# Type II Diabetes and Insulin Resistance
## Evidence for Lack of Inherent Cellular Defects in Insulin Sensitivity

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**SUMMARY**

**To determine if inherent cellular differences in insulin sensitivity account for the insulin resistance of non-insulin-dependent diabetes, the effect of insulin on several aspects of cell glucose metabolism was compared in fibroblasts from diabetics and matched nondiabetic controls. The response of total cell glucose metabolism to insulin was assessed by measurement of $^{14}$C-glucose uptake. Insulin stimulated cell glucose incorporation in nondiabetic cells up to twofold with half-maximal stimulation at approximately $3 \times 10^{-9}$ M insulin. This was similar to that observed in diabetic cells. Insulin stimulation of I glycogen synthase activity was also compared in the cells from diabetics and nondiabetics. Both groups demonstrated a threefold increase in %I activity in the presence of insulin with half-maximal stimulation at approximately $2 \times 10^{-9}$ M. There were no differences between diabetics and nondiabetics in either magnitude of response or insulin concentration for half-maximal stimulation. Finally, insulin stimulation of hexose transport was compared in the two cell types using 2-deoxyglucose. In both groups hexose transport was elevated approximately 40% over baseline in the fibroblast in the presence of insulin, with half-maximal stimulation at approximately $2 \times 10^{-9}$ M insulin. No differences were found in insulin action on glucose metabolism in fibroblasts from diabetics and nondiabetics; these results may indicate that there are no inherent differences in cell sensitivity to insulin's glucoregulatory action in non-insulin-dependent diabetics. DIABETES *30*:562–567, July 1981.**

Resistance to insulin-mediated glucose uptake is a well-documented component of non-insulin-dependent diabetes.[1,2] Direct measurements of insulin action using insulin infusion[3–6] or clamping techniques[7] have documented decreased sensitivity to insulin in diabetics, and this decreased sensitivity has been shown to occur, in part, in peripheral rather than hepatic tissues.[7] The insulin resistance has been correlated with decreased receptor binding in some studies,[7,8] but others have implicated a post-receptor site of resistance to insulin action.[5] It is not possible from in vivo studies, even if accompanied by measurements of freshly collected blood cells or tissues, to determine if the observed insulin resistance is a secondary response to the hyperglycemia and/or other changes associated with the hyperglycemic state, or if it is a reflection of inherent cellular differences in response to insulin that contribute to the development of the disorder.

Diploid fibroblast cultures established from skin biopsies afford an easily accessible system for the examination of cellular metabolic capacity that is isolated from physiologic variables. Recent evidence from this and other laboratories indicate that the human fibroblast possesses a specific insulin receptor[9,10] and that several points of carbohydrate metabolism respond to physiologic levels of insulin.[11–15] We have recently established fibroblast lines from genetically characterized diabetics and nondiabetics.[16] For this the Pima Indian population has been utilized, because they have a high prevalence of well characterized non-insulin-dependent diabetes mellitus.[17] Extensive clinical and demographic data on several generations are available for selection of subjects, and the homogeneity of the population[18] is an advantage of eliminating other genetic variables that might confound the comparison of diabetics and nondiabetics. This report compares the action of insulin on hexose transport, glucose metabolism, and glycogen synthase activity in fibroblasts from diabetics and nondiabetics. The results show no evidence for the presence of impaired sensitivity to the action of insulin in cells from diabetics.

### MATERIALS AND METHODS

**Subjects.** Skin biopsies were obtained with informed consent from eight diabetic and nine nondiabetic Pima Indians

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between the ages of 20 and 49 yr (Table 1). Criteria for classification of diabetics and nondiabetics in the Pimas were based on previous studies of plasma glucose levels and their relation to complications in this population.[19] All diabetics had symptomatic non-insulin-dependent (type II) diabetes with no evidence of ketoacidosis. They were selected as having severe glucose intolerance and relatively early age of onset. In addition, both parents of all but two patients were diabetic, as documented by glucose levels greater than 200 mg/dl 2 h after a 75-g oral carbohydrate load. The nondiabetic Indians had no symptoms of diabetes and 2-h glucose levels were less than 160 mg/dl; both parents had normal oral glucose tolerance.

