Enzyme-Linked Immunosorbent Assay Method for Human Autophosphorylated Insulin Receptor

Applicability to Insulin-Resistant States

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The insulin receptors from erythrocytes of 50 patients with non-insulin-dependent diabetes mellitus were tested for their ability to autophosphorylate. The assay was performed by a new enzyme-linked immunosorbent assay system that used monoclonal anti-insulin receptor antibodies absorbed to microtiter plates as a first antibody and polyclonal antiphosphotyrosine antibody as a labeled second antibody. By this assay, 3 patients were identified with defects in their insulin receptor kinase, although their defects appeared heterogeneous. Patient 1 had 85% less maximal autophosphorylation with a normal ED50 (1.6 x 10^{-9} M insulin). Patient 2, who had polycystic ovary disease, had a 49.2% decrease in maximal autophosphorylation of insulin receptors, and the ED50 was shifted to the right (5.6 x 10^{-8} M). Patient 3 with acanthosis nigricans had a normal maximal autophosphorylation, but the ED50 shifted to the right (2.9 x 10^{-8} M). The mechanisms for the diversity detected in this assay is not known, but this technique has sufficient specificity and sensitivity to be used to screen for insulin-resistant patients who have a lack of kinase activity. Diabetes 43:274–80, 1994

The insulin receptor is a transmembrane glycoprotein composed of two α-subunits and two β-subunits. On the binding of insulin to the α-subunit, the β-subunit is autophosphorylated (1–3). By this autophosphorylation, the intrinsic tyrosine kinase activity of the receptor is activated and phosphorylates various substrates on tyrosine residues (4,5). Several lines of evidence now indicate that this tyrosine kinase activity is a critical early step in insulin action (6–8). Insulin resistance is a feature of non-insulin-dependent diabetes mellitus (NIDDM) that is generally distal from the insulin-binding step (9,10). A decrease in receptor tyrosine kinase activity could therefore be one candidate for the development of NIDDM. Indeed, a decrease in receptor tyrosine kinase activity has been reported in several tissues of NIDDM (11–15). These prior attempts to quantitate the autophosphorylation and tyrosine kinase activity of the insulin receptor mainly relied on 32P incorporation into the receptor β-subunit and substrates as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (11–16). However, this technique is time consuming and requires a large quantity of samples because its sensitivity is low, thereby limiting the number of subjects that can be studied. We have developed a sensitive and simple enzyme-linked immunosorbent assay (ELISA) for the detection of autophosphorylated insulin receptor. This assay allowed us to study the ability of the insulin receptor to autophosphorylate in 50 NIDDM patients. We have found that three of these subjects had a decreased insulin receptor autophosphorylation activity.

RESEARCH DESIGN AND METHODS

[γ-32P]ATP was purchased from ICN (Lisle, IL), peroxidase-conjugated streptavidin was obtained from Tago...
from Sigma (St. Louis, MO). A wheat-germ agglutinin from Dako Japan (Kyoto, Japan). Porcine insulin and bovine serum albumin (BSA) (fraction V) were obtained from Sigma (St. Louis, MO). A wheat-germ agglutinin (WGA)-coupled sepharose 4B column was purchased from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade and were obtained from standard suppliers. Chinese hamster ovary cells that overexpress the human insulin receptor (CHO.T) were a gift from Dr. Richard A. Roth (Stanford University, Stanford, CA). The monoclonal insulin receptor antibodies used in this study were developed in our laboratory and will be described in detail elsewhere. Briefly, 3B6 recognized the extracellular domain of the human insulin receptor (HIR) distal to the insulin-binding site and did not cross-react with the human insulin-like growth factor-I receptor. 1F9 recognized an epitope on the intracellular domain of the HIR. Its binding to the receptor was not affected by prior autophosphorylation of the receptor. Polyclonal anti-phosphotyrosine antibody was prepared as described (17).

Twenty healthy control subjects (12 men and 8 women; 32–60 years of age, mean age 46.8 years; mean fasting blood glucose 5.2 mM) and 50 NIDDM patients, treated with oral drugs or diet or both (28 men and 22 women; 19–79 years of age, mean age 59.9 years; mean fasting blood glucose 7.5 mM), were studied for insulin receptor autoradiography. These patients were classified as having NIDDM on the basis of a standard 75 g oral glucose tolerance test with the use of the criteria of the National Diabetes Data Group (18).

