Autophosphorylation of Cultured Skin Fibroblast Insulin Receptors From Patients With Severe Insulin Resistance and Acanthosis Nigricans

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The severe insulin resistance with acanthosis nigricans seen in young women without insulin-receptor autoantibodies is characterized by hyperinsulinemia and decreased in vivo responsiveness to insulin. We evaluated the potential cellular defects in insulin-receptor binding and autophosphorylation in 12 subjects with this syndrome. When evaluated as a group, insulin binding to freshly isolated monocytes was 55% that of controls. Specific binding of insulin to skin fibroblasts in monolayer culture was 49% that of controls. Maximal insulin-stimulated receptor autophosphorylation was only 27% that of controls. Individual data demonstrated that the diminished autophosphorylation activity was out of proportion to the diminished fibroblast insulin binding in cell lines from most subjects and was <50% of the predicted activity in 6 of the 12 studied cell lines. These data are consistent with genetically determined defects leading to diminished numbers of cell surface insulin receptors with intact tyrosine kinase autophosphorylation in many of our cell lines. However, in at least half, there appeared to be an additional defect beyond insulin binding, resulting in a disproportionate decrease in insulin-sensitive phosphorylation of the insulin-receptor β-subunit.
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The syndrome of type A severe insulin resistance is characterized by endogenous hyperinsulinemia, insensitivity to administered insulin, hyperandrogenemia, and acanthosis nigricans in young females without insulin-receptor antibodies (1–4). Investigation of the mechanism of insulin resistance has demonstrated decreased insulin binding to circulating monocytes (1,2), erythrocytes (5,6), fat cells (7), and cultured fibroblasts (2,8) in most but not all affected individuals. In vivo assessment of insulin action via euglycemic clamp studies has given results consistent with a combined insulin-receptor and postreceptor defect (2). Evaluation of insulin-sensitive tyrosine kinase as a potential site of the postreceptor defect has consistently demonstrated decreased phosphorylation (9–13). However, it is unclear whether the decrease in insulin-receptor tyrosine kinase activity is more than can be explained by decreased numbers of normally functioning receptors. Because previous studies describe evaluations of cells from very few affected individuals and are inconsistent in their results, we sought to characterize the insulin-sensitive insulin-receptor autophosphorylation activity in a relatively large group of type A patients. The results of our studies of insulin-receptor autophosphorylation in cultured skin fibroblasts from 12 affected individuals demonstrate that the tyrosine kinase activity was proportionate to the decreased insulin binding in ~50% of the cell lines but was much less than could be explained by decreased receptor numbers in the remaining cell lines.

MATERIALS AND METHODS

Minimum essential medium (MEM) and Hanks' balanced salt solution were purchased from Gibco (Grand Island, NY). Sodium dodecyl sulfate (SDS) and polyacrylamide gel reagents were purchased from Bio-Rad (Richmond, CA). SB-5 X-ray film was purchased from Kodak (Rochester, NY). Fetal calf serum was purchased from Hazelton (Lenexa, KA). Wheat-germ lectin–sepharose 6-MB was purchased from Pharmacia (Piscataway, NJ). Phenylmethylsulfonyl fluoride (PMSF) and *N*-acetylglucosamine (NAG) were purchased from Sigma (St. Louis, MO). [γ-^{32}P]ATP (sp act 3000 Ci/mmol) and carrier-free [^{125}I]NaI were purchased from Du Pont New England Nuclear (Cambridge, MA). All other chemicals were reagent grade.

Insulin 1 pM = 0.139 μU/ml

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Subjects. After written informed consent was obtained, each subject was admitted to the University of Texas Medical Branch at Galveston General Clinical Research Center for evaluation of insulin responsiveness as previously described (2). These studies were reviewed and approved by the University of Texas Medical Branch Institutional Review Board. Each subject underwent an oral glucose tolerance test with 75 g glucose, assessment of insulin binding to freshly isolated circulating mononuclear cells, assessment of plasma glucose response (% decrease/min) to a bolus of 0.1 U/kg i.v. human insulin, and skin biopsy for long-term culture of fibroblasts.

