Journal of Supramolecular Structure 14:499–509 (1980)
Control of Cellular Division and Development: Part B: 279–289

# Alterations in the Responsiveness of Diabetic Fibroblasts to Insulin

**Mohan K. Raizada and Robert E. Fellows**

*Department of Physiology and Biophysics, The University of Iowa College of Medicine, Iowa City, Iowa 52242*

Fibroblastic cultures from the skin of nondiabetic and diabetic (db/db) mice have been used to investigate alterations in the biological responses of diabetic cells to insulin. Confluent cultures from the skin of both nondiabetic and diabetic animals possess specific receptors for insulin. Diabetic fibroblasts exhibit only 36% as much specific binding of insulin as nondiabetic fibroblasts, because of a decrease in the total number of binding sites, without a change in binding affinity. Insulin caused a time- and dose-dependent increase in the rate of 2-deoxy D-glucose (dGlc) uptake and in ornithine decarboxylase (ODC) activity of both nondiabetic and diabetic fibroblasts. In nondiabetic cells, half-maximal increase in dGlc uptake was obtained with 0.3 nM insulin, and a maximum increase of 120% was obtained with 4.1 nM insulin. In contrast, diabetic cultures required 0.8 nM insulin for a half-maximal increase in dGlc uptake, and maximum stimulation with 4.1 nM insulin was only 50% above control levels. With 4-fold higher insulin concentrations, ODC activity of diabetic cells was only 40% that of nondiabetic cells. In nondiabetic cells, down regulation of insulin receptors by insulin abolished the ability of insulin to stimulate dGlc uptake. These results demonstrate that cells cultured from diabetic animals, which possess a decreased number of insulin receptors, also exhibit decreased stimulation of deoxy D-glucose uptake and ornithine decarboxylase activity by insulin.

**Key words: fibroblasts, diabetic mice, insulin, deoxy D-glucose, ornithine decarboxylase**

Decreased insulin binding in tissues and membrane fractions from the genetically obese (ob/ob) mouse [1, 4], diabetic db/db mouse [4, 5], and obese and diabetic human subjects [6, 7] may be due to down regulation, insulin deficiency, or genetic factors. This defect has been shown to be due to a decrease in the total number of insulin binding sites per cell, per milligram protein or per unit surface area. Changes in receptor numbers are also associated with the development of insulin resistance in obesity [8]. A decrease in the number of insulin receptors has been associated with decreased uptake of glucose and stimulation of glycogen synthesis in isolated soleus muscles from obese rats [9]. Similarly, a decreased ability of insulin to stimulate dGlc uptake has been observed in diaphragm and in soleus muscles of ob/ob mice [10, 11]. Czech et al [12] showed that even at a 100-fold

Abbreviations: dGlc, 2-deoxy D-glucose; ODC, ornithine decarboxylase; TMEM, Temin's modification of Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

Received April 7, 1980; accepted September 2, 1980.

0091-7419/80/1404-0499$03.50 

greater concentration, insulin was only about 50% effective in stimulating glucose metabolism and hexose transport in fat cells from fa/fa Zucker rats. Although these findings demonstrate that altered tissue responses to insulin are associated with obesity, little is known about these responses of diabetic animals. In previous studies, we found that fibroblasts from the skin of genetically diabetic db/db mice maintained in culture have a decreased number of insulin receptors when compared to similarly prepared cultures from nondiabetic mice [13]. As a first step toward an understanding of the mechanism involved in biological responses to insulin in diabetic cells, we have studied the relationship between the alteration in insulin receptors and insulin responses in these cells. We have found that cells from diabetic mice require higher insulin concentrations than do cells from nondiabetic animals in order to produce half-maximal stimulation of dGlc uptake and ODC activity. Furthermore, maximal stimulation of these activities by insulin is comparatively lower in diabetic fibroblasts than in those from nondiabetic littermates.

## MATERIALS AND METHODS

Four-week-old nondiabetic and diabetic male mouse littermates of C57BL/KS JM-db (db/db) strain were obtained from the Jackson Laboratory, Bar Harbor, ME. Temin's modification of Eagle's medium (TMEM) and fetal bovine serum (FBS) were purchased from Grand Island Biological Company (Grand Island, NY); lyophilized trypsin (190 units/mg) was obtained from Worthington Biochemical Corporation (Freehold, NJ). Porcine insulin (lot 104 M 95 AH, 27.3 USP/mg) was obtained from Elanco (Indianapolis, IN). The glucagon content of this preparation is one part per million. 2-Deoxy-D-glucose, pyridoxal phosphate, and dithiothreitol were purchased from Sigma Chemical Company (St. Louis, MO); 2-[$^3$H](G)] deoxy D-glucose (specific activity 8.1 Ci/mmole) was from New England Nuclear (Boston, MA), and DL-[1-$^{14}$C] ornithine monohydrochloride (specific activity 40–60 mCi/mmole) was from Amersham Corporation (IL).