Preparation of samples. Heparinized blood (1 ml) was washed twice with 6 ml of RPMI-1640 medium at 20°C and then resuspended in 1 ml of RPMI-1640 containing 0.1% BSA and various concentrations of insulin. The erythrocytes were incubated for 15 min at 37°C with gentle shaking. The autophosphorylation reaction was stopped by adding 16 ml of 5 mM Tris-HCl (pH 8.0) containing 2 mM sodium orthovanadate, 1 mg/ml bacitracin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM EDTA. After centrifugation at 28,000 g for 30 min, the supernatant was discarded. The erythrocyte ghosts thus obtained were washed twice with 20 mM Tris-buffered saline (pH 7.4) containing 0.1% BSA for 30 min at 56°C. After washing, 100 µl of the samples was added and incubated overnight at 4°C. The wells were washed five times with buffer A, after which 100 µl of biotinylated antiphosphotyrosine antibody was added (0.3 µg/ml in 50 mM HEPES-buffered saline containing 0.05% Tween 20, 1% BSA, 2 mM sodium orthovanadate, 1 mg/ml bacitracin, and 1 mM PMSF). After incubation for 2 h at room temperature, the wells were washed again, 100 µl of peroxidase-conjugated streptavidin (diluted to 1:30,000 in the same buffer as for the phosphotyrosine antibody) was added, and incubation was continued for 1 h at room temperature. Finally, the wells were washed six times, and the peroxidase activity was determined colorimetrically by adding 100 µl of OPD (0.67 mg/ml in 0.1 M citrate-phosphate buffer, pH 5.0 with 0.4 µl/ml of 30% H2O2). Then the reaction was stopped by adding 100 µl of 5 N H2SO4, and the absorbance at 490 nm was measured.

ELISA for the total insulin receptor number. The ELISA for the total insulin receptor number was performed exactly as for the autophosphorylated insulin receptor ELISA, except that a peroxidase-conjugated anti-insulin receptor monoclonal antibody (1F9, 0.3 µg/ml) was used instead of the biotinylated antiphosphotyrosine antibody and peroxidase-conjugated streptavidin.

Preparation of an autophosphorylated insulin receptor standard. CHO.T cells (5 x 10⁵) in 15 cm culture dishes were first preincubated for 30 min at 37°C with serum-free Ham's F-12 medium and then were treated with 10⁻¹⁷ M insulin for 10 min. The insulin-treated CHO.T cells were next lysed with 1% Triton X-100 (300 µl/dish) in 50 mM HEPES (pH 7.4) containing protease and phosphatase inhibitors. After 40 min on ice, the lysate was centrifuged at 100,000 g for 15 min at 4°C. The supernatant thus obtained was applied to a column composed of 5 ml of antiphosphotyrosine antibody (PY1D7; 17) coupled with Affi-Gel 10 (Bio-Rad, Richmond, CA) and pre-equilibrated with 50 mM HEPES-buffered saline containing 0.05% Tween 20. After washing with 100 ml of HEPES-buffered saline and Tween 20, the protein fraction containing phosphotyrosine was eluted with 15 ml of 40 mM disodium phenyl phosphate in HEPES-buffered saline and Tween 20. Next, the eluate was applied to a WGA (1 ml)-coupled sepharose 4B column and eluted in turn with 5 ml of 0.3 M N-acetyl-D-glucosamine in 50 mM HEPES-buffered saline and Tween 20 containing protease and phosphatase inhibitors. This eluate was diluted to 20% with fetal calf serum, divided into aliquots, and frozen at -85°C. The total number of the autophosphorylated insulin receptor thus obtained was defined as 5,000,000 autophosphorylated insulin receptor unit (Auto-IRU) or 5,000,000 insulin receptor unit (IRU) or both. Samples from the erythrocyte lysates were compared with this standard insulin receptor preparation, and the Auto-IRU and IRU in the erythrocyte lysates were determined by this comparison.

Quantitation of 32P incorporation by insulin receptors. Semiconfluent CHO.T cells in 60 mm culture dishes were treated for 10 min with various concentrations of insulin (0, 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, 10⁻⁸ or 10⁻⁷ M). The cells were then lysed; the lysates were immunoprecipitated on microtiter plates (Falcon, Lincoln Park, NJ) precoated with 3B6. After washing, the insulin receptors adsorbed...
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FIG. 1. Correlation between $^{32}$P incorporation into the receptor and the values obtained for autophosphorylation of the receptor by the ELISA. Insulin-treated (0, 10$^{-11}$, 10$^{-10}$, 10$^{-9}$, or 10$^{-8}$ M) CHO.T cell lysates were adsorbed on microtiter plates coated with 50 µl/well of a monoclonal anti-insulin receptor antibody (3B6). Then $^{32}$P incorporation into the β-subunit of the insulin receptor was quantitated as described in METHODS. At the same time, 150-fold diluted lysates were examined by our ELISA system. The units for autophosphorylation of receptor detected by the ELISA are Auto-IRU and are defined in METHODS.

