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Stimulation of glucose incorporation and amino acid transport by insulin and an insulin-like growth factor in fibroblasts with defective insulin receptors cultured from a patient with leprechaunism

(multiplication-stimulating activity)

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ABSTRACT **Fibroblasts cultured from an infant with leprechaunism and insulin resistance have been reported to exhibit a profound, selective defect in insulin binding. We now examine the effect of this defect on two acute metabolic actions of insulin thought to be mediated by the insulin receptor, glucose incorporation and *N*-methyl-α-aminoisobutyric acid (Me-AiBu) uptake. In the patient's fibroblasts, maximal insulin-stimulated glucose incorporation was <25% of that in control fibroblasts, whereas stimulation by hydrogen peroxide, an insulinomimetic agent that acts distal to the insulin receptor, was normal. By contrast, insulin stimulated Me-AiBu uptake to the same extent in patient's and control fibroblasts. Impaired glucose incorporation and relatively normal Me-AiBu uptake also were observed in the patient's cells with multiplication-stimulating activity, an insulin-like growth factor, despite the fact that multiplication-stimulating activity appeared to stimulate both responses in normal fibroblasts via an insulin-like growth factor receptor. The divergent effects on two hormone-stimulated functions in the patient's cells suggest differences in the coupling of a receptor to different effectors. The same coupling mechanisms appear to be used by insulin receptors and receptors for insulin-like growth factors.**

Leprechaunism is a rare genetic disorder characterized by low birth weight, unusual facies, hirsutism, poor growth, and death at an early age (reviewed in ref. 1). Tissue resistance to insulin may play a prominent role in the pathophysiology of this disorder because hyperinsulinemia, resistance to exogenous insulin, and beta-cell hyperplasia are commonly observed (1–6). The basis of this insulin resistance appears to be heterogeneous. Defects have been described that occur distal to the insulin receptor (2, 3) or that affect insulin receptors on circulating erythrocytes (4) or cultured cells (5, 6).

A profound and selective abnormality of insulin binding to insulin receptors in fibroblasts cultured from an infant with characteristic features of leprechaunism has been reported (1, 5). The patient's fibroblasts exhibited <20% as much ^{125}I-labeled insulin binding per cell as did control fibroblasts (5). The present study examines the effect of this insulin receptor defect on two acute metabolic effects of insulin in fibroblast cultures thought to be mediated by insulin receptors: [^{14}C]glucose incorporation (7) and *N*-[^{14}C]methyl-α-aminoisobutyric acid (Me-AiBu) uptake (8, 9). We also have examined the ability of an insulin-like growth factor (IGF), multiplication-stimulating activity (MSA) (10), to stimulate these biological functions. A preliminary account of these results has been presented (11).

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MATERIALS AND METHODS

Fibroblast Cultures. Fibroblast cultures from the leprechaunism patient (1, 5) and a normal male control of the same age (5) were established and propagated as described (5). Handling of stock fibroblast cultures and subcultivation of cells for experiments were performed in parallel for patient's and control fibroblasts. Cultures from normal 21- to 22-year old female volunteers also were used as controls (12).

[^{14}C]Glucose Incorporation. Incorporation of D-[^{14}C]glucose into fibroblast cultures, reflecting both transport and metabolism, was studied by using the method of Howard *et al.* (7) with modifications. Fibroblasts (250,000 cells) were plated in 60-mm plastic dishes in growth medium (5) and incubated at 37°C in 95% air/5% CO_2. Cultures typically became confluent by day 5 and were used for experiments 2–7 days later. The monolayers were washed and preincubated with 3 ml of serum-free, glucose-free incubation medium (pH 7.4) (7) for 15–18 hr. The medium was replaced with 3 ml of incubation medium without bicarbonate but containing peptides. Incubation was continued for 3–4 hr at 37°C in the absence of CO_2. D-[^{14}C]Glucose (New England Nuclear; uniformly labeled, 345 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) and carrier D-glucose were added (final concentrations, 1 μCi/ml and 100 μM, respectively) for 20 min at 37°C. The cells were washed (7) and solubilized in 2 ml of 0.1% sodium dodecyl sulfate/0.5 mM EDTA, and aliquots of lysate were used for scintillation counting (≈50% efficiency) and protein determination (13). Experimental points were determined in triplicate. Alternatively, fibroblasts (50,000 cells per 16-mm well) were plated in 24-well Costar cluster plates (Cambridge, MA) and fed with growth medium on the next day. Preincubation and incubation volumes were 1.5 ml (or 0.5 ml).

