Functional Characteristics of Decreased Insulin Receptors on Fibroblasts Obtained from a Subject with Severe Insulin Resistance and Acanthosis Nigricans

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SUMMARY

In a patient with severe insulin resistance and acanthosis nigricans, a decrease in the number of insulin receptors has been found on freshly isolated monocytes and cultured fibroblasts compatible with a primary or genetic decrease in cell-surface insulin receptors. To determine the functional characteristics of the remaining receptors on these cells, insulin-stimulated glucose uptake, insulin internalization, and insulin-induced receptor loss were evaluated in monolayer fibroblasts obtained from this subject. Maximal insulin stimulation of 2-deoxyglucose was markedly blunted, compatible with abnormal insulin responsiveness due to a functional impairment of the remaining receptors. In the presence of chloroquine, the acanthotic subject's fibroblasts internalized more insulin per available receptor compared with the normal cell line, suggesting an accelerated rate of insulin internalization. When the rate of insulin internalization was more directly determined by assessing the rate of appearance of acid-resistant, cell-associated radioactivity at 37°C, a similar increase in insulin internalization rate was evident. When downregulation was assessed, insulin's ability to induce receptor loss in the acanthotic subject's cell line was augmented.

Thus, a primary or genetic decrease in insulin receptors on cultured fibroblasts from a patient with acanthosis nigricans and insulin resistance is associated with functional impairment of the remaining receptors leading to significant alterations in ligand processing and subsequent insulin action. DIABETES 1986; 35:148–54.

The syndrome of insulin resistance occurring in young females with acanthosis nigricans and signs of excess androgen effects has been well described.[1,2] This syndrome represents a heterogeneous group of patients consisting of at least three separate groups: those individuals with antireceptor antibodies and other autoimmune features, patients with defects in insulin receptors unassociated with receptor antibodies, and those without any binding abnormalities.[1-4] In those individuals with decreased receptors on circulating cells unassociated with receptor antibodies, a similar decrease in insulin receptors has been identified in their cultured cells, consistent with a primary or genetic defect at the level of the insulin receptor.[5] To date, however, little information is available regarding the functional capacity of these receptors.

We have identified a 15-yr-old female with severe insulin resistance, hirsutism, and acanthosis nigricans with decreased insulin receptors on circulating monocytes as well as on cultured fibroblasts. To determine the functional capacity of these receptors, insulin stimulation of 2-deoxyglucose transport, insulin internalization, and insulin-induced receptor loss were evaluated in cultured fibroblasts obtained from this subject.

MATERIALS AND METHODS

Materials. Eagle's Minimal Essential Medium (MEM), biotin, and trypsin were purchased from Gibco (Grand Island, New York); fetal calf serum and bovine serum albumin (fraction V) from Reheis (Kanakee, Illinois); Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), Tricine (N-Tris[hydroxymethyl-methyl]glycine), 2-deoxy-D-glucose, L-glucose, and chloroquine from Sigma Chemical (St. Louis, Missouri); and 2-deoxy-D-[1-^{3}H]glucose (19.5 Ci/mmol), L-[1-^{3}H] glucose (10.7 Ci/mmol), 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 Company, Indianapolis, Indiana.

Clinical features. A 15-yr-old Black female presented to the University of Texas Medical Branch at Galveston in 1983 with menstrual irregularity, mild hirsutism, and marked acanthosis nigricans involving her neck, axillae, antecubital fossa, umbilicus, and groin that was first noted at age 4 yr. An oral

