Impaired Activation of Phosphoinositide 3-Kinase by Insulin in Fibroblasts from Patients with Severe Insulin Resistance and Pseudoacromegaly

A Disorder Characterized by Selective Postreceptor Insulin Resistance

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Abstract

Some patients with severe insulin resistance develop pathological tissue growth reminiscent of acromegaly. Previous studies of such patients have suggested the presence of a selective postreceptor defect of insulin signaling, resulting in the impairment of metabolic but preservation of mitogenic signaling. As the activation of phosphoinositide 3-kinase (PI 3-kinase) is considered essential for insulin's metabolic signaling, we have examined insulin-stimulated PI 3-kinase activity in anti-insulin receptor substrate (IRS)-1 immunoprecipitates from cultured dermal fibroblasts obtained from pseudoacromegalic (PA) patients and controls. At a concentration of insulin (1 nM) similar to that seen in vivo in PA patients, the activation of IRS-1-associated PI 3-kinase was reduced markedly in fibroblasts from the PA patients $(32\pm7\%$ of the activity of normal controls, P < 0.01). Genetic and biochemical studies indicated that this impairment was not secondary to a defect in the structure, expression, or activation of the insulin receptor, IRS-1, or $p85\alpha$. Insulin stimulation of mitogenesis in PA fibroblasts, as determined by thymidine incorporation, was indistinguishable from controls, as was mitogen-activated protein kinase phosphorylation, confirming the integrity of insulin's mitogenic signaling pathways in this condition.

These findings support the existence of an intrinsic defect of postreceptor insulin signaling in the PA subtype of insulin resistance, which involves impairment of the activation of PI 3-kinase. The PA tissue growth seen in such patients is likely to result from severe in vivo hyperinsulinemia activating intact mitogenic signaling pathways emanating from the insulin receptor. (*J. Clin. Invest.* 1998. 101:1111– 1120.) Key words: insulin resistance • pseudoacromegaly • phosphoinositide 3-kinase • signal transduction • insulin receptor

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Introduction

Extreme insulin resistance is found in a number of rare familial disorders. To date, insulin receptor mutations have been found in the majority of patients studied with leprechaunism and the Rabson-Mendenhall syndrome (1-3). 10-20% of patients with the type A syndrome of insulin resistance have also been reported to have insulin receptor mutations, as have occasional patients with non-insulin-dependent diabetes (2, 4, 5). However, in the great majority of patients with various other clinical phenotypes of insulin resistance, including those with lipodystrophic syndromes, polycystic ovary disease, and syndrome X, the insulin receptor gene has been found to be normal (2). An unusual form of severe insulin resistance associated with pathological tissue growth reminiscent of acromegaly has been described by Flier et al. (6). In vivo studies of this patient suggested that there was severe resistance to insulin-mediated glucose disposal but that insulin's effects on amino acid metabolism were relatively preserved. In this patient's cultured dermal fibroblasts, insulin-stimulated mitogenesis and amino acid uptake were within normal limits, whereas insulin stimulation of glucose uptake was impaired. Although a heterozygous mutation in the insulin receptor β subunit (Val985Met) was found, this mutation was also present in a number of normal subjects, and functional studies of the mutant receptor revealed it to be indistinguishable from the wild-type receptor. It was concluded that there was evidence for a postreceptor defect in insulin signaling which selectively affected metabolic but not mitogenic actions of insulin (6). This was considered to provide a plausible mechanism for the pathological tissue growth in the subjects, with very high circulating insulin concentrations providing the stimulus to growth through an intact mitogenic signaling pathway. However, no information was provided regarding the precise site of disruption of insulin's metabolic signaling pathway in this patient.

The capacity to investigate defective insulin action has been greatly enhanced by recent advances in the understanding of the signaling mechanisms distal to the insulin receptor (7). A considerable body of evidence has accumulated which gives a central role to the activation of phosphoinositide 3-kinase (PI 3-kinase)¹ in the mediation of insulin signaling to metabolically relevant end-points such as the translocation of glucose transporters. Thus, for example, overexpression of a constitutively active form of PI 3-kinase is sufficient to promote GLUT4 translocation in rat adipocytes, whereas constitutive activation

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^{1.} *Abbreviations used in this paper:* IRS, insulin receptor substrate; MAP, mitogen-activated protein; PA, pseudoacromegaly; PI 3-kinase, phosphoinositide 3-kinase; SSCP, single-stranded conformation polymorphism; WGA, wheat germ agglutinin.

of the mitogen-activated protein (MAP) kinase cascade is inactive in this regard (8).

We now report the results of studies on three patients with extreme insulin resistance and pseudoacromegaly (PA) which provide firm evidence for a selective impairment of PI 3-kinase activation by insulin in this disorder, a defect that occurs despite the normal structure, expression, and function of the insulin receptor and its major substrate, IRS-1.

