as muscle diseases such as muscular dystrophy (5). Myoblast cultures are easily established from muscle biopsies from normal individuals as well as persons with non-insulin-dependent diabetes mellitus (NIDDM).<sup>1</sup> In a uniform culture environment, an underlying biochemical defect in skeletal muscle should be retained. In contrast insulin resistance, induced by outside or environmental factors, should be rapidly lost in culture. Recently, Henry et al. (6) demonstrated that “defective insulin-stimulated glucose transport persists in cultures from NIDDM subjects.” The loss of insulin-stimulated glucose transport in diabetic muscle cultures supports the notion that insulin resistance is intrinsic to skeletal muscle, but could also result secondarily from the NIDDM, or as a consequence of chronic hyperglycemia.

To test the hypothesis that insulin resistance is intrinsic in skeletal muscle cells and would be maintained in cell culture, primary myoblast cell cultures were established from non-diabetic Pima Indians with varying degrees of insulin resistance and insulin-stimulated glucose to glycogen was measured in these cell lines and compared with various in vivo measures of insulin action. The myoblast cell lines expressed muscle phenotypes and both in vivo and in vitro measures of insulin action were correlated. In addition, these cultures maintained their ability to respond to insulin indicating that insulin resistance is a defect of cells in the skeletal muscle lineage and does not result from external factors.

## Methods

All studies were approved by the National Institute of Diabetes and Digestive and Kidney Diseases, as well as the tribal council of the Gila River Indian Reservation. After informed consent, healthy Pima volunteers were admitted to the research ward of the Clinical Diabetes and Nutrition Section of the National Institutes of Health. After several days of a weight maintenance diet, the volunteers underwent percutaneous muscle biopsies of the vastus lateralis. The muscle biopsies were collected in cold Dulbecco's modified eagle media (DME). On a separate day, and in 11 subjects on a separate admission, maximal rates of insulin-stimulated glucose disposal in vivo were mea-

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1. Abbreviations used in this paper: DPBS, Dulbecco's phosphate buffered saline; NIDDM, non-insulin-dependent diabetes mellitus.

Table I. Volunteer Characteristics

	Mean $\pm$ SE	Range
Insulin mediated glucose disposal	8.8 $\pm$ 2.6*	5.5–14.2
Percent fat	27 $\pm$ 10	11–42
Age	29 $\pm$ 7 yr	21–41
Fasting glucose	85 $\pm$ 7 mg/dl	76–96
2-h glucose postload plasma glucose	117 $\pm$ 33 mg/dl	64–170

\*mg/min·kg estimated metabolic body size (20)

sured with a hyperinsulinemic euglycemic clamp (7). Characteristics of the volunteers are presented in Table I.

100–500 mg of skeletal muscle tissue was finely minced, and the cells dissociated with 0.25% (wt/vol) trypsin, 0.1% (wt/vol) type IV collagenase, and 0.1% (wt/vol) bovine serum albumin (BSA) incubating at 37°C for 30 min with agitation. The cells were collected by centrifugation at 150 g. The cells were plated in uncoated 25-cm<sup>2</sup> flasks for 1 h at 37°C to remove fibroblasts. The residual cellular material was transferred to fresh 60-mm plates, coated with either 0.01% human type I collagen or 1% gelatin. Myoblasts were allowed to grow until 60–70% confluent ( $\sim$  2–4 wk) in DME supplemented with 25 mM Hepes, 20% fetal calf serum, 2 mM glutamine, 0.5% chick embryo extract, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B (5, 8). The 20% fetal calf serum contained 2 nM insulin and 5.5 mM glucose. To subculture, cells were trypsinized, counted, and plated at  $1 \times 10^4$  cells in six-well coated culture plates, or  $2 \times 10^5$  cells/25 cm<sup>2</sup> coated flask. All experiments used cells cultured in 6 well coated culture plates and cells were passed in 25-cm<sup>2</sup> flasks. The primary monolayer was designated the first cell population doubling. Subsequent population doublings were calculated using the formula:  $N_H/N_I = 2^x$  where  $N_H$  is the number of cells harvested,  $N_I$  is the cell inoculum number, and  $x$  is the number of population doublings (9). Cell cultures were considered senescent when subcultivations did not result in at least one cell population doubling. Cultures used for experimental purposes were between the fourth and eighth population doubling.