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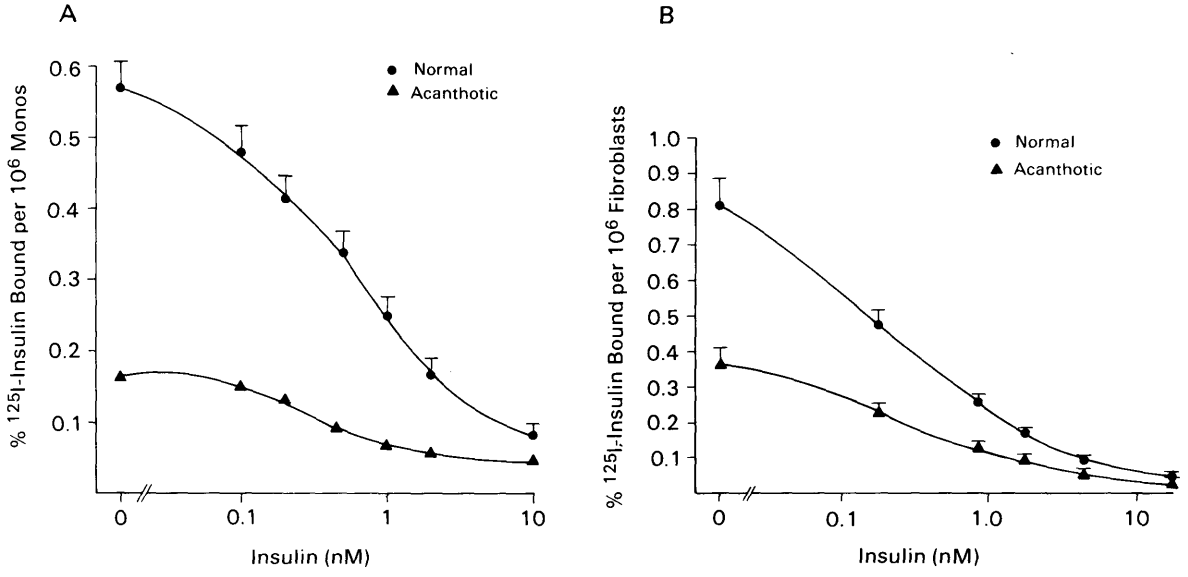

FIGURE 1. (A) Ability of isolated monocytes from the acanthotic subject and a group of 22 normal control subjects to bind insulin. All data are corrected for nonspecific binding and represent the mean ± SEM. (B) Comparison of ^{125}I-insulin binding in fibroblasts from the acanthotic subject and normal control subjects. The closed circles represent the mean ± SEM of 28 separate experiments performed with control cells from six normal volunteers. The closed triangles represent the mean ± SEM of 4 separate experiments performed with cells obtained from the acanthotic subject.

glucose tolerance test was within normal limits; however, her fasting insulin level was significantly elevated at 60 μU/ml. Her response to exogenous insulin was markedly blunted on three separate occasions (K_{ITT} = 0.8–1.9%/min, normal range = 6.7 ± 0.3%/min). Pelvic ultrasound showed no ovarian enlargement. Androgen profile revealed a testosterone level of 38 mg/ml, androstenedione of 109 ng/ml, and dehydroepiandrosterone of 312 ng/dl, which were normal for our laboratory in postpubertal females.

Monocyte insulin binding. Mononuclear cells were isolated from 50 ml of blood after an overnight fast and insulin binding to freshly isolated monocytes was performed as previously described.[6] Briefly, cells were incubated with ^{125}I-insulin (0.2 ng/ml) and a variable amount of unlabeled insulin. After incubation at 15°C for 90 min, cells were separated from the medium by centrifugation in a Beckman Microfuge and cell-associated radioactivity was assayed. Nonspecific binding was assayed in the presence of an excess of unlabeled insulin (10 μg/ml). In all binding experiments, triplicate determinations were performed to assess total and/or nonspecific binding.

Cell culture. Human fibroblasts were obtained from a punch biopsy of the volar aspect of the forearm and maintained in culture as previously described.[7] Briefly, stock cultures of cells were maintained in medium containing 10% fetal calf serum and were routinely subcultured every 6–7 days in 75-cm^2 flasks. For experiments, confluent cells were subcultured (1:3 split) into 60 × 15-mm plastic dishes and were used on the seventh day after subculture.