### Preparation of Fibroblastic Cultures From the Skin of Nondiabetic and Diabetic (db/db) Mice

Four-week-old nondiabetic and diabetic mice were anesthetized with Nembutal, their backs were shaved, and the skin was moistened with 70% ethanol. Pieces of skin (0.2–0.5 cm) were removed and washed twice with 4 ml of TMEM containing 200 units penicillin G, and 200 μg streptomycin per milliliter. Pieces were distributed over the moist surface of a 100 mm tissue culture dish and covered with 0.5 × 0.5 cm cover glasses previously moistened with TMEM. TMEM containing 100 units of penicillin G, 100 μg streptomycin/ml, and 20% FBS was added to each dish, and the dishes were placed in a humidified incubator at 37°C with 5% $CO_2$ and 95% air. The medium was changed every third day. Fibroblasts started to grow out of the explants after 7–10 days and covered the dish within 3–4 weeks. The monolayer was washed once with Tris-isotonic salt solution, pH 7.4, and cells were dissociated from the dish by incubation of the monolayer with 0.25% trypsin in Tris-isotonic salt solution, pH 7.4, for 10 min at room temperature. Dissociated cells were washed once with 40 ml TMEM containing 15% FBS and centrifuged at 800g for 7 min. Finally, 1 × $10^6$ cells were plated in 100 mm tissue culture dishes in TMEM containing 15% FBS and grown at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. These cells will be referred to as passage number one. On day 4, when cultures became confluent as determined by phase-contrast microscopic examination, cells were transferred to 35 mm or 100 mm tissue culture

dishes for experiments. Fibroblasts derived from skin explants were transferred by the procedure described above up to 18 times, after which the cultures were discarded and fresh cultures were started.

### Determination of Cell Numbers

Cell densities in seeding suspension or monolayers were determined in triplicate samples either by directly suspending the cells in Isoton dilutent (Curtin-Matheson) or by dissociating the monolayer with 0.25% trypsin before suspension in dilutent. Cell counts were determined with a hemocytometer and/or with a Coulter Counter.

### Measurement of [$^3$H]-2 Deoxy D-Glucose Uptake

The uptake of [$^3$H] dGlc by attached confluent cultures of nondiabetic and diabetic cells was determined as described previously [14]. Nondiabetic and diabetic cells ($0.2 \times 10^6$) were plated in 35 mm tissue culture dishes and grown in TMEM containing 15% FBS at 37°C. On day 3 cells were washed twice with 2 ml TMEM without serum and incubated in the same medium for 18–24 hr at 37°C. Serum-deprived cultures were used for [$^3$H] dGlc uptake studies as follows.

Monolayers were rinsed twice with 2 ml phosphate-buffered saline containing 0.9 mM $Ca^{2+}$ and 0.49 mM $Mg^{2+}$ (PBS), pH 7.4 [15]. They were then incubated with one ml PBS, pH 7.4, containing 0.5 mM [$^3$H] dGlc for 5 min at 37°C. The radioactive medium was removed, cells were rinsed 5 times with 2 ml ice-cold PBS, and digested with 0.5 ml 1.0 N NaOH. Portions of cell digests were counted in a Beckman liquid scintillation spectrometer [16], and protein content determined [17]. In some experiments, cultures were incubated for varying time periods in the presence of varying concentrations of porcine insulin prior to measurement of dGlc uptake, as well as during dGlc uptake.

### Measurement of Ornithine Decarboxylase Activity

Nondiabetic and diabetic fibroblasts ($1 \times 10^6$) were plated in 100 mm diameter Falcon tissue culture dishes and allowed to grow for 3 days in TMEM containing 15% FBS. Monolayers of cells, washed twice with 8 ml of TMEM without serum, were incubated in the same medium for 16–24 hr. Cultures were washed twice with ice-cold PBS (pH 7.4), and subsequent steps were performed at 4°C. Cells were removed from the dishes with a rubber policeman, collected by centrifugation at 2,000g, and homogenized in 10 ml TES buffer (pH 7.6) containing 0.5 mM dithiothreitol (DTT) and 0.2 mM pyridoxal 5-phosphate. Homogenates were centrifuged at 27,000g for 15 min, and supernatant fractions were collected for ODC activity. The assay mixture, containing 10 mM TES, pH 7.6, 0.5 mM DTT, 0.2 mM pyridoxal 5-phosphate, and 0.1 $\mu$Ci DL [1-$^{14}$C]-ornithine, was placed in a $17 \times 100$ mm polypropylene tube and sealed with a rubber stopper with a hanging plastic center well containing 0.2 ml Hyamine. The supernatants (100–200 $\mu$l) were added to the assay mixtures and incubated at 37°C with constant shaking. This reaction was terminated after 60 min by addition of 1 ml of 2 M citric acid. Incubation continued for another hour to ensure complete trapping of $^{14}CO_2$ by Hyamine. The center well was transferred to a scintillation vial, and radioactivity was counted in a Beckman liquid scintillation spectrometer [16].