**Reagents.** Eagle's minimal essential medium (MEM), Dulbecco's phosphate-buffered saline (DPBS), and other cell culture supplies were purchased from Grand Island Biological Company (Grand Island, New York). Fetal bovine serum (Rehatuin) was obtained from Reheis Chemical Co. (Phoenix, Arizona), and was heat inactivated at 56°C for 30 min. 2-$^{3}$H(G) deoxy-D-glucose (5–10 Ci/mmol), uridine diphosphate U-$^{14}$C-glucose (250–360 mCi/mmol), and 1-$^{14}$C L-glucose (45–55 mCi/mmol) were purchased from New England Nuclear (Boston, Massachusetts), and D-U-$^{14}$C-glucose (>230 mCi/mmol) from Amersham Searle (Arlington Heights, Illinois). 2-Deoxy-D-glucose, uridine diphosphate glucose, glucose-6-phosphate, fatty acid-free bovine albumin (BSA), and glycogen were purchased from Sigma Chemical Co. (St. Louis, Missouri). Porcine insulin (615-D56-10) was a gift of Eli Lilly and Co. (Indianapolis, Indiana).

The preincubation and labeling medium used in the hexose uptake and glycogen synthase studies consisted of a modification of Eagle's basal medium (BME). This was prepared containing NaCl (0.14M), KCl (2.7 mM), $CaCl_2$ (0.90 mM), $KH_2PO_4$ (1.47 mM), $Na_2HPO_4$ (8.06 mM), $MgCl_2$ (0.49 mM), glutamine (2 mM), BME amino acids, BME vitamins, and BSA (1 mg/ml). This mixture will be designated subsequently as IM.

**Cell cultures.** Cell cultures were established from 3-mm full thickness punch biopsies obtained from the medial aspect of the forearm after cleansing with PhisoHex and ethanol and administration of a local anesthetic (xylocaine). The skin specimen was placed in Eagle's minimal essential medium (MEM) supplemented with penicillin (50 U/ml) and streptomycin (50 μg/ml) and fibroblast cultures were derived from explants as described previously.[16] Cultures were grown as monolayers in MEM supplemented with nonessential amino acids (1% v/v), fetal bovine serum (10% v/v) and Hepes buffer (10 mM). Cells were subcultured using a 0.05% trypsin/0.02% EDTA solution in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (DPBS). Stock cultures were maintained in closed vessels at 37°C, and they were routinely screened for mycoplasma (Flow Labs, Rockville, Maryland). Cultures were used for experimental procedures between the 5th and 15th passages.

**Assay of total cell glucose incorporation.** Replicate cultures of cells were grown to confluence in 60-mm petri dishes containing 5 ml of medium and were studied on day 7–8, after the cells had been confluent for several days. Cell monolayers were preincubated by rinsing in DPBS and incubating at 37°C for 18 h in IM containing $NaHCO_3$ (21 mM) in a humidified atmosphere of 5% $CO_2$ in air. Insulin was added 3 h before the assay after adjusting the volume to 3 ml. Insulin action on glucose incorporation in this system can be observed in less than 15 min and is maximal in 2 h.[12] Insulin degradation during this period, as determined by recovery of TCA-soluble $^{125}$I-insulin, was less than 10% at concentrations below $10^{-7}$ M. Total cell glucose incorporation

TABLE 1
Characteristics of biopsy donors

| | Age (yr) | Age of onset (yr) | Plasma glucose (mg/dl) | | Diabetic status | | Relative weight (kg/m²) |
|---|---|---|---|---|---|---|---|
| | | | Fasting | 2-H | Father | Mother | |
| Nondiabetic | | | | | | | |
| PT | 28 | — | 106 | 143 | – | – | 44.2 |
| RW* | 23 | — | 99 | 127 | – | – | 39.2 |
| CC | 20 | — | 89 | 103 | – | – | 26.8 |
| GH | 49 | — | 86 | 135 | – | – | 52.5 |
| GC | 35 | — | 93 | 154 | – | – | 41.9 |
| SA | 22 | — | 81 | 136 | – | – | 22.8 |
| CS | 21 | — | 92 | 102 | – | – | 40.5 |
| KD | 41 | — | 95 | 157 | – | – | 29.0 |
| CT | 22 | — | 99 | 127 | – | – | 42.9 |
| Mean ± SEM | 29 ± 3.1 | — | 93 ± 2.4 | 132 ± 6.1 | | | 37.8 ± 9.5 |
| Diabetics | | | | | | | |
| CL | 25 | 20 | 231 | 506 | + | + | 22.2 |
| EJ | 37 | 27 | 221 | 304 | + | + | 26.8 |
| PJ* | 23 | 21 | 152 | 270 | + | + | 29.0 |
| FE | 26 | 16 | 239 | 356 | + | + | 31.3 |
| ML | 24 | 20 | 103 | 255 | + | + | 34.0 |
| DP* | 23 | 18 | 253 | 367 | + | – | 27.4 |
| JM* | 27 | 25 | 246 | 403 | + | + | 41.3 |
| GS | 42 | 22 | 265 | 383 | – | + | 31.3 |
| Mean ± SEM | 28 ± 2.5 | 21 ± 1.3 | 214 ± 20 | 365 ± 29 | | | 30.4 ± 5.7 |

