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Human Insulin Receptors Expressed in Insulin-Insensitive Mouse Fibroblasts Couple with Extant Cellular Effector Systems to Confer Insulin Sensitivity and Responsiveness*

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ABSTRACT. When cDNA for human kidney insulin receptors was used to transfect NIH3T3 mouse fibroblast cells with few or no endogenous insulin receptors, a resultant cell line, 3T3/HIR, expressed more than 6 million receptors/cell. Results of the present study demonstrated that these human receptors in murine cells mediated a diverse group of responses, including insulin binding and internalization as well as insulin-stimulated tyrosine phosphorylation of the receptor and a putative cellular substrate pp185. In addition, the cells were stimulated by insulin in various acute and long term metabolic processes, including glucose transport, glycogen formation, amino acid uptake, and thymidine uptake and incorporation into DNA. There were weak or no responses to insulin in control fibroblasts transfected only with the pSV2Neo plasmid containing a bacterial gene for neomycin resistance (3T3/NEO cells). These findings indicated that transfection of insulin receptor cDNA conferred insulin sensitivity to the target cells in a broad range of cellular responses and further demonstrated that effector molecules for mediating such responses were present in cells that normally lacked sensitivity to this hormone. Expressed receptors readily coupled with the effector systems to become fully functional. (*Endocrinology* **124**: 257–264, 1989)

INSULIN is a major regulatory hormone for carbohydrate, lipid, and protein metabolism in mammalian tissues (1, 2). When insulin's normal process of receptor binding and signal transduction is disrupted, the result is an abnormal state of cellular resistance to the essential actions of insulin. Such impaired sensitivity to insulin is characteristic of many patients with type II diabetes mellitus and obesity (3). To understand, and possibly correct defects in these patients, it will be necessary to understand the normal cellular mechanisms of insulin actions.

An approach to determining postreceptor mechanisms involved in signal transmission would be to overexpress insulin receptors in cells that are insensitive to insulin to ascertain whether insulin sensitivity and responsiveness can be induced. Comparison of such cell lines with and without receptors should facilitate understanding of the biochemical mechanism(s) involved in postreceptor events. In the present study, NIH3T3 Swiss mouse embryo fibroblasts were used as recipient cells for insulin receptor cDNA transfection. These cells have few or no endogenous insulin receptors and are insensitive to insulin. Our recent report described expression of a human kidney insulin receptor cDNA in these NIH3T3 cells to produce a cell line with an extremely high number of insulin receptors (6×10^6/cells; 3T3/HIR); preliminary observations suggested that receptor function was grossly normal, and that receptor tryrosine kinase activity in particular was retained (4).

In the present study we examined the acute and long term effects of insulin on metabolism and growth of these NIH3T3 cells expressing high levels of insulin receptors. We found that such cells responded to insulin binding with tyrosine phosphorylation of the receptor and a putative cellular substrate for the receptor kinase called pp185. The cells also became highly sensitive to insulin for acute and chronic regulation of cell metabolism and growth, and their receptors were linked to an

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endocytic degradative pathway for insulin processing. These findings suggested that the mechanisms and substrates requisite for mediating a broad array of insulin responses were present in parent cells lacking sensitivity to the hormone and further indicated that expressed receptors coupled readily with these effector systems.

Materials and Methods

Materials

D-[U-^{14}C]Glucose (275 mCi/mmol), [6-^{3}H]thymidine (22 Ci/mmol), NCS tissue solubilizer, and OCS scintillation fluid were purchased from Amersham Corp. (Arlington Heights, IL). 2-Deoxy-D-glucose-1-^{14}C (57 mCi/mmol) and type III glycogen from rabbit liver, dexamethasone, and controlled process serum replacement-type I (CPSR-1) were obtained from Sigma Chemical Co. (St. Louis, MO). [*Methyl*-^{3}H]Aminoisobutyrate (33 Ci/mmol) and carrier-free [^{32}P]orthophosphate were purchased from New England Nuclear (Boston, MA). Ready-Solv CP scintillation fluid was obtained from Beckman Instruments, Inc. (Palo Alto, CA). Culture medium and serum were purchased from Hazelton Research Products (Denver, PA) or Gibco (Grand Island, NY). Pansorbin was obtained from Calbiochem (La Jolla, CA). Monoiodinated [^{125}I-Tyr-A^{14}]human insulin (350 μCi/μg) was generously provided by Dr. Bruce Frank (Eli Lilly Co., Indianapolis, IN).

Cells

A prior report described the derivations of the cell lines 3T3/HIR, NIH3T3 cells expressing high levels of human insulin receptors (6×10^6/cell) after transfection with a bovine papilloma virus/insulin receptor cDNA construct (4), and 3T3/NEO, NIH3T3 cells transfected with the plasmid pSV2Neo containing the bacterial gene for neomycin resistance (4, 5). Preliminary studies showed that 3T3/NEO and wild-type NIH3T3 cells had identical low levels of insulin binding and relative insensitivity to insulin.