## RESULTS

### Morphological and $^{125}$I-Insulin Binding Characteristics of Fibroblasts Cultured From the Skin of db/db Mice

Cells cultured from the skin of nondiabetic and diabetic (db/db) mice have flat, fusiform morphology, a rather smooth cell surface and, in general, resemble fibroblastic cells (Fig. 1). The plating efficiency of both cell types was 99% or better; the doubling time for both cell types was 18–20 hr when cultured in TME medium containing 15% FBS. The number of cells and total amount of protein per dish were comparable for both cell types. Both cell types bind $^{125}$I-insulin specifically. The binding was time-, temperature-, and pH-dependent and was 85–90% specific (Table I). Dissociation rates of $^{125}$I-insulin bound to nondiabetic and diabetic cultures were similar, and 50% dissociation was attained in 7.5 min. Analysis of binding data according to the method of Scatchard revealed curvilinear plots with similar low and high affinities for nondiabetic and diabetic fibroblasts. The total number of binding sites for nondiabetic cultures was calculated to be $21 \times 40^4$ per cell. In contrast, diabetic cultures had only $4.5 \times 10^4$ binding sites per cell (Fig. 2). Of many hydrolytic enzymes tested, only trypsin, at 10 μg/ml at 24°C for 10 min, caused a significant (170–200%) increase in the specific binding of insulin by fibroblasts from nondiabetic and diabetic mice. These results clearly demonstrate that binding of insulin to nondiabetic and diabetic cells is specific, rapid, and reversible, and can be designated a true hormone-receptor interaction. An average decrease of 62% in the binding of insulin to diabetic fibroblasts was obtained in cultures prepared from all 8 groups of diabetic mice compared to thin littermates. Cultures from nondiabetic and diabetic animals bound 0.5–0.9 fmoles and 0.23–0.37 fmoles $^{125}$I-insulin per milligram protein.

### Effect of Insulin on 2-Deoxy D-Glucose Uptake in Fibroblastic Cultures From Nondiabetic and Diabetic Mice

Uptake of [$^3$H] dGlc by serum-deprived confluent cultures of nondiabetic fibroblasts was time-dependent and linear up to 5 min at a concentration of 0.5 mM dGlc (Fig. 3). All uptake experiments therefore were performed with 0.5 mM dGlc for 5 min. A significant decrease in the basal transport rate of dGlc has been reported in many tissues obtained from obese and diabetic animals [18, 19]. This decrease was associated with a decrease in the specific binding of insulin. Since cells cultured from diabetic mice showed 1/2–1/3 the number of insulin receptors, we investigated the basal uptake rate of dGlc in nondiabetic and diabetic cells to see if similar differences existed in the cell culture system.

Unstimulated uptake of dGlc by confluent cultures of nondiabetic and diabetic fibroblasts was comparable (Table II), and no significant difference in uptake was observed with cells from different passage numbers up to 17. Incubation of 83 nM porcine insulin for 15 min at 37°C with nondiabetic cells produced a time-dependent increase in the sugar uptake (Table III). A 25% stimulation of uptake was observed as early as 2 min and reached a maximal 61% stimulation in 15 min. No significant increase in the uptake of dGlc was observed in untreated cultures during this time.

Incubation of insulin with nondiabetic and diabetic fibroblasts for 10 min caused a dose-dependent increase in dGlc uptake (Fig. 4). A similar basal rate of dGlc uptake was detected in both diabetic and nondiabetic cells. At 4.1 nM, insulin caused a maximal stimulation of 2.2-fold in nondiabetic and 1.5-fold in diabetic fibroblasts, and the $ED_{50}$ for nondiabetic and diabetic cells was 0.3 nM and 0.8 nM, respectively. Concentrations of insulin at this level caused a 15–20% inhibition of $^{125}$I-insulin binding.