Methods

Subjects

Insulin-resistant PA patients. The clinical and biochemical characteristics of the three PA patients are described in Table I. All had marked hyperinsulinemia (or very high insulin requirement for treatment of diabetes), abnormal glucose tolerance, and acanthosis nigricans. In addition, all three had additional acromegaloid features, such as increasing hand and foot size during adult life and/or macroglossia. All subjects had undetectable plasma growth hormone on at least one occasion, either in a random sample or during an oral glucose tolerance test, and plasma IGF-1 concentrations were within normal agerelated limits in all three subjects.

Normal control subjects. Dermal fibroblasts from five healthy control subjects (three females and two males) with no personal or family history of diabetes and normal fasting plasma glucose and insulin concentrations were studied in parallel. See Table I for further details.

Subjects with insulin receptor mutations. Dermal fibroblasts from two patients with severe insulin resistance due to the presence of mutant insulin receptors were also included in some of the studies. Subject Lep was a patient with leprechaunism who was homozygous for the mutation Leu233Pro in the insulin receptor, which has been reported to result in severely impaired transport of the receptor to the plasma membrane (9). Subject Type A was a patient with the type A syndrome of insulin resistance who had a heterozygous mutation in the tyrosine kinase domain of the insulin receptor (Ala1134Thr) (10). These patients had no features of PA (see Table I for further details).

Methods

PI 3-kinase activity. PI 3-kinase activity was assayed using a protocol adapted from Jackson et al. (11). Briefly, cells were grown to confluence in 4-well plates (60 mm) and serum-starved overnight, before

treatment with 1 nM insulin for 5 min. Cells were then rinsed once with ice-cold PBS, lysed, precleared by centrifugation, and IRS-1 was immunoprecipitated using the anti–IRS-1 antibody, CT (1 in 200; a gift from K. Siddle, University of Cambridge, UK). PI 3-kinase activity in the immunoprecipitates was assayed by measuring phosphorylation of phosphatidylinositol. Experiments performed using NP-40 as a specific inhibitor confirmed that PI 3-kinase and not PI 4-kinase activity was being measured in these studies. Reaction products were separated using TLC and analyzed using a PhosphorImager (Fujix BAS 2000; Fuji Photo Film Co., Tokyo, Japan).

Insulin binding to EBV-transformed lymphocytes. EBV-transformed lymphocytes were pelleted by centrifugation at 1,100 g, washed once in binding buffer composed of 100 mM Hepes (pH 7.8), 120 mM NaCl, 1 mM EDTA, 15 mM sodium acetate, 1.2 mM MgSO₄, 10 mM glucose, 1% BSA (RIA grade), and resuspended at a concentration of 7×10^6 cells/ml in a final volume of 250 µl of binding buffer. ¹²⁵I-insulin (20,000 cpm) in 50 µl of binding buffer was added to each sample without or with the addition of cold insulin (10^{-10} – 10^{-5} M), and the final mixture was incubated in a shaking water bath at 16°C for 4 h. The cells were then pelleted by centrifugation at 1,100 g for 10 min and washed 3 times with PBS. ¹²⁵I-insulin bound to the cells was counted using a γ counter (model NE1600; Nuclear Enterprises Ltd., Edinburgh, UK).

Insulin receptor autophosphorylation. Wheat germ agglutinin (WGA)-purified receptors (3 μ g) were incubated in the absence or presence of 1 nM insulin for 18 h at 4°C in buffer A (50 mM Hepes [pH 7.6], 150 mM NaCl, 0.08% Triton X-100, and 0.1% BSA). Receptor phosphorylation was initiated by addition of MnCl₂ (4 mM) and $[\gamma^{-32}P]ATP$ (50 mM, 30 μ Ci/assay). After a 20-min incubation at room temperature, the reaction was terminated by addition of an equal volume of stop solution (50 mM NaF, 10 mM Na₄P₂O₇, 2 mM Na₃VO₄, 5 mM EDTA, and 5 mM ATP). Immunoprecipitation was performed for 18 h at 4°C using the anti-insulin receptor antibody Ros-1 (1 in 100; a gift from K. Siddle) and protein A-agarose, or the antiphosphotyrosine antibody P3300 (1:60; Sigma Chemical Co., Poole, Dorset, UK) and protein G-agarose, respectively. Immune complexes were washed four times in ice-cold wash buffer (50 mM Hepes [pH 7.4], 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM Na₃VO₄) and then resuspended in Laemmli sample buffer containing 100 mM DTT and subjected to SDS-PAGE followed by autoradiography. Autoradiographs were quantified using a densitometer (Chromoscan 3; Joyce Loebl, Gateshead, Tyne and Wear, UK).

