Defect in Phosphorylation of Insulin Receptors in Cells from an Insulin-Resistant Patient with Normal Insulin Binding

Abstract. *Mononuclear blood cells were obtained from a patient with type A insulin resistance. The cells showed a normal ability to bind iodine 125–labeled insulin. Analysis of solubilized insulin receptors from the patient's cells revealed a defect in insulin-stimulated tyrosine kinase activity, which is closely associated with the receptor itself. The enzyme failed to phosphorylate the insulin receptor and showed a markedly reduced ability to phosphorylate exogenously added substrates. It appears that receptors from this insulin-resistant patient have a defect distal to the insulin-binding site (the α subunit of the receptor). The defect could be located in the β subunit, which has an adenosine triphosphate–binding site, or in another receptor component that transfers a signal of insulin binding into kinase activity. This dissociation between the normal binding and the defective protein kinase component of the insulin receptor represents the first biochemical defect of the receptor distal to ligand binding.*

The insulin receptor is an important locus for studying the nature of insulin resistance in animals and man. Defects in the binding component of the receptor have been the primary focus of study; other causes of insulin resistance include autoantibodies to the receptor, defects in receptor regulation, or structural genetic defects of the receptor itself (*1*).

We previously described a syndrome of insulin resistance (type A) in young, nonobese women characterized by hyperinsulinemia, polycystic ovaries, acanthosis nigricans, elevation of plasma testosterone, and android habitus (*2, 3*). Insulin binding in five of the seven patients has been below normal due to a reduced number of insulin receptors. In the two remaining patients, binding to both freshly isolated and cultured cells has been normal (*4*). Thus, type A insulin resistance is associated with low or normal insulin binding.

The insulin receptor is generally viewed as an integral glycoprotein of the plasma membrane consisting of disulfide-linked subunits in an α_2-β_2 configuration; the α subunit has an apparent molecular size of 130 kilodaltons (kD) and the β subunit 95 kD. Covalent cross-linking techniques have shown the α subunit to be the major insulin-binding component (*5*), while the β subunit exhibits adenosine triphosphate (ATP) binding activity (*6*) and is autophosphorylated (*7*). Thus the binding function of the receptor and the protein kinase activity of the receptor can be studied separately (*7, 8*).

In typical type A insulin resistance, in which insulin binding is markedly diminished, the number of insulin receptors is reduced and protein kinase–specific activity would be expected to be diminished. We studied a patient with insulin resistance (*9*) and other phenotypic features of the type A syndrome but with normal insulin binding, as measured in freshly isolated and cultured cells.

Solubilized, lectin-purified receptor preparations were extracted from circulating mononuclear cells from normal volunteers and the insulin-resistant patient. After phosphorylation of these preparations in the presence and absence of insulin ($10^{-7}M$), the phosphorylated proteins were immunoprecipitated with receptor antibody and identified on autoradiograms of a sodium dodecyl sulfate (SDS)–polyacrylamide gel. Insulin stimulated phosphorylation of the 95-kD (β) subunit of the insulin receptor in the cell extracts from normal subjects (Fig. 1, lanes C and D), as has been reported in other cell types (*8, 10, 11*). In contrast, when the same experiment was carried out with receptor preparations from the insulin-resistant patient, no phosphorylated bands were detected at 95 kD in the presence or absence of insulin (Fig. 1, lanes A and B). To measure the amount of ^{32}P incorporated into the bands at 95 kD, we excised this region of each lane of the gel and counted the radioactivity. While the radiation in lanes A and B was indistinguishable from background, insulin stimulated phosphorylation (by 61 percent) of the 95-kD subunit of receptors from the normal subjects (Fig. 1, lanes C and D).

To further evaluate the function of the receptor kinase, we assessed the ability of insulin to stimulate phosphorylation of added substrates. It was previously shown that the insulin-dependent kinase associated with the insulin receptor phosphorylates such exogenous substrates as casein, histone, and synthetic copolymers containing tyrosine residues (*12, 13*). The substrate used here contained glutamate and tyrosine residues in a 4:1 ratio (No. P-0275, Sigma). This property is useful when measuring the kinase activity independent of its receptor substrate.