Monocyte insulin binding. Mononuclear cells were isolated from whole blood by the method of Boyum (14), and specific binding of ^{125}I-labeled insulin was quantitated as previously described (15). Purified porcine insulin (provided by R. Chance, Lilly, Indianapolis, IN) was iodinated by a modification of the method of Freychet et al. (16) to achieve a specific activity of ~150 μCi/μg. Binding was related to the number of monocytes in the mononuclear cell preparation as determined by visual inspection of Wright-Giemsa–stained slides (~25%). Monocyte insulin-receptor affinity and number per cell were quantitated from the slope and *x*-axis intercept of a line tangent to the high-affinity segment of a Scatchard plot of the binding data (17).

Skin fibroblast cultures. Skin fibroblasts were obtained from the subjects from a 3-mm punch biopsy of the volar forearm as previously described (18). Cells were maintained in 75-cm^2 culture flasks with MEM at pH 7.4 and supplemented with 10 mM HEPES, 26 mM $HaHCO_3$, 1 mg/L biotin, and 10% fetal calf serum. Cells were subcultured every 6 days (1–3 split) and reached confluence in 4 days. There was no difference in confluent cell density or time to confluence in cells cultured from normal subjects or from subjects with acanthosis nigricans. All experiments were performed on cell cultures within 10 population doublings from the time of initial confluency.

Fibroblast insulin binding. For insulin-binding studies, confluent cells were subcultured to 60 × 15-mm plastic dishes 7 days before the assay. On the day of the study, the growth medium was aspirated, and the monolayers were washed twice with binding buffer at 22°C (MEM, pH 7.4, 25 mM HEPES, 25 mM tricine, and 1% bovine serum albumin). ^{125}I-insulin (0.05 pmol/ml), unlabeled insulin standards, and binding buffer were added to a total of 2 ml, and dishes were incubated in a shaking water bath for 3 h at 16°C. At 3 h, the dishes were washed repetitively with ice-cold Hanks' buffer, and the monolayers were solubilized in 3 ml I N NaOH and counted in a γ-counter. Cell counts were performed in parallel to the binding studies on trypsinized dishes with a Coulter counter calibrated against hemocytometer counts. In a given study, each insulin concentration was performed in triplicate dishes. In each cell line, insulin binding was performed in at least three separate assays. Binding parameters were analyzed as described for monocytes.

Fibroblast autophosphorylation. Fibroblasts were cultured in 75-cm^2 flasks to confluence. On the day before study, growth medium was replaced with serum-free medium. Twelve to 15 flasks (25 to 30 × 10^6 cells) were decanted, and 5 ml solubilization buffer was added to each flask and incubated at 20°C for 60 min. Solubilization buffer consisted of 150 mM NaCl, 50 mM HEPES, 2 mM PMSF, and 0.5% Triton X-100 at pH 7.6. After solubilization, the buffer was removed from each flask, pooled, and centrifuged at 18,000 × *g* for 60 min. The supernatant was applied to a 1-ml bed volume wheat-germ lectin–Sepharose 6-MB column and recycled continuously overnight at 4°C. The column was then washed with 100 ml wash buffer (150 mM NaCl, 50 mM HEPES, 2 mM PMSF, 0.1% Triton X-100, pH 7.6), followed by addition of 5 ml wash buffer containing 0.3 M NAG. The first 3-ml eluant after adding NAG was used for phosphorylation. Aliquots of the partially purified insulin receptor were incubated in absence or presence of insulin (final concn 0, 0.1, 0.5, 1, 5, 10, 100, and 1000 nM) for 60 min at 20°C. Phosphorylation buffer (20 μM ATP, 8 mM $MnCl_2$, 50 mM HEPES, pH 7.4) was then added to each tube, and finally 20 μl [^{32}P]ATP was added to initiate the reaction. After 20 min, the reaction was terminated with stop buffer (10 mM $Na_4P_2O_7$, 10 mM NaF, 4 mM EDTA). The receptor was immunoprecipitated with a purified polyclonal chicken antibody L6 (19) and the tubes were incubated overnight at 4°C, followed by addition of rabbit anti-chicken IgG. The tubes were centrifuged at 3000 × *g* for 10 min, and the pellet was suspended in SDS-sample buffer (1% SDS, 1% glycerol, 0.03% bromphenol blue, 50 mM Tris, pH 6.9). After 3 min at 90°C with 3% mercaptoethanol, the mixtures were applied