Myoblasts were subcultured into six-well plates. Monolayers were rinsed with Dulbecco's phosphate buffered saline (DPBS) and blocked with 10% normal goat serum in DME. Cells were rinsed and incubated with monoclonal anti  $\alpha$ -sarcomeric actinin (1/800 dilution) or monoclonal anti-myosin (1/400 dilution) for 120 min at 37°C. The cells were washed and treated with FITC conjugated rabbit anti-mouse IgG (1/100) (10). The cells were fixed in 3.7% formaldehyde and observed under a fluorescence microscope at an excitation wavelength of 546 nm.

Total RNA was extracted from six-well plates using TRIzol (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Slot blotting was carried out according to Maniatis et al. (11). The Northern slot blots were probed with  $\alpha$ -<sup>32</sup>P end-labeled oligonucleotide probes directed against coding regions of *GLUT4*, *MYOD1*, *MYF5*, and *MYOGENIN* to confirm the presence of messenger RNA of a muscle-specific lineage.

Measurement of total cellular conversion of glucose to glycogen was performed according to the method of Howard et al. (12) with the following modifications. 42 h before glucose to glycogen measurements, cells were rinsed with DPBS and preincubated in Basal Medium Eagle (BME) supplemented with BME amino acids, BME vitamin solution, 2 mM glutamine, 1 mg/ml BSA, and 25 mM glucose (final concentration) for 24. At 18 h, before the experiment the cells were rinsed in DPBS and incubated in modified BME media, without glucose. 1 h before the addition of the labeled glucose, insulin was added to a final concentration of 100 nM in the modified BME, without glucose. Total cellular glycogen production was measured using D-U-<sup>14</sup>C-glucose (1  $\mu$ Ci/ml in 5.5 mM glucose). After a 30-min incu-

bation period, the cells were rinsed with 0.2 mM phloretin and aspirated, followed by two washes with DPBS. Cells were digested with 0.3 ml of 5 M NaOH containing 20 mg/ml of glycogen then heated at 100°C for 30 min. Liquid scintillation spectroscopy was used to quantify labeled glycogen (13). Protein was measured according to the method of Lowery as modified by Bensadoun (14). Creatine kinase activity was measured using Sigma's Creatine Kinase kit (Sigma Chemical Co., St. Louis, MO). Within each experiment, triplicate determinations were performed and the results are expressed as the mean  $\pm$  standard error. Regression analyses were performed using Statistical Analysis System (Cary, NC) computer software.

## Results

After the preparation of myoblasts, several months were required to establish enough viable primary cell cultures for experimental purposes. Once established, the cell lines were maintained on 20% serum to prevent differentiation into myocytes. Although some fusion and differentiation of myoblasts into myotubes took place in culture, the frequency of myotube formation was low and did not differ between cultures. Monoclonal antibodies directed against  $\alpha$ -actinin and myosin de-

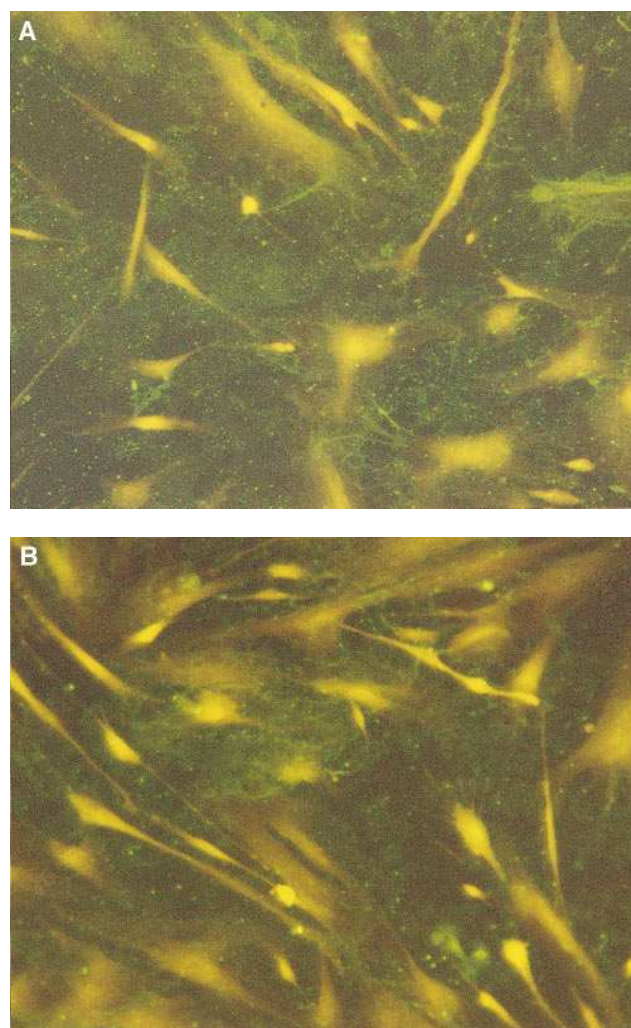


Figure 1. Photomicrographs of myoblasts stained with either  $\alpha$ -actinin monoclonal antibody (A), or myosin monoclonal antibody (B).  $\times 100$ .