Fibroblast insulin binding. Binding experiments were performed with cultured human fibroblast monolayers in 60 × 15-mm plastic dishes as previously described.[7] Cell monolayers were incubated with ^{125}I-insulin (0.2 ng/ml) and

TABLE 1
Monocyte and fibroblast insulin binding summary

	B_o (%/10^6 cells)	K_a (10^9 L/mol)	R_o (receptors/cell)
Monocytes			
Normal	0.571 ± 0.036	1.84	3078 ± 474
Acanthotic	0.165	1.70	600
Fibroblasts			
Normal	0.809 ± 0.074	0.93	5186 ± 421
Acanthotic	0.366 ± 0.042	0.75	2776 ± 235

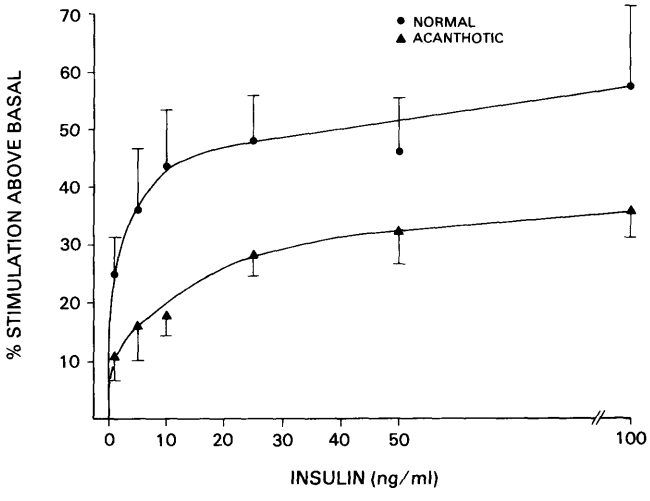

FIGURE 2. Effect of insulin concentration on 2-deoxy-D-glucose transport in fibroblasts from the acanthotic subject and normal control subjects. The closed circles represent the mean ± SEM of 13 separate experiments performed with control cells from five normal volunteers. The closed triangles represent the mean ± SEM of 8 separate experiments performed with cells obtained from the acanthotic subject. The insulin-stimulated 2-deoxy-D-glucose uptake data are expressed as a percentage over basal (non-insulin-exposed) uptake values.

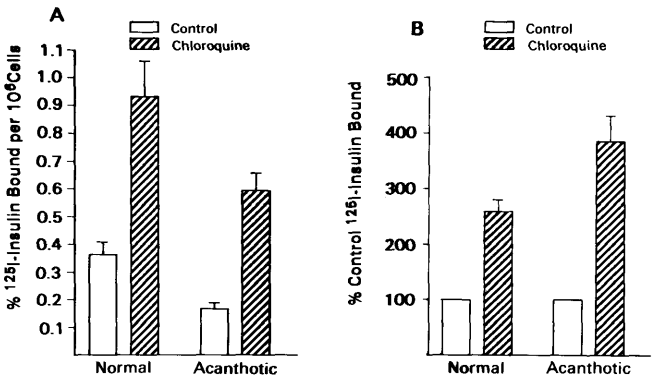

FIGURE 3. (A) Effect of chloroquine on cell-associated ^{125}I-insulin in fibroblasts from the acanthotic subject and normal volunteers. ^{125}I-insulin (0.2 ng/ml) was incubated with fibroblasts for 120 min at 30°C in the absence or presence of 0.2 mM chloroquine. Results represent the mean ± SEM of 10 experiments performed with control cells from four normal volunteers versus cells from the acanthotic subject. (B) The amount of ^{125}I-insulin bound in the presence of chloroquine is expressed as a percentage of that bound in the absence of chloroquine.

a variable amount of unlabeled insulin at 16°C for 3 h unless otherwise stated. The reaction was terminated by repetitive washing with ice-cold modified Hanks' buffer. After this, the monolayers were extracted and solubilized in 2 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 10 μg/ml unlabeled insulin. All data were corrected for nonspecific binding and normalized to a cell concentration of 10^6 cells/dish.

