Insulin Receptors in Cultured Human Fibroblasts

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SUMMARY

In order to study human insulin resistance, we have first characterized the interaction of insulin with specific insulin receptors in cultures of normal human fibroblasts. ^{125}I-insulin bound rapidly to human fibroblasts in suspension at 15°, achieving steady state between one and three hours. Insulin was not degraded during the binding assay. In competitive binding experiments, 2 ng./ml. (3.3 × 10^{-10} M) of unlabeled insulin reduced ^{125}I-insulin binding by 50 per cent. Insulin analogues competed for binding in proportion to their biologic potencies. A curvilinear Scatchard plot was obtained, suggesting the existence of negatively cooperative site-site interactions among the insulin receptors. This was confirmed directly by studies of the dissociation kinetics. The high affinity, specificity, and negative cooperativity of the fibroblast insulin receptor closely resemble the properties of other human insulin receptors. The cultured human fibroblast should prove a useful tissue for the study of insulin-resistant states in man. DIABETES 25:250-55, April, 1976.

Altered sensitivity to insulin has been described in a variety of physiologic and disease states.[1] Although the mechanisms by which insulin acts on cells are complex and poorly understood, the first step in insulin action, its binding to specific plasma membrane receptors, is amenable to study in vitro. Insulin receptors have been demonstrated in a number of human cell types, including cultured lymphocytes[2,3] and placental cells,[4] as well as in circulating monocytes[2,3,5] and in adipocytes.[6] Insulin binding to monocytes and adipocytes must be studied soon after the cells are removed from the patient. Such studies, therefore, reflect the physiologic milieu of the cells at the time they were obtained.[7] In order to examine genetic components of insulin resistance and insulin sensitivity, we have established fibroblast cultures from skin biopsies of individual patients. Human fibroblasts can be cultivated for over 50 generations without undergoing neoplastic transformation[8] and faithfully reflect the genetic endowment of the donor.[9] In this report, we describe the interaction of insulin with specific insulin receptors in cultures of normal human fibroblasts.

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MATERIALS AND METHODS

Hormones. Crystalline zinc porcine insulin, 25 U./mg., was purchased from Eli Lilly. The following polypeptides were generous gifts: desalanine-desasparagine (DAA) bovine insulin, F. H. Carpenter; porcine proinsulin, R. E. Chance; guinea pig insulin, L. F. Smith; S-sulfonated insulin β-chain, Mann Research Laboratories; glucagon, M. Rodbell; human growth hormone 1523D, A. E. Wilhelmi.

Fibroblast cultures. Fibroblast cultures were initiated from punch biopsies of skin from the volar surface of the mid-forearm of normal adult volunteers. Details of cell propagation have been previously described.[10] Fibroblasts were grown at 37° in modified Eagle's Minimal Essential Medium supplemented with 10 mM HEPES (N-2-hydroxyethyl piperazine-N′-2-ethane sulfonic acid, pH 7.4) and 20 per cent fetal calf serum (Flow, lot 4055838). Cultures were trypsinized, divided 1:3 each week, and used between the third and 15th weekly passages. Within these limits, the passage number of the culture did not appear to affect insulin binding.

Insulin binding assay. For insulin-binding experiments, fibroblasts were seeded four to 10 days in advance at a density of 10^6 cells/75 cm.^{2} flask in 12 ml. of medium and used without additional medium change. Fibroblasts were detached from the monolayer by treatment with 0.05 per cent trypsin (Nutritional Biochemicals, lot 1849)—0.5 mM EDTA at 37° for seven to 12 minutes (see Results). Soybean trypsin inhibitor (Sigma), 2 gm./gm. trypsin, was added, and the cells were sedimented and washed several times in pH 8.0 HEPES Binding Buffer [0.1 M HEPES, 0.12 M NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 10 mM glucose, 1 mM EDTA, 10 mg./ml. bovine serum albumin[4]]. The standard binding assay consisted of 1-5 × 10^6 cells, ^{125}I-porcine insulin (150-230 Ci./gm., 50 pg.), unlabeled porcine insulin, or other

polypeptides when indicated in a total volume of 0.5 ml. HEPES Binding Buffer. The cells were incubated for two hours at 15° and collected by centrifugation as previously described.[4] In general, approximately 80 per cent of the cells remained viable at the end of the incubation, based on trypan blue exclusion. Radioactivity in the cell pellets was determined by counting for 10 minutes in a Nuclear Chicago Autogamma Counter at 85 per cent efficiency.[4] The counting error was less than 3 per cent for incubations with tracer insulin alone. Duplicate determinations agreed within 5 per cent.