Insulin receptor tyrosine kinase activity. WGA-purified receptors $(1.5 \ \mu g)$ were incubated for 18 h without or with insulin (1 nM or 1

Table I. Clinical Details of PA Patients (P1–P3), Normal Control Subjects (C1–C5), and Subjects with Insulin Receptor Mutations (Lep and Type A)

Subject	Sex	Age at presentation	Age at evaluation	Body mass index	Fasting plasma glucose	2 h Plasma glucose	Fasting plasma insulin	2 h Plasma insulin	Acanthosis nigricans	Acromegaloid features
		yr	yr	kg/m ²	mmol/liter	mmol/liter	pmol/liter	pmol/liter	Present/Absent	Present/Absent
P1	F	19	22	25	6.6	11.2	287	2200	Present	Present
P2	F	12	39	48	14.8	19.0	*	*	Present	Present
P3	М	5	15	47	6.3	14.2	6540	N/A	Present	Present
C1	М	N/A	27	21	4.5	N/A	45	N/A	Absent	Absent
C2	F	N/A	25	24	4.5	N/A	60	N/A	Absent	Absent
C3	F	N/A	39	22	5.6	N/A	69	N/A	Absent	Absent
C4	F	N/A	24	22	4.6	N/A	39	N/A	Absent	Absent
C5	М	N/A	27	26	5.8	N/A	49	N/A	Absent	Absent
Lep	М	Neonate	Neonate	N/A	0.8	15.0	868	N/A	N/A	Absent
Type A	F	15	20	N/A	4.9	12.2	536	5056	Present	Absent

N/A, data not available (or not applicable). 2 h Plasma glucose refers to the value of plasma glucose after the oral administration of 75 g of glucose. *Patient P2 had diabetes treated with 600 U insulin/d. μ M) at 4°C in buffer A (see above). The reaction was started by the addition of the copolymer poly(Glu-Tyr) (2.5 mg/ml), MgCl₂ (12 mM), MnCl₂ (4 mM), ATP (50 μ M), and 10 μ Ci/assay of [γ -³²P]ATP. After 30 min at room temperature, 25 μ l aliquots were spotted on squares of 3MM paper (Whatman International Ltd., Kent, UK). The papers were dried and washed overnight in 10% TCA with 10 mM sodium pyrophosphate. The retained radioactivity was counted using a liquid scintillation counter.

Quantitation of IRS-1 expression, insulin-stimulated tyrosine phosphorylation of IRS-1, and insulin-stimulated association of $p85\alpha$ with IRS-1. Confluent cultured fibroblasts from normal control subjects and PA patients were serum-starved overnight. For the studies of insulin-induced tyrosine phosphorylation of IRS-1, cells were stimulated with insulin (1 nM or 1 µM) for 5 min. Fibroblasts were washed with ice-cold PBS, lysed in lysis buffer (100 mM Tris-HCl [pH 7.6], 1% Triton X-100, 100 mM NaF, 1 mM Na₃VO₄, 10 mM Na₄P₂O₇, leupeptin [1 µg/ml], aprotinin [250 kallikrein inhibitor U/ml], pepstatin [1 µg/ml], antipain [1 µg/ml], benzamidine [2.5 mM], and PMSF [1 mM]) and clarified by centrifugation. Protein concentrations were estimated according to the method of Schaffner and Weissman (12). Lysates containing 1.9 mg of protein were immunoprecipitated with the anti-IRS-1 antiserum (1 in 200), and immune complexes were washed and resuspended in Laemmli sample buffer containing 100 mM DTT. Samples were then electrophoresed on 7.5% polyacrylamide-SDS gels, and proteins were transferred to Immobilon-P (Millipore Corp., Bedford, MA) or nitrocellulose (Schleicher & Schuell, Dassel, Germany) membranes by constant current (150 mA) for 2 h (13).

For quantification of IRS-1 and $p85\alpha$, membranes were blocked in blocking buffer for 8 h (5% nonfat dried milk in 50 mM Tris-HCl [pH 7.5], 0.15 M NaCl, and 0.01% sodium azide) followed by overnight incubation in blocking buffer containing anti–IRS-1 or antip85 α antisera (1 in 500), then 2 h incubation with ¹²⁵I–protein A (200,000 cpm/ml).

For the study of IRS-1 tyrosine phosphorylation, Immobilon-P membranes were blocked in blocking buffer containing 3% BSA overnight. Membranes were then incubated in blocking buffer containing antiphosphotyrosine antibody (4G10, 1 in 10,000; Upstate Biotechnology Inc., Lake Placid, NY) for 1 h followed by incubation with blocking buffer containing peroxidase-conjugated mouse immunoglobulins (1 in 10,000; Dakopatts A/S, Glostrup, Denmark). Proteins were visualized using enhanced chemiluminescence according to the manufacturer's protocol (Amersham International, Little Chalfont, Bucks, UK). Membranes were exposed to HyperFilm MP (Amersham International), and autoradiograms were quantified using a Chromoscan 3 densitometer.

MAP kinase phosphorylation. Confluent fibroblasts in 4-well plates (60 mm) were serum-starved overnight. Cells were stimulated with insulin at the appropriate concentration for 5 min then harvested as described above. Lysates containing 160 μ g protein were resolved by 10% SDS-PAGE, and proteins were transferred to Immobilon-P membrane. Membranes were probed essentially as described above. Phospho-specific p44/42 MAP kinase antibody (New England Biolabs Ltd., Hertfordshire, UK) was used at 1 in 500 dilution. Approximately 1 μ Ci of ¹²⁵I-labeled goat anti–rabbit antibody was used as secondary antibody (Calbiochem, La Jolla, CA). Results were visualized and quantified using a Fujix BAS 2000 PhosphorImager.