FIG. 2. Phosphoamino acid specificity of the ELISA. Autophosphorylated Insulin receptor standards were assayed by the ELISA system as described in METHODS, except that the biotinylated polyclonal antiphosphotyrosine antibody was incubated in the presence of 1 mM of the various phosphoamino acids. P-THR, phosphothreonine; P-SER, phosphoserine; P-TYR, phosphotyrosine.

RESULTS

The specificity and sensitivity of the autophosphorylated insulin receptor ELISA was evaluated as follows. When insulin-treated CHO.T cells were quantitatively assessed for receptor autophosphorylation by either the ELISA or by $^{32}$P incorporation, a strong linear correlation was found between the two assays ($r^2 = 0.98$, Fig. 1). However, because the sample volume required for the ELISA was 150-fold less than that for autoradiography, our new assay was much more sensitive than the standard one. In addition, when the immunoprecipitants obtained from insulin-treated CHO.T cells by incubation with 3B6 were analyzed by immunoblotting with the antiphosphotyrosine antibody, a single polypeptide band was detected at 95 kiloDalton (kD) that corresponded to the insulin receptor β-subunit (data not shown). Furthermore, the binding of the antiphosphotyrosine antibody was completely inhibited by 1 mM phosphotyrosine but not by phosphoserine or phosphothreonine in our ELISA system (Fig. 2). Thus, this assay was shown to be both sensitive and specific for the autophosphorylated HIR.

To investigate the clinical application of this ELISA, we chose erythrocytes as the sample, because they are the most easily and repeatedly accessible clinical material. When erythrocytes were treated with insulin, maximal autophosphorylation occurred within 5 min and remained at the same level for at least 30 min (data not shown). Therefore, an incubation time of 15 min was selected for this study. The sensitivity and specificity of this ELISA were confirmed further by dilution and recovery tests. In the dilution test, an identical curve was obtained for the purified autophosphorylated insulin receptor standard and the erythrocyte lysate, and the detection limit was as low as 80 µl of whole blood (Fig. 3). In the recovery
TABLE 1

Recovery test

<table>
<thead>
<tr>
<th>CHO.T standard (μl)</th>
<th>Erthrocyte sample (μl)</th>
<th>Obtained values (Auto-IRU)</th>
<th>Expected values (Auto-IRU)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>505</td>
<td>505</td>
<td>100%</td>
</tr>
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<td>100%</td>
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<td>435</td>
<td>98%</td>
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<td>50</td>
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<td>102%</td>
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<td>103%</td>
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</tr>
<tr>
<td>10</td>
<td>100</td>
<td>190</td>
<td>190</td>
<td>105%</td>
</tr>
<tr>
<td>0</td>
<td>470</td>
<td>155</td>
<td>155</td>
<td>100%</td>
</tr>
</tbody>
</table>

Various amounts of erythrocyte samples were added to the indicated amounts of standard autophosphorylated insulin receptor and recovery (% obtained values/expected values) was assessed. The original concentration of standard autophosphorylated insulin receptor and erythrocyte sample is shown at the top and the bottom row as CHO.T standard (100 μl) and erythrocyte sample (100 μl), respectively.

FIG. 3. Standard and sample serial dilution curves of autophosphorylated insulin receptor detected by the ELISA. The human erythrocyte sample was made from 400 μl of whole blood that had been treated with 10⁻⁷ M insulin. The original concentration of standard autophosphorylated insulin receptor was 500 Auto-IRU/100 μl; y-axis absorbance at 490 nm, x-axis dilution 1.5⁻¹. RBC, red blood cell.

The glucose disposal rate under euglycemic-hyperinsulinemic glucose clamp and glucose and insulin levels during glucose tolerance tests are shown. Glucose tolerance tests were performed with a standard (75 g) oral glucose load. Plasma glucose and insulin concentration were measured after an overnight fast and glucose ingestion.