Dissociation experiments. ^{125}I-insulin, 100-225 pg./ml., was bound to fibroblasts, 5-9 × 10^6/ml., for two hours at 15°; 3.2-3.9 per cent of the tracer was bound. The cells were harvested by centrifugation and resuspended in an equal volume of cold HEPES Binding Buffer. Aliquots of the cell suspension were diluted 20- or 50-fold in buffer alone, or buffer and unlabeled insulin or desalanine-desasparagine-insulin, and were incubated at 15°. At the indicated times after dilution, duplicate tubes were centrifuged and the cell-associated radioactivity determined. In control experiments (not shown) dilutions of 20-fold or greater were sufficient to prevent rebinding of the dissociated ^{125}I-insulin.

RESULTS

The binding of ^{125}I-insulin to cultured human fibroblasts was studied in cell suspension. Fibroblasts were detached from the cell monolayer by mild trypsin treatment (0.05 per cent trypsin, 0.5 mM EDTA, 37°). ^{125}I-insulin binding was maximal in cells trypsinized for seven to 12 minutes (figure 1). Prolonged trypsin treatment (figure 1) or higher trypsin concentrations (not shown), however, resulted in a complete loss of specific insulin binding.

^{125}I-insulin bound rapidly to cultured human fibroblasts at 15° (figure 2). Half-maximal binding was achieved in 20 minutes, with a plateau of binding maintained between one and three hours. Subsequent experiments were performed under steady-state conditions, two-hour incubation at 15°. Incubation with a large excess of unlabeled insulin (10-40 μg./ml.) reduced the ^{125}I-insulin bound at steady state by approximately 80 per cent. This compatible component of total insulin binding has been designated "specific" binding. Fibroblasts incubated with ^{125}I-insulin at 37°, by contrast, were not at steady state after two hours. Although the total insulin bound at 37° was 250 per cent of that bound at 15°, less than 20 per

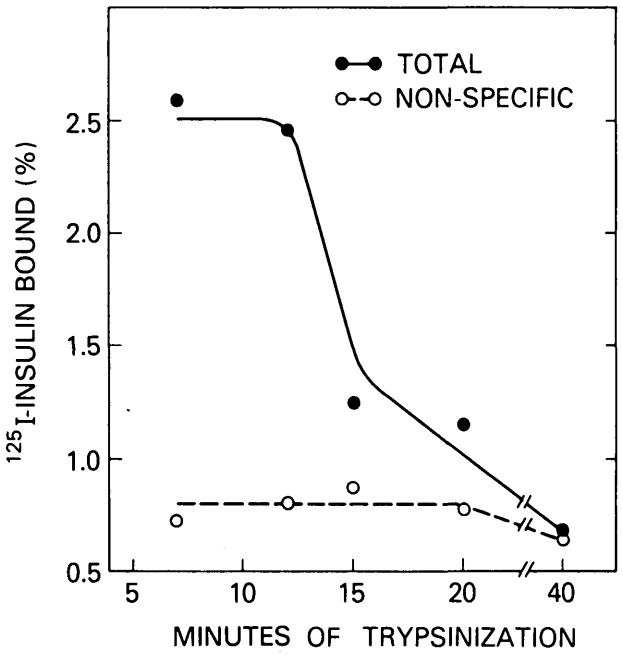

FIG. 1. Effect of the duration of trypsin treatment on ^{125}I-insulin binding. Fibroblasts were detached from the cell monolayer by incubation with 0.05 per cent trypsin—0.5 mM EDTA at 37° for different times. ^{125}I-insulin binding was determined in the absence (●——●) and presence (o——o) of 40 μg./ml. unlabeled insulin as described in Materials and Methods. Background counts were not subtracted in this experiment and account for most of the nonspecific binding.