Experimental procedures

[^{125}I]-Insulin binding and degradation assays. Confluent monolayer cultures of 3T3/HIR cells ($4–9 \times 10^5$ cells/35-mm well) were rinsed with PBS and incubated for 30 min at 30 C in 950 μl assay medium [Dulbecco's Modified Eagle's Medium (DMEM) containing 20 mM HEPES, 5 mM sodium bicarbonate, and 1% BSA] without or with 100 μM chloroquine added. [^{125}I-Tyr-A^{14}]Insulin (0.25 nM) was then added in 50 μl, and cells were further incubated at 30 C for the indicated time interval. After this incubation, a 750-μl aliquot of medium was removed to 750 μl ice-cold 12% trichloroacetic acid (TCA) in tubes, vortexed, and allowed to precipitate. After centrifugation, radioactivity in 1-ml aliquots of supernatant was counted as a measure of insulin degradation products. To determine the amount of [^{125}I]insulin bound, the monolayers were washed five times with PBS, solubilized with 0.5 N sodium hydroxide, and counted for radioactivity.

Internalization of bound [^{125}I]insulin. Binding experiments were performed as described above, and internalization of [^{125}I]insulin was determined as reported previously and essentially according to the technique developed by Haigler *et al.* (6, 7) for quantitation of epidermal growth factor internalization. Briefly, after aspiration of tracer hormone and binding medium, parallel sets of cell monolayers were washed three times with PBS. One set of cells was then subjected to two 1-ml acid washes (0.2 M acetic acid and 0.5 M sodium chloride, pH 2.5). Each set of cell samples was solubilized in 0.1% sodium dodecyl sulfate (SDS) and counted for radioactivity. The acid-resistant radioactivity represented the internalized fraction of insulin. The surface-bound insulin was determined by subtracting acid-resistant radioactivity from total cell-associated radioactivity.

^{32}P phosphorylation of the intact cells and immunoprecipitation of phosphotyrosine-containing proteins. Confluent cells in 15-cm dishes were incubated for 2 h with 5 ml phosphate-free and serum-free RPMI-1640 medium containing carrier-free [^{32}P]orthophosphate (0.5 mCi/ml). Insulin was added, and the incubation was continued for the indicated times. The experiments were stopped quickly by removing the incubation medium and freezing the cell monolayers with liquid nitrogen. The monolayers were thawed immediately, and 2 ml of a 4 C solution containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate, 1 mg/ml apoprotinin, and 2 mM phenylmethylsulfonylfluoride were added. The cells were scraped from the dishes, and insoluble material was sedimented by centrifugation at 50,000 rpm in a Beckman 70.1 Ti rotor for 60 min. The supernatant was immunoprecipitated with anti-phosphotyrosine antibodies (αPY) on Pansorbin, as described previously (8). The proteins were eluted from washed precipitates with Laemmli sample buffer, reduced with dithiothreitol, and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% resolving polyacrylamide gels (9). The phosphoproteins were identified by autoradiography at −70 C of the stained and dried gels using Kodak X-Omat film and an intensifying screen. The radioactivity in the gel fragments was quantified by Cerenkov counting or scanning densitometry.

Measurement of 2-deoxy-[^{14}C]glucose uptake. Twenty-four hours before assay, confluent monolayers of 3T3/HIR or 3T3/NEO cells were prefed with low glucose DMEM containing 0.05% BSA. For assay, cell monolayers ($4–9 \times 10^5$ 3T3/HIR cells/well or $1–3 \times 10^5$ 3T3/NEO cells/well) were rinsed with PBS and incubated in assay medium (Swim's no glucose, 5 mM sodium bicarbonate, 20 mM HEPES, and 1% BSA, pH 7.4) without or with insulin added at indicated concentrations for 15 min at 37 C. Cells were rinsed again and incubated for 15 min at 37 C in medium containing 0.5 μCi 2-deoxy-[^{14}C]glucose. Medium was aspirated, and cells were rinsed with buffer containing 20 μM cytochalasin-B and 10 mM Tris, pH 7.4. Cells were solubilized with 0.9 ml 0.5 N NaOH and transferred to vials. The mixture was neutralized by the addition of 105 μl glacial acetic acid, and radioactivity was counted using 10 ml Beckman Ready-Solv CP.

Measurement of D-[U-^{14}C]glucose incorporation into glycogen. Cell monolayers (3T3/HIR or 3T3/NEO cells in 35-mm wells) were grown in glucose- and serum-free medium for 24 h before

the assay. For assay, cells were incubated for 90 min at 37 C in glucose-free medium containing 1% BSA, 5 mM sodium bicarbonate, 20 mM HEPES (pH 7.4), and 1 μCi [^{14}C]glucose without or with insulin added at indicated concentrations. After incubation, monolayers were rinsed and solubilized for 30 min at 37 C with 2 ml 30% KOH containing 4 mg carrier glycogen. The solubilized mixture was transferred to glass tubes and boiled for 30 min. After cooling, glycogen was precipitated by the addition of 4 ml 95% ethanol. The precipitate was pelleted at 1000 × *g*, redissolved, reprecipitated in ethanol, and pelleted again. The pellets were dissolved in 0.4 ml water, and such samples were transferred to vials and counted for radioactivity using 5 ml Beckman Ready-Micro scintillation fluid.