2-Deoxy-D-glucose transport studies. Measurement of 2-deoxyglucose transport was performed on confluent cell monolayers grown in 60 × 15-mm plastic culture dishes as previously described by Berhanu et al.[8] Briefly, cell monolayers were rinsed twice with 3 ml of buffer (37°C, pH 7.45) containing 116 mM NaCl, 5.4 mM KCl, 1.0 mM $NaH_2PO_4 \cdot H_2O$, 2.0 mM Na_2HPO_4, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1.0 mM $CaCl_2$, 0.2% BSA, and 25 mM Tris. Cells were preincubated in this buffer with or without insulin in a final volume of 1.8 ml for 60 min at 37°C in a shaking water bath. The transport reaction was initiated by the addition of 200 μl of buffer containing [^{3}H]-2-deoxy-D-glucose (0.4 μCi) and unlabeled 2-deoxy-D-glucose resulting in a final 2-deoxy-D-glucose concentration of 0.1 mM. The reaction was terminated after 3 min by rapid removal (vacuum aspiration) of the reaction media and washing each monolayer four times with ice-cold Krebs-Ringer bicarbonate buffer containing 10 mM Hepes and 0.3 mM phloretin (pH 7.4). After solubilization with 1 ml of 1 N NaOH, and the rinsing of each dish with 1 ml of 1 N HCl, the combined radioactivity of the total solubilized material from each dish was determined in a liquid scintillation counter after the addition of 15 ml of aqueous scintillant. Correction for diffusion and extracellular trapping of radioactivity was determined by subtracting the value for L-[1-^{3}H]glucose uptake measured in parallel with each 2-deoxyglucose transport measurement.

Statistical analysis. The unpaired *t*-test was used to test significance.

RESULTS

Figure 1A summarizes insulin binding data in freshly isolated monocytes obtained from 22 normal controls and the acanthotic subject. Insulin binding to monocytes from the acanthotic subject is significantly decreased by 71% compared with the mean of normal control cells. The ability of fibroblast monolayers from control subjects and the acanthotic patient to bind insulin is compared in Figure 1B. As is demonstrated, insulin binding to the acanthotic subject's fibroblasts is reduced by 55% compared with normal subject fibroblasts. Receptor affinity and number generated by Scatchard analysis[9] are shown in Table 1 and reveal that the reduction in insulin binding seen in both freshly isolated monocytes and cultured fibroblasts obtained from the acanthotic subject is due to a decrease in receptor number. Thus, the persistence of the binding defect in cultured cells several generations removed from the in vivo milieu is compatible with a primary or genetic decrease in insulin receptors in this patient.

To determine the functional capacity of these receptors, the ability of insulin to stimulate the uptake of 2-deoxyglucose was assessed in fibroblast monolayers in subject and control cells as demonstrated in Figure 2. Despite a decreased number of receptors in the subject's cell line, insulin sensitivity was similar in control versus subject's cells, with the concentration of insulin leading to half-maximal stimulation being 8.5 versus 7.0 ng/ml, respectively. However, maximally stimulated transport was markedly blunted (44% of control) in the subject's cell line. When the percent increase in glucose transport was plotted as a function of the amount of cellular bound insulin, a lower biologic effect was elicited in the acanthotic subject's cells by a given amount of bound insulin (data not shown). This indicates that a defect in the steps of insulin action distal to the binding event is present in the acanthotic subject's cells.

Chloroquine is a drug that inhibits intralysosomal proteolysis, thereby inhibiting intracellular insulin degradation.[10] If

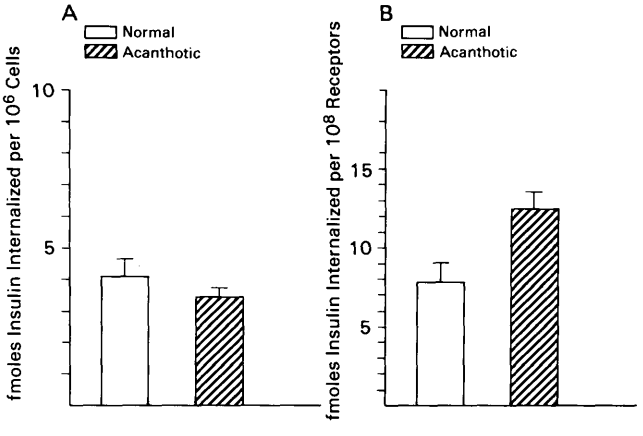

FIGURE 4. (A) Insulin internalization during chloroquine exposure. The increase in cell-associated radioactivity seen in the presence of chloroquine is expressed as fmoles insulin internalized per 10^6 cells and compared in the normal and acanthotic subjects' cell lines. Results represent the mean ± SEM of 10 experiments performed with control cells from four normal volunteers versus cells from the acanthotic subject. (B) The data are normalized for receptor number and expressed as fmoles insulin internalized per 10^8 receptors.