Thymidine incorporation into DNA in cultured dermal fibroblasts. Subconfluent fibroblasts in 6-well dishes were incubated in serum-free medium overnight. The indicated concentrations of insulin were then added for a further 16 h before the addition of 1 μ Ci of [³H]thymidine for 90 min. Cells were then washed three times with ice-cold PBS and solubilized in 0.03% SDS. TCA was added (final concentration 10% wt/vol), and the precipitate was collected by filtration onto glass fiber filters (Whatman International Ltd.) which were washed with 3 × 5 ml of 10% TCA and 1 × 5 ml of 95% ethanol before drying. Acid-precipitable radioactivity was then determined by liquid scintillation. Glycogen synthesis in cultured dermal fibroblasts. Confluent fibroblasts were incubated in serum-free low-glucose DME (1,000 mg/ liter of glucose) for 4 h. Insulin was added at the indicated concentrations for 30 min, and then 1 μ Ci of [¹⁴C]glucose was added to each well, and cells were incubated at 37°C for a further 90 min. Dishes were placed on ice and washed three times with PBS. Cells were solubilized in 0.03% SDS and transferred to 15-ml centrifuge tubes. Glycogen was precipitated by adding 100 μ l of carrier glycogen (20 mg/ ml) and boiling the tubes for 30 min before adding 3 vol of cold ethanol and shaking at 4°C overnight. Pellets were collected by centrifugation at 1,100 g and washed once with 70% ethanol. Radioactivity incorporated into glycogen was then determined by liquid scintillation.

Molecular scanning of genes encoding the insulin receptor, IRS-1, and the p85a subunit of PI 3-kinase. All exons and intron/exon boundaries (except for the highly GC-rich exon 1) encoding the insulin receptor were examined by PCR-single-stranded conformation polymorphism (SSCP) as described previously (2). The single exon encoding IRS-1 was examined using a set of overlapping primers, each amplifying 200-250 bp (14). For p85α SSCP, total RNA was extracted from both dermal fibroblasts and EBV-transformed lymphocytes derived from the subjects and reverse-transcribed into cDNA. p85a cDNA was amplified by PCR in 12 overlapping segments of ~ 250 bp using primer pairs specific to the human p85 α cDNA sequence. All radiolabeled amplified products were studied under two electrophoresis conditions with positive controls on each individual gel. Gels in which the positive control did not show a discernibly different migration pattern were repeated. The nucleotide sequence underlying each variant conformer was determined by direct sequencing of amplified PCR products as described (2).

Statistical analysis. For normally distributed data, comparisons were made using the unpaired Student's t test. For nonnormally distributed data, comparisons between subjects were made using the Wilcoxon rank sum test. All analyses were performed on an IBM-compatible PC using the C-Stat program.

Results

Insulin-stimulated PI 3-kinase activity in anti–IRS-1 immunoprecipitates from PA patients, normal controls, leprechaun and type A fibroblasts was compared in four independent experiments at an insulin concentration of 1 nM (Fig. 1 *A*). In two of the four experiments, PI 3-kinase activity in all three PA patients was lower than that seen in any of the controls. In the other two experiments, the results for one of the controls (C2) fell within the range of the PA patients. In each of the experiments, the mean activity of the PA patients was substantially lower than the mean control values. Combining the results of all four experiments, PA patients showed a stimulation of PI 3-kinase activity that was $32\pm7\%$ of that seen in control fibroblasts (P < 0.01) (Fig. 1, *B* and *C*).

As expected, minimal stimulation of PI 3-kinase by 1 nM insulin was seen in leprechaun or type A fibroblasts, confirming that at this concentration of insulin, PI 3-kinase activation is being mediated through the insulin rather than the IGF-1 receptor (Fig. 1 *A*). At 10 nM and 100 nM insulin, significant stimulation of PI 3-kinase activity was seen in fibroblasts from the patients with insulin receptor mutations, indicating that significant activation of IGF-1 receptors was occurring at these concentrations and that responses in control and PA subjects at these concentrations were not significantly different (data not shown). To determine whether the defect in PI 3-kinase activation was due to a more proximal defect in the insulin signaling cascade, functional and genetic studies of the insulin receptor and IRS-1 were undertaken.