Measurement of [methyl-^{3}H]aminoisobutyrate uptake. Twenty-four hours before assay, serum-supplemented DMEM on confluent cultures was replaced with medium containing 1 μM dexamethasone and 0.05% BSA or 0.5% CPSR-1 serum substitute. At the time of the assay, cell monolayers were rinsed with PBS and incubated for 6 h at 37 C in 1 ml Krebs-Ringer-HEPES buffer (pH 7.4), 20 mM glucose, and 1% BSA, without or with insulin added at the indicated concentrations. Monolayers were then rinsed with PBS and incubated at 37 C for 20 min with 0.5 μCi [*methyl*-^{3}H]aminoisobutyrate (MeAIB) in 1 ml assay buffer. After rinsing with PBS, cells were solubilized in 0.9 ml 0.5 N NaOH. Samples were then transferred to vials and counted for radioactivity using 10 ml Beckman Ready-Micro scintillation fluid and 105 μl glacial acetic acid for neutralization.

Measurement of [^{3}H]thymidine incorporation into DNA. Before the assay, confluent monolayers of 3T3/HIR or 3T3/NEO cells (in 35-mm wells) were cultured for 24 h at 37 C in DMEM lacking serum, but containing 0.05% BSA. Cells were then further treated for 18 h in this medium without or with insulin added at indicated concentrations. Cell monolayers (4–9 × 10^{5} 3T3/HIR3.5 cells/well or 1–3 × 10^{5} 3T3/NEO cells/well) were rinsed and incubated for 30 min at 37 C in 1.0 ml DMEM containing 20 mM HEPES (pH 7.4), 5 mM sodium bicarbonate, 1% BSA, and 0.5 μCi [^{3}H]thymidine. Monolayers were rinsed and solubilized with 2 ml 0.1% SDS for 1 h at 37 C. Solubilized material was transferred to glass tubes, cooled to 4 C, and precipitated by the addition of 2 ml cold TCA (200 mg/ml) to each tube. Tubes were vortexed, incubated for 30 min at 4 C, and centrifuged at 500 × *g* for 20 min at 4 C. Supernatant was aspirated, and the pellet was dissolved completely in 0.1 ml water and 1.0 ml NCS for 3–5 h. The solubilized material was transferred to vials and counted for radioactivity by liquid scintillation using 10 ml OCS.

Results

Insulin binding, internalization, and degradation

After binding of insulin to cell surface receptors, the insulin-receptor complexes are rapidly internalized to endosomes (10). In these organelles, the ligand and receptor may be uncoupled and sorted to separate pathways, *i.e.* the lysosome for ligand degradation and the cell surface for recycled receptors (11, 12). To determine whether the human insulin receptors expressed in the 3T3/HIR cells were appropriately coupled to such processes, the time course of binding and internalization of insulin at 37 C was determined (Table 1) using an acid wash technique (6). [^{125}I-Tyr-A^{14}]Insulin bound rapidly to cells, such that by 5 min 7.4% of the input radioactivity was bound to the cell surface (removable by acid treatment). By 10–20 min a peak of 9.4% radioactivity associated with the cell surface was reached, and this level declined thereafter. Internalized (acid-resistant) radioactivity followed a similar time course. These data are consistent with rapid insulin binding to cell surface receptors with consequent internalization of the insulin-receptor complex. By contrast, [^{125}I]insulin bound only minimally and slowly to the cell surface of 3T3/NEO cells, reaching just 0.27% of input radioactivity at 60 min. Internalized radioactivity also accumulated slowly, and the capacity for internalization in 3T3/NEO cells was only about 1% of that observed in 3T3/HIR cells. 3T3/HIR and 3T3/NEO cells, however, were able to bind [^{125}I]IGF-I similarly, and preliminary analyses of such data indicated that there were about 200,000 insulin-like growth factor I (IGF-I) receptors/cell in both cell lines.[1]

To further demonstrate that the insulin receptors of 3T3/HIR cells were coupled to a degradative pathway, the time course of insulin binding and degradation was measured in the presence or absence of chloroquine (Fig. 1); this drug appears to block the pH-dependent dissociation of receptor-ligand complexes by inhibiting acidification of endosomes (13, 14). Chloroquine increased 2-

TABLE 1. Insulin internalization by 3T3/HIR and 3T3/Neo cells

Cells	Time (min)	Total radioactivity (% of total)	Acid-resistant radioactivity (% of total)	Acid-dissociable radioactivity (% of total)
HIR	5	10.21	2.84	7.37
	10	15.02	5.60	9.42
	20	14.85	5.47	9.38
	30	12.36	4.76	7.60
	40	10.91	4.73	6.18
	60	9.44	4.21	5.23
NEO	5	0.21	0.05	0.16
	10	0.24	0.05	0.19
	20	0.25	0.06	0.19
	30	0.30	0.06	0.24
	40	0.32	0.07	0.25
	60	0.34	0.07	0.27