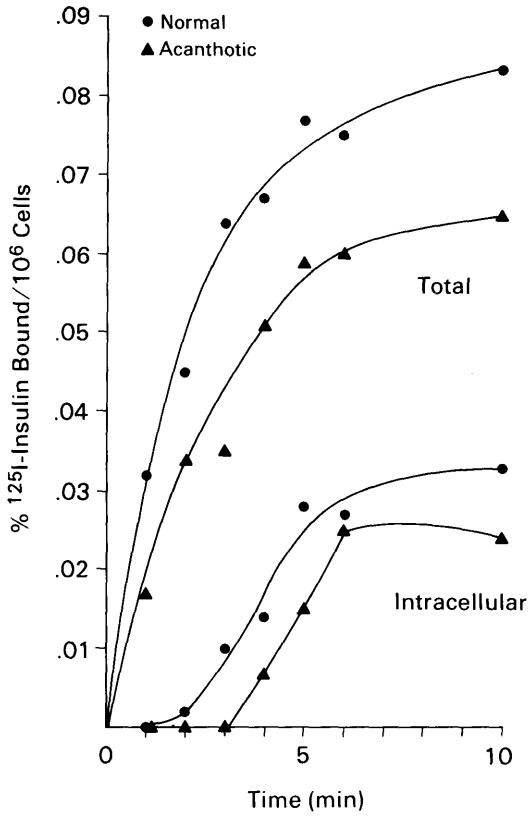

FIGURE 5. Time course of total and intracellular cell-associated radioactivity. Normal and acanthotic subjects' fibroblasts were incubated with ^{125}I-insulin (0.2 ng/ml) at 37°C. Intracellular radioactivity was defined as cell-associated radioactivity remaining after acute acid exposure (0.05 M glycine containing 0.15 M NaCl, pH 2.5, 2 min) to strip surface-associated ligand. Results represent the mean of three experiments performed with control cells from a normal volunteer versus cells from the acanthotic subject. All data are corrected for nonspecific binding.

chloroquine-treated cells are incubated with ^{125}I-insulin, release of internalized radioactivity is inhibited and intact insulin accumulates within the cell, thus allowing for an indirect assessment of insulin internalization.[7,11] When cells are incubated in the presence of chloroquine, a marked increase in cell-associated radioactivity is seen in both normal and acanthotic subjects' cells (Figure 3A), with chloroquine leading to a significantly ($P < 0.01$) greater percent increase in cell-associated radioactivity in the acanthotic subject's cells (Figdure 3B).

In Figure 4A, the increase in cell-associated radioactivity seen in the presence of chloroquine is expressed as fmoles insulin internalized per million cells and compared in the two cell lines. Despite 55% fewer receptors, comparable amounts of insulin were internalized under these experimental conditions. However, when the data are normalized for receptor number as shown in Figure 4B, a significantly ($P < 0.01$) greater amount of insulin is internalized per available receptor in the acanthotic subject's cell line, suggesting an augmented rate of insulin internalization.