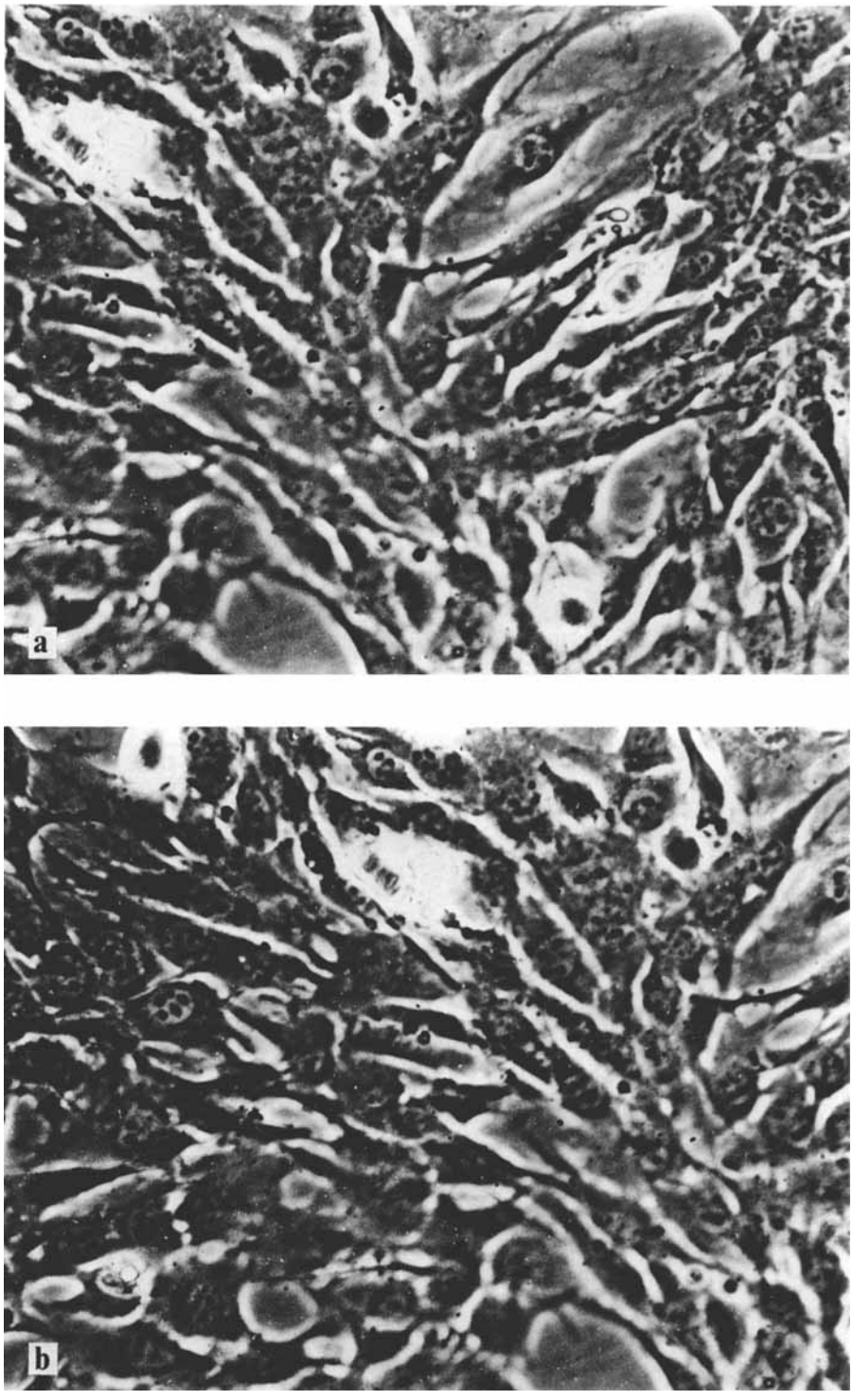


Fig. 1. Phase-contrast micrographs of fibroblastic cultures from the skin of nondiabetic and diabetic (db/db) mice. Nondiabetic (a) and diabetic (b) cells, $0.2 \times 10^6$, were plated in 35 mm tissue culture dishes in TMEM containing 15% FBS and grown at 37°C as described in Methods (magnification 250 ×).

**TABLE I. Characteristics of $^{125}$I-Insulin Binding to Fibroblastic Cultures From the Skin of Nondiabetic and Diabetic Mice***

| | Cell type | |
|---|---|---|
| | Nondiabetic | Diabetic |
| pH optimum | 8.0 | 8.0 |
| Time dependence | Linear to 30 min | Linear to 30 min |
| Specific binding | 85–90% | 85–90% |
| Scatchard plot | Curvilinear | Curvilinear |
| $T_{1/2}$ of dissociation | 7.5 min | 7.5 min |
| Affinities | | |
| High | $2–8 \times 10^{10}$ $M^{-1}$ | $2–8 \times 10^{10}$ $M^{-1}$ |
| Low | $2–8 \times 10^{9}$ $M^{-1}$ | $2–4.8 \times 10^{9}$ $M^{-1}$ |
| Number of binding sites per cell | $7.7 –21 \times 10^{4}$ | $2.9–4.5 \times 10^{4}$ |
| Neuraminidase (20 μg/ml, 40 min) | No effect | No effect |
| α-Chymotrypsin (15 μg/ml, 10 min) | No effect | No effect |
| Phospholipase $A_2$ (40 μg/ml, 40 min) | No effect | No effect |
| Phospholipase C (40 μg/ml, 40 min) | No effect | No effect |
| Trypsin (10 μg/ml, 10 min) | 170–200% increase in binding | 160–200% increase in binding |

*Nondiabetic and diabetic cells ($0.2 \times 10^6$ per 35 mm dish) were incubated at 37°C in TME medium containing 15% FBS. Confluent cultures were used for $^{125}$I-insulin binding. Triplicate plates were incubated with 0.5 ml PBS containing 0.28 nM $^{125}$I-insulin and 1.6% BSA for 30 min at 24°C. Specific binding of $^{125}$I-insulin was obtained by subtracting nonspecific binding in the presence of 16.6 μM unlabeled insulin from total binding. Confluent cultures of fibroblasts were washed with PBS and incubated with indicated concentrations of hydrolytic enzymes at 24°C.