Monolayer cultures of cells in six-well culture dishes were incubated at 37 C with [^{125}I]insulin (33 pmol/L) in a total volume of 1 ml in the presence or absence of an excess of unlabeled insulin (10^{-6} M). Total cell-associated radioactivity and acid-resistant radioactivity were determined as described in *Experimental procedures*. Acid-dissociable radioactivity was derived from these values by subtraction. All data are expressed as percent input (counts per min/10^{5} cells · ml).

[1]Hofmann, C., and J. Whittaker, manuscript in preparation.

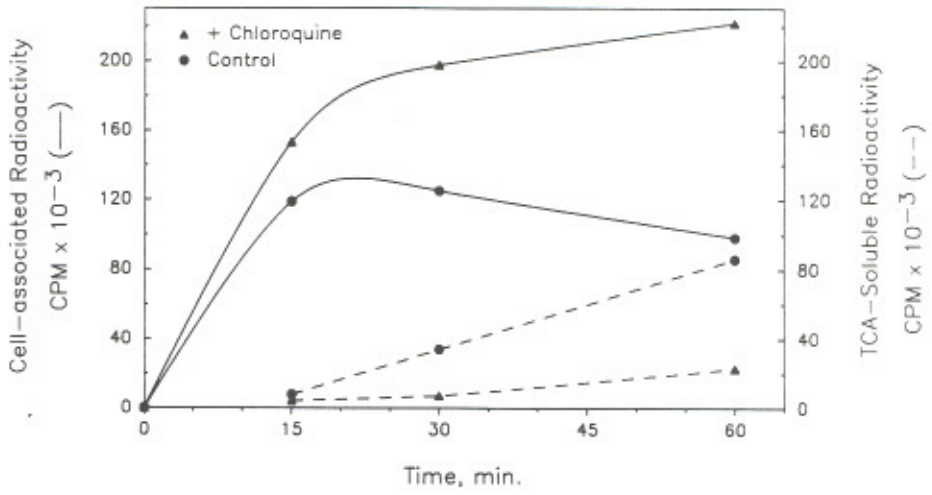

FIG. 1. Time course of [^{125}I]insulin binding and degradation with or without chloroquine present. Confluent monolayer HIR cells were preincubated for 30 min at 30 C in medium with or without 100 μM chloroquine. [^{125}I]Insulin (0.25 nM) was then added to the plates for the indicated incubation interval. Radioactivity associated with the cells and TCA-soluble radioactivity in the medium were determined. *Data points* represent the mean value for triplicate points. Deviation was too small to be represented graphically.

fold the amount of [^{125}I]insulin radioactivity associated with 3T3/HIR cells and decreased the amount of degradation products in the medium. Binding and degradation of [^{125}I]insulin by 3T3/NEO cells were qualitatively similar, although the amount of hormone bound and degraded was less than 1% of that in 3T3/HIR cells (data not shown). Thus, in 3T3/HIR cells, human insulin receptors mediated internalization and degradation of insulin.

Insulin-stimulated tyrosine phosphorylation of the insulin receptor and pp185 in 3T3/HIR cells

Insulin stimulates tyrosyl phosphorylation of the β-subunit of the insulin receptor immediately after insulin binding, and αPY immunoprecipitate this and other phosphotyrosine-containing proteins from a variety of cells. However, no phosphotyrosine-containing proteins were detected in Triton X-100 extracts of 3T3/NEO cells before or after insulin stimulation (Fig. 2, lanes a and b). By contrast, the 3T3/HIR cells contained three distinct phosphoproteins that were immunoprecipitated with the αPY (Fig. 2, lanes c and d). In the absence of insulin, a pp120 protein was detected, and as previously described, it was not stimulated by insulin (8, 15). After insulin stimulation of 3T3/HIR cells for 2 min, a 95K phosphoprotein was detected in the αPY immunoprecipitate during SDS-PAGE under reducing conditions (Fig. 2, lane d). This protein was confirmed to be the β-subunit of the insulin receptor by tryptic peptide mapping and immunoprecipitation with antiinsulin receptor antibodies (data not shown). In addition to the β-subunit, a 175K phosphoprotein was observed after insulin stimulation (Fig. 2, lane d). This protein was distinct from the insulin receptor, as it was extracted from the cytosol fraction of the 3T3/HIR cells, whereas the insulin receptor was found only after whole cell solubilization with Triton X-100 (data not shown); moreover, the tryptic phosphopeptide map was unique, indicating that it was not a precursor of the insulin receptor (data not shown). The characteristics of this protein correspond to those of pp185,