Me-[^{14}C]AiBu Transport. Insulin stimulates transport of the nonmetabolizable amino acid analog Me-AiBu through the A transport system (14). Me-[^{14}C]AiBu uptake by fibroblast cultures was determined by using the method of Hollenberg and Cuatrecasas (8, 9) with minor modifications. Cells were plated (50,000 per 16-mm well) in Costar cluster plates, fed with growth medium on day 1, 2, or 3, and used 4–7 days after the last feeding (>2 days after confluency). Cultures were preincubated with Earle's balanced salt solution (GIBCO) supple-

Abbreviations: MSA, multiplication-stimulating activity; Me-AiBu, *N*-methyl-α-aminoisobutyric acid; IGF, insulin-like growth factor.

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mented with 25 mM sodium bicarbonate, 25 mM Tris·HCl, and 1 mg of bovine serum albumin per ml (pH 7.4) for 1–2 hr at 37°C in 95% air/5% CO_2. The medium was then aspirated, peptides were added to three replicate wells in 1.5 ml of either the same medium (without bicarbonate) or medium containing 25 mM Hepes (pH 7.60–7.65), and the mixture was incubated at 37°C in air for 2.5–4 hr. Me-[1-^{14}C]AiBu (New England Nuclear; 53.5 mCi/mmol; final concentration, 8 μM) was added for 12 min at 37°C. Uptake was terminated by aspiration and rapid washing with iced incubation buffer. Cells were processed as described above.

Materials. MSA (Sephadex G-75 peak II, M_r 8700) was purified from BRL 3A conditioned medium (15). Porcine zinc insulin (25 units/mg) was purchased from Eli Lilly; porcine proinsulin was from NOVO (Copenhagen, Denmark); bovine serum albumin (gamma-globulin-free for MeAiBu transport and fatty acid-free for glucose incorporation assays) were from Sigma.

RESULTS

Biological Effects of Insulin and Proinsulin in Normal Fibroblasts. Insulin stimulated [^{14}C]glucose incorporation and Me-[^{14}C]AiBu uptake in normal fibroblasts with a sensitivity and specificity appropriate for insulin receptor-mediated responses (Fig. 1). Maximal stimulation of glucose incorporation was 160 ± 10% of basal levels; maximal stimulation of Me-AiBu uptake was 230 ± 2% of basal. Half-maximal stimulation of both responses was observed with 3–3.5 ng of insulin per ml. Proinsulin stimulated both effects to the same extent as insulin but required 20- to 50-fold higher concentrations. Similar results have been reported (7–9, 16).

Stimulation of Glucose Incorporation by Insulin in Patient's and Control Fibroblasts. Insulin-stimulated glucose incorporation was compared in patient's and control fibroblasts in five paired experiments (Table 1; Fig. 2). Mean basal glucose incorporation was 10.3 ± 1.3 and 10.9 ± 1.1 nmol of glucose per mg of protein per 20 min for control and patient's fibroblasts, respectively. In control fibroblasts treated with insulin, the mean maximal incorporation was 170 ± 20% of basal incorporation. By contrast, the stimulation of glucose incorporation by insulin in patient's fibroblasts was greatly reduced: mean maximal incorporation was 112 ± 5% of basal levels. This stimulation was too low to allow precise comparison of the dose–response curve for insulin with that in control fibroblasts. Patient's fibroblasts did not inactivate insulin: insulin that had been incubated with patient's fibroblasts for 4 hr, recovered, and applied to control fibroblasts for an additional 4 hr stimulated glucose incorporation to 178% of basal levels (results not shown).

The decreased stimulation of glucose incorporation in the patient's fibroblasts did not result from a post-receptor defect in the effector pathway. Hydrogen peroxide, acting distal to the insulin receptor, mimicked the effects of insulin on glucose transport and glucose metabolism in adipocytes (17, 18) and stimulated [^{14}C]glucose incorporation in normal human fibroblasts (B. Howard, personal communication; Table 2). At maximally effective concentrations, H_2O_2 stimulated [^{14}C]glucose incorporation to a similar extent in patient's and control fibroblasts (Table 2).