* Biopsies from these individuals were processed and the cell line stored frozen at the Institute of Medical Genetics, Camden, New Jersey (culture numbers GM3234, GM2440, GM2574, and GM2556, respectively).

was measured for 20 min using D-U-$^{14}$C-glucose (1 μCi/ml, 0.5 mM). Incubation was terminated by rapidly aspirating the medium containing the radiolabeled sugar and rinsing 3 times with 15–20 ml of cold (4°C) DPBS (total rinsing time was approximately 20 s). Assay of radioactivity in a fourth rinse indicated that less than 5% of the intracellular radioactivity is lost during this rinsing procedure. The cells were digested for 2 h at room temperature in 2 ml of NaOH (0.1 M) in $Na_2CO_3$ (2%) and aliquots were then obtained for protein determination[20] and assay of radioactivity. Zero time values for uptake were determined by adding the mixture containing radiolabeled sugar to a dish of cells placed on ice and then immediately aspirating the radiolabeled medium and rinsing as described above.

**Assay of glycogen synthase activity.** Replicate cultures of cells were grown to confluence in 100-mm petri culture dishes containing 10 ml of medium and studied on days 7 or 8. Cell monolayers were preincubated without glucose for 18 h as described for glucose incorporation studies. After preincubation, cultures were rinsed with DPBS and incubated in 5 ml of IM containing the indicated concentrations of insulin at 37°C for 2 h. Insulin action on glycogen synthase in this system is observed within 5 min and reaches maximum in 1 h.[14] Cells were then rinsed with ice cold DPBS three times and harvested using a Teflon policeman. Cells were homogenized in 0.5 ml glycylglycine, 2 mM EDTA, 1 mM DTT, pH 7.0, using a Polytron homogenizer for 15 s at a setting of 6. The homogenates were centrifuged at 12,000 × *g* for 10 min and the supernatants were used for enzyme assay. The activity of glycogen synthase in extracts was measured by the method of Thomas, Schlender, and Larner.[21] Protein concentration of all extracts was determined using a BioRad Protein Assay Kit (Bio-Rad Laboratories, Richmond, California).

**Assay of hexose uptake.** Kinetics of hexose transport and the effect of insulin on hexose transport were assessed using 2-deoxyglucose. Monolayers were prepared and preincubated with or without insulin as described for glucose incorporation studies, except that the preincubation medium contained 16.7 mM glucose. For estimation of $K_m$ and $V_{max}$ the initial rates of deoxyglucose uptake were determined for 1 min at 37° in IM containing 1 μCi/ml $^3$H-deoxy-D glucose from 0.1 to 10 mM. Phloridzin (1 mM) was included in the rinse to prevent transport out of the cell during rinsing, and rates of uptake were corrected for diffusion and nonspecific uptake by subtracting the rate of uptake estimated from concentrations between 2 and 10 mM.[12] For the determination of insulin sensitivity, monolayers received concentrations of insulin from $10^{-10}$–$10^{-6}$ M for 2 h and were pulsed for 5 min with 0.1 mM 2-deoxyglucose at 1 μCi/ml. Rates of uptake were corrected for diffusion and nonspecific uptake by the simultaneous determination of uptake of 0.1 mM $^{14}$C-L-glucose. Insulin stimulation of deoxyglucose uptake under these conditions can be observed in 5 min and reaches a maximum in 1 h (data not shown).

**Radioactivity.** Radioactivity was assayed in a Packard Liquid Scintillation Spectrometer equipped with an external standard for quench correction. Cell extracts were neutralized with HCl before counting. Glycogen precipitates on paper were counted in LSC (Yorktown Research, S. Hackensack, New Jersey) and cell suspensions were counted in a 2:1 mixture of LSC and Triton X-100.