Insulin ($10^{-7}M$) stimulated phosphorylation of casein and 4:1 glutamate and tyrosine 50 and 80 percent, respectively, when receptor preparations from normal subjects were used (Fig. 2). By contrast, both basal and insulin-stimulated phosphorylation of casein and 4:1 glutamate

Table 1. Binding of [^{125}I]insulin to various cells of the insulin-resistant patient. Binding was tested with a tracer concentration of the radioisotope (0.2 ng/ml) and various concentrations of unlabeled insulin (*17*). Specific binding is defined as the difference between total and nonspecific (done in the presence of 100 μg of insulin per milliliter) binding of labeled insulin. Insulin binding to the patient's cells was normal with respect to receptor concentration per cell, receptor affinity profile, and negative cooperativity. Experiments were performed on three occasions over 3 years. Results are means ± standard deviations for the three experiments. Binding of labeled insulin to the transformed lymphocytes was determined once; the normal range is 29 ± 15.6 percent (N = 8) in studies with the same tracer. Values in parentheses are percentages of paired control preparations run concurrently. Binding of insulin to solubilized, lectin-purified preparations of insulin receptors from mononuclear blood cells was carried out as described by Hedo *et al.* (*18*). Portions of receptor preparations equivalent to ~10 μg of protein were incubated with [^{125}I]insulin (40 to 60 pg) in the presence or absence of unlabeled insulin (100 μg/ml) for 15 hours at 4°C. Nonspecific binding represented 30 to 40 percent of total radioactivity bound. In a typical experiment, the ratio of absorbance at 280 nm to absorbance at 260 nm was 0.93 (identical for the patient and a normal subject), corresponding to about 0.75 mg of protein per milliliter.

Preparation	Specific binding of [^{125}I]insulin (percent)	
	Per 10^7 cells per milliliter	Per 0.1 unit of absorbance at 280 nm
Intact cells		
Fibroblasts (cultured)	6.2 ± 1.76 (106)	
Monocytes (fresh)	6.4 ± 1.48 (100)	
Erythrocytes (fresh)	5.3 ± 0.7 (100)	
Lymphocytes (EBV-transformed)	33.4 (100)	
Cell-free system		
Mononuclear blood cells		
Experiment 1		1.31 (99)
Experiment 2		0.33 (110)