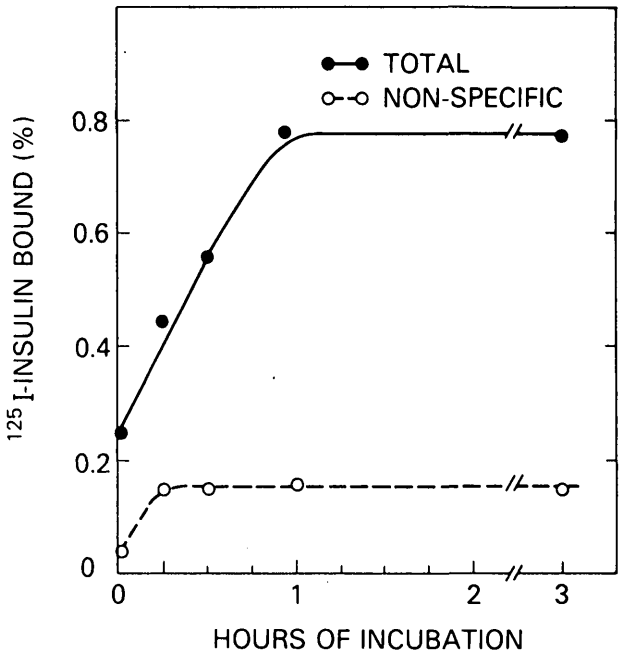

FIG. 2. Time course of ^{125}I-insulin binding. The per cent of tracer insulin bound/10^6 fibroblasts is shown for different times of incubation in the absence (●——●) and presence (o——o) of 40 μg./ml. of unlabeled insulin.

cent of it was bound specifically (data not shown). At 15°, specifically bound ^{125}I-insulin increased lineraly with the cell concentration between 1 and 8 × 10^6 cells/ml. (data not shown).

^{125}I-insulin was not degraded during the binding assay. The ability of tracer insulin to be precipitated by 10 per cent trichloroacetic acid and its ability to bind to cultured human lymphocytes,[3,11] a highly sensitive index of hormone integrity, were not diminished by a two-hour incubation at 15° with 3 × 10^6 fibroblasts/ml. In contrast, 40 per cent of the ^{125}I-insulin tracer was degraded when 2 × 10^6 fibroblasts were incubated for 45 minutes at 37° in phosphate-buffered saline.*

The specificity of ^{125}I-insulin binding to cultured human fibroblasts was demonstrated in competition experiments using insulin analogues and unrelated polypeptides. A representative experiment is shown in figure 3. Half-maximal binding was observed at 2 ng./ml. (3.3 × 10^{-10}M) porcine insulin. Glucagon, human growth hormone, and insulin β-chain—all essentially devoid of insulin biologic activity—did not compete for ^{125}I-insulin binding at concentrations as great as 10-50 μg./ml. Polypeptides with insulin-like biologic activity—proinsulin, desalanine-desasparagine insulin, guinea pig insulin, and multiplication-stimulating activity[4] (data not shown)—competed for insulin tracer binding but were less potent than porcine insulin. The ability of these insulin analogues to inhibit ^{125}I-insulin binding paralleled their relative insulin-like activities in a fat-cell glucose-oxidation bioassay.[12]

Competitive binding data, plotted according to Scatchard,[13] were curvilinear (figure 4), as has been observed in most insulin-binding systems.[14] Although the existence of several classes of insulin receptors with different intrinsic affinities cannot be excluded, the curvilinearity can be attributed to the presence of site-site interactions[14] among the insulin receptors (see below). In the presence of interacting binding sites,[15] a fixed K_d cannot be determined. The shallow slope and low counts at high insulin concentrations preclude a reliable estimation of the binding capacity, Ro, from the x-intercept of the Scatchard plot.