**TABLE II. Uptake of $^3$H-2 Deoxy D-Glucose in Fibroblastic Cultures From Nondiabetic and Diabetic Mice***

| | Nondiabetic | | Diabetic | |
|---|---|---|---|---|
| Passage number | [$^3$H] dGlc uptake (nmoles/mg/min) | Protein (μg/plate) | [$^3$H] dGlc uptake (nmoles/mg/min) | Protein (μg/plate) |
| 3 | 3.0 ± 0.30 | 295 ± 7.8 | 2.7 ± 0.40 | 305 ± 13.2 |
| 8 | 2.2 ± 0.10 | 433 ± 16.8 | 2.6 ± 0.10 | 474 ± 23.2 |
| 11 | 2.6 ± 0.09 | 727 ± 16.8 | 2.5 ± 0.16 | 689 ± 51.8 |
| 17 | 2.7 ± 0.07 | 404 ± 5.2 | – | – |

*Explants of skin from nondiabetic and diabetic (db/db) mice were placed in 10 mm diameter culture dishes and incubated with TMEM containing 20% FBS. After 4 weeks, cells were dissociated by 0.25% trypsin and plated in 100 mm diameter culture dishes (first passage). They were fed every third day and subcultured on day 4.

**TABLE III. Insulin Stimulation of 2-Deoxy D-Glucose Uptake as a Function of Time in Fibroblastic Cultures of the Nondiabetic Mouse***

| | [$^3$H] dGlc uptake (nmoles/min/mg protein | | |
|---|---|---|---|
| Time (min) | – Insulin | + Insulin | % Stimulation |
| 0 | 2.4 ± 0.12 | – | – |
| 2 | 2.6 ± 0.07 | 3.3 ± 0.09 | 125 |
| 5 | 2.6 ± 0.13 | 3.6 ± 0.05 | 138 |
| 7.5 | 2.7 ± 0.04 | 4.0 ± 0.05 | 148 |
| 15 | 2.8 ± 0.20 | 4.5 ± 0.30 | 161 |

*Serum-deprived nondiabetic fibroblasts were incubated without or with 83 nM porcine insulin in PBS, pH 7.4. At indicated times, 0.5 mM [$^3$H] dGlc was added, and incubation was continued for an additional 5 min. Results are means of triplicate samples ± SEM.

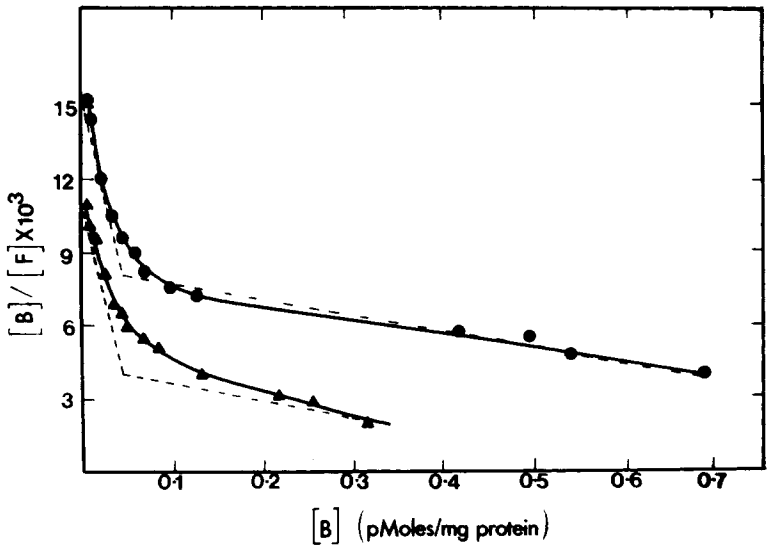


Fig. 2. Scatchard plot analysis of $^{125}$I-insulin binding to fibroblastic cultures of nondiabetic and diabetic (db/db) mice. Binding of $^{125}$I-insulin to confluent fibroblastic cultures of nondiabetic (•) and diabetic (db/db) (▲) mice was measured by incubation of cultures for 30 min at 24°C with 0.22 nM $^{125}$I-insulin in PBS containing 1.6% BSA in the presence of increasing concentrations (167 pM to 167 nM) of unlabeled porcine insulin. The bound [B]/free [F] ratio of insulin was plotted as a function of insulin bound [B]. These curves were resolved into high- and low-affinity components graphically (broken lines). Total number of binding sites was 21 X $10^4$ for nondiabetic and 4.5 X $10^4$ for diabetic fibroblasts. High and low affinities for nondiabetic fibroblasts were calculated to be 8 X $10^{10}$ $M^{-1}$ and 8 X $10^9$ $M^{-1}$; for diabetic fibroblasts they were 8 X $10^{10}$ $M^{-1}$ and 4.8 X $10^9$ $M^{-1}$, respectively. Data, which are means of triplicate determination, are adapted from reference 34.