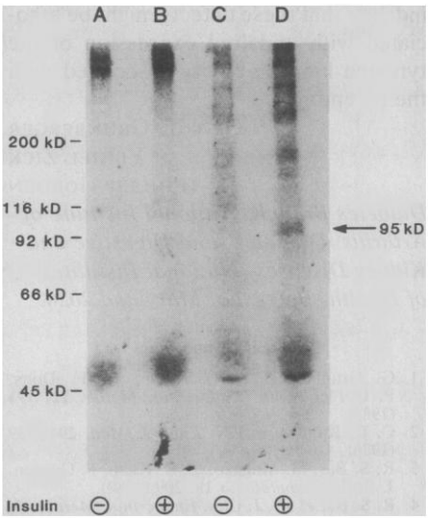

Fig. 1. Autoradiograph of an SDS–polyacrylamide gel of phosphorylated and immunoprecipitated receptor preparations from mononuclear cell extracts from the insulin-resistant patient and a normal subject. Lanes A and B show receptor preparations from the patient, incubated in the absence and presence of insulin, respectively; lanes C and D, receptor preparations from a normal subject, incubated without and with insulin, respectively. Blood (100 ml) was drawn and the mononuclear cell layer was harvested from a Ficoll-Hypaque gradient (*19*). Solubilized, lectin-purified insulin receptors were then prepared at 4°C as follows (*11*). Cells were homogenized in the presence of protease inhibitors, phenylmethylsulfonyl fluoride, and aprotinin and centrifuged at 550*g*, and the supernatant was centrifuged at 190,000*g* for 120 minutes. The pellet was solubilized by 1 percent Triton-X and centrifuged at 120,000*g* for 60 minutes. The supernatant was then applied on the wheat germ agglutinin column and eluted by 0.3*M* *N*-acetyl-D-glucosamine. Absorbance of the preparation at 280 nm was determined. Receptor preparations (400 μl) were first incubated for 30 minutes at 22°C in the absence or presence of 40 μl of insulin (final concentration, $10^{-7}M$). The final volume of 600 μl was made up by 50 m*M* Hepes buffer (*p*H 7.6). Phosphorylation reaction was initiated at 22°C by adding 150 μl of a solution containing 0.45 m*M* [γ-^{32}P]ATP (2.2 Ci/mmole; New England Nuclear), 25 m*M* manganese acetate, 25 μ*M* adenosine triphosphate, and 5 m*M* cytidine triphosphate and terminated 10 minutes later by adding 250 μl of "stopping" solution (*7*, *10*). Phosphorylated receptor preparations were incubated with 250 μl of protein A [Pansorbin, 20 percent (weight to volume); Calbiochem-Behring] for 16 hours at 4°C. The samples were centrifuged at 8000*g* for 5 minutes, the supernatant was reincubated with serum containing antibody to the insulin receptor (final dilution, 1:200) for 16 hours at 4°C, and immune complexes were precipitated with 200 μl of protein A for 60 minutes at 4°C. The precipitates were washed, suspended in a solution containing β-mercaptoethanol (*10*), and boiled for 5 minutes. The supernatants were applied to 7.5 percent SDS–polyacrylamide gel (*8*). After electrophoresis, the gels were stained, destained, dried, and autoradiographed exactly as described by Zick *et al.* (*10*). The region corresponding to 95 kD was excised from each lane of the gel and the ^{32}P content counted (net counts per minute: lane A, 0; lane B, 0; lane C, 230; and lane D, 370). The 45-kD band in lanes A, B, C, and D is considered nonspecific and has not been further characterized. In similar experiments in which normal serum was used for immunoprecipitation, no phosphorylated bands were seen when the insulin receptor preparations from the patient's or a normal subject's cells were used.

and tyrosine were markedly decreased when receptors from the patient were used as the source of the kinase.

Although the patient's kinase preparation was also deficient in phosphorylating artificial substrates, we cannot exclude the possibility that the patient's cell extract was also defective as a substrate. Interestingly, the basal kinase activity was also diminished; we do not know whether this was due to a more generalized defect (such as a chronic lack of insulin), an inhibitor, or other factors.

The insulin receptor is presumably the principal source of insulin-dependent kinase activity. Therefore, we would expect to find decreased kinase activity in cells from an insulin-resistant patient with a diminished receptor concentration (*14*). The patient we studied showed normal insulin binding to both circulating and cultured cells (Table 1). In addition, binding of [^{125}I]insulin to the solubilized, wheat germ agglutinin–enriched receptor used in the phosphorylation experiments was normal (Table 1). Thus, diminution in the kinase activity cannot be ascribed to a decreased concentration of the receptor as defined by ligand binding parameters. It is not known whether the defect in the phosphorylation is related to a quantitative or a qualitative defect in the β subunit or its coupling to the α subunit.

The role of autophosphorylation of the β subunit in transducing insulin binding into insulin's biological effects has not been determined (*15*). If phosphorylation is physiologically important, one would expect to find defects in receptor phosphorylation in some cases of insulin resistance. We have described a biochemical defect in insulin receptors of peripheral blood mononuclear cells from a patient with normal insulin binding but defective protein kinase activity (*16*).

In many cases, cells from patients with various syndromes of insulin resistance have normal insulin binding and affinity. These syndromes are frequently attributed to postreceptor defects. Our findings