Insulin-Stimulated Me-AiBu Uptake in Patient's and Control Fibroblasts. In contrast to glucose incorporation, insulin stimulation of Me-[^{14}C]AiBu uptake in patient's fibroblasts was not significantly different from that observed in control fibroblasts (Fig. 2 *Right*). Maximal stimulation of uptake was 230 ± 20% of basal levels in control and 270 ± 40% of basal in patient's fibroblasts in 11 paired experiments. The dose–response curves obtained with the patient's cells were shifted 4- to 5-fold to the right. In patient's fibroblasts, as in normal fibroblasts, proinsulin stimulated Me-AiBu uptake with approximately 5% the potency of insulin.

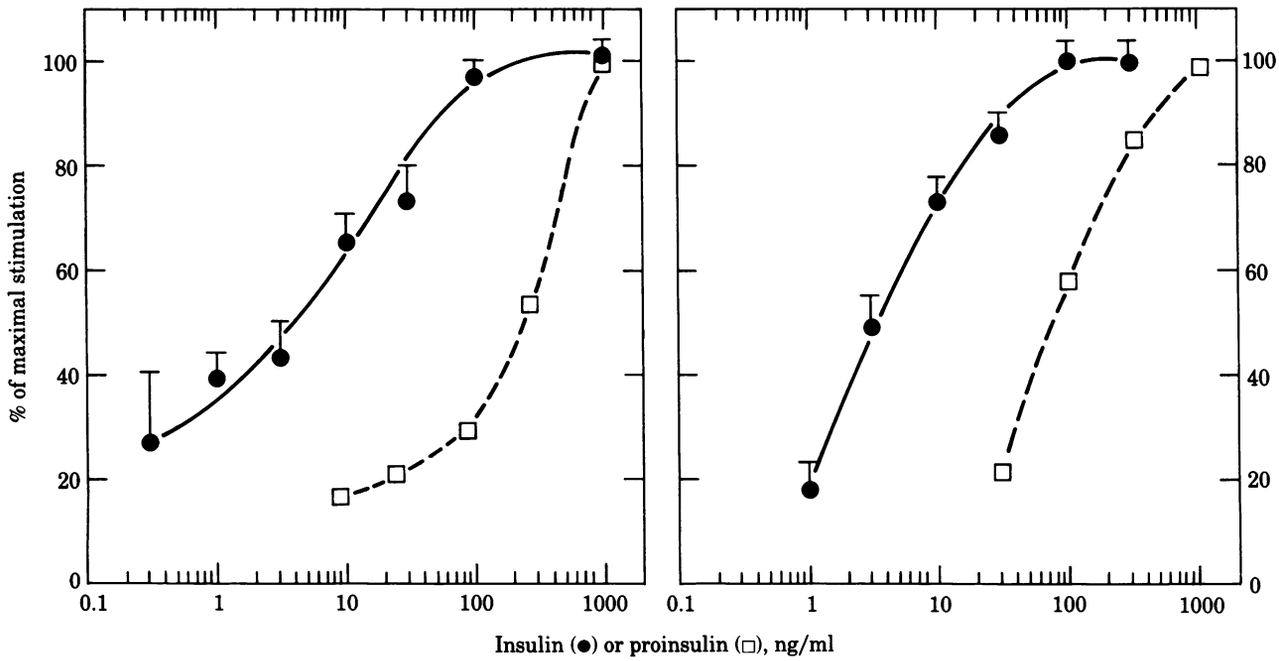

FIG. 1. Stimulation of [^{14}C]glucose incorporation and Me-[^{14}C]AiBu uptake by insulin and proinsulin in normal fibroblast cultures. (*Left*) Glucose incorporation by normal infant fibroblasts (n = 8) and normal adult fibroblasts (n = 4) plated in 60-mm dishes (n = 10; 390 ± 20 μg of cell protein) or 16-mm wells (n = 2). Incorporation at different insulin concentrations is plotted as percentage of maximal stimulation after subtraction of basal incorporation (mean ± SEM). Proinsulin was studied in a representative experiment in which insulin- and proinsulin-stimulated incorporation was 160% of basal. (*Right*) Me-AiBu uptake was measured in normal infant fibroblasts (n = 15) or normal adult fibroblasts (n = 15) with either Tris (n = 18) or Hepes buffer (n = 12). Wells contained 50–150 μg of protein in different experiments. Results are plotted as in *Left*. Proinsulin was studied in a representative experiment in which maximal incorporation stimulated by insulin and proinsulin were 230% and 260% of basal, respectively.