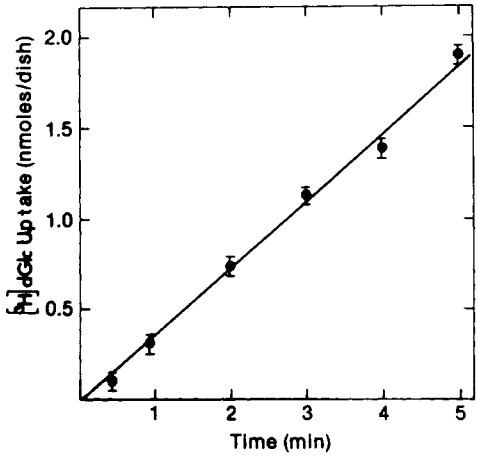


Fig. 3. [$^3$H]-2 deoxy D-glucose uptake by confluent fibroblastic cultures of the nondiabetic mouse. Confluent cultures of nondiabetic fibroblasts were washed twice with serum-free TMEM and incubated for 16 hr at 37°C in TMEM without serum. The cells were washed and incubated with 0.5 mM [$^3$H]-2 deoxy D-glucose (dGlc) for indicated times. Uptake of [$^3$H] dGlc was measured after washing the cells with ice-cold PBS and dissolving the cells in 1 N NaOH.

Incubation of confluent cultures of nondiabetic fibroblast with unlabeled insulin caused a time- and dose-dependent decrease in the specific binding of insulin [unpublished observation]. The decrease in binding has also been observed in other systems [14, 20–22] and has been referred to as "down regulation" of insulin receptors. We have measured the ability of insulin to stimulate dGlc uptake in insulin-treated nondiabetic cells in order to determine whether down regulation is associated with an altered response of the cells to insulin. Treatment of confluent cultures of nondiabetic fibroblasts for 24 hr with 16.6 nM insulin (see Fig. 5) resulted in a 2.4-fold increase in the basal dGlc uptake. Incubation with concentrations of insulin up to 16.6 nM for 10 min resulted in no further stimulation of dGlc uptake. Significant stimulation was seen only at high concentrations of insulin (166 nM).

### Effect of Insulin on Ornithine Decarboxylase Activity

Ornithine decarboxylase (ODC), the first enzyme in the pathway for synthesis of polyamines, plays an important but ill-defined role in the regulation of cell function. We have demonstrated that in cultured cells insulin causes a time- and dose-dependent increase in ODC activity, presumably due to de novo synthesis of the enzyme [23]. ODC stimulation by insulin can be used to detect differences in the response to insulin by nondiabetic and diabetic cells. Insulin (16.6 nM) caused a 131% stimulation of ODC activity in confluent cultures of nondiabetic fibroblasts (see Fig. 6). The $ED_{50}$ of stimulation was found to be 5 nM insulin. In contrast, the same concentration of insulin caused only 56% stimulation in nondiabetic fibroblasts. The $ED_{50}$ for stimulation of ODC in confluent cultures of diabetic fibroblasts was approximately 20 nM. Our results agree with previous observations that sug-

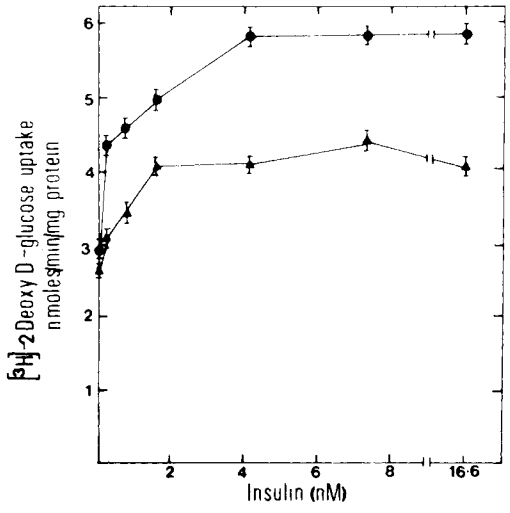


Fig. 4. Effect of insulin concentration on stimulation of 2 deoxy D-glucose uptake by confluent fibroblastic cultures from nondiabetic and diabetic mice. Serum-deprived cultures of nondiabetic (•) and diabetic (▲) cells were incubated for 15 min at 37°C with indicated concentrations of porcine insulin. Sugar uptake was determined as described in Figure 2.

gest the existence of two major alterations in diabetic cells. First, maximal stimulation of dGlc uptake and ODC activity by insulin is significantly reduced. Second, diabetic fibroblasts require more insulin to elicit a half-maximal response than do nondiabetic cells.