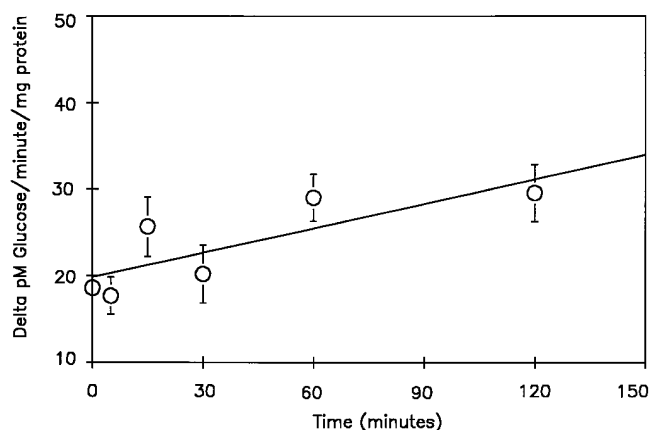


Figure 2. Time course of incorporation of [ $^{14}\text{C}$ ]glucose into glycogen. Cell cultures were incubated with 1  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]glucose. Glycogen was isolated from replicate cultures at the time points indicated, and the amount of radiolabeled glycogen quantified by liquid scintillation spectroscopy.

tected both of these protein components of skeletal muscle in the myoblast cultures (Fig. 1). These cell lines also expressed the muscle-specific RNAs encoding *GLUT4*, *MYOD1*, *MYF5*, and *MYOGENIN* confirming the presence of cells of muscle-specific lineage (data not shown). All the cell lines expressed creatine kinase activity ( $0.89 \pm 0.79$   $\mu\text{mol}$  creatine/min per mg protein), a muscle-specific marker.

A measure of non-oxidative glucose metabolism, glycogen production, was used to assess insulin action in vitro. The myoblast cell lines were pretreated with 100 nM insulin for 1 h and then incubated in [ $^{14}\text{C}$ ]glucose for 30 min. Subsequently, the labeled glucose incorporated into glycogen was measured. The insulin-stimulated cell cultures produced glycogen in a time-dependent manner (Fig. 2). Pretreatment with increasing amounts of insulin was accompanied by a dose dependent increase in glycogen production (Fig. 3).

In vivo measures of insulin-mediated glucose disposal (M value) correlated with the increase, over basal, of insulin-stimulated glycogen production in culture ( $r = 0.58$ ,  $P = 0.007$ )

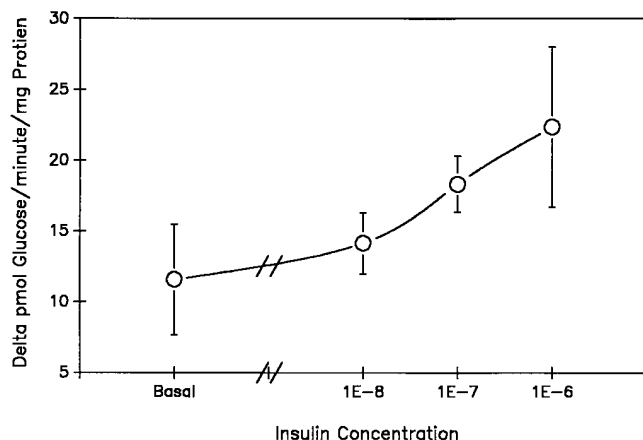


Figure 3. Dose response curve using increasing concentrations of insulin. Cell cultures were treated with varying Molar amounts of insulin 1 h before initiation of the glucose to glycogen assay.