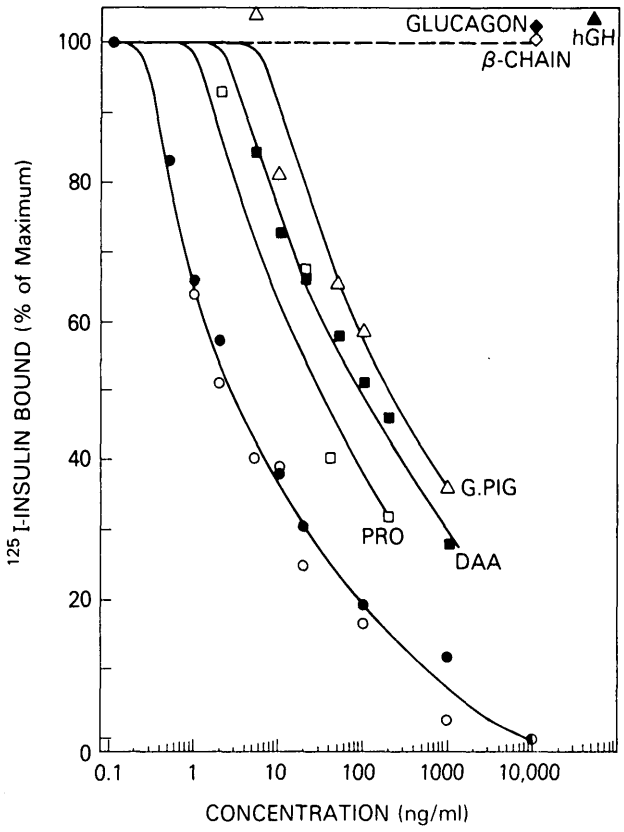

FIG. 3. Specificity of ^{125}I-insulin binding. ^{125}I-insulin was bound to fibroblasts in the presence of the indicated concentrations of different polypeptide hormones: desalanine-desaparagine bovine insulin (DAA, ■); porcine proinsulin(□); guinea pig insulin (Δ); S-sulfonated insulin β-chain (◇); glucagon (◆); human growth hormone 1523D (hGH, ▲). The maximum specific binding, 1.9 per cent of tracer ^{125}I-insulin bound per 10^6 was taken as 100 per cent. Two experiments with porcine insulin are shown (o, ●).

The kinetics of dissociation of ^{125}I-insulin from human fibroblasts were studied following dilution of the hormone-receptor complexes in the presence and absence of excess unlabeled insulin.[14] At high insulin concentrations, a greater fraction of the insulin receptors were occupied and the rate of dissociation of bound ^{125}I-insulin increased (figure 5). This loss of affinity of insulin receptors for insulin with increased binding of insulin has been attributed to negatively

*The stability of insulin receptors during the binding assay is more difficult to assess, since the receptor concentration cannot be measured directly. When cells were preincubated in HEPES Binding Buffer for one or two hours at 15° before addition of ^{125}I-insulin, the specifically bound insulin was decreased by approximately 40 per cent. The interpretation of this experiment is not clear-cut. For example, the number or the affinity of insulin receptors might have been reduced by the preincubation, or, alternatively, inhibitors of binding (e.g., receptor-degrading proteases) might have been released. Furthermore, it is not known whether empty receptors (as exist under preincubation conditions) have the same stability as occupied receptors (and receptors are rapidly filled under binding conditions). Additional work is required to resolve this complex problem.

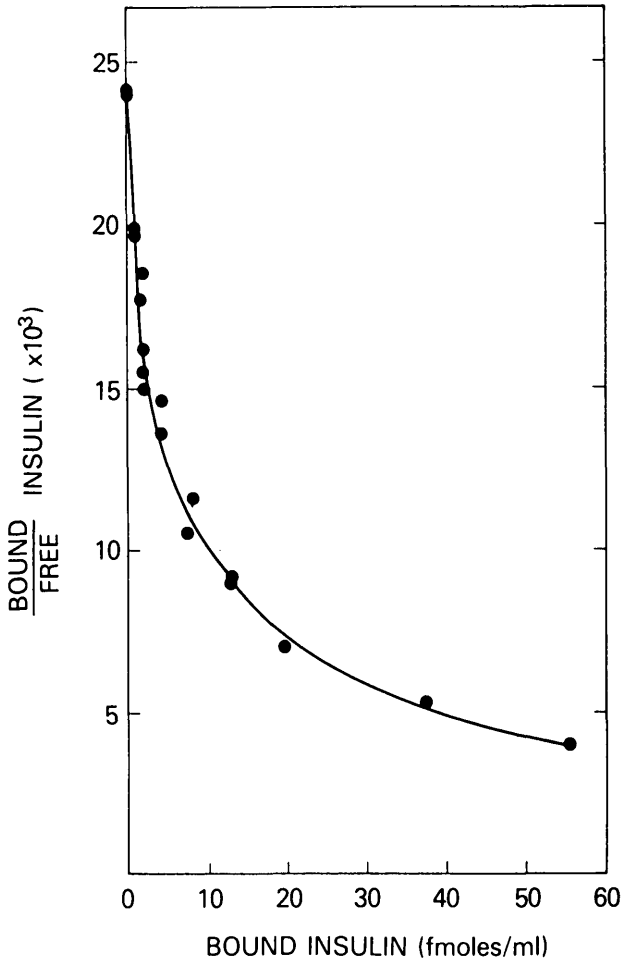

FIG. 4. Scatchard plot of the competitive binding data. Nonspecific binding has been subtracted. The binding shown is for 10^6 fibroblasts.

