# Insulin Binding, Internalization, and Insulin Receptor Regulation in Fibroblasts from Type II, Non-insulin-dependent Diabetic Subjects

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**SUMMARY**

**The ability of insulin to bind, internalize, and regulate its own receptor was investigated in cultured human fibroblasts obtained from 8 normal subjects and 8 patients with type II, non-insulin-dependent diabetes mellitus (NIDDM). The ability of the cells from the two groups to bind insulin was the same, and Scatchard analysis demonstrated identical curvilinear plots. When cells were incubated at 37°C with the lysosomotropic agent, chloroquine, and $^{125}$I-insulin, the drug led to a marked, but comparable, increase in cell-associated radioactivity in both control and diabetic fibroblasts (236 and 245% increase, respectively). Insulin pretreatment leads to a loss of insulin receptors in cultured human fibroblasts and preincubation with insulin led to a comparable dose-dependent decrease in subsequent insulin binding in both normal and diabetic fibroblasts. Scatchard analysis demonstrated that this decrease in binding was entirely due to a decrease in receptor number with no change in receptor affinity. These data demonstrate normal insulin binding, insulin internalization, and insulin-mediated receptor loss in fibroblasts from patients with NIDDM, and these cells are several generations removed from the in vivo milieu. Thus, these results provide direct evidence that the well-known decrease in insulin binding in freshly isolated cells from patients with NIDDM is a reflection of environmental factors rather than an intrinsic (genetic) cellular abnormality. DIABETES 30:596–600, July 1981.**

Insulin resistance is a characteristic feature of patients with type II, non-insulin-dependent diabetes mellitus (NIDDM).[1,2] While the cause of this insulin resistance has not been fully elucidated, it has now been widely described that freshly isolated monocytes,[3] adipocytes,[4] and erythrocytes[5] from type II diabetic patients possess decreased numbers of insulin receptors. Since binding to its receptor is an important rate-determining step in overall insulin action, it is apparent that this decrease in insulin binding may contribute to the in vivo insulin resistance. However, it is unclear whether this decrease in insulin receptors seen in freshly isolated cells is a reflection of environmental factors or an intrinsic (genetic) cellular abnormality. Recently, Helderman and Raskin[6] have demonstrated that T-lymphocytes derived from NIDDM patients developed fewer insulin receptors in response to a mitogen after 48 h in culture, as compared with control cells, and this raises the possibility that some genetic factor may be responsible for the reduced cellular insulin receptors observed in this form of diabetes. Human fibroblasts in tissue culture represent an ideal system to compare environmental and genetic factors, since fibroblasts faithfully reproduce genetically determined donor characteristics, and the cells can be grown in a controlled environment several generations removed from the in vivo milieu.

To evaluate the role of genetic versus environmental factors in the binding characteristics and regulation of insulin receptors in NIDDM, insulin binding, insulin internalization, and insulin-mediated loss of insulin receptors (downregulation) were studied in cultured fibroblasts obtained from normal subjects and patients with type II diabetes mellitus.

## MATERIALS AND METHODS

**Materials.** Minimal essential medium (MEM #1401500) and trypsin were purchased from Gibco (Grand Island, New York); fetal calf serum and bovine serum albumin (BSA) from Reheis (Kankakee, Illinois); Hepes (N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid), Tricine (N-Tris [hydroxymethyl-methyl] glycine), and chloroquine from Sigma Chemical (St. Louis, Missouri); and Na-$^{125}$I (carrier free) from New England Nuclear (Boston, Massachusetts). Single component crystalline porcine insulin was the kind gift of Dr. Ronald Chance, Eli Lilly and Co. (Indianapolis, Indiana).

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**Study group.** Cultured fibroblasts were obtained from skin biopsies from normal subjects and patients with NIDDM. The normal group consisted of 8 subjects: 5 nonobese adult males (mean age 43 yr) and 3 nonobese adult females (50 yr). All subjects in the control group had a negative personal and family history for diabetes mellitus and normal fasting serum glucose levels. The diabetic group consisted of 8 type II, non-insulin-dependent diabetic patients, and selected clinical characteristics of this group are provided in Table 1. None of the patients had any complications of diabetes and all patients were being treated with diet alone at the time of biopsy.