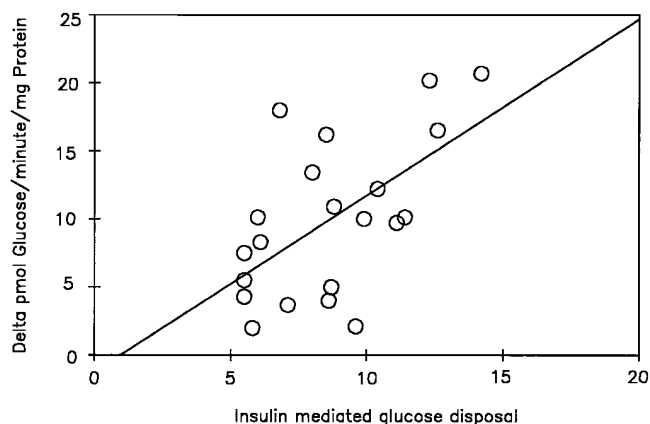


Figure 4. Relationship between insulin-mediated glucose uptake measured in vivo (mg/min  $\cdot$  kg estimated metabolic body size) and the difference between basal and insulin-stimulated pmol of [ $^{14}\text{C}$ ]glucose/minute/mg protein incorporated into glycogen.

(Fig. 4). The insulin-stimulated increases in glycogen production in the cell lines were negatively correlated with fasting plasma glucose concentrations ( $r = -0.48$ ,  $P = 0.04$ ), as well as plasma glucose concentrations 2 h after an oral glucose tolerance test ( $r = -0.65$ ,  $P = 0.001$ ) (Fig. 5). The in vitro measure of glycogen production also correlated with percent body fat ( $r = -0.44$ ,  $P = 0.05$ ) (data not shown). The relationship between in vivo measures of glucose disposal and the in vitro measures of glycogen production is not the result of percent body fat, since correction of in vivo measures of glucose disposal for percent body fat in the regression analysis did not abolish the relationship ( $P = 0.001$ ).

The in vitro insulin sensitivity was maintained in culture through 9–10 population doublings (Fig. 6). After this time the phenotype was progressively lost so that by 12 population doublings, there was less than a 20% difference in insulin-stimulated glucose to glycogen over basal levels at low population doublings in several cell lines. The loss of the stimulatory effect of insulin on glycogen production was not a function of in

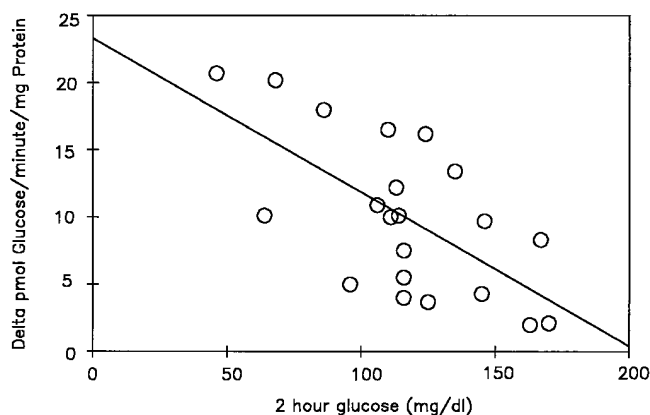
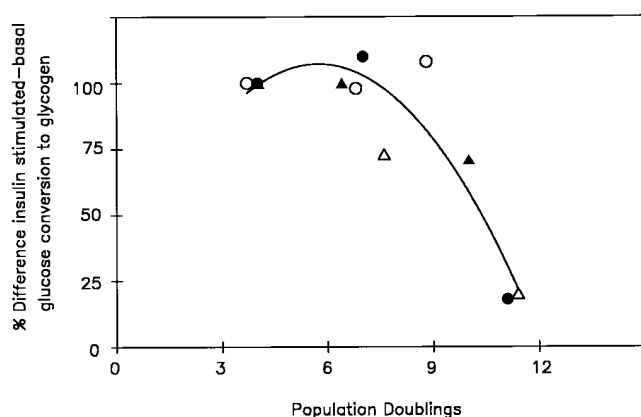


Figure 5. Relationship between plasma glucose concentration 2 h after an oral glucose tolerance load, and insulin-stimulated levels of glycogen production in the myoblast cell lines.



**Figure 6.** The loss of insulin-stimulated glucose conversion to glycogen with increasing population doublings in culture. Cells lines were tested at increasing population doublings for the ability to convert [ $^{14}$ C]glucose to glycogen with insulin stimulation. A progressive loss of insulin stimulation is noted with increasing population doubling that is not related to the *in vivo* measures of insulin sensitivity. The *in vivo* glucose disposal rates of subjects are: (○) 8.9 mg/min  $\cdot$  kg estimated metabolic size; (●) 10.4 mg/min  $\cdot$  kg estimated metabolic size; (▲) 5.5 mg/min  $\cdot$  kg estimated metabolic size; (△) 5.5 mg/min  $\cdot$  kg estimated metabolic size.