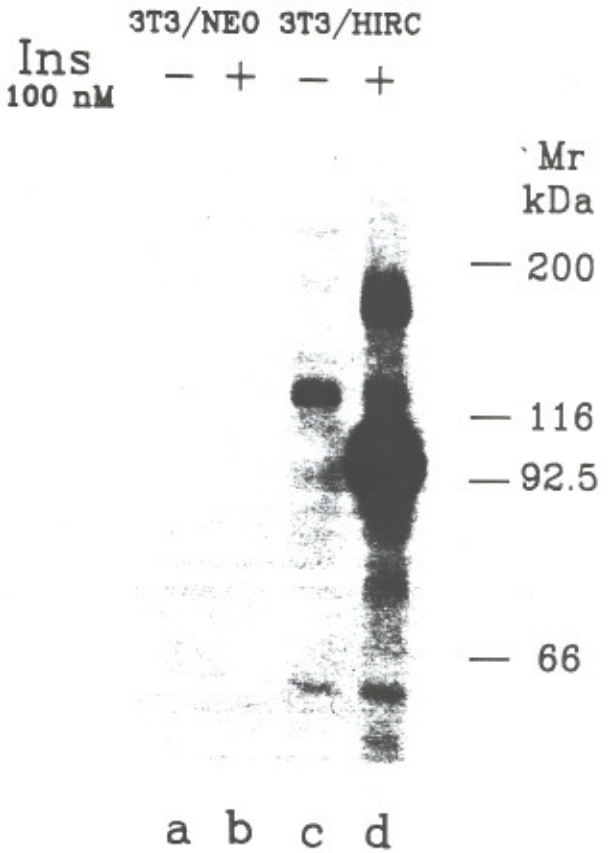

FIG. 2. Immunoprecipitation of phosphotyrosine-containing proteins from [^{32}P]phosphate-labeled NIH3T3 cells. Control 3T3/HIR cells (lanes c and d) were labeled for 2 h with [^{32}P]orthophosphate. The cells were incubated without (−) or with 100 nM insulin (+; Ins) for 2 min and solubilized as described in *Experimental procedures*. The phosphotyrosine-containing proteins were immunoprecipitated with αPY, reduced with dithiothreitol, and separated by SDS-PAGE. The autoradiogram was obtained during a 6-h exposure. Mr, Mol wt.

a putative cytoplasmic substrate for the insulin receptor kinase characterized originally in Fao hepatoma cells (8, 15). Thus, the human insulin receptor in NIH/3T3 cells recognizes the murine pp185 and catalyzes its tyrosyl phosphorylation during insulin stimulation.

Sensitivity to insulin for regulation of acute metabolic and long term growth effects

An early metabolic response of target cells to insulin is an increase in the rate of glucose transport. Uptake of 2-deoxy-[^{14}C]glucose was, therefore, measured in 3T3/HIR and 3T3/NEO cells. The 3T3/NEO cells did not respond to insulin in concentrations up to 10 nM, although a slight stimulation of 8–12% was seen with 100 nM insulin (Fig. 3) (our unpublished data). In contrast, half-maximal stimulation of deoxyglucose uptake occurred in 3T3/HIR cells at 0.1 nM insulin, with maximal stimulation (50%) occurring at 1 nM (Fig. 3). Insulin stimulation declined at insulin concentrations of 10–1000 nM (Fig. 3, and data not shown). The basal level of glucose transport was elevated 65% in 3T3/HIR cells compared to 3T3/NEO cells even after normalization with respect to cell number (8×10^5 3T3/HIR cells *vs.* 1.5×10^5 3T3/NEO cells/well). Of further interest was the observation that 3T3/NEO cells showed some response to IGF-I for stimulation of glucose transport, and the cells became even more sensitive and responsive to IGF-I after insulin receptor transfection, *i.e.* in 3T3/HIR cells (see Footnote 1).

Another acute action of insulin is the stimulation of glycogen synthesis. Although glycogen synthase is mainly associated with liver and muscle, all cells possess this enzyme (16). Glycogen synthesis, however, was not stimulated by insulin in 3T3/NEO cells (Fig. 4). By contrast, insulin stimulated glycogen synthesis in 3T3/HIR cells, with a half-maximal response at 0.01 nM; maximal stimulation was observed with 0.1 nM insulin (Fig. 4). The level of insulin stimulation declined at insulin concentrations above 1 nM. In some experiments the basal incorporation of [^{14}C]glucose into glycogen was elevated in 3T3/HIR cells compared to that in 3T3/NEO cells.

Insulin stimulates the influx of neutral amino acids into a variety of mammalian cells through transport system A (17). The stimulation by insulin of amino acid uptake in cells represents an intermediate effect, as it requires several hours for onset and is dependent on the synthesis of mRNA and protein (18). The effect of insulin on system A activity was studied by measuring uptake of the alanine analog MeAIB. There was no stimulatory effect of insulin on [Me-^{3}H]AIB uptake in 3T3/NEO cells (Fig. 5). In contrast, 0.1 nM insulin induced a detectable increase in [Me-^{3}H]AIB uptake, and maximal stimulation (46% above basal uptake) resulted from treatment with 10 nM insulin. When results were normalized for cell number, basal uptake of MeAIB was elevated in 3T3/HIR cells compared to that in 3T3/NEO cells.