cooperative site-site interactions.[14]†

Physiologic insulin concentrations induced negative cooperativity in human fibroblast insulin receptors (figure 6). In contrast, a 1,000-times higher concentration of desalanine-desasparagine insulin, a concentration that resulted in the occupancy of more than 70 per cent of the insulin receptors, failed to induce the cooperative interactions (figure 6). Desalanine-

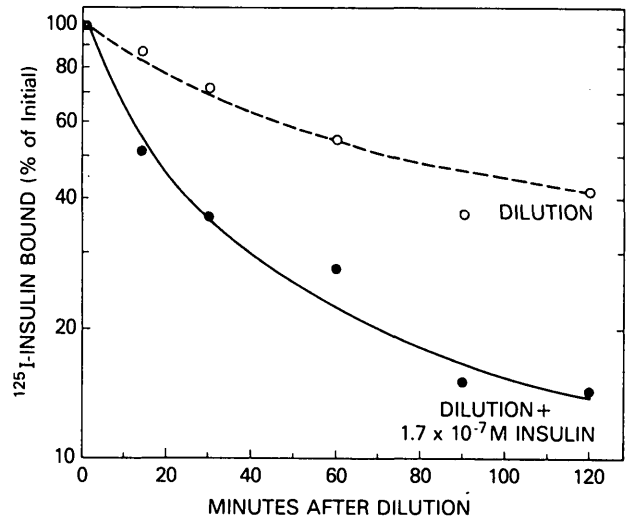

FIG. 5. Kinetics of dissociation. The ^{125}I-insulin remaining bound to fibroblasts at different times after dilution in buffer ± 1 μg./ml. (1.7×10^{-7} M) of unlabeled insulin was determined (see Materials and Methods).

desasparagine insulin and desoctapeptide insulin were unique among an extensive series of modified insulins tested in cultured human lymphocytes in that they failed to induce cooperativity even at concentrations that saturated the insulin receptors.[14,16] Our data

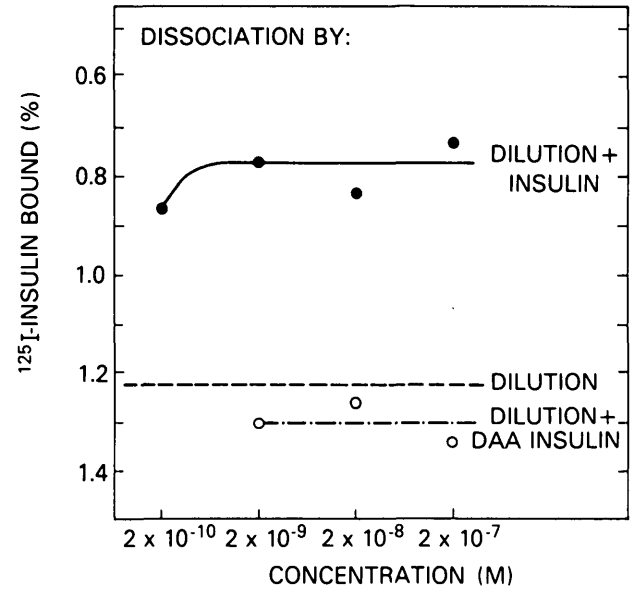

FIG. 6. Effect of different concentrations of insulin and DAA-insulin on the dissociation of ^{125}I-insulin. Bound ^{125}I-insulin was dissociated from fibroblasts by 20- or 50-fold dilution alone(-|-|-) or dilution in the presence of the indicated concentrations of insulin (•——•) or DAA-insulin (o——o). The ^{125}I-insulin remaining bound after a 90-minute incubation at 15° is shown as the per cent of initial tracer radioactivity.