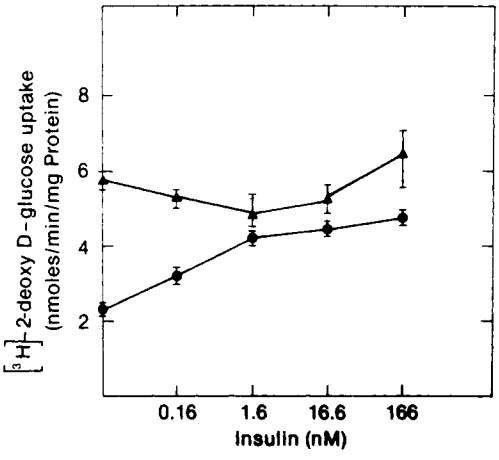


Fig. 5. Effect of preincubation with insulin on stimulation of 2 deoxy D-glucose uptake by confluent fibroblastic cultures from nondiabetic mice. Confluent fibroblastic cultures from nondiabetic mice were serum deprived for 16 hr. One group of cells was incubated without (•) and the other with 16.6 μM insulin (▲) for 24 hr. This caused a decrease in insulin receptors of nondiabetic fibroblasts by 50–60% in later group of cells. Cultures were washed twice with PBS and incubated with several concentrations of porcine insulin for 15 min at 37°C. Uptake of [$^3$H] dGlc was measured as described in Figure 2.

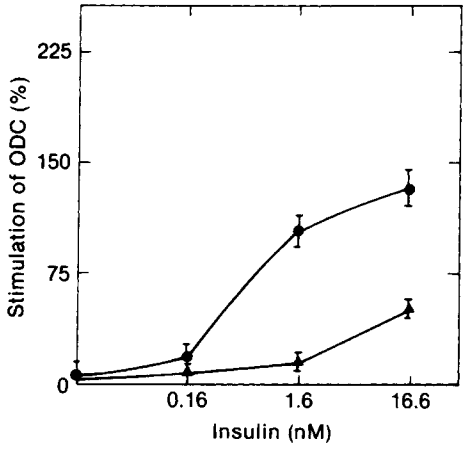


Fig. 6. Dose response of insulin stimulation of ornithine decarboxylase activity in confluent fibroblastic cultures of nondiabetic and diabetic mice. Nondiabetic (•) and diabetic (▲) fibroblasts were plated in 100 mm diameter culture dishes, grown for 3 days, serum deprived for 16 hr, and incubated with indicated concentrations of insulin for 6 hr. Cells were scraped from the dishes and homogenized, and the supernatant was assayed for ODC activity.

## DISCUSSION

A number of investigators have used fibroblastic cultures from the skin of nondiabetic and diabetic humans [24–28] to investigate mechanisms related to the pathogenesis of diabetes. These studies have produced conflicting observations with respect to doubling time, life-span, plating efficiency, and metabolic effects of insulin in diabetic cells. As an example, Goldstein and associates reported that fibroblasts from diabetic humans exhibited altered growth properties related to the diabetic trait [24, 26], whereas, in well controlled experiments, Howard et al [29] found no difference in growth properties of diabetic cells and suggested that altered growth properties were not related to diabetic status. Whereas changes in the responsiveness of diabetic fibroblasts to stimulation of glucose oxidation by insulin were not detected by some investigators [24, 30], others [27, 28] found that insulin stimulated glucose oxidation in nondiabetic fibroblasts but not in diabetic fibroblasts. These conflicting results may be due to the use of different experimental protocols and the difficulty of obtaining carefully matched diabetic and nondiabetic volunteers. In order to overcome some of these problems, we have used fibroblastic cultures from the skin of nondiabetic and diabetic (db/db) mice.

In addition to finding that cultures from diabetic animals exhibit a consistent decrease in number of insulin-binding sites compared with cultures from nondiabetic animals, our investigation also indicates that cells cultured from the skin of diabetic (db/db) mice express altered metabolic responses to insulin. Basal uptake of dGlc is similar in diabetic cells and nondiabetic cells and can be maintained at the same levels for many passages. These data are in conflict with findings reported for muscle and fat cells in which a significant decrease in the basal rate of glucose uptake was observed in diabetic rats [12].

Insulin caused a time- and dose-dependent increase in dGlc uptake in nondiabetic fibroblasts. A dose of 0.3 nM insulin was required to generate 50% of maximum stimulation. This concentration of insulin was similar to that required for 50% stimulation of α-amino isobutyric acid uptake in human fibroblasts [31]. Similarly, Fujimoto and Williams [32,33] demonstrated that comparable doses of insulin stimulate glucose uptake in human fibroblasts. Maximum stimulation of 2.2-fold was observed with 4.1 nM insulin. In contrast, the same concentration of insulin caused only a 1.5-fold stimulation of dGlc uptake in diabetic fibroblasts. As a consequence the maximum stimulation of dGlc uptake by insulin was 68% of that of nondiabetic cells. These results are consistent with a preliminary report of Fujimoto and Williams [28] that reports a decrease in the response of insulin to glucose oxidation in skin fibroblasts from diabetic patients. The amount of insulin required to produce 50% stimulation varied between 0.3 nM and 0.7 nM in nondiabetic cells, while diabetic cells required 2–4 times higher concentration of insulin to produce similar effect. The significance of this difference is not clear, although the effect is reproducible in separate experiments with cells prepared from different mice. Insulin caused a similar dose-dependent stimulation of ODC activity, and again, the maximum stimulation of diabetic cells was only 56%, compared with 131% in nondiabetic cells. An increase in $ED_{50}$ was observed in the former.