**Cell culture.** Human fibroblasts were obtained from a punch biopsy of the volar aspect of the forearm using a 3-mm Baker punch. The specimen was immediately placed in a sterile solution containing MEM, pH 7.4, supplemented with 10 mM Hepes, 26 mM $NaHCO_3$, 1 mg/L biotin, 20% fetal calf serum, penicillin (20 U/ml), streptomycin (20 mg/ml), and Fungizone (1.2 mg/ml). Under sterile conditions, the punch biopsy was cut into multiple small pieces and small tissue fragments were placed on the surface of sterile 25-$cm^2$ culture flasks. A small drop of the above medium was placed over each tissue fragment and the cells were incubated 6–8 h at 37°C, 100% humidity, and under 5% $CO_2$ to allow for attachment of the fragment to the surface of the flask. Following this, 4 ml of the same medium was added to the flask and the cells were maintained at the above conditions with refeeding every 5–7 days. With this method, an outgrowth of fibroblasts from the tissue fragment was seen by 2 wk and a confluent monolayer was evident by 3–4 wk. Stock cultures of cells were maintained in medium containing 10% fetal calf serum and no antibacterial or antifungal agents. Cells were routinely subcultured (1–3 split) every 6 days in 75-$cm^2$ flasks and reached confluence in 4 days. For experiments, confluent cells were subcultured (1–3 split) into 60 × 15-mm plastic dishes and were used on the sixth day after subculture. After complete trypsinization, documented by visualization under light microscopy, cell counts were determined in all experiments in control and diabetic dishes by use of a Coulter Counter (Model ZB). The mean cell density at confluence for all cultures was $1.1 \pm 0.06 \times 10^6$ cells/dish with a range of $0.8–1.3 \times 10^6$ cells/dish; no difference in the mean, range, or variability in confluent cell density was noted between normals and cells from the NIDDM patients. Individual experiments were always performed on dishes within the same subculture, and these dishes yielded cell counts with a mean coefficient of variation of 3%. Cell viability was always > 95% as determined by incubating cells with 0.05% trypan blue (5 min, 37°C) and assessing exclusion of the dye under the microscope. Neither insulin nor drug treatment resulted in a difference in cell number or viability in control or diabetic cells. All experiments were performed on cell cultures within 10 population doublings from the time of initial confluency.

**Experimental procedure.** Binding experiments were performed with cultured human fibroblast monolayers in 60 × 16-mm plastic dishes as previously described. After aspiration of growth medium, monolayers were washed twice with 3 ml of 22°C binding buffer (MEM, pH 7.4, with 27 mM Hepes, 25 mM Tricine, and 1% BSA). Then 0.2 ng/ml $^{125}$I-insulin, insulin standards, other reagents, and buffer were added to a total volume of 2 ml and the dishes were incubated in a shaking water bath (50 oscillations/min) for 3 h at 16°C. The reaction was terminated by repetitive washing with ice-cold modified Hanks' buffer. After this the monolayers were extracted and solubilized in 3 ml of 1 N NaOH, and this solution, containing all cell-associated radioactivity, was counted in an automatic gamma counter. Nonspecific binding was determined in the presence of 100 μg/ml native porcine insulin and averaged 20 ± 2% of the total counts bound in both control and diabetic cells. All data were corrected for nonspecific binding and normalized to a cell concentration of $10^6$ cells/dish. Insulin degradation was studied by measuring the ability of radioactivity present in the medium to precipitate with 10% trichloracetic acid. The amount of insulin degradation was minimal. Thus, in the binding experiments less than 1% of the medium insulin was degraded after incubation for 3 h at 16°C, while in the insulin preincubation experiments, less than 10% of the medium insulin was degraded after incubation for 16 h at 37°C.