TABLE 1
Clinical characteristics of subjects with acanthosis nigricans and insulin resistance

Subjects	Age (yr)	Percent of ideal body weight	Fasting insulin (μU/ml)	Glucose response (% decrease/min)
Acanthosis nigricans				
A1	14	192	65	0.2
A3	15	164	65	1.3
A5	14	158	45	1.3
A6	33	210	32	2.2
A7	18	183	63	1.9
A11	15	178	47	2.2
A12	16	187	65	1.2
A14	12	174	40	1.1
A15	12	207	89	1.6
A19	34	231	88	1.6
A21	9	189	24	3.5
A27	18	197	36	1.9
Mean ± SD	18 ± 8	189 ± 20	55 ± 21	1.7 ± 0.8
Control (*n* = 10, mean ± SD)	30 ± 8	104 ± 10	5 ± 1	7.1 ± 1.2

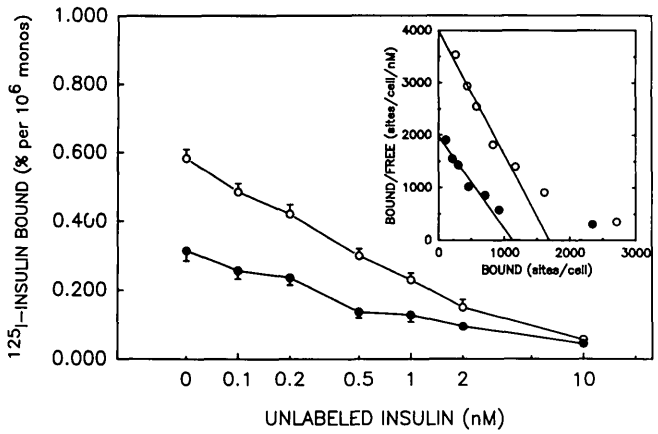

FIG. 1. Specific binding of ^{125}I-labeled insulin to freshly isolated mononuclear cells from 12 subjects with acanthosis nigricans (●, *n* = 12) is compared with similar studies from 36 normal subjects (○, *n* = 36). Data are means ± SE for binding of radiolabeled insulin in presence of increasing concentrations of unlabeled insulin. Units are percents of total counts specifically bound per million monocytes. *Inset*: mean data for same studies plotted as bound-to-free versus bound insulin. *Curves* in *inset* represent linear regressions through first 5 points.

to slab gels with 7.5% polyacrylamide separating gels (20). After electrophoresis, the gels were fixed, stained, dried, and subjected to autoradiography. Autoradiographs were quantitated by photodensitometry with a Quickscan thin-layer chromatograph (Helena, Beaumont, TX), and phosphorylation is expressed as percent of basal (absence of insulin) phosphorylation. Each cell line had insulin-stimulated autophosphorylation quantitated at least three times, and the data represent the means of these separate determinations.

RESULTS

The clinical characteristics of 12 female subjects with acanthosis nigricans are given in Table 1. For comparison, the mean and standard deviation of the same parameters for 10 normal female controls were included. As a group, the subjects with acanthosis nigricans had at least moderate obesity, marked hyperinsulinemia (10-fold elevated), and markedly decreased glucose responsiveness to exogenous insulin (24% of mean control value).

Curves showing competition of unlabeled insulin for binding of radiolabeled insulin to monocytes are shown in Fig. 1. Cells from subjects with acanthosis nigricans displayed ~50% less insulin binding than those from control subjects. Scatchard analysis of the specific binding demonstrated decreased receptor number but normal receptor affinity for insulin (Fig. 1, *inset*). Five subjects had insulin binding to monocytes within 1SD of the control mean.

Assessment of insulin binding to long-term cultured skin fibroblasts should quantitate genetically determined receptor levels (7). If diminished insulin binding seen in freshly isolated cells was due to in vivo humoral factors alone, then insulin binding should return to normal levels when cells are maintained in defined culture media. Figure 2 displays competition curves and Scatchard plots for insulin binding to skin fibroblasts. Insulin binding was decreased by ~50% on average due to decreased numbers of receptors with no alteration in affinity.