Table 1. Glucose incorporation in patient's and control fibroblasts

Experiment	Control Incorporation† No insulin	Control Incorporation† With insulin	Control Stimulation,‡ -fold	Patient Incorporation† No insulin	Patient Incorporation† With insulin	Patient Stimulation,‡ -fold	Relative stimulation,* %
1	7.9	16.4	2.07	10.7	13.3	1.24	22
2	10.1	18.9	1.87	11.3	13.7	1.21	24
3	14.8	18.5	1.25	9.6	9.3	1.00	0
4	10.5	14.5	1.38	14.9	14.6	1.00	0
5§	8.0	16.0	2.01	8.2	9.3	1.14	14
Mean	10.3	16.9	1.7	10.9	12.0	1.12	
±SEM	±1.3	±0.8	±0.2	±1.1	±1.1	±0.05	

The experiments are the same five paired experiments summarized in Fig. 2 *Left*. The insulin concentrations that gave maximal stimulation varied from 300 to 1000 ng/ml.

* Relative stimulation = [(patient fold stimulation − 1.00)/(control fold stimulation − 1.00)] × 100 for each paired experiment.

† Incorporation is shown as nmol of glucose per mg of protein per 20 min.

‡ Fold stimulation = with insulin/without insulin.

§ Performed in Costar cluster plate.

MSA-Stimulated Biological Functions. The IGFs are polypeptides, chemically similar to insulin, that can exhibit the same biological activities as insulin by interacting with either insulin receptors or IGF receptors (19, 20). MSA, one of the IGFs (20, 21), stimulated glucose incorporation and Me-AiBu uptake in control fibroblasts with high potency (Fig. 3), suggesting that it acted through an IGF receptor. Half-maximal responses were observed at 35 and 15 ng of MSA per ml, corresponding to 9% and 27% of the potency of insulin, respectively; MSA is only 0.2% as potent as insulin in its interaction with insulin receptors (20). Another IGF, somatomedin A, also appears to stimulate AiBu uptake in fibroblasts via an IGF receptor (22).

Surprisingly, the stimulation of glucose incorporation by MSA in the patient's fibroblasts was impaired. Mean maximal stimulation was 18% of control incorporation in five paired experiments (Fig. 3 *Left*) and never exceeded 30% of control. Inhibition was observed at MSA concentrations up to 3 μg/ml (data not shown). Normalized dose–response curves for MSA in the patient's and control fibroblasts were similar (data not shown), although precise comparison was not possible.

As with insulin, MSA-stimulated Me-AiBu uptake was relatively unaffected in the patient's fibroblasts: 245 ± 44% of basal incorporation for control fibroblasts and 332 ± 47% of basal in the patient's fibroblasts. The dose–response curve was shifted approximately 2-fold to the right.

DISCUSSION

We have examined two biological effects of insulin in fibroblasts cultured from a leprechaunism patient that exhibit a profound