$^{125}$I-insulin was prepared to a specific activity of 80–150 μCi/μg by a modification[7] of the method of Freychet et al.[8] as previously described.[9]

## RESULTS

Figure 1 summarizes insulin binding data using freshly isolated adipocytes from the 8 patients with NIDDM and from a group of 21 nondiabetic controls. Insulin binding is decreased in cells from the diabetic subjects, and Scatchard analysis (Figure 1B) reveals parallel curvilinear plots indicating a decrease in insulin receptor number with no

TABLE 1
Clinical characteristics

| Patient No. | Age | Sex | Relative weight | Fasting serum glucose (mg/dl) | Fasting serum insulin (μU/ml) | Duration of diabetes (mo) |
|---|---|---|---|---|---|---|
| 1 | 62 | M | 1.00 | 152 | 13 | 60 |
| 2 | 29 | F | 2.40 | 143 | 42 | 3 |
| 3 | 64 | F | 0.94 | 213 | 53 | 11 |
| 4 | 52 | F | 1.32 | 252 | 26 | 72 |
| 5 | 60 | M | 0.98 | 182 | 12 | 2 |
| 6 | 39 | F | 1.36 | 221 | 16 | 36 |
| 7 | 64 | F | 1.33 | 218 | 15 | 96 |
| 8 | 35 | M | 1.04 | 236 | 27 | 24 |
| Controls (N = 8) | 45.6 ± 5 (22–66) | 5 M, 3 F — | 1.01 ± 0.06 (0.88–1.06) | 86 ± 3 (78–92) | 8 ± 1 (4–10) | — — |

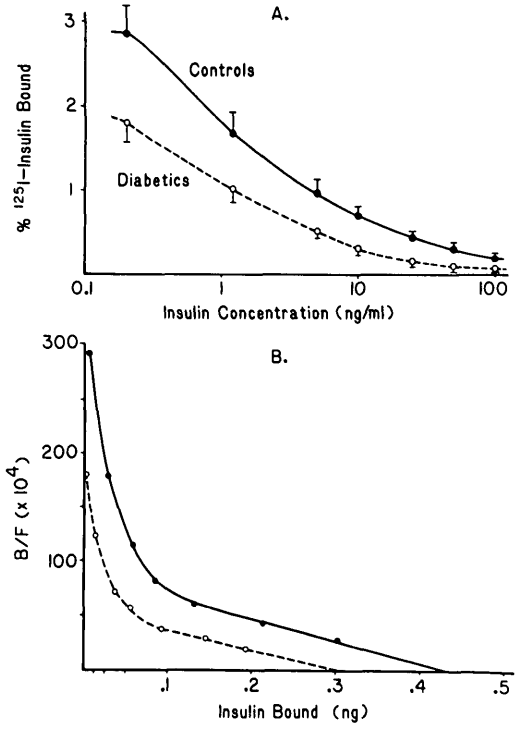



**FIGURE 1. (A) Ability of isolated adipocytes from the 8 patients with NIDDM and a group of 21 control subjects to bind insulin. All data are corrected for nonspecific binding and represent the mean ± SE. Results are normalized to a cell concentration of 2 × $10^5$ cells/ml. (B) Scatchard plots of the data from Figure 1A.**

change in binding affinity. These results show that, as in previous reports,[3,4] insulin binding is decreased in freshly isolated cells obtained from the 8 diabetic patients included in this study.

The ability of fibroblast monolayers from control and NIDDM patients to bind insulin is compared in Figure 2A. Cells were incubated in the presence of $^{125}$I-insulin and various concentrations of unlabeled insulin for 3 h at 16°C. It is apparent that insulin binding to fibroblast receptors is identical in both groups of cells at all insulin concentrations, and Scatchard analysis yielded comparable curvilinear plots (Figure 2B).

Current thinking holds that, after the initial binding event, some proportion of cell surface-bound insulin is internalized by an endocytotic mechanism. The internalized insulin is then subsequently degraded either wholly or partly within lysosomes, and the degraded material is released from the cells.[10,11] Chloroquine is a lysosomotropic agent that inhibits intracellular insulin degradation.[11,13] If chloroquine-treated cells are incubated with $^{125}$I-insulin, release of internalized radioactivity is inhibited and intact $^{125}$I-insulin accumulates within the cell.[11–13] Figure 3 summarizes the ability of chloroquine to cause an increase in cell-associated radioactivity in control and diabetic fibroblasts. Cells were incubated with $^{125}$I-insulin (0.2 ng/ml) for 2 h at 30°C in the presence or absence of 0.2 mM chloroquine. Chloroquine led to a marked increase in cell-associated radioactivity in both control (236 ± 23) and diabetic (245 ± 33) fibroblasts with no significant difference between the two groups.