†Other interpretations of these data are possible. De Meyts et al.[16,17] have extensively and cogently presented the evidence favoring cooperativity as the explanation for their results with insulin receptors of cultured human lymphocytes. Two of the most crucial points in their argument—the effectiveness of low insulin concentrations and the requirement for specific structural features of the insulin molecule—hold as well for human fibroblasts (see below) as for lymphocytes. Because of the analogies between the two systems, we have provisionally interpreted our dissociation experiments as indicative of negative cooperativity.

suggest that the cooperative sites of human fibroblast insulin receptors may have a similar specificity.

DISCUSSION

In this report we have characterized the interaction of insulin with specific insulin receptors in cultured human fibroblasts. Insulin bound to fibroblasts with high affinity—2 ng./ml. of insulin gave half-maximal displacement. Approximately 80 per cent of the total ^{125}I-insulin bound could be displaced by excess unlabeled hormone. Insulin analogues competed for ^{125}I-insulin binding according to their biologic potencies. Insulin binding was rapid, reached a steady state during which the hormone was not degraded, and was readily reversed by dilution. Site-site interactions among human fibroblast insulin receptors were evident at low insulin concentrations. The sensitivity at physiologic insulin concentrations, specificity, and negative cooperativity of the human fibroblast insulin receptors closely resemble the properties of other human insulin receptors.[2-6]

Our results considerably extend the previous accounts of insulin binding to human fibroblasts.[2,18] Gavin et al.[2] described an insulin receptor with appropriate specificity (except for the equal potency of proinsulin and desoctapeptide insulin) but low affinity (400 ng./ml. of insulin gave half-maximal displacement). Their binding experiments were performed at 37° for 45 minutes. In our hands, under these incubation conditions, binding was not at steady state, nonspecific binding was high, and hormone degradation was extensive. Hollenberg and Cuatrecasas[18] bound ^{125}I-insulin to fibroblast monolayers in plastic culture vessels at 24°. The affinity of their insulin receptor was high (half-maximal saturation at 6 ng./ml. of insulin). They did not, however, establish the specificity of their receptor by the use of insulin analogues. Moreover, nonspecific binding was high (>60 per cent), and total binding was low (approximately 0.5 per cent of tracer radioactivity). The low binding is of particular concern, since Hollenberg and Cuatrecasas[18] estimated that in their experiments as much as 0.4 per cent of the ^{125}I-insulin tracer could bind to the plastic surface. We too have observed that ^{125}I-insulin binds quite well to plastic. In addition, excess unlabeled insulin competes effectively for this binding to plastic (M.M.R., unpublished results), as reported by Cuatrecasas and Hollenberg[19] for other nonreceptor materials.

It should be stressed that our binding studies were performed in cell suspension, rather than binding ^{125}I-insulin directly to the fibroblast monolayer. This enabled us to examine insulin binding at different cell concentrations and temperatures and facilitated the processing of multiple samples. More important, it eliminated the potential artifact of ^{125}I-insulin binding to the plastic culture vessel. In our previous experiments with human placental cell cultures, cells were detached from the monolayer by mechanical scraping in Ca++-free media.[4] This approach was not successful with human fibroblast cultures. Instead, we have used gentle trypsinization to suspend the fibroblasts. We are well aware that insulin receptors can be destroyed by trypsin.[20] The carefully controlled mild trypsin treatment, however, detached both cultured placental cells (unpublished observations) and fibroblasts without affecting their ability to bind insulin.

Insulin exerts biologic effects in human fibroblast cultures—stimulation of glucose oxidation[21] and uptake,[22] DNA[10] and RNA[22] synthesis, and α-aminoisobutyric acid transport[18]—although fibroblasts are not regarded as a primary target tissue for insulin. It appears, however, that insulin receptors in nontarget tissues accurately reflect the state of insulin receptors in traditional target tissues. In the ob/ob mouse,[23] an obese animal with marked insulin resistance, profoundly reduced insulin binding has been observed in thymic lymphocytes[24] as well as in isolated hepatocytes and in plasma membranes prepared from liver and fat.[23] Likewise, circulating monocytes from a unique group of diabetic females with extraordinary clinical resistance to insulin have greatly depressed insulin binding.[25,26] We are currently studying insulin binding to fibroblast cultures established from several of these patients, as well as from other patients with genetic syndromes associated with insulin resistance.[27]

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