Figure 1. Insulin-stimulated PI 3-kinase activity in anti-IRS-1 immunoprecipitates from cultured dermal fibroblasts. Insulin-stimulated (1 nM) PI 3-kinase activity in anti-IRS-1 immunoprecipiates was measured as described in Methods. Dermal fibroblasts from the three PA patients, normal controls, the leprechaun (Lep) and the type A insulin-resistant patient were examined in four separate experiments. All assays were performed in duplicate. PI 3-kinase activity is expressed as arbitrary phosphorimager units. (A)Results of individual subject responses in all four experiments. (B)Mean±SEM of response for each PA patient vs. controls vs. insulin receptor mutation patients. (C) Mean \pm SEM of response for PA patients as a group vs. controls vs. insulin receptor mutation patients. *P < 0.01 for comparison of PA patients vs. normal control fibroblasts. **P < 0.001for comparison of leprechaun plus type A vs. normal control fibroblasts (Wilcoxon rank sum test).

In cultured fibroblasts from normal control subjects and PA patients, specific insulin binding was very low (data not shown). To allow comparison of insulin binding between PA patients and normal control subjects, EBV-transformed lymphocytes were obtained from the three PA patients and four of the normal control subjects. The concentration of unlabeled

Figure 2. Insulin binding to EBV-transformed lymphocytes from normal control and PA subjects. EBV-transformed lymphocytes from controls (O) and PA patients (7 \times 10⁶ cells, \bullet) were incubated with tracer amounts of ¹²⁵I-insulin (20,000 cpm) either in the absence or presence of increasing concentrations of cold insulin. Specific binding is expressed as a percentage of that in the absence of unlabeled ligand (100%). Nonspecific binding in the presence of $10 \,\mu\text{M}$ insulin has been subtracted (< 5% of total binding). Data points are the means±SEM of triplicate determinations in three separate experiments for each of the PA patients and four normal control subjects.



A



Figure 3. Insulin receptor autophosphorylation. Receptors (3 µg) were semipurified from dermal fibroblasts from PA patients and controls by WGA chromatography and incubated in the absence (*white bars*) or presence (*black bars*) of insulin (1 nM) and $[\gamma^{-32}P]$ ATP. Insulin receptors were immunoprecipitated by (*A*) antiinsulin receptor antibody or (*B*) antiphosphotyrosine antibody and analyzed by SDS-PAGE followed by autoradiography and densitometry. *ns*, Nonsignificant.

insulin required for half-maximal inhibition of ¹²⁵I-insulin binding (IC₅₀) to EBV-transformed lymphocytes was determined from competition experiments (Fig. 2). The IC₅₀ value for inhibition of ¹²⁵I-insulin binding to EBV-transformed lymphocytes from PA patients and normal control subjects was indistinguishable ($\sim 2-5$ nM), and the total amount of labeled insulin bound in the absence of unlabeled insulin was also closely comparable (data not shown).



Figure 4. Tyrosine kinase activity of partially purified receptors. Receptors (1.5 µg) purified from cultured fibroblasts from either control subjects or PA patients were incubated in the presence of $[\gamma^{-32}P]$ ATP and with the artificial substrate poly(Glu-Tyr) (4:1) as described in Methods. After 30 min, 50 µl of aliquots were spotted on 3MM paper. The paper was washed extensively overnight, and the retained radioactivity was counted by liquid scintillation. The tyrosine kinase activity assays were performed in duplicate in each control (n = 5) and PA patient (n = 3). The activity is expressed as the mean±SEM of cpm incorporated × 10^{-3/}mg protein. ns, Nonsignificant (PA patients vs. controls at 1 nM or 1 µM insulin).

The autophosphorylation of insulin receptors, semipurified from dermal fibroblasts by WGA chromatography, in response to 1 nM insulin was comparable in PA patients and controls (Fig. 3, A and B). Similarly, the tyrosine kinase activity of semipurified insulin receptors, using poly(Glu-Tyr) as a substrate, was equivalent in PA subjects and controls (Fig. 4). Finally, although SSCP variants in the insulin receptor gene were detected in two of the three PA patients, these represented silent polymorphisms, and no missense or nonsense mutations were detected (Table II).

The expression of IRS-1 in cultured dermal fibroblasts was examined by immunoblotting of anti–IRS-1 immunoprecipitates (Fig. 5). Levels of IRS-1 protein were indistinguishable in controls and PA patients. To determine whether a major defect in IRS-1 tyrosine phosphorylation in response to insulin was present in PA fibroblasts, antiphosphotyrosine blotting of anti–IRS-1 immunoprecipitates obtained from cells stimulated with 1 μ M (Fig. 6) and 1 nM insulin (Fig. 7, *A* and *B*) was performed. Minimal basal tyrosine phosphorylation was detectable in any of the subjects studied. Although the extent of

Table II. Sequence Variants in the Insulin Receptor, IRS-1, and p85a Genes Detected by PCR-SSCP Direct Sequencing

Subject	Insulin receptor	IRS-1	p85α
P1	C-T at position +32 of intron 3' to exon 6 (het) Ala ⁵²³ GCG-GCA (het)	None	Tyr ²⁶¹ TAC-TAT (het)
P2	None	None	None
Р3	T-A at position -19 of intron 5' to exon 2 (het) A-C at position $+28$ of intron 3' to exon 7 Pro ⁶¹⁷ CCA-CCC (het) Phe ⁶⁴² TTC-TTT (het)	Arg ⁹⁰ GAC-GAT(het)	Ile ⁶³³ ATT-ATC (het)

No mutations altering amino acid sequence or splice sites were detected. het, Heterozygous.