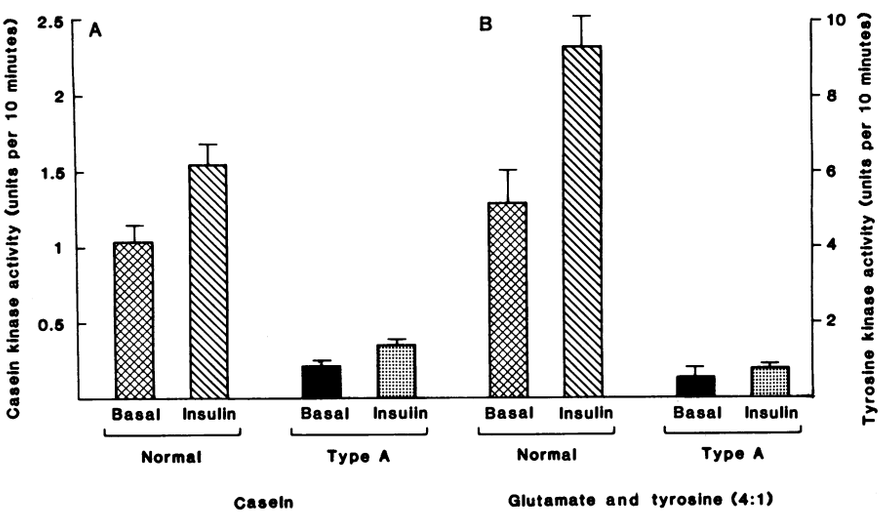

Fig. 2. Kinase activity of the solubilized insulin receptor from mononuclear blood cells. (A) Casein phosphorylation in the absence and presence of insulin ($10^{-7}M$), using receptor preparations from cells from a normal subject and the insulin-resistant patient. (B) Phosphorylation of the copolymer sodium glutamate and tyrosine (4:1) in the absence and presence of insulin. For the phosphorylation of exogenous substrates, the lectin-purified receptors (40 μl) were incubated with insulin (20 μl; final concentration $10^{-7}M$) or 50 m*M* Hepes buffer (20 μl; *p*H 7.6) for 30 minutes at 22°C. Substrates [casein or glutamate and tyrosine (10 mg/ml) in 40 μl of 50 m*M* Hepes (pH 7.6)] and $MgCl_2$ (20 μl; final concentration, 20 m*M*) were added and the phosphorylation reaction was initiated by adding 40 μl of a solution containing 0.45 m*M* [γ-^{32}P]ATP (2.2 Ci/mmole) and ATP (final concentration, 100 μ*M*). The reaction was terminated at 10 and 20 minutes by applying 75-μl portions to filter papers (Whatman No. 3) and soaking the papers in a bath of 10 percent (weight to volume) trichloroacetic acid and 0.01*M* sodium pyrophosphate. After being washed for 24 hours, radioactivity of the trichloroacetic acid–precipitable material was measured in a liquid scintillation counter. One unit of kinase was defined as the amount of enzyme catalyzing incorporation of 1 pmole of phosphate into 1 mg of artificial substrate in 10 minutes. Results are means ± standard errors for three separate experiments.

indicate that these defects might be associated with impaired expression of the tyrosine kinase activity associated with the receptor.

GEORGE GRUNBERGER
YEHIEL ZICK
PHILLIP GORDEN
Diabetes Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

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14. Using the erythrocyte insulin receptor, F. Grigorescu and C. R. Kahn [*Diabetes* **32** (Suppl. 1), 1A (1983)] showed that a patient with diminished [^{125}I]insulin binding has decreased receptor kinase activity. In addition, we have shown, in another patient with type A insulin resistance and very low [^{125}I]insulin binding, a marked decrease in insulin-stimulated phosphorylation of the β subunit.
15. It has been speculated that binding of insulin to the α subunit of its receptor leads to phosphorylation of the β subunit, which in turn initiates a cascade of events leading to the terminal action of the hormone [Y. Zick, R. W. Rees-Jones, J. Roth, in *Proceedings of the 11th Congress of the International Diabetes Federation* (Excerpta Medica, Amsterdam, 1982), p. 161; M. Kasuga, Y. Fujiti-Yamaguchi, D. L. Blithe, C. R. Kahn, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2137 (1983); O. M. Rosen, R. Herrera, Y. Olowe, L. M. Petruzzelli, M. H. Cobb, *ibid.*, p. 3237].
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20. We thank C. Culwell and J. R. Young for excellent secretarial assistance, J. Podskalny, B. Marcus-Samuels, and J. R. Young for binding studies, and S. I. Taylor and J. Roth for valuable discussions. Y.Z. is a Fulbright Scholar and a recipient of a Chaim Weizmann postdoctoral fellowship.