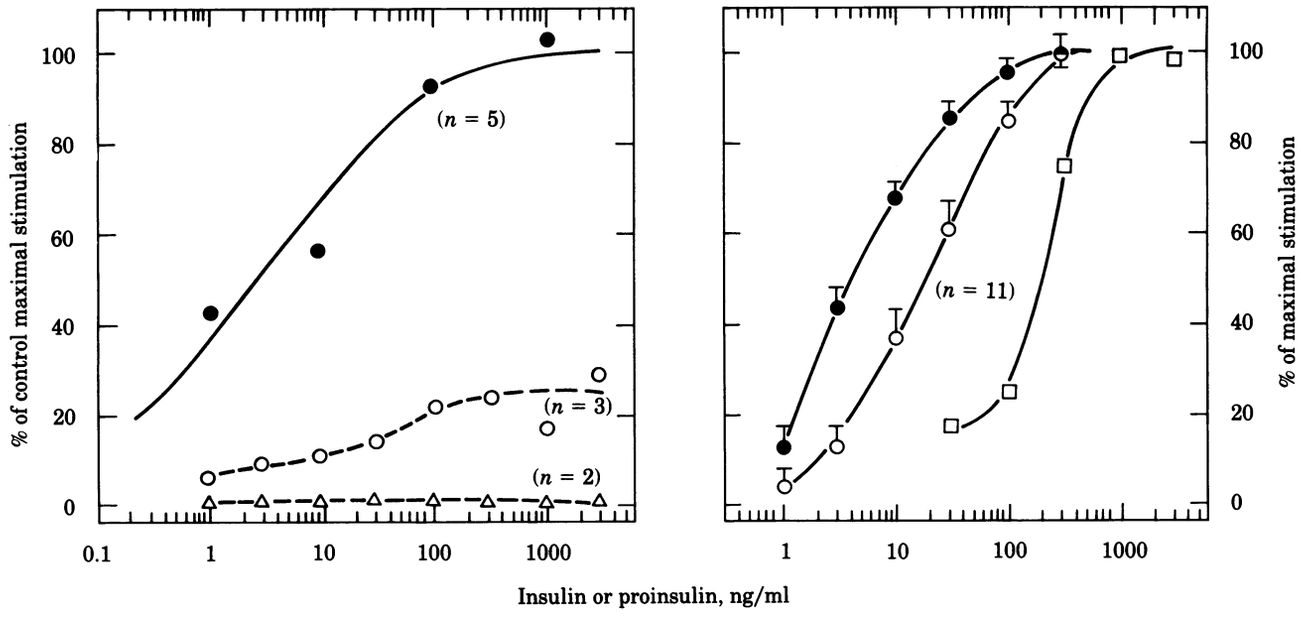

FIG. 2. Insulin-stimulated biological functions in patient's and control fibroblasts. (*Left*) The dose–response of glucose incorporation was studied in the five experiments with paired patient's and control fibroblasts shown in Table 1. The mean stimulation (above basal incorporation) by different concentrations of insulin in control fibroblasts (●) and patient's fibroblasts (△ in experiments 3 and 4; ○ in experiments 1, 2, and 5) is plotted as percentage of maximal stimulation in control fibroblasts in the same experiment. In experiments 1–4, the mean protein content per 60-mm dish was 397 μg for control and 270 μg for patient's fibroblasts. The control dose–response curve (solid line) is redrawn from Fig. 1. (*Right*) The dose–response curves of Me-AiBu uptake for 11 paired experiments of patient's and control fibroblasts. Mean protein content per well was 127 μg for control and 61 μg for patient. Basal incorporation was 0.78 ± 0.17 and 1.6 ± 0.41 nmol/mg of protein per 12 min in control and patient, respectively. The dose–response curves for control (●) and patient (○) are plotted as percentage of their own maximal uptake above basal. The dose–response curve for proinsulin in patient's cells (□) is from a representative experiment in which proinsulin and insulin stimulated uptake to 160% of basal levels.

Table 2. Effects of H_2O_2 on [^{14}C]glucose incorporation in control and patient's fibroblasts in paired experiments

Experiment		Incorporation, cpm/well		Stimulation,* -fold
		No H_2O_2	With H_2O_2	
1	Control	8,810	12,600	1.43
	Patient	3,890	6,920	1.78
2	Control	10,300	14,000	1.36
	Patient	4,160	6,660	1.60
3	Control	8,120	12,600	1.55
	Patient	4,130	6,150	1.49
4†	Control	7,730	18,940	2.45
	Patient	4,840	9,250	1.91

Fibroblasts were plated in Costar cluster plates, grown to confluency, and preincubated in glucose-free medium. Freshly diluted H_2O_2 at different concentrations and [^{14}C]glucose were added simultaneously. After 12 min, cells were washed with warmed phosphate-buffered saline containing 1% ethanol and freshly added 0.15 mM phloretin. Maximally effective H_2O_2 concentration varied from 6 to 12 mM. The dose–response curve was quite steep, as observed in adipocytes (17).

* Fold stimulation = with H_2O_2/ no H_2O_2.

† Conditions were identical to insulin-stimulation experiments. [^{14}C]Glucose and H_2O_2 were added simultaneously and incubated for 20 min. In this experiment, insulin (100 ng/ml) stimulated incorporation 1.60-fold in control fibroblasts but did not stimulate incorporation in patient's cells.

defect in insulin binding (1, 5): [^{14}C]glucose incorporation and Me-[^{14}C]AiBu uptake. In normal fibroblasts, the sensitivity to insulin ($ED_{50} \approx 0.5$ nM) and relative potency of proinsulin suggest that both biological responses are mediated by insulin receptors (20, 23).