Figure 5. IRS-1 expression in dermal fibroblasts. Cultured fibroblasts from normal controls and PA patients were serum-starved for 18 h before study. Cells were then lysed, and the same amount of total protein was subjected to immunoprecipitation using the anti-IRS-1 antibody CT. The immunoprecipitated fraction was solubilized, resolved on a 7.5% gel, transferred to a nitrocellulose membrane, and incubated with the same anti-IRS-1 antibody. 125 I-protein A was used for detection (as described in Methods). (A) IRS-1 protein expression in control subjects (C1-C5) and PA patient fibroblasts (P1-P3) in a representative experiment. (B) The level of IRS-1 immunoreactivity was quantified by densitometry of autoradiograms. Data are expressed as the mean values \pm SEM of normal controls (n = 5) and PA patients (n = 3). ns, Nonsignificant (PA patients vs. control cells).

tyrosine phosphorylation of IRS-1 varied widely between subjects in both control and PA groups, there was no evidence for a systematic reduction in IRS-1 phosphorylation at either insulin concentration studied. The association of the $p85\alpha$ regulatory subunit of PI 3-kinase with tyrosine-phosphorylated IRS-1 in response to stimulation with 1 nM insulin was studied using anti- $P85\alpha$ immunoblotting of anti-IRS-1 immunoprecipitates (Fig. 7, *C* and *D*). The amount of $p85\alpha$ associated with IRS-1



Figure 6. Insulin-stimulated tyrosine phosphorylation of IRS-1. Confluent cultures of dermal fibroblasts from PA patients (P1-P3) and normal controls (C1-C5) were serum-starved for 18 h before stimulation with insulin $(1 \mu M)$. After 3 min, a cell lysate was prepared, and the same amount of total protein was subjected to immunoprecipitation using the anti-IRS-1 antibody as in Fig. 5. The immunoprecipitated fraction was solubilized, resolved on a 7.5% gel, and transferred onto a nylon membrane. The membrane was blotted with an antiphosphotyrosine antibody. Enhanced chemiluminescence was used for detection as described in Methods. (A)Autoradiographic analysis of the tyrosine phosphorylation of IRS-1 protein in a representative experiment. The blot was exposed for 3 min. (B) Densitometric analysis of autoradiograms. The level of IRS-1





Figure 7. Insulin-stimulated tyrosine phosphorylation of IRS-1 at 1 nM insulin and association of the p85 α subunit of PI 3-kinase with IRS-1. Experiments were performed as described in Fig. 6, except that stimulation was with 1 nM insulin. Anti-IRS-1 immunoprecipitates were solubilized, resolved on a 7.5% gel, and transferred onto a nylon membrane. The membrane was divided, and one half was blotted with an antiphosphotyrosine antibody (A), while the other was blotted with an anti-p85 antibody (D). The degree of IRS-1 phosphorylation (B) and p85a association with IRS-1 (D) was quantified by densitometry (ex-

pressed in arbitrary units). The values represent the mean ± SEM of controls and PA patients. -, Unstimulated. +, 1 nM insulin. *ns*, Non-significant (PA patients vs. control cells).

in the PA subjects was similar to that seen in controls. The insulin-stimulated association of p110 α and p110 β in IRS-1 immunoprecipitates was also similar between PA subjects and controls (data not shown). Finally, the entire coding regions of the IRS-1 and p85 α genes were examined by PCR-SSCP in the PA patients. A single polymorphism was identified in the IRS-1 gene in one of the PA subjects, and two variants were detected in the p85 α gene in two of the PA subjects. Upon sequencing, these variants were found to represent silent polymorphisms (Table II).

To examine whether the original observations of Flier et al. (6) relating to the preservation of normal insulin-stimulated mitogenesis and reduction in insulin-stimulated glucose transport in cultured dermal fibroblasts from a patient with PA insulin resistance also pertained to our subjects, further studies were undertaken. Insulin-stimulated mitogenesis was indistinguishable in PA versus control fibroblasts across a range of insulin concentrations (Fig. 8), whereas this was severely impaired in the cells from the leprechaun patient. Consistent with these findings, insulin-stimulated MAP kinase activation, as assessed by anti-phospho-MAP kinase blotting, was indistinguishable between controls and PA fibroblasts (Fig. 9). In contrast to the small stimulation of glucose transport demonstrated in normal fibroblasts by Flier et al. (6), in nine independent experiments, consistent insulin stimulation of 2-deoxyglucose uptake could not be demonstrated in normal control fibroblasts (data not shown). Therefore, it was not possible to assess whether this was abnormal in PA patient fibroblasts. No differences were seen in insulin-stimulated glycogen synthesis between PA and normal control subjects, whereas in leprechaun fibroblasts, a modest stimulation of glycogen synthesis was apparent only at higher insulin concentrations (Fig. 10).