Stimulation of DNA synthesis is a long term action of insulin, requiring 18–24 h for onset. In 3T3/HIR cells, stimulated uptake and incorporation of [^{3}H]thymidine

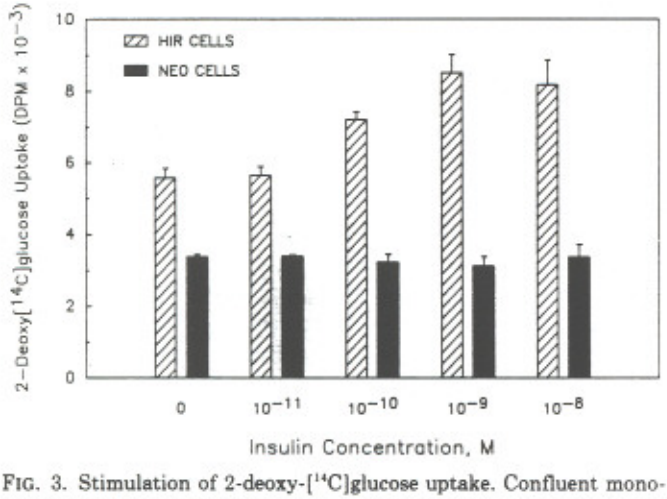

FIG. 3. Stimulation of 2-deoxy-[^{14}C]glucose uptake. Confluent monolayers of HIR or NEO cells were preincubated for 24 h at 37 C in serum-free medium. For assay, cells were incubated for 15 min at 37 C in the presence of the indicated concentrations of insulin. After rinsing, uptake of 2-deoxy-[^{14}C]glucose was determined as described in *Experimental procedures*. Data were normalized to uptake per 10^5 cells. Each value is the mean ± SD of triplicate points from a representative experiment from one of six similar experiments. Where error bars are not shown, deviation is too small to be graphically represented.

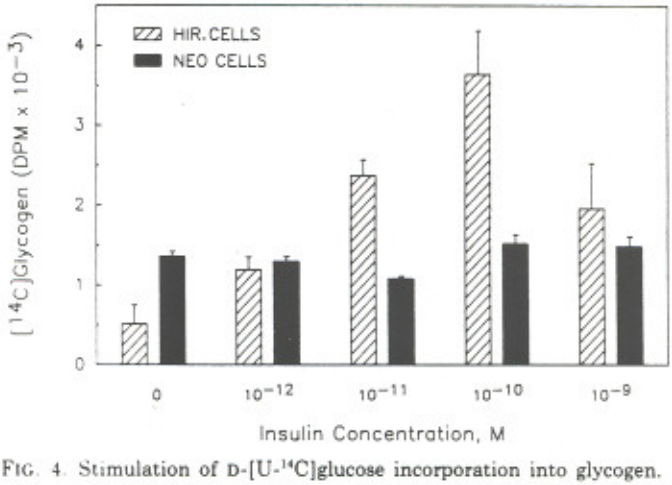

FIG. 4. Stimulation of D-[U-^{14}C]glucose incorporation into glycogen. Cell monolayers (HIR or NEO) were grown in glucose- and serum-free medium for 24 h before the assay. For assay, cells were incubated for 90 min at 37 C in glucose-free medium containing 1 μCi [^{14}C]glucose without or with insulin added at indicated concentrations. After incubation, monolayers were rinsed and solubilized, and glycogen was extracted and counted for incorporated radioactivity, as described in *Experimental procedures*. Data were normalized to incorporation per 10^5 cells. Each value is the mean ± SD of triplicate points from one of six such assays with similar results.

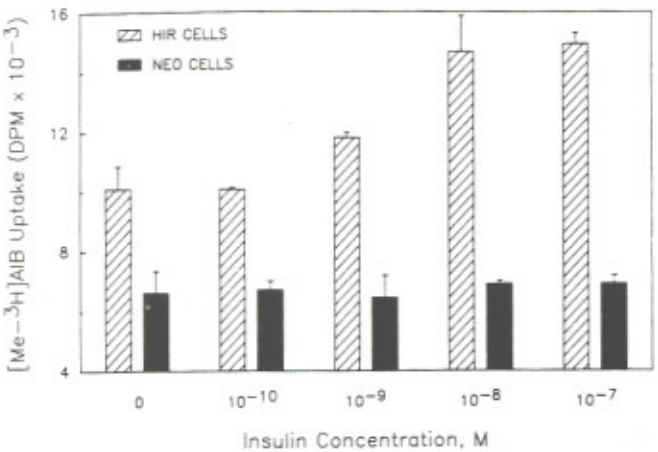

FIG. 5. Stimulation of [Me-^{3}H]AIB uptake. Confluent monolayers of HIR or NEO cells were preincubated for 24 h at 37 C in serum-free medium. Cells were rinsed and further incubated for 6 h in KRH, 20 mM glucose, and 1% BSA without or with insulin added at the indicated concentrations. After this incubation, uptake of [Me-^{3}H]AIB was measured as described in *Experimental procedures*. Data were normalized to uptake per 10^5 cells. *Data points* represent the mean ± SD for triplicate points. Where error bars are not shown, deviation is too small to be graphically represented. The experimental data reported is for one of six such assays with similar results.