Figure 4 demonstrates the ability of insulin to cause a decrease in cell surface receptors (downregulation) in fibroblasts from normal and NIDDM subjects. Cells were prein-

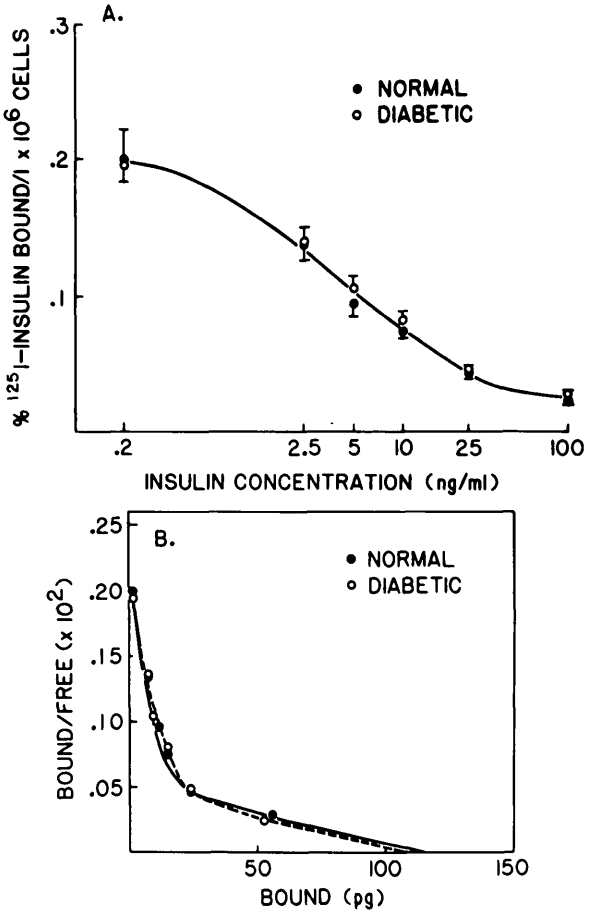
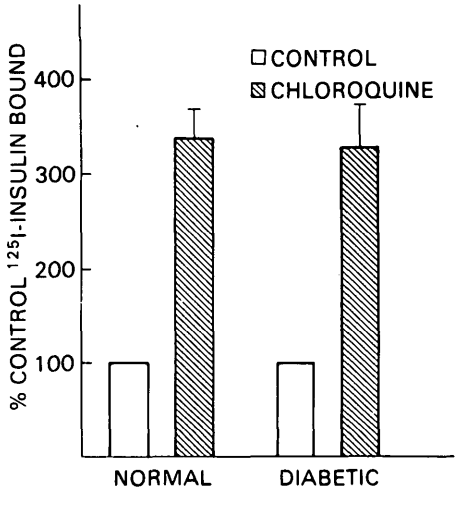



**FIGURE 2. (A) Comparison of $^{125}$I-insulin binding in fibroblasts from control and NIDDM subjects. Fibroblasts monolayers from 8 control (●) and 8 NIDDM diabetic patients (○) were incubated in the presence of $^{125}$I-insulin (0.2 ng/ml) plus various concentrations of unlabeled insulin for 3 h at 16°C. Each point represents the mean ± SEM, and data are corrected for nonspecific binding. The data are expressed as % specific $^{125}$I-insulin bound/$10^6$ cells. (B) Scatchard analysis of $^{125}$I-insulin binding to fibroblasts from control and NIDDM subjects.**

**FIGURE 3. Effect of chloroquine on cell-associated $^{125}$I-insulin in cells from normal and NIDDM subjects. $^{125}$I-insulin (0.2 ng/ml) was incubated with fibroblasts from normal (open bars) and NIDDM subjects (hatched bars) for 120 min at 30°C in the absence or presence of 0.2 mM chloroquine. Results represent the mean (±SE) of 5 experiments.**