Discussion

The occurrence of acromegaloid manifestations is an unexplained phenomenon seen in a subset of patients with extreme insulin resistance. An improved understanding of the molecular basis for the pathological tissue growth seen in these rare



Figure 8. Insulin-stimulated DNA synthesis in dermal fibroblasts. Dermal fibroblasts from controls (\bigcirc) , PA patients (●), and the leprechaun subject (\blacksquare) were incubated for 16 h with insulin before measurement of $[^{3}H]$ thymidine incorporation as described in Methods. The values represent the mean±SEM of three to five separate experiments carried out in triplicate.



Figure 9. Insulin-stimulated MAP kinase phosphorylation in dermal fibroblasts. Confluent cultures of dermal fibroblasts from PA patients and normal controls were serum-starved overnight before stimulation with insulin for 5 min at the indicated concentrations. After SDS-PAGE, proteins were transferred onto a polyvinylidene difluoride membrane and probed with phospho-specific MAP kinase antibody and ¹²⁵I-labeled secondary antibody. (*A*) Representative blots showing dose response of MAPK phosphorylation in control and PA fibroblasts. (*B*) Quantitation of results using a PhosphorImager. The values represent the mean±SEM of controls (*white bars, n = 5*) and PA patients (*black bars, n = 3*) from three separate experiments. *ns*, Nonsignificant.

patients may provide insights into more common associations of hyperinsulinemia with abnormalities of tissue growth and remodeling such as are seen in insulin resistance-associated polycystic ovarian change and in the atherosclerosis associated with syndrome X. One hypothesis that has been suggested as an explanation for the nonmetabolic clinical features of insulin resistance is that at pathologically elevated plasma insulin concentrations, promiscuous activation of the IGF-1 receptor is likely to occur (15). This might be expected to transduce a greater "mitogenic" signal to target tissues. However, contrary to this mechanistic explanation, at the equivalently elevated (or even greater) levels of plasma insulin seen in patients with insulin receptor mutations, no such PA tissue changes are observed, despite the integrity of IGF-1 receptor signaling pathways in such patients. Thus, it appears unlikely that promiscuous activation of IGF-1 receptors is the mechanism of PA tissue growth in insulin-resistant states.

The observation by Flier et al. (6) that cultured dermal fibroblasts from a severely insulin-resistant PA patient showed a normal mitogenic response to insulin but were impaired in their glucose uptake in response to insulin suggested the possible existence of a selective form of insulin resistance, presumably occurring at a postreceptor level. However, no studies of insulin signal transduction pathways in patients with this phenotype have been reported to date.

PI 3-kinase is an attractive candidate for the site of a defect that preferentially impairs insulin signaling to metabolic rather than mitogenic events. Upon insulin stimulation, IRS-1 is phosphorylated on multiple tyrosine residues in YXXM motifs (16), several of which have been shown to bind to the SH2 domain of the p85 regulatory subunit of PI 3-kinase (17). This binding appears both to induce the activation of the p110 catalytic subunit of PI 3-kinase (18) and also possibly to target the active enzyme to membrane-bound lipid substrate (19). Inhibition of PI 3-kinase activity, either using chemical inhibitors or dominant-negative PI 3-kinase constructs, severely impairs insulin-stimulated glucose transport in adipocytes and muscle (20-23). The role of PI 3-kinase in insulin's stimulation of mitogenic events is less clear, although there is an increasing body of evidence which suggests that it may be of importance. Inhibition of PI 3-kinase, by addition of the inhibitor LY294002 or antibodies directed against the amino-terminal SH2 domain of p85, blocks insulin stimulation of DNA synthesis in 3T3-L1 adipocytes (22) and in Rat 1a fibroblasts, respectively (24). However, in 32D cells which lack endogenous IRS-1, expression of a mutant IRS-1 lacking all PI 3-kinase binding sites was able to at least partially restore insulin-stimulated mitogenesis (25). Notwithstanding, an endogenous defect of insulin-stimulated PI 3-kinase activation might be expected to lead to a more severe defect in insulin's stimulation of glucose transport (the rate-limiting step for glucose disposal) than in insulinstimulated mitogenesis.

Therefore, we examined insulin-stimulated PI 3-kinase activity in anti–IRS-1 immunoprecipitates in cultured dermal fibroblasts from three insulin-resistant PA patients and compared this with normal controls and patients with known mutations in the insulin receptor. Although dermal fibroblasts are not an optimal model system in which to study insulin signaling, they have the advantage of surviving multiple passages in culture. This minimizes any secondary effects of the hyper-



Figure 10. Insulin-stimulated glycogen synthesis in dermal fibroblasts. Insulin-stimulated glycogen synthesis in dermal fibroblasts from controls (\bigcirc), PA patients (●), and the leprechaun subject (\blacksquare) was measured as described in Methods. Data represent the mean±SEM of three to five separate experiments carried out in triplicate.

insulinemic environment in the patient and should allow intrinsic defects of insulin signaling to be detected.