Insulin-receptor autophosphorylation was moderately to markedly decreased in cultured fibroblasts from the 12 subjects. Figure 3 shows insulin dose-response curves for each subject compared with 18 studies performed in three cell lines from normal control subjects. Only 2 subjects approached normal maximal insulin-stimulated autophosphorylation, but their dose-response curves suggest a shift to the right in insulin responsiveness.

Comparison of group data suggested that phosphorylation was decreased out of proportion to the decrease in insulin receptors (27 vs. 49% of control, respectively). Individual data for binding to cultured fibroblasts and maximal stimulation of autophosphorylation are shown in Table 2. A ratio of phosphorylation to insulin binding should be equal to 1.00 if phosphorylation activity were decreased in proportion to the decrease in insulin receptors. Five of the 12 cell lines had ratios within 1SD of the mean of the control studies. The 6 cell lines with the most severely defective tyrosine kinase activity were <50% of the activity predicted by the decrease in their cell surface receptors.

DISCUSSION

We evaluated the in vivo insulin responsiveness of 12 subjects with acanthosis nigricans and assessed the insulin-receptor levels on freshly isolated monocytes and on long-term cultured skin fibroblasts. Insulin-sensitive–receptor autophosphorylation was compared to receptor quantitation and found to be proportional in ~50% of the fibroblasts. However, in the remainder, phosphorylation was more severely defective than could be explained by decreased numbers of receptors alone.

Previously published evaluations of insulin-receptor autophosphorylation in patients with type A severe insulin resistance have described few patients and have employed erythrocyte insulin receptors (10,12,13) or monocytes and transformed lymphocytes (11). Grigorescu et al. (10) described studies of fibroblasts from one patient. Grunberger et al. (11) described studies of monocytes and Epstein-Barr virus–transformed lymphocytes from six subjects where the

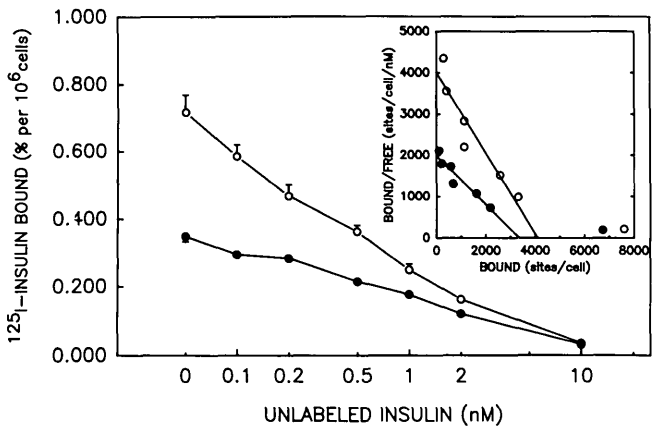

FIG. 2. Specific binding of ^{125}I-labeled insulin to skin fibroblasts maintained in monolayer culture. Data are means ± SE of labeled insulin bound in presence of indicated concentration of unlabeled insulin (abscissa). Error bars not shown are smaller than symbol size. *Curves* for normal subjects (○, *n* = 29) represent 7 separate binding studies. *Curves* for subjects with acanthosis nigricans (●, *n* = 36) represent 3 separate binding studies. *Inset*: Scatchard plots of means of same binding studies. *Curves* in *inset* represent linear regressions through first 6 points.

TABLE 2
Relationship of fibroblast insulin binding and insulin-stimulated autophosphorylation

Subjects	Total specific ^{125}I-insulin binding (%/10^6 cells)	Maximal receptor phosphorylation (% of basal)	Insulin binding (% of normal)	Receptor phosphorylation (% of normal)	Phosphorylation: insulin binding*
Acanthosis nigricans					
A7	0.392	110	68	5	0.08
A3	0.503	133	87	18	0.21
A1	0.324	123	56	13	0.22
A14	0.455	135	79	19	0.24
A21	0.400	147	69	26	0.37
A11	0.416	157	72	31	0.43
A12	0.265	144	46	24	0.52
A27	0.153	130	27	16	0.61
A19	0.434	192	75	50	0.66
A15	0.256	156	44	30	0.69
A6	0.267	165	46	35	0.76
A5	0.330	194	57	51	0.89
Mean ± SD†	0.350 ± 0.096	149 ± 25	61 ± 17	27 ± 13	0.47 ± 0.25
Control (n = 18, mean ± SD)	0.577 ± 0.108	284 ± 77	100 ± 19	100 ± 26	1.02 ± 0.43