To more directly assess the rate of insulin internalization, the time course of ^{125}I-insulin binding at 37°C in normal and acanthotic subjects' cells was assessed as demonstrated in Figure 5. Intracellular radioactivity was defined as radioactivity remaining after acute acid exposure (0.05 M glycine containing 0.15 M NaCl, pH 2.5, 2 min) to strip surface-associated ligand. This acute acid exposure has been shown to remove >99% of all surface-bound ligand when performed after ^{125}I-insulin binding at 4°C (data not shown). As is demonstrated, ^{125}I-insulin binding to the cell surface was apparent within 1 min and rapidly approached a steady-state level. In acid-treated cells, there was an initial lag in the appearance of internalized radioactivity followed by a wave of internalization, reflected by the linear portion of the curve. Comparison of the rate of insulin appearance in the cell during the linear segment of the curve allowed direct assessment of the insulin internalization rate for each cell line. As was seen in the chloroquine studies, despite a 55% decrease in cell-surface receptors in the acanthotic subject's cell line, similar amounts of insulin were internalized per unit time in the two cell lines (Figure 6A). When normalized for receptor number (Figure 6B), the acanthotic subject's cells demonstrated a significantly ($P < 0.05$) greater rate of insulin internalization per available receptor as compared with normal cells.

Insulin-induced receptor loss, or downregulation, has been demonstrated in a variety of cell types, including cultured fibroblasts.[12,13] To determine if this important regulatory phenomenon persisted in the acanthotic subject's cells with an intrinsic decrease in receptor number, normal and acanthotic subjects' cells were incubated in the absence and presence of 1, 2.5, or 5 ng/ml insulin for 24 h at 37°C. After extensive washing to remove all extracellular unbound insulin and after dissociation of bound insulin, ^{125}I-insulin binding was measured over a 3-h period at 16°C. Insulin binding was significantly decreased in the basal state as previously demonstrated; however, insulin treatment led to a dose-dependent decrease in subsequent insulin binding in both cell types (Figure 7A). To compare the insulin effect, in Figure 7B the data were expressed as percent of ^{125}I-insulin bound in the absence of insulin preincubation for each cell line. As is shown, the insulin effect was significantly greater at all insulin

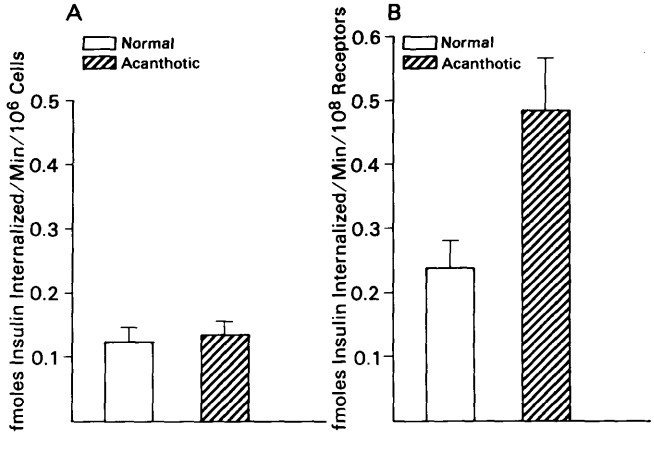

FIGURE 6. (A) Rate of insulin internalization. Based on the rate of insulin appearance in the cell during the linear segment of the time course of intracellular radioactivity, the rate of insulin internalization for the two cell lines was determined and expressed as fmoles insulin internalized per minute per 10^6 cells. (B) Data are expressed as fmoles insulin internalized per minute per 10^8 receptors.

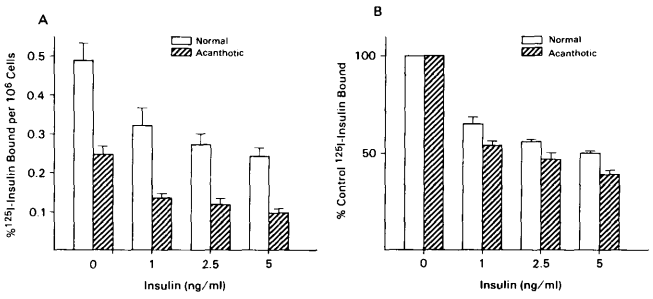

FIGURE 7. (A) Insulin-induced receptor loss. The normal and acanthotic subjects' fibroblasts were incubated in the absence or presence of either 1, 2.5, or 5 ng/ml insulin for 24 h at 37°C. After extensive washing to remove all extracellular unbound insulin and after dissociation of bound insulin, ^{125}I-insulin binding was measured over a 3-h period at 16°C. Results represent the mean ± SEM of three separate experiments performed in control cells from two normal volunteers versus cells from the acanthotic subject. (B) To compare the insulin effect, the data are expressed as the percentage of ^{125}I-insulin bound in the absence of insulin preincubation for each cell line.