TABLE 2
Glucose incorporation in diabetic versus nondiabetic fibroblasts

| | Diabetic (6) | Nondiabetic (6) |
|---|---|---|
| Preincubation with glucose | | |
| μg/10$^6$ cells | 0.18 ± 0.04* | 0.20 ± 0.05 |
| μg/mg protein | 0.92 ± 0.40 | 1.3 ± 0.30 |
| After glucose starvation | | |
| μg/10$^6$ cells | 0.45 ± 0.13 | 0.39 ± 0.07 |
| μg/mg protein | 2.3 ± 0.54 | 2.2 ± 0.71 |

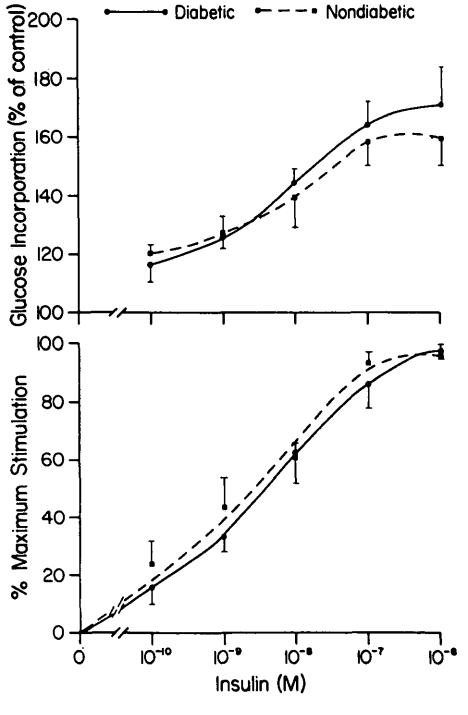
Cultures were grown to confluence as described in MATERIALS AND METHODS and preincubated for 18 h in serum-free IM with or without glucose (5.5 mM). After washing with glucose, incorporation was determined in 3 ml IM for a 20-min incorporation period using 0.5 mM [$^{14}$C]glucose.
* Mean ± SEM.

## RESULTS

Basal rates of glucose utilization in diabetic and nondiabetic fibroblasts were compared (Table 2) after overnite incubation in the serum-free incubation medium (employed for all subsequent assays). Glucose starvation, which enhances insulin action on glucose incorporation and glycogen synthase,[12,14] resulted in similarly elevated basal rates of glucose incorporation in diabetic and nondiabetic cells.

An assessment of the overall effect of insulin on glucose metabolism of the cultured fibroblast was made by measuring total cell glucose accumulation over a 20-min period in

**FIGURE 1. Insulin stimulation of glucose utilization in diabetic and nondiabetic fibroblasts. Monolayers were preincubated in glucose-free medium and received the indicated concentrations of insulin 3 h before the determination of glucose utilization. Total cell [$^{14}$C]glucose (0.5 mM, 1 μCi/ml) incorporation was measured for 20 min at 37°C. ■---■, Nondiabetics, N = 6; ●——●, diabetics, N = 6. (A) Incorporation after insulin treatment is expressed as percent of incorporation in non-insulin-treated control; basal incorporation was 22 ± 3.3 nmol/mg protein for the nondiabetics and 21 ± 3.0 for diabetics. (B) Data from A are expressed as % of maximal stimulation achieved at either $10^{-7}$ or $10^{-6}$ M insulin.**