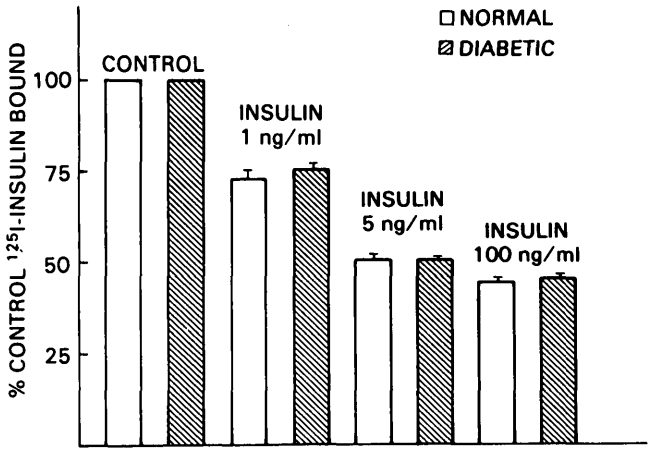



**FIGURE 4. Insulin-mediated loss of insulin receptors in fibroblasts from normal and NIDDM subjects. Cells from normal (open bars) and diabetic subjects (hatched bars) were incubated with either 1, 5, or 100 ng/ml insulin for 15 h at 37°C. After extensive washing to remove all extracellular unbound insulin and dissociation of bound insulin, $^{125}$I-insulin binding was measured over a 3-h period at 16°C. Results represent the mean ± SE of 4 experiments.**

cubated with either 1, 5, or 100 ng/ml insulin for 15 h at 37°C. Under these conditions, <10% of the insulin present in the medium was degraded at the end of the 16-h preincubation with insulin. After insulin preincubation, the cells in monolayer were washed twice with 3 ml of the binding buffer to remove all extracellular unbound insulin. Fresh insulin-free medium was then added and the cells were incubated at 37°C for 1 h to allow dissociation of all bound insulin. Control studies using $^{125}$I-insulin have demonstrated > 95% dissociation of all bound insulin after a 1-h incubation at 37°C. Subsequently, the cells were again washed twice with 3 ml of the binding buffer, and then $^{125}$I-insulin binding was measured over a 3-h period at 16°C. As can be seen, insulin treatment led to a dose-dependent decrease in subsequent insulin binding in both groups of cells, with no significant differences between the two cell types at any insulin concentration. Scatchard analysis demonstrated that the decrease in insulin binding was due to a decrease in insulin receptor number with no change in receptor affinity (data not shown).

## DISCUSSION

The presence of insulin resistance in NIDDM patients has been well described using a variety of experimental techniques.[1,2,14] Although the mechanisms are not entirely clear, it is apparent that the insulin resistance significantly contributes to the overall pathophysiology in this form of diabetes mellitus. It is also known that cells from NIDDM patients contain fewer insulin receptors than normal, and this observation has been made using freshly isolated monocytes,[3] adipocytes,[4] and erythrocytes.[5] It is evident that this decrease in cellular insulin receptors contributes, at least partly, to the overall in vivo insulin resistance in NIDDM.[14,15] However, the cause of this decrease in insulin receptors is poorly understood.

One important factor regulating the number of cellular insulin receptors is the circulating plasma insulin concentration.[16] Gavin et al.[17] were the first to provide evidence for this concept by showing that insulin treatment led to a reduced number of insulin receptors on cultured human lymphocytes. Subsequent to this observation, an inverse relationship has been observed between the plasma insulin level and the number of insulin receptors in a variety of in vivo situations.[1,14,16] In NIDDM, a close inverse relationship between the circulating insulin level and the number of cellular insulin receptors has been demonstrated,[15] suggesting that this is at least one factor leading to the reduced insulin binding. In addition, a number of other factors that have not been evaluated in NIDDM may influence the cell's complement of insulin receptors.[18,19] On the other hand, Helderman and Raskin have presented evidence consistent with the formulation that a genetic factor is responsible for the defect in insulin binding in NIDDM.[6] These workers have shown that circulating T-lymphocytes do not possess insulin receptors in the native state, but develop insulin receptors after mitogenic stimulation in vitro.[20] When studied in this manner, T-lymphocytes from NIDDM patients develop fewer insulin receptors than normal during a 48-h period in tissue culture.[6] These data raise the possibility that a primary cellular defect may be responsible for the reduction in insulin receptors in NIDDM. Additional support for this concept is found in the work of Goldstein et al.,[21] who reported reduced insulin receptors in circulating monocytes from prediabetic individuals.