concentrations (1 ng/ml, $P < 0.05$; 2.5 ng/ml, $P < 0.05$; and 5 ng/ml, $P < 0.005$) in the acanthotic subject's cells. Scatchard analysis demonstrated that the decrease in insulin binding was due to a decrease in receptor number. These results demonstrate that insulin's ability to mediate receptor loss was augmented in the acanthotic subject's fibroblasts.

DISCUSSION

Several insulin-resistant syndromes have been described in which a decrease in the number of insulin receptors has been demonstrated on both freshly isolated cells as well as on cultured cells, compatible with an intrinsic or genetic decrease in the insulin receptor in these subjects.[5,14,15] Despite an increase in the identification of such cases, little is known regarding the functional ability of these genetically decreased insulin receptors.

In this report, a young female with severe insulin resistance and acanthosis nigricans was found to have a persistent decrease in insulin receptors on her cultured fibroblasts. To determine the functional ability of these remaining receptors, insulin-stimulated glucose uptake, insulin internalization, and insulin-induced receptor loss were evaluated in monolayer fibroblasts obtained from this subject.

When the ability of insulin to stimulate 2-deoxyglucose was assessed, maximally stimulated transport was markedly blunted in the subject's cells. When the percent increase in glucose transport was assessed as a function of the amount of cellular-bound insulin, it was evident that a given amount of bound insulin led to less of a biologic effect in the acanthotic subject's cells. Thus, the decrease in maximally insulin-stimulated transport was due to a defect in insulin action distal to the binding of insulin to its receptor. Despite a decreased number of receptors in the subject's cells, a shift to the right in the insulin dose-response curve was not evident. The reason for this lack of diminished insulin sensitivity in the presence of decreased numbers of insulin receptors is not readily apparent. One possibility might be an actual enhancement of the linkage mechanism between insulin binding to its receptor and initiation of glucose transport. The nature of this "coupling mechanism" or signal is not known; however, several hypotheses have been put forward, including internalization of the insulin receptor itself.[16,17] In the present study, we have shown that the acanthotic subject's cell line has an increased rate of insulin internalization and that insulin's ability to induce cell-surface receptor loss (i.e., internalization) is augmented. Thus, if the internalized insulin receptor itself is involved in the initiation of insulin-stimulated glucose transport, then one might predict, under these circumstances, the maintenance of normal sensitivity despite a decreased number of cell-surface receptors. However, since maximal transport is blunted in the subject's cell line, one must assume that there still remains a defect(s) in insulin action distal to the binding event. Podskalny and Kahn evaluated the ability of insulin to stimulate glycogen synthase activity in fibroblasts obtained from two patients with the type A syndrome of severe insulin resistance.[18] A marked shift to the right in the insulin dose-response curve was seen, but maximally stimulated levels were normal. In a preliminary report, Chaiken et al. found that maximal insulin stimulation of 2-deoxyglucose and alpha-amino isobutyric acid uptake was markedly blunted in fibroblasts with decreased insulin receptor affinity obtained from a type A patient.[19] Thus, primary defects at the receptor level include both alterations in receptor number as well as receptor function and are associated with subsequent alterations in insulin action resulting in diminished insulin sensitivity and/or responsiveness.