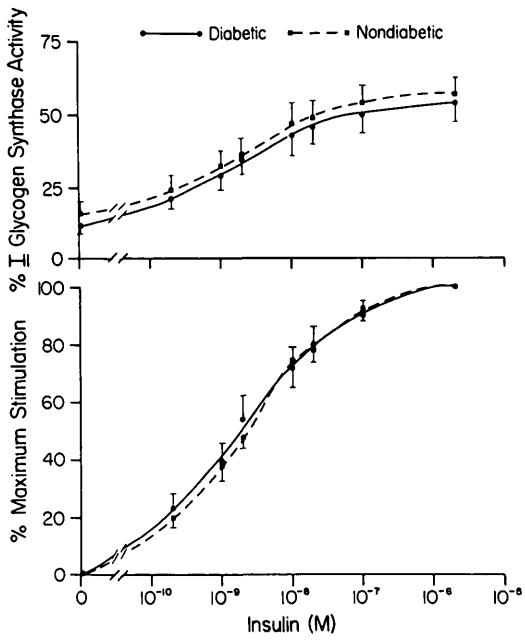



**FIGURE 2. Effect of insulin on glycogen synthase activity in diabetic and nondiabetic fibroblasts. Replicate monolayers (approximately 3 × $10^5$ cells, 1.5 mg protein) were grown to confluency and preincubated for 18 h in glucose-free, serum-free medium. Insulin was added 2 h before the end of the preincubation period. Glycogen synthase I and total activities were determined in supernatants of homogenized cells as described in** MATERIALS AND METHODS. **■---■, Nondiabetics, N = 7; ●——●, diabetics, N = 5. (A) % I activity. (B) Data from A are expressed as % of maximal stimulation achieved at $10^{-6}$ M insulin.**

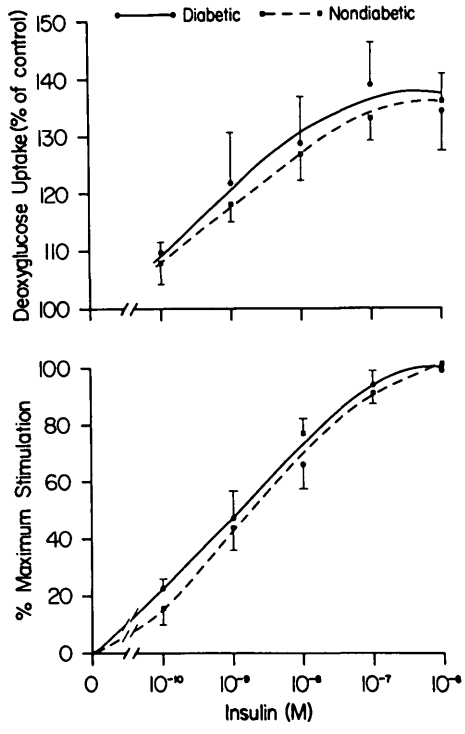



**FIGURE 3. Insulin stimulation of 2-deoxyglucose transport in diabetic and nondiabetic fibroblasts. Monolayers were prepared and preincubated as in Figure 1. $^3$H-2-deoxyglucose uptake (0.1 mM, 1 μCi/ml) was measured for 5 min as described in** MATERIALS AND METHODS. **■---■, Nondiabetics; ●——●, diabetics. Values are the mean ± SEM of 6 duplicate or quadruplicate experiments for each group. (A) Uptake after insulin treatment is expressed as percent of uptake in non-insulin-treated controls; basal uptake was 2.8 ± 0.20 nmol/mg protein for the nondiabetics and 2.4 ± 0.37 nmol/mg for the diabetics. (B) Data from A are expressed as % of maximal stimulation achieved at either $10^{-7}$ or $10^{-6}$ M insulin.**

the presence and absence of insulin (Figure 1). We have shown previously that the glucose incorporated over the 20-min incubation is converted primarily to glycogen, lactate, and nucleotides, and that insulin stimulates incorporation into all 3 fractions.[12] In nondiabetic fibroblasts insulin caused a significant increase in glucose incorporation, with a maximal response of approximately 160% above basal. The effect of insulin was observed with as little as $10^{-10}$ M insulin. When data were expressed as percent of maximal stimulation (Figure 1B), half-maximal stimulation was observed at approximately 3 × $10^{-9}$ M. When glucose incorporation was measured in the diabetics there were no significant differences in sensitivity or maximal response to insulin.

Under the conditions of preincubation employed, it has been previously shown that a large proportion of the glucose incorporated in 20 min is found in glycogen[12] and that insulin stimulates the I activity of glycogen synthase under these conditions.[14] Insulin stimulation of glycogen synthase in the diabetic and nondiabetic fibroblasts is compared in Figure 2. Insulin caused an increase in the I activity in the cultures from nondiabetics from a value of 16% without insulin to a maximum of 56% (Figure 2A). An insulin concentration of approximately 2 × $10^{-9}$ M gave a half-maximal stimulation (Figure 2B). In fibroblasts from diabetics, basal % I activity was similar and the effect of insulin on I activity was not significantly different at any of concentrations of insulin employed.

Insulin stimulation of hexose transport was also directly compared in fibroblasts from diabetics and nondiabetics. Insulin caused a small but significant increase in 2-deoxyglucose uptake in fibroblasts (Figure 3A) and this has been shown previously to be due to an increment in the $V_{max}$ of transport.[12] In nondiabetics the mean maximum effect was approximately 135% of baseline. Response was observed with physiologic levels of insulin, and the concentration of insulin for half-maximal effect was approximately 2 × $10^{-9}$ M (Figure 3B). The cells from the diabetics did not differ from controls in the magnitude of response or in concentration of insulin required for half-maximal effect.