From the above discussion, it is apparent that decreased insulin receptors are an important component of the NIDDM state, but that the role of genetic versus environmental factors in the etiology of this abnormality is controversial. Of course, this question can be broadened to other aspects of diabetes mellitus in which the role of genetic versus acquired environmental factors is debated. Human fibroblasts in tissue culture represent a unique system to ascertain the role of environmental versus inherited factors, since they consistently express genetically determine donor characteristics and can be grown in a controlled situation in which they have been removed from the in vivo milieu of the donor for several generations. We and others have recently demonstrated that cultured human fibroblast monolayers contain specific high affinity insulin receptors that display all of the binding, functional, and immunologic characteristics of the insulin receptors on other well-studied target tissues.[22–25]

For these reasons, we have studied the interaction of insulin with fibroblast monolayers obtained from patients with type II diabetes mellitus (NIDDM) and have compared these results with similar studies in normal fibroblasts. The data demonstrate that insulin binding, insulin internalization, and insulin-mediated receptor loss are entirely normal in fibroblasts from diabetic patients. Thus, diabetic fibroblasts possess insulin receptors that are normal in number and affinity. Furthermore, these receptors also have a normal ability to initiate the usual events that follow the binding event between insulin and its receptor. For example, after cell surface binding, insulin becomes internalized via endocytosis, is degraded within cells, and the degraded products are rapidly released.[10,11] This process can be assessed by the use of chloroquine, which inhibits intralysosomal proteolysis[11] and impairs fusion of endocytotic vesicles with lysosomes.[25,26] When $^{125}$I-insulin is incubated with chloroquine-treated hepatocytes,[13] adipocytes,[11,12] or fibroblasts,[27] there is a marked increase in cell-associated radioactivity compared with control cells. Since previous work has demon-

strated that all of the chloroquine-induced increase in radioactivity represents intracellular intact insulin,[11,12] this indicates that chloroquine inhibits processing and degradation of internalized insulin, leading to an intracellular accumulation of $^{125}$I-insulin.[28] When fibroblasts from normal and NIDDM patients were incubated in the presence of chloroquine and $^{125}$I-insulin, the chloroquine-induced increase in cell-associated radioactivity was comparable in both groups of cells. This indicates that the processes underlying insulin internalization and intracellular degradation are normal in diabetic fibroblasts. Insulin treatment leads to a loss of cell surface insulin receptors in a variety of cell types,[17,29,30] including cultured fibroblasts.[23,24,27] This process was also examined in fibroblasts from NIDDM individuals. The results demonstrated that insulin's ability to mediate receptor loss was normal, indicating that this important regulatory phenomenon is intact in cells from diabetic subjects.

In a recent report, Raizada et al.[31] have demonstrated that cultured fibroblasts obtained from genetically diabetic *db/db* mice have reduced numbers of insulin receptors. In addition, fibroblasts obtained from these mice do not demonstrate a normal rate of insulin-induced receptor loss. Clearly these results in genetically diabetic mice differ from the current results obtained in human diabetes. It seems likely that the marked species differences between the two studies account for the experimental differences. From these results, one might suggest that the pathophysiologic mechanisms underlying the diabetic state in the *db/db* mouse are not fully representative of human NIDDM.

In summary, the current studies demonstrate normal insulin binding, hormone internalization, and insulin-mediated receptor loss in cultured fibroblasts that are several generations removed from the in vivo environment of the diabetic donor. These studies provide direct evidence that the decrease in cellular insulin receptors associated with NIDDM is a reflection of acquired environmental factors rather than a genetically determined cellular abnormality.

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