Insulin-stimulated glucose incorporation was profoundly impaired in the patient's fibroblasts. In five paired experiments, stimulation by insulin in the patient's cells was <25% of the stimulation in control fibroblasts; in two of these experiments, no stimulation was detected. The decreased ability of the patient's cells to respond to insulin appeared to be specific to the insulin receptor rather than the effector pathway because the patient's fibroblasts responded normally to H_2O_2.

It is difficult to relate the insulin binding defect and the impairment in glucose incorporation quantitatively. Insulin binding to patient's fibroblasts is too low (5) to determine whether the decreased binding resulted from a change in receptor affinity or in number or in both. In addition, binding and biological experiments were performed under different experimental conditions. However, it is unlikely that the abnormal biological response resulted solely from a decreased affinity of the receptor for insulin because it was not overcome by a high concentration of insulin. A decrease in receptor concentration (if the binding reaction were limiting) or a post-receptor block would appear to be involved.

Surprisingly, the dramatic defect in insulin-stimulated glucose incorporation was not observed for Me-AiBu uptake. Instead, insulin stimulated Me-AiBu uptake to the same extent in patient's and control fibroblasts, although the dose–response curve was shifted 4- to 5-fold to the right. The potency of proinsulin relative to insulin in the patient's cells was the same as in control cells, suggesting a normal insulin receptor-mediated response.

The profoundly decreased insulin binding and the divergent effects on two presumably insulin receptor-mediated biological functions might arise from a general biochemical alteration,