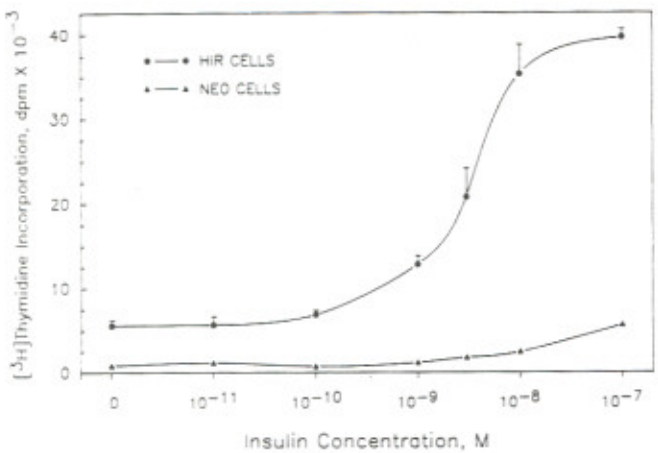

FIG. 6. Insulin stimulation of [^{3}H]thymidine incorporation into DNA. Before assay, confluent monolayers of HIR or NEO cells were cultured for 24 h at 37 C in serum-free medium. Cells were then further incubated for 18 h in medium without or with insulin added at the indicated concentrations. Monolayers were rinsed and incubated for 30 min at 37 C in medium containing 0.5 μCi [^{3}H]thymidine. Cells were then rinsed and solubilized, and [^{3}H]thymidine incorporated into TCA-precipitable material was measured, as described in *Experimental procedures*. Data were normalized to uptake per 10^5 cells. *Data points* represent the mean ± SD for triplicate points. This assay was performed six times with similar results.

into TCA-precipitable products were half-maximal at the insulin dose of 3 nM; maximal stimulation (7-fold over basal) required an insulin dose of 10 nM (Fig. 6). In 3T3/NEO cells, insulin elicited a slight dose-dependent stimulation of [^{3}H]thymidine incorporation. However, both basal and hormone-stimulated levels of thymidine incorporation were substantially lower in 3T3/NEO cells than in 3T3/HIR cells, *i.e.* values stimulated by high levels of insulin (100–1000 nM) were only about 15% the stimulated values in 3T3/HIR cells. 3T3/NEO cells also responded to IGF-I stimulation of thymidine incorporation, and both sensitivity and responsiveness to IGF-I were increased upon insulin receptor transfection, *i.e.* in 3T3/HIR cells (see Footnote 1).

Discussion

While binding of [^{125}I]insulin to receptors of human skin fibroblasts and continuously cultured fibroblast cell lines has been reported (19–21), estimates of insulin receptor numbers by Scatchard analysis methods (22) have been difficult due to relatively low levels of binding to these cells (20). Fibroblast cells were, however, reported to be sensitive to insulin stimulation in a broad range of responses, including glucose transport (23), glycogen synthesis (21), potassium uptake (24), induction of ornithine decarboxylase (25), aminoisobutyrate uptake (18, 26), poly-ADP ribosylation (26), and thymidine uptake and incorporation into DNA (19, 26). For these fibroblasts, insulin at high doses (100–1000 nM) was generally required to stimulate the responses maximally (19–21, 23–28), whereas insulin doses of 1–10 nM generally stimulated maximal responses in target liver or fat cells (29). Such findings suggest that reported insulin actions may occur at least in part via related IGF-I receptors (26). In our present study, 3T3/HIR cells responded maximally to insulin at 0.1 nM for glycogen synthesis, 1 nM for glucose transport, and 10 nM for MeAIB uptake as well as thymidine uptake and incorporation into DNA. Taken together, our results indicate that 3T3/HIR cells were at least 100 times more sensitive to insulin than other fibroblasts. Differing sensitivities and time courses for various cellular responses to insulin have been described (1) and may be due to differential branching of postreceptor pathways.