Table 3 summarizes the values obtained for insulin sensitivity from individual experiments on diabetic and nondiabetic fibroblasts. Concentrations of insulin required for half-maximal stimulation of glucose incorporation, glycogen synthase, and hexose transport were similar. When mean values for diabetics were compared with those from nondia-

TABLE 3
Insulin concentrations for half-maximal stimulation of steps in glucose metabolism in diabetic versus nondiabetic fibroblasts

| | Nondiabetic | Diabetic |
|---|---|---|
| Total cell glucose incorporation | 3.1 ± 1.4 × $10^{-9}$ | 3.9 ± 1.2 × $10^{-9}$ |
| Hexose transport | 2.6 ± 1.4 × $10^{-9}$ | 1.9 ± 0.7 × $10^{-9}$ |
| Glycogen synthase | 2.4 ± 0.6 × $10^{-9}$ | 3.8 ± 2.5 × $10^{-9}$ |

Experimental conditions are described in Figures 1–3. Each individual experiment was graphed as % maximal stimulation. Values are the mean ± SEM of the concentrations in each experiment resulting in 50% of maximal stimulation.

betics, there were no differences in sensitivity in any of the parameters of insulin action.

## DISCUSSION

This study is the first in vitro comparison of insulin action on glucose metabolism in fibroblasts from diabetics and nondiabetics. The use of cells many generations removed from environmental influences allows the direct evaluation of inherent metabolic potential. The advantage of the Pima Indians as a study population is that their relative genetic homogeneity[18] limits variables that might influence such cellular comparisons. Several aspects of insulin action on cell glucose metabolism have been examined, and no evidence of insulin insensitivity was found in cells from the diabetics.

Although the fibroblast is not a major target organ for insulin-mediated glucose disposal in vivo, the data suggest that this system may be qualitatively reflective of insulin's glucoregulatory action. The measurement of glucose incorporation employed in this study is an assessment of insulin's effect on total cell glucose metabolism. The concentration dependence for insulin action on glucose utilization was similar to that observed in studies on intact muscle preparations such as rat diaphragm;[22] it is also similar to the range of insulin required in in vivo infusion studies[23] to assess insulin-mediated glucose disposal. The data indicated that cells from diabetics did not differ from those from nondiabetics in insulin stimulation of glucose utilization. Fractionation of the cells after glucose uptake indicated that under the conditions of glucose deprivation employed, the glucose is converted mainly to glycogen, lactic acid, and nucleotides (ribose) and that all three respond to insulin;[12] presumably this reflects several sites of insulin control.

Insulin action on glycogen synthase activity paralleled in time course and sensitivity the action on total cell glucose utilization, but the direct measure of enzyme activity provides a more specific measure of insulin regulation. The characteristics of insulin regulation of glycogen synthase in the fibroblast[14] are similar to those observed in adipocytes[24] and hepatocytes.[25] The response of I glycogen synthase activity to insulin was identical in the diabetic and nondiabetic cells.

Insulin action on hexose transport in the fibroblast is more difficult to study because the response is characteristically small.[12,13,26] A large number of experiments consistently showed a response to insulin in diabetic cells well within the range observed in controls. Insulin action on transport in the fibroblast differs from that observed in the young rat adipocyte[27,28] in that the response is of lower magnitude and somewhat slower. Recent studies, however, have indicated that the hexose transport response of insulin can be influenced by metabolic alterations.[29,30] Thus, the fibroblast may not differ qualitatively, but simply in the state of other metabolic properties that also regulate hexose transport.

In conclusion, this study has compared several parameters of insulin action on glucose metabolism in fibroblasts from diabetics and nondiabetics, and no evidence of insulin insensitivity was found in cells from the diabetics. On the other hand, in vivo insulin resistance has been directly demonstrated in this population in diabetics compared with normals.[5] Further study will be required to determine whether mechanisms of insulin action in fibroblasts are similar to those in the major tissues involved in glucose homeostasis. If defects in glucose disposal in the major target tissues are reflected in the fibroblast, then the results of the present study indicate that there are no inherent differences in cell sensitivity to insulin's glucoregulatory action in non-insulin-dependent diabetics.

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