*Quantitated as percent of mean of control studies (18 separate studies in 3 cell lines from normal subjects).
†$P < .001$ vs. controls, by Student's t test for independent samples.

decreased phosphorylation was proportional to decreased insulin binding (11). Similar proportional decreases were described by Grigorescu et al (13). In another report, Grigorescu et al. (12) found one of three subjects to have normal insulin binding but a 50% decrease in autophosphorylation. The results of our studies of cultured fibroblasts from a much larger group of subjects suggest that many subjects (~50%) have a modest decrease in insulin receptors and a postbinding defect in autophosphorylation.

Evaluation of potential defects in insulin-receptor tyrosine kinase activity in other insulin-resistance syndromes such as obesity and non-insulin-dependent diabetes mellitus (NIDDM) have been largely limited to freshly isolated tissues (21–25). Freidenberg et al. (22) evaluated insulin binding, insulin-receptor autophosphorylation, and insulin-sensitive phosphorylation of the exogenous substrate poly-Glu^{80}-Tyr^{20} in fat cells from normal, obese, and NIDDM subjects. They found diminished tyrosine kinase activity in proportion to decreased insulin binding. Comi et al. (23) evaluated erythrocyte insulin-receptor tyrosine kinase activity with exogenous substrate in 14 subjects with NIDDM and found a proportionate decrease in insulin binding and tyrosine kinase activity. Caro et al. (21,24,25) evaluated tyrosine kinase activity in several tissues from obese subjects and obese NIDDM patients undergoing gastric bypass surgery. Their results differed from tissue to tissue. Hepatocytes had normal insulin binding (21), but muscle and fat had decreased binding (24,25). In muscle, receptor autophosphorylation was normal, but use of the exogenous substrate Glu^{80}-Tyr^{20} revealed decreased tyrosine kinase activity (25). In hepatocytes and fat cells, diabetic patients had less tyrosine kinase activity than obese individuals (21,24), but in muscle, the tyrosine kinase activity was not different between obese and NIDDM patients (25). Even though these reports are not entirely consistent, in the two most prevalent insulin-resistant syndromes, insulin-receptor tyrosine kinase activity generally appears diminished in proportion to the decrease in receptors seen in these conditions. These studies thus suggest there is no severe defect in insulin action that involves the coupling of insulin binding to activation of tyrosine kinase in obesity and NIDDM.

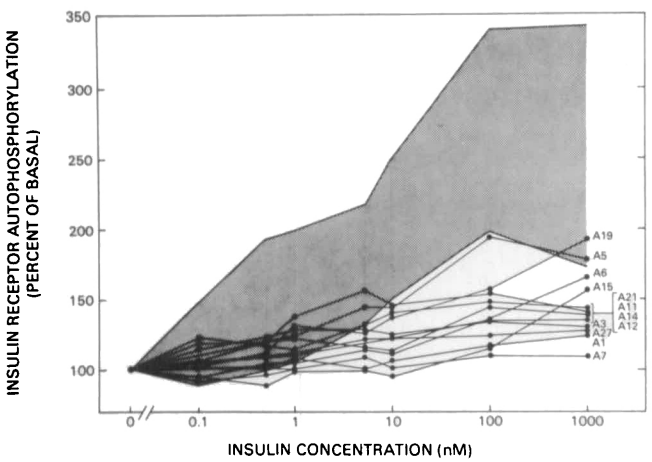

FIG. 3. Insulin dose-response curves of insulin-receptor autophosphorylation determined as described in MATERIALS AND METHODS. Each *curve* represents means of at least 3 separate studies. *Shaded area* represents mean ± SD for 18 separate studies in 3 cell lines from normal individuals.

In summary, in evaluating the insulin resistance of patients with acanthosis nigricans, we found decreased insulin binding to freshly isolated monocytes and to long-term cultured skin fibroblasts. Quantitation of insulin-receptor autophosphorylation in cells from 6 of 12 subjects demonstrated a marked decrease, out of proportion to the diminished insulin binding. These data suggest that in addition to decreased insulin-receptor levels in cells from patients with type A insulin resistance, many affected individuals have a hereditary postbinding defect in insulin-receptor autophosphorylation activity.

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