NIH3T3 mouse fibroblasts transfected with the plasmid pSV2Neo, but not with the insulin receptor cDNA, were relatively insensitive to insulin. Autophosphorylation of the receptor β-subunit and phosphorylation of the putative cytosolic substrate pp185 were not detected. Only slight increases in glucose, amino acid, and thymidine uptake could be elicited by insulin doses of 100 nM or greater. Such minimal responses to insulin at high doses may have been elicited via related IGF-I receptors or through a very low number of insulin receptors. If a low number of receptors is present, then there may exist either limited coupling between these receptors and cellular effectors or effector deficiencies. In contrast to NIH3T3 cells, Chinese hamster ovary cells or rat 1 fibroblasts were recently reported to have fewer than

3000 receptors/cell, but were fully responsive to insulin stimulation of 2-deoxyglucose uptake (30, 31), thus indicating that these few receptors were well coupled with the effector system. While the NIH3T3 cell receptor/effector system may be poorly coupled, the effector system itself appeared normal, since human insulin receptors expressed in these NIH3T3 cells exhibited hormone-sensitive receptor kinase activity, were coupled to an endocytic degradative pathway for insulin, and were also capable of mediating a full array of metabolic as well as growth responses to the hormone. It seems likely that minimal insulin responsiveness in 3T3/NEO cells, as in the parent NIH3T3 mouse fibroblasts, may be mediated via related IGF-I receptors, as previously suggested for insulin responses in human skin fibroblasts (26). Our preliminary findings indicated that 3T3/NEO cells displayed IGF-I receptors and responded to IGF-I stimulation of glucose transport and DNA synthesis. Another recent report also described binding of [^{125}I]IGF-I to Swiss mouse 3T3 fibroblasts and highly sensitive stimulation of DNA synthesis by this growth factor; less sensitive stimulation of DNA synthesis by insulin was suggested to occur via insulin receptors, although interaction of insulin with the IGF-I receptor could not be ruled out (32).

Overexpression of insulin receptors in NIH3T3 cells rendered the cells insulin sensitive and elicited responses of substantial magnitude. While increased sensitivity can be attributed to an increased receptor number, increased responsiveness suggests that postbinding events may also have been altered in these cells. The high expression of insulin receptors in 3T3/HIR cells may result in a different receptor functional state, *e.g.* more clustering and improved coupling with effectors. Alternatively, 3T3/HIR cells may become sensitive to insulin by maintaining synthesis of enzymes or other cellular components requisite for metabolic responses to the hormone. Such concepts are supported by the finding that basal levels of responses studied were generally elevated in 3T3/HIR cells compared to those in 3T3/NEO cells. Increased basal receptor phosphorylation, however, was not observed, indicating that elevated basal bioactivity may be mediated through a mechanism independent of insulin receptor tyrosine autophosphorylation. A 120K phosphotyrosine-containing protein was detected in 3T3/HIR cells, but not in 3T3/NEO cells. Although insulin does not stimulate the phosphorylation of this protein in 3T3/HIR cells or other cells (8), its phosphorylation may play a role in the maintenance of basal cellular metabolism and growth. A further possibility is that transformation with bovine papilloma virus construct alone can alter basal activity or induce insulin responsiveness. This appears unlikely, as NIH3T3 cells remained nonresponsive after expression of a bovine papilloma virus construct encoding only the binding domain (α-subunit) of the insulin receptor.[2]

Transmission of the insulin signal may occur in cells by tyrosine phosphorylation. After insulin binding to the receptor α-subunit (33), the β-subunit of the receptor undergoes autophosphorylation of tyrosine residues, which activates a tyrosine-specific phosphotransferase (34, 35). Although some details of the enzyme activation are understood (36), the subsequent steps in the mechanism of insulin action are poorly described. One pathway may involve the phosphorylation of cellular substrates on tyrosine residues by the insulin receptor (8, 15, 37), and a growing list of criteria suggests that pp185 may be an important substrate (8, 15). Its phosphorylation occurs immediately after insulin stimulation in all cell types studied so far, and it appears to be a substrate for the IGF-I receptor (38), but not the epidermal growth factor receptor (39). In contrast to the insulin receptor, the pp185 is probably located in the cytosol (8). The phosphorylation of cytosolic pp185 increases significantly in 3T3/HIR cells, which express high levels of insulin receptor, thus suggesting that phosphorylation of pp185 is closely related to the kinase activity of the receptor. Similar results have been found for Chinese hamster ovary cells (8). Although the 3T3/HIR cells express over 10^6 insulin receptors/cell, other phosphotyrosine-containing proteins were not detected with the αPY. Thus, pp185 appears to be an insulin receptor substrate found in all cells studied so far.

In summary, we demonstrated that expression of high levels of insulin receptors in NIH3T3 cells produced insulin sensitivity and enhanced responsiveness in a cell type that was normally only minimally sensitive and responsive to insulin. Such findings indicated that effector molecules for insulin responses were present in these insulin-insensitive cells, and that expressed receptors readily coupled with these effector systems to become fully functional. It seems possible that such effector systems may in fact be those already in place for related IGF-I receptors, since insulin and IGF-I stimulate a similar array of biological responses (40–42), display a high degree of structural homology in the intracellular kinase domains of their respective receptors (43), and share a common intracellular substrate for tyrosine phosphorylation by the receptor kinases (8, 38, 39). Further exploration is needed to clarify possible receptor and postreceptor overlap for insulin and IGF-I.

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[2]Whittaker, J., unpublished results.

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