Short Communications

Immunoprecipitation of ^{125}I -Insulin Crosslinked Receptor

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Summary. ^{125}I -insulin was covalently crosslinked to its receptor on human placental membrane fractions with disuccinimidyl suberate. The ^{125}I -insulin crosslinked receptor was solubilized with Triton X-100 and used as a probe to determine autoantibodies to the insulin receptor in sera from patients with insulin resistance (type B syndrome). When the solubilized ^{125}I -insulin: receptor complex was incubated with these sera and then with anti-human IgG serum, the complex was precipitated as a function of the amount of anti-receptor serum. Unlabelled insulin at concentrations $\leq 10\text{ }\mu\text{g/ml}$ did not affect immunoprecipitation of the complex, suggesting that a subpopulation of anti-receptor antibodies recognizes determi-

nants outside the insulin binding region of the receptor molecule. ^{125}I -insulin: receptor complex was precipitated also by the addition of anti-insulin antibodies. The effect of anti-insulin antibody was eliminated by the addition of excess unlabelled insulin. The immunoprecipitation assay using ^{125}I -insulin crosslinked receptor was sensitive and convenient for detecting anti-receptor antibodies.

Key words: Crosslinking, disuccinimidyl suberate, insulin receptor, anti-receptor antibody, immunoprecipitation, type B syndrome.

It is well documented that the patients with an unusual type of insulin resistant diabetes (type B syndrome) have autoantibodies to the insulin receptor in their sera [1–4]. These antibodies can be detected by their ability to inhibit the binding of ^{125}I -insulin to its receptor. Recently, Harrison et al. [5] developed a sensitive immunoprecipitation assay for the anti-receptor antibody, showing that the solubilized insulin receptor labelled with ^{125}I -insulin could be precipitated quantitatively by anti-receptor antibodies. In this assay, however, anti-receptor antibodies may displace receptor-bound ^{125}I -insulin, since ^{125}I -insulin is not covalently bound to the receptor. Anti-insulin antibodies, if present in the anti-receptor serum, may also reduce the precipitation of the ^{125}I -insulin: receptor complex by removing the receptor-bound ^{125}I -insulin. In the present study, we crosslinked ^{125}I -insulin to the receptor on human placental membranes using disuccinimidyl suberate (DSS) and examined the immunoprecipitation of the solubilized complex by anti-receptor and anti-insulin serum. This affinity crosslinking technique was developed by Pilch and Czech [6] and has proved useful for studying the subunit structure of insulin receptors [7, 8].

Materials and Methods

Hormones and Reagents

Monocomponent porcine insulin (25 U/mg, Eli Lilly, Indianapolis, Indiana, USA) was radioiodinated to a spec. act. of 100–150 Ci/g by a modification of the chloramin T method. DSS was purchased from Pierce Chemicals (Rockford, Illinois, USA). Protein-A Sepharose CL-4B was a product of Pharmacia (Uppsala, Sweden).

Membrane Preparations

Crude membrane fractions were prepared from fresh or frozen-stored human placenta by modifying the procedure reported by Posner [9]. The membrane fractions were solubilized with Triton X-100 as described previously [10].

Crosslinking of ^{125}I -Insulin

The membrane fractions (2–5 mg protein) were incubated with ^{125}I -insulin (5 nmol/l) for 18 h at 4°C. The mixture was then diluted five- to tenfold with ice-cold Krebs Ringer phosphate (KRP) buffer (pH 7.4) and centrifuged at 10,000 g for 30 min at 4°C. The pellet was resuspended in ice-cold KRP buffer at a concentration of 1–2 mg protein/ml. Crosslinking was performed according to the method of Pilch and Czech [6]. The freshly prepared DSS (0.1 mol/l in dimethyl

sulphoxide) was added at a dilution of 1:100. Crosslinking was allowed to proceed for 20 min at 4°C, and was terminated by the addition of ten volumes of ice-cold Tris (0.01 mol/l) containing EGTA (0.001 mol/l), pH 7.4. The mixture was centrifuged at 10,000 g for 30 min at 4°C and the pellet collected.

Solubilization of ^{125}I -Insulin Crosslinked Receptor

The ^{125}I -insulin crosslinked membranes were solubilized for 60 min at 24°C in Tris-HCl buffer (0.05 mol/l), pH 7.4 containing 2% (v/v) Triton X-100, 100 U/ml bacitracin, and 1000 U/ml aprotinin with stirring. The mixture was centrifuged at 100,000 g for 90 min and the insoluble pellet discarded. Approximately 50% of the protein and 40% of the radioactivity were solubilized into the supernatant. To separate ^{125}I -insulin crosslinked receptor from unbound ^{125}I -insulin, the supernatant was gel-filtered on a column of Sepharose 6B (1.5 × 85 cm) equilibrated with Tris-HCl buffer (0.05 mol/l, pH 7.4) containing 0.1% (v/v) Triton X-100.

Immunoprecipitation of Receptor

Aliquots of the solubilized ^{125}I -insulin: receptor complex (1000–2000 cpm) were incubated for 4 h at 24°C with 10 μl serially diluted sera from patients with type B syndrome in Tris-HCl buffer (pH 7.4, 0.5 ml, 0.05 mol/l) containing 0.5% BSA and 0.05% (v/v) Triton X-100. At the end of the incubations rabbit anti-human IgG serum was added and tubes were incubated for 16 h at 4°C and centrifuged at 1000 g for 30 min at 4°C. The supernatants were removed and the radioactivity of the pellets determined. In another series of experiments, ^{125}I -insulin: receptor complex was incubated with various dilutions of guinea pig anti-porcine insulin serum in a similar manner. Immunoprecipitation was achieved by the addition of protein-A Sepharose (10 mg). The immunoprecipitation assay for anti-receptor antibodies was performed also by the method of Harrison et al. [5].

Patients

Sera from three patients with type B syndrome were used in the study. The sera contained antibodies that inhibited the binding of ^{125}I -insulin to the receptor on human placental membranes. Anti-insulin antibodies were not detected in these sera.

Fig. 1. Immunoprecipitation of ^{125}I -insulin crosslinked receptor. Aliquots of the solubilized ^{125}I -insulin crosslinked receptor (2000 cpm) were incubated with serial dilutions of human anti-receptor sera (A, B, C; diluted with normal human serum) as described in the text. Immune complexes were precipitated by the addition of anti-human IgG serum followed by centrifugation. The mean value of duplicate determinations is shown

Fig. 2. Immunoprecipitation of ^{125}I -insulin bound receptor. Triton-solubilized human placental membrane (100 μg protein) was incubated with ^{125}I -insulin (10,000 cpm) at 20°C in Tris-HCl buffer (200 μl , 