The glucose disposal rate in healthy control subjects was 6–10 mg · kg⁻¹ · min⁻¹ and that in NIDDM patients was 4–6 mg · kg⁻¹ · min⁻¹ at our hospital. The autophosphorylation of insulin receptors per 300 μl of erythrocytes is shown in Fig. 4. All four subjects had lower autophosphorylated insulin receptor levels than normal control subjects, although the actual decrease in autophosphorylation was quite different. When receptor autophosphorylation was adjusted per unit receptor, this became clearer (Fig. 5). Table 3 shows the amount of total insulin receptor for these four patients. Patients 2, 3, and 4 had fewer insulin receptors than the normal subjects, possibly because of the downregulation of their receptors because of their hyperinsulinemia. However, patient 1 had twice as many receptors as the normal subjects. Autophosphorylation per unit receptor is shown in Fig 5. The dose-response curve for patient 1 was parallel to that of the normal subjects, but at each point this patient had 85% less autophosphorylated receptor than the control subjects. Patient 2 showed a 49.2% decrease in maximal autophosphorylation of insulin receptors, and the ED₅₀ was also shifted to the right.

TABLE 2

Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Glucose disposal rate (mg · kg⁻¹ · min⁻¹)</th>
<th>Insulin (pM) Fasting</th>
<th>Glucose (mM) Fasting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>6–10</td>
<td>&lt;90</td>
</tr>
<tr>
<td>Patient number</td>
<td></td>
<td>2 h</td>
</tr>
<tr>
<td>1</td>
<td>56</td>
<td>1.88</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>1.98</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>1.05</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
<td>0.71</td>
</tr>
</tbody>
</table>

The glucose disposal rate in healthy control subjects was 6–10 mg · kg⁻¹ · min⁻¹ and that in NIDDM patients was 4–6 mg · kg⁻¹ · min⁻¹ at our hospital.
Patient 3 had normal maximal autophosphorylation, but the ED$_{50}$ was shifted ~10 times to the right. Patient 4 had insulin receptors that exhibited normal dose-dependent autophosphorylation. In the residual 46 NIDDM patients, autophosphorylation per unit receptor was significantly higher than the control subjects (0.86 ± 0.15 of NIDDM patients vs. 0.66 ± 0.09 of normal control subjects, P < 0.01, Fig. 6).

**DISCUSSION**

Previously, the autophosphorylation of the insulin receptor has been measured by the incorporation of $^{32}$P phosphorous into the $\beta$-subunit of the insulin receptor. Because the insulin receptor constitutes a small fraction of the protein in cells, earlier studies have had to use 20–100 ml of erythrocytes to access in vitro insulin-induced insulin receptor phosphorylation (16). For in vivo phosphorylation studies, the impermeability of plasma membrane to ATP necessitates the incubation of the cells with $[^{32}P]$orthophosphate to label the cellular pool of ATP. However, because the efficacy of $[^{32}P]$orthophosphate to label cellular ATP is quite low, it is almost impossible to use erythrocytes as the sample in these studies. Most in vivo phosphorylation studies of cells from patients have therefore used EB virus transformed B-lymphocytes. However, this technique also requires large amounts of blood to get lymphocytes for transformation.

After transformation, lymphocytes must be cultured for 2–6 months to get enough cells for assay. In this study, we have described a new simple ELISA system for measuring in vivo autophosphorylation of the insulin receptor that uses only 100 μl of erythrocytes as the sample. The specificity of this sandwich method has been confirmed by several criteria. First, a single polypeptide band was detected at 95 kD that corresponded to the insulin receptor $\beta$-subunit on an immunoblot with phosphotyrosine antibody after immunoprecipitation of the CHO.T cell lysates with the insulin receptor antibody. Second, when receptor autophosphorylation in insulin-treated CHO.T cells was quantitated by either the ELISA or by $^{32}$P incorporation, a strong linear correlation was found between the two assays (Fig. 1). Third, the binding of the antiphosphotyrosine antibody to the receptor in the ELISA was completely inhibited by 1 mM phosphotyrosine but not by phosphoserine or phosphothreonine (Fig. 2). Thus, this assay was shown to be both sensitive and specific for the autophosphorylated HIR.