At 1 nM insulin (a concentration similar to that seen in vivo in these patients), there was a highly significant reduction in PI 3-kinase activity in the PA patients' cells, with their responses reduced by almost 70% compared with control fibroblasts. At higher insulin concentrations, significant stimulation of PI 3-kinase activity was seen in the patients with insulin receptor mutations, implying that activation in this setting was likely to be occurring through interaction of insulin with IGF-1 receptors. Therefore, further studies to elucidate the possible location of this defect were undertaken at 1 nM insulin.

Detailed functional and genetic studies of the insulin receptor and IRS-1 suggest that the impaired PI 3-kinase activity seen in the PA fibroblasts is unlikely to result from impairment of the structure, expression, or function of the insulin receptor or IRS-1. As our experimental system only permitted assessment of global tyrosine phosphorylation of IRS-1, it is of course possible that site-specific defects in the tyrosine phosphorylation of IRS-1 might be present. However, the fact that the insulin-stimulated association of the p85 α subunit of PI 3-kinase with IRS-1 was comparable in PA and control fibroblasts provides some assurance that the tyrosine phosphorylation of IRS-1 at the sites responsible for recruitment of PI 3-kinase was not impaired in the PA subjects. What possible molecular mechanisms might underlie such a defect? One obvious possibility is a genetic defect in the insulin-stimulated isoforms of the p85 and/or p110 subunits of the PI 3-kinase enzyme itself. At least three human isoforms of the p85 (26) and two of the p110 subunits (27) have been cloned, and there are, in addition, alternatively spliced versions of the p85 subunits (28). Screening of the p85 α gene indicates that mutations in the coding region of this gene are not responsible for the observed defective insulin-stimulated PI 3-kinase activity in the PA patients. Further studies of the genes encoding other p85 isoforms and p110 subunits are clearly a priority.

Our data rules out the likelihood that different amounts of $p85\alpha$ and $p110\alpha/\beta$ associated with IRS-1 are responsible for the observed differences in IRS-1–associated PI 3-kinase activity. However, possibilities other than a mutation in a catalytic subunit of PI 3-kinase may account for the defect seen in the PA patients' fibroblasts, as it has been shown that the catalytic activity of the p110 subunit is susceptible to inhibition. Such inhibition may be brought about by phosphorylation of a serine residue on the p85 subunit (29), or by the association of an inhibitory protein with the p110 subunit (30). It will be of interest to test whether these latter mechanisms of inhibition of PI 3-kinase activity could explain the defect of insulin-stimulated PI 3-kinase activity in PA patients' fibroblasts.

In the index case described previously (6), defective insulin-stimulated glucose transport was detected in the PA patients' fibroblasts. In contrast, despite multiple experiments and various manipulations of the test system, we could not demonstrate consistent stimulation of glucose transport by insulin in normal control fibroblasts. Therefore, we were unable to compare this activity in control versus PA patients' fibroblasts. As an alternative marker of insulin's metabolic signaling, we examined insulin-stimulated glycogen synthesis. Surprisingly perhaps, we were unable to detect any significant differences between the responses seen in the PA patients' fibroblasts and controls. In this regard, it should be pointed out that although the signaling pathways to glucose transport and glycogen synthesis are similar, they are not thought to be identical (31). It is also of note that at 1 nM insulin, the concentration at which the difference in PI 3-kinase activity was seen, the stimulation of glycogen synthesis is limited. At higher insulin concentrations, where the stimulation of glycogen synthesis is greater, PI 3-kinase activity was comparable in the PA patients' fibroblasts and controls. Thus, the inability to detect any difference in insulin-stimulated glycogen synthesis between the PA patients' fibroblasts and controls may be due to the relative insulin insensitivity of dermal fibroblasts. Whether this would also be the case in a more insulin-responsive tissue such as adipocytes or skeletal muscle remains to be established.

In summary, we have extended the observations on PA insulin resistance by studying a larger number of patients and by examining the insulin signal transduction pathways in detail. Although, unlike Flier et al. (6), we were unable to demonstrate a defect in insulin-stimulated glucose transport in cultured dermal fibroblasts, we did detect a novel abnormality of potential relevance to insulin's defective metabolic signaling, namely impaired activation of IRS-1–associated PI 3-kinase by insulin. As a considerable body of evidence supports the central role of PI 3-kinase in insulin's signaling to glucose transport in metabolically responsive tissue, this finding provides a plausible explanation for the severe in vivo insulin resistance seen in these patients.

Additionally, as we have confirmed the previous observation of preservation of normal insulin-stimulated mitogenesis in PA patients' fibroblasts, our findings provide support for the notion that the pathological tissue growth seen in these patients is explicable on the basis of a selective postreceptor defect in insulin signaling. Thus, the extreme hyperinsulinemia seen in vivo in these patients, occurring in compensation for the impaired metabolic signaling to glucose disposal, is likely to be the agent responsible for the stimulation of pathological tissue growth through the excessive activation of insulin signaling pathways that remain competent for mitogenesis.

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