The ability of insulin to be internalized into the cell is an important postbinding event; however, little information is available regarding the ability of these genetically depleted receptors to perform this important function. Chloroquine is a lysosomatropic agent that inhibits the intracellular processing and degradation of insulin by lysosomes and other subcellular fractions.[10,20] If chloroquine-treated cells are incubated with ^{125}I-insulin, release of internalized radioactivity is inhibited and intact insulin accumulates within the cell, thus providing a good estimate of the amount of insulin internalized.[7,11] As demonstrated in Figure 4A and 4B, despite 55% fewer receptors, the acanthotic subject's fibroblasts internalized similar amounts of insulin under these conditions. When the data were normalized per receptor number, more insulin was internalized per available receptor in the acanthotic subject's fibroblasts. These data suggest an augmented rate of insulin internalization per available receptor; however, chloroquine has multiple cellular effects and other interpretations are possible. For instance, since the insulin concentrations were the same between both groups of cells, the total amount of ligand coming into the cells was probably reduced in the acanthotic cells. It is possible that chloroquine more effectively traps incoming insulin when the amount of incoming insulin is less. Alternatively, chloroquine may have a more dramatic effect on insulin clearance from the cell in the abnormal cell line. However, when insulin internalization was more directly evaluated by assessing the rate of appearance of cell-associated, acid-resistant radioactivity (Figures 6 and 7), an augmented rate of insulin internalization per available receptor was apparent in the acanthotic subject's fibroblasts.

Evidence now supports the concept that, after the binding of insulin to its receptor, the insulin-receptor complex is internalized via a temperature- and energy-dependent endocytotic process.[21,22] Thus, our finding of an accelerated rate of insulin internalization per available receptor suggests the

possibility of an accelerated rate of surface receptor internalization and subsequent degradation. While insulin receptor internalization and/or degradation was not assessed in our subject, McElduff et al. recently evaluated the rate of insulin receptor degradation in Epstein-Barr-virus–transformed lymphocytes obtained from patients with several syndromes of extreme insulin resistance, including the syndrome of acanthosis nigricans and severe insulin resistance.[23] They found that accelerated insulin receptor degradation was an additional feature of cells from patients with these genetic forms of insulin resistance, suggesting that accelerated insulin receptor degradation may explain the low normal surface receptor concentrations that were seen in some of these patients.

Insulin-induced receptor loss, or downregulation, has been demonstrated to be an important postbinding regulatory phenomenon.[24] Recently, ^{125}I-insulin binding to circulating cells has been reported as being normal in some patients with certain forms of extreme insulin resistance despite marked hyperinsulinemia.[3,4,25] Postbinding defects have been proposed that may give rise to a defect in downregulation in vivo.[3,4,25] Wagman et al. employed cultured Epstein-Barr-virus–transformed lymphocytes from insulin-resistant patients with lipoatrophic diabetes, leprechaunism, and "S-kindred" to directly assess the downregulation process in vitro.[26] Surprisingly, despite a lack of evidence for in vivo downregulation, downregulation in vitro proceeded normally in cultured cells obtained from these subjects. This apparent discrepancy between the in vivo and in vitro observations has remained unexplained.

When we directly assessed insulin-induced downregulation in fibroblasts obtained from our subject, there was an augmented ability of insulin to induce cell-surface receptor loss (Figure 7A and 7B). The mechanism of insulin-induced receptor loss has varied based on cell type studied; however, at least three mechanisms have been described: receptor degradation, receptor redistribution to an intracellular site, and extracellular shedding of receptors.[27-29] We have previously demonstrated that, in human skin fibroblasts, insulin increased the rate of cell-surface receptor loss and that this process was energy and temperature dependent.[13] This energy and temperature dependence of insulin-mediated receptor loss in human fibroblasts was compatible with the concept that this regulatory process proceeds by an endocytotic internalization pathway. Thus, the augmented effect of insulin to induce cell-surface receptor loss in these cells may be via the ability of insulin to further accelerate an already augmented rate of insulin receptor internalization. However, this is conjecture, since the basal rate of receptor internalization has not been assessed.

In summary, a primary or genetic decrease in insulin receptors on cultured fibroblasts obtained from a patient with acanthosis nigricans and insulin resistance is associated with normal insulin sensitivity but decreased responsiveness as measured by glucose transport, an augmented chloroquine-induced increase in cell-associated radioactivity, an increased rate of insulin internalization by available receptors, and an augmented ability of insulin to induce receptor loss. This mutant cell line with a primary defect in receptor number as well as receptor function should serve as a useful tool for further investigation of insulin action.

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