The simplicity of this ELISA allowed us to rapidly quantify insulin receptor phosphorylation in the group of 50 NIDDM patients. We found three patients with de-

**TABLE 3**

Total number of insulin receptors per 300 μl of erythrocytes in control subjects and patients

<table>
<thead>
<tr>
<th>Total IR number (IRU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects (n = 20)</td>
</tr>
<tr>
<td>Patients (number)</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

Data are means ± SD.
creased insulin receptor autophosphorylation/total insulin receptor by testing these patients at a single insulin concentration. One additional patient with hyperinsulinemic insulin resistance and these three patients were tested further to their insulin receptor autophosphorylation at six different insulin concentrations. For all four patients, the amount of autophosphorylated insulin receptors per 300 μl of erythrocytes was found to be low compared with those in normal subjects (Fig. 4). However, when the activity was adjusted per unit receptor (Fig. 5), the receptors of patient 4 were found to have the same amount of autophosphorylated receptor as control subjects because the receptor level of patient 4 was almost 25% that of the control subjects (Table 3). This 75% decrease in receptor number may have caused the insulin resistance in this patient.

Patient 1 had twice as many insulin receptors as normal (Table 3). A possible explanation for the increase of receptors in this patient could be that the loss of kinase activity prevented the mutant receptor from being internalized and degraded (20). Another possibility is that the mutant receptor may contain some kind of signal-promoting accelerated receptor synthesis. The first explanation seems unlikely because we have previously found a 60% decrease in receptor number in the erythrocytes from a patient with a Val996 mutation of the insulin receptor (21,22), a mutation that caused a loss of ATP binding and receptor internalization. Additional studies such as the quantitation of receptor mRNA are necessary to determine the reason for the increase in receptor numbers in this patient.

The erythrocyte insulin receptors from patient 1 also exhibited an 80% decrease in maximal autophosphorylation activity, whereas sensitivity to insulin was the same as the normal subjects (Fig. 5). The family of this patient has also been studied extensively, and we have found four relatives with the same kinase deficiency (80–85% decrease in autophosphorylation activity). Recent studies have indicated that hybrid receptors with one kinase-deficient subunit are completely inactive (23). Thus, if a patient expresses a single mutant insulin receptor that is kinase deficient and readily forms hybrid receptors with the other normal receptor gene product, only the normal-normal form (compromising 25% of the receptor molecules) would possess tyrosine kinase activity (23). Thus, the data obtained in patient 1 are consistent with the presence of a simple heterozygous mutation in the kinase domain of the insulin receptor. In fact, the proband and four relatives were recently found to be heterozygous for a point mutation (Arg1131→Gln1131) located in kinase catalytic loop (Miyako Kishimoto, unpublished observations).

Patient 2 had erythrocyte insulin receptors that exhibited a 51% decrease in maximal autophosphorylation and had an ED50 shifted to the right. The family of this patient also was studied, and we found two relatives with decreased insulin receptor autophosphorylation activity (50–60% decrease). Thus, this dysfunction of the insulin receptor also was genetic, and the sequence of the receptor of this family is now being determined.

Patient 3, who had acanthosis nigricans, had a normal autophosphorylation activity at maximum insulin stimulation, but the ED50 was shifted ~10 times to the right. This displacement of the curve might indicate that the decreased autophosphorylation activity was caused by a decrease of α-subunit insulin-binding capacity. However, the 125I-labeled insulin-binding displacement curves of the erythrocyte insulin receptors were normal in these patients, so patient 3 was also considered to have type C syndrome (syndrome accompanied with post-insulin-binding receptor abnormality) of insulin resistance.

When the autophosphorylation activity per unit receptor is compared with the normal subjects, the other 46 NIDDM patients we studied had slightly higher values (Fig. 6). Previous studies have suggested that a decrease occurs in insulin receptor kinase activity in NIDDM patients, and it correlates with insulin resistance (11–15). The difference in our results may be explained by several points. First, possibly, the insulin receptors of the erythrocyte do not reflect what is happening in the insulin receptor in other tissues. Second, in the prior studies, the number of insulin receptors were often measured by 125I-labeled insulin binding and Scatchard analysis. Because this is difficult when the receptor number is low, these prior studies may have incorrectly assessed receptor number, and this could have affected their estimates of receptor kinase per receptor. Third, most of the prior studies have measured insulin receptor...
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tyrosine kinase activity by in vitro assays, which may differ from the in vivo assay we have used.

In conclusion, we have developed a new ELISA for measuring insulin receptor autophosphorylation. The exact mechanism that produces receptor tyrosine kinase deficiency cannot be detected by this assay. However, the simplicity and sensitivity of this ELISA suggest that it will be a useful tool for the detection of insulin receptor mutations and for analyzing receptor tyrosine kinase activity in various diabetic states.

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