Incubation of nondiabetic cells with 16.6 μM concentrations of insulin caused a time-dependent decrease in the specific binding of insulin due to a decrease in the number of binding sites. Incubation of cells with the same concentration of insulin caused a 2.6-fold increase in the basal rate of dGlc uptake. The mechanism of this increase is not known, but it may be related to insulin-stimulated synthesis of glucose-transport sites. Experiments presented in this communication also indicate that the effect was followed by a shift in the dose-response curve of dGlc uptake. A significant effect could be seen only with a high con-

centration of insulin (166 nM). This down regulation of insulin receptors is associated with nonresponsiveness of nondiabetic cells to physiological concentrations of insulin, although the basal transport rate in these cells is increased. These experiments indicate that the decreased responsiveness of diabetic fibroblasts from the skin of db/db mice is associated with a decrease in the number of insulin receptors. The effect is rapid, consistent, and reproducible in cells prepared from different mice. The putative mechanism of alterations in post-receptor responses to insulin is not clear, but the fibroblastic culture system provides an opportunity to investigate further the role of insulin receptors and post-receptor responses to insulin in diabetes mellitus.

## ACKNOWLEDGMENTS

We wish to thank Ms Glendy Tan for expert technical assistance and Ms Sharla Williams for typing this manuscript. This project was supported by NIH grant AM21018 to M. K. Raizada.

## REFERENCES

1. Freychet P, Laudat NH, Laudat P, Rossellin G, Kahn CR, Gorden P, Roth J: FEBS Lett 25:339, 1972.
2. Freychet P, Forgue E: Diabetes 23 (Suppl 1):354, 1974.
3. Soll AH, Goldfine ID, Roth J, Kahn CR, Neville DM Jr: J Biol Chem 259:4127, 1974.
4. Soll AH, Kahn CR, Neville DM Jr, Roth J: J Clin Invest 56:769, 1975.
5. Chang KJ, Huang D, Cuatrecasas P: Biochem Biophys Res Commun 64: 566, 1975.
6. Olefsky JM, Reaven GM: J Clin Endocrinol Metab 43:232, 1976.
7. DePirro R, Fusco A, Lauro R, Testa I, Ferreti F, DeMartinis C: Diabetes 29:96, 1980.
8. LeMarchand Y, Freychet P, Jeanrenand B: Endocrinology 102:74, 1978.
9. LeMarchand Y, Freychet P: Metabolism 27:1982, 1978.
10. Genuth SM, Przybylski RJ, Rosenberg DM: Endocrinology 88:1230, 1971.
11. Cuendet GS, Louten EG, Jeanrenaud B, Renold AE: J Clin Invest 58:1078, 1976.
12. Czech MP, Richardson DK, Becker SG, Walters CG, Gitomer W, Heinrich J: Metabolism 27 (Suppl 2):1967, 1978.
13. Raizada MK, Wu B, Fellows RE: J Cell Biol 79:46a, 1978 (abstr).
14. Raizada MK, Perdue JF: J Biol Chem 251:6445, 1976.
15. Dulbecco R, Vogt M: J Exp Med 99:167, 1954.
16. Kahlenberg A: Biochem Biophys Res Commun 36:690, 1969.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
18. Olefskey JM, Kobayashi M: Metabolism 27 (Suppl 2):1917, 1978.
19. Fricke RF, Longmore WJ: J Biol Chem 254:5092, 1979.
20. Gavin JR III, Roth J, Neville DM Jr, DeMeyts P, Buell DN: Proc Natl Acad Sci USA 71:84, 1974.
21. Kobayaski M, Olefsky JM: Am J Physiol 235:E53, 1978.
22. Mott DM, Howard BV, Bennett PH: J Biol Chem 254:8762, 1979.
23. Yang JW, Raizada MK, Fellows RE: J Neurochem (in press).
24. Goldstein S, Littlefield JW, Soeldner JS: Proc Natl Acad Sci USA 65:155, 1969.
25. Vracko R, Benditt EP: Fed Proc 34:68, 1975.
26. Goldstein S, Moerman EJ, Soeldner JS, Gleason RE, Barnett DM: J Clin Invest 63:358, 1979.
27. Wolf SA, Gertner M, Hirschhorn K, Knittle JL: Diabetes 20 (Suppl 1):383, 1971 (abstr).
28. Fujimoto WY, Williams RH: Diabetes 21 (Suppl 1):360, 1972 (abstr).
29. Howard BV, Fields RM, Mott DM, Savage PJ, Nagulesparan M, Bennett PH: Diabetes 29:119, 1980.
30. Goldstein S, Littlefield JW: Diabetes 18:545, 1969.
31. Hollenberg MD, Cuatrecasas P: J Biol Chem 250:3845, 1975.
32. Fujimoto WY, Williams RH: Diabetes 23:443, 1974.
33. Fujimoto WY, Williams RH: In Vitro 13:268, 1977.
34. Raizada MK, Tan G, Deo R, Fellows RE: Endocrinology (in press).