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Perfluorochemical Emulsions Can Increase Tumor Radiosensitivity

Abstract. *An oxygen-carrying perfluorochemical emulsion enhanced the effectiveness of radiation therapy in two transplantable solid tumors in mice. The perfluorochemical emulsion had no effect on tumor growth after x-irradiation, but delayed tumor growth significantly when administered to oxygen-breathing mice before or during irradiation.*

The cytotoxicity of ionizing radiation is modified by the presence of molecular oxygen (*1*). Hyperbaric oxygen treatment augments the therapeutic effect of radiation in animals and humans (*2, 3*), but is difficult to implement clinically (*3*). Although several chemicals, including nitroimidazole derivatives (*4*) and platinum complexes (*5*), sensitize hypoxic cells to cytotoxic agents, none has proved to be clinically useful (*6*).

In both normal and tumor tissue, oxygen tension is dependent on delivery through the vascular system and on utilization by tissues (*7*). Tumors often have inadequate vasculature and areas of intermittent and irregular blood flow because blood vessels in tumors can constrict and collapse (*8*). This leads to zones of necrosis and areas of hypoxia.

Radiotherapeutic efficacy may be limited by the radioresistance of hypoxic cells (*1*). Three times more radiation is required to kill fully anoxic cells than oxygenated cells. Perfluorochemicals dissolve large amounts of oxygen (*9*), and perfluorochemical emulsions are being tested as oxygen transport agents for use during surgery after hemorrhage or to minimize ischemic damage after stroke or myocardial infarction (*10*). The volume of oxygen dissolved in perfluorochemical emulsions changes linearly with the partial pressure of oxygen. Therefore, to fully exploit the oxygen-carrying capacity of these materials in vivo, high partial pressures of oxygen are used. As with hemoglobin, the uptake and release of oxygen from perfluorochemical emulsions are completely reversible, and the rate is twice as fast as that of hemoglobin. Ninety percent or more of the emulsion particles in the preparation that we used are less than 0.2 μm in diameter—much smaller than red blood cells (average diameter, 5 to 10 μm). We reasoned that these small oxygen-carrying particles would be able to deliver sufficient oxygen to hypoxic regions of solid tumors to significantly increase the radiosensitivity of these tumor regions. we report that a perfluorochemical emulsion and a 95 percent oxygen atmosphere markedly delayed the growth of two solid tumor lines exposed to x-rays in vivo. Using a tumor excision assay, we were able to measure the enhancement of cell killing in one of the lines.

Lewis lung tumor (*11*) was carried in C57BL/6J male mice (Jackson Laboratory). FSa-II fibrosarcoma (*12*), which had been adapted for growth in culture (FSa-IIC), was carried in C3H/Be/FeJ male mice (Jackson Laboratory). For the experiments 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted intramuscularly in the legs of mice. The Lewis lung tumor was grown in B6D2F1/J male mice (Jackson Laboratory) and the FSa-IIC fibrosarcoma was grown in C3H/Be/FeJ male mice 8 to 10 weeks of age. When the tumors were approximately 50 mm^3 in volume (about 1 week after tumor cell implantation), the perfluorochemical emulsion, Fluosol-DA (20 percent) (*13*), in volumes ranging from 0.2 to 0.5 ml, was injected into the tail vein. The animal was then allowed to breathe air or was placed in a circulating atmosphere of 95 percent oxygen and 5 percent carbon dioxide. One hour later the tumor-bearing limb was given a single dose of x-rays of 1000, 2000, or 3000 rads with a Gamma Cell 40 (Atomic Energy of Canada; dose rate, 88 rads per minute). The shielded portion of the animal received less than 2 percent of the delivered dose. Animals were anesthesized during the radiation treatment.

In experiments to determine the delay in tumor growth, the progress of each tumor was measured thrice weekly until it reached a volume of 500 mm^3. Untreated Lewis lung tumors reach 500 mm^3 in about 14 days and untreated FSa-IIC tumors reach 500 mm^3 in about 12 days. Statistical comparisons were made with the Dunn multiple comparisons test (*14*).

In experiments to determine the surviving fraction of FSa-IIC fibrosarcoma cells, tumors were excised 24 hours after x-ray treatment and single-cell suspen-