*in vivo* measures of insulin action. Cell lines derived from persons with both high and low M values lost insulin-stimulated glycogen production in a similar fashion. The cell lines also lost their ability to continue to divide.

## Discussion

Primary skeletal muscle tissue culture has several advantages over *in vivo* experiments of insulin action and have been used to measure glucose transport *in vitro* (15, 16). Myoblasts were chosen over terminally differentiated myotubes because of their ease in handling and maintenance. In addition, the culture environment is uniform between cell lines. *In vivo* experiments of glucose uptake are complicated by other factors such as pre-existing hyperinsulemia and hyperglycemia which could have unforeseen effects on skeletal muscle. Second, cell lines offer the opportunity to perform studies not possible *in vivo*, such as genetic transformation experiments (17). *In vivo* measures of insulin resistance using the hyperinsulinemic euglycemic clamp technique requires the constant infusion of glucose to maintain euglycemia. This does not allow a clear distinction between insulin effects and the glucose effects *in vivo*, since while plasma glucose levels are constant, the flux of glucose across the skeletal muscle cell membrane can be large. In culture however, insulin effects can be differentiated from glucose by treatment of cell cultures with one or the other compound. Contamination of cultures with nonmuscle cells, specifically fibroblasts, is a concern. By preplating the muscle biopsies, fibroblasts are selectively removed and the residual cells are enriched for myoblasts. Expression of muscle-specific proteins, enzymatic activities, and muscle-specific differentiation factor RNAs also indicate the cultured cells are of a muscle-specific lineage. Although not terminally differentiated, primary cultured myoblasts retain and express the insulin resistant phenotype.

Cells used in these experiments were confined to population doublings 4–8. After approximately 10 population doublings some cell lines began to lose the ability to express insulin action *in vitro*. To eliminate the possibility that the results were the result of slow growth by insulin-resistant cell cultures, cultures were grown for 7 d in six-well plates and any cultures not at 80–90% confluence were discarded. The loss of the ability of the myoblast cell lines to express insulin action *in vitro* with increasing population doublings is not the consequence of the dilution of intercellular factors. After nine population doublings any intercellular factors that might account for differential insulin-stimulated glycogen production would be diluted over 500-fold. Also, the nine population doublings do not account for the number of population doublings required to produce the first monolayer of myoblasts, which would further increase the dilution of any intercellular factors. The two lowest points, at  $\sim 12$  population doublings, are cell lines from persons with M values of 6.6 and 12.4 mg/kg fat-free mass per minute. Cultures which showed reduced or absent insulin-stimulated glycogen production could not be subcultured indicating that cellular senescence was contributing to the loss of the insulin responsive phenotype.

The significant relationship between *in vivo* measures of insulin action and the *in vitro* measures of insulin-stimulated glycogen production indicate that the insulin resistance phenotype is maintained in culture and intrinsic to the cells. In addition to the positive relationship between the *in vivo* measure of insulin mediated glucose disposal there is also the negative relationship with fasting glucose and plasma glucose concentrations 2 h after an oral glucose tolerance test. Since plasma glucose concentrations partially reflect the level of insulin resistance, these relationships are not unexpected. Cell lines derived from individuals with high fasting or two hour glucose concentrations had low levels of glycogen production. However, if this were due to the effects of *in vivo* exposure to increased plasma glucose levels, these effects would have to carry over and be maintained in the culture environment. This is unlikely, since in both *in vitro* and *in vivo* models of hyperglycemic induced insulin resistance, normal glucose tolerance can be restored by glucose starvation (18). This does not rule out a contributory role for hyperglycemia in worsening insulin resistance, but it does not appear to be primary.

Recently, two papers (6, 19) reported a relationship between 2-deoxyglucose transport and *in vivo* glucose disposal rates in myotubes from diabetics and individuals with normal glucose tolerance. They demonstrated insulin dependent increases in glycogen production as well as in glycogen synthase activity in myotubes. In addition, by treating cells with either insulin or glucose they were able to differentiate the effects of each on myotubes. Our experiments extended these observations by examining the relationship between glycogen production, *in vitro*, and glucose disposal, *in vivo*, and by demonstrating that undifferentiated myoblasts also retain defects in insulin action.

The time and dose dependent increase in glycogen synthesis demonstrate that these myoblasts respond to insulin in a manner similar to intact skeletal muscle. The strong relationship between *in vivo* measures of insulin resistance and *in vitro* measures of glycogen production demonstrate that the defect in insulin resistance is intrinsic to myoblasts and is maintained in culture. In this respect these cell lines are unique models for the study of insulin resistance *in vitro*.

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