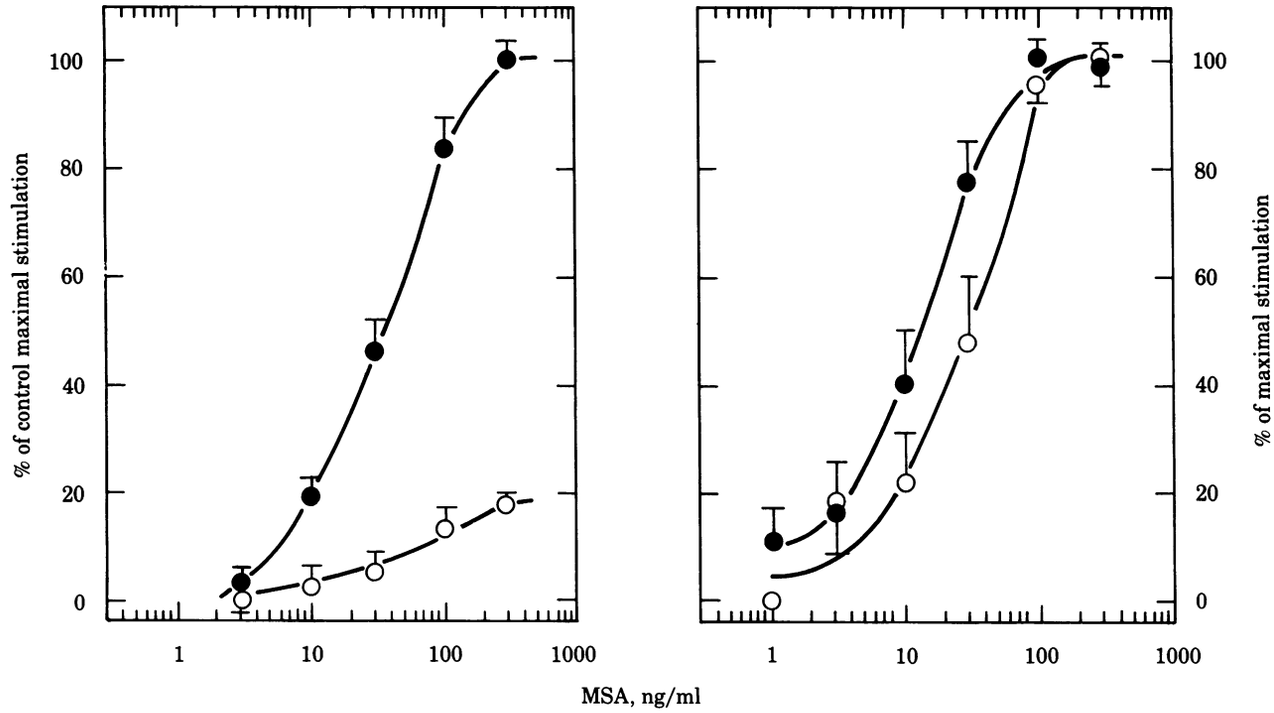

FIG. 3. MSA-stimulated biological functions in control and patient's fibroblasts. (*Left*) Dose–response curves for [^{14}C]glucose incorporation were studied in five paired experiments performed in 60-mm dishes. Basal incorporation was 8.9 ± 1.2 and 10.5 ± 1.6 nmol/mg of protein per 20 min in control and patient's fibroblasts, respectively. Mean maximally stimulated incorporation was 194 ± 17% of basal for control fibroblasts and 116 ± 4% for patient's cells. The mean stimulation by different concentrations of MSA in control fibroblasts (●) and patient's fibroblasts (○) are plotted as percentage of maximal stimulation in control fibroblasts in the same experiments. (*Right*) Dose–response curves for Me-AiBu uptake are shown for five paired experiments. Basal incorporation was 0.54 ± 0.23 nmol/mg of protein per 12 min for control fibroblasts and 0.60 ± 0.23 nmol/mg of protein per 12 min for patient's cells. The stimulation by different concentrations of MSA in control fibroblasts (●) and patient's fibroblasts (○) are plotted as percentage of their own maximal uptake after subtraction of basal uptake.

multiple separate defects, or a common molecular defect. Some possible mechanisms by which a single defect could generate the constellation of observed abnormalities will be considered. (*i*) Maximal stimulation of glucose incorporation might require greater fractional occupancy of insulin receptors than maximal stimulation of Me-AiBu uptake. The similar dose–response curves for binding (12, 24–26) and biological responses, however, argue against "spare" (23) insulin receptors. (*ii*) Insulin stimulation of Me-AiBu uptake might not be mediated by insulin receptors. (*iii*) Different populations of insulin receptors might be coupled to discrete biological functions, with those receptors linked to glucose incorporation being selectively impaired. The severity of the insulin binding defect is against this hypothesis. (*iv*) Recent evidence suggests that insulin receptors are complex structures consisting of a binding subunit and other (regulatory) proteins (27–30). Different components might form part of the binding site but be coupled to different specific biological functions. Those components that mediate glucose incorporation and form part of the insulin binding site would be most severely affected. Precedent for this mechanism occurs in the adenylate cyclase complex in which binding of GTP to the nucleotide regulatory protein affects both enzyme activity and hormone binding to the receptor (31). (*v*) The defects in the insulin receptor might differentially affect its ability to couple to different effectors (32, 33). (*vi*) The efficiency of coupling of the receptors to Me-AiBu effectors might be preferentially restored by a post-receptor compensatory event (direct or secondary to altered glucose metabolism). (*vii*) The defect in the insulin binding site might impair the ability of the receptors to aggregate. Aggregation of insulin receptors might be required for stimulation of glucose incorporation (34) but not for Me-AiBu uptake.

MSA, an IGF, stimulated glucose incorporation and Me-AiBu uptake in control fibroblasts with a high potency, suggesting that it acted via an IGF receptor. Remarkably, MSA-stimulated biological functions and insulin-stimulated biological functions were affected similarly in the leprechaunism patient's fibroblasts. MSA-stimulated glucose incorporation was markedly decreased in magnitude; MSA-stimulated Me-AiBu uptake was normal in magnitude but slightly shifted in sensitivity. These results suggested that insulin receptors and IGF receptors are linked similarly to specific effector pathways.

The impaired biological response to MSA suggested that IGF receptors also might be affected in the patient's fibroblasts. In fact, binding of ^{125}I-labeled insulin-like growth factor I, one of the insulin-like growth factors, to patient's fibroblasts was decreased by 70–80% (35) as a result of an altered affinity for IGF-I (unpublished data). Because high concentrations of MSA do not stimulate glucose incorporation by the patient's fibroblasts, a post-binding site alteration (possibly involving the coupling components) also may be involved.

In summary, our results suggest that: (*i*) insulin receptors may be coupled differently to effectors of specific biological responses; (*ii*) IGF receptors are coupled to the same effectors in an analogous manner; and (*iii*) the genetic lesion in this patient with leprechaunism may affect a structure that is common to insulin and IGF receptor complexes and is specifically linked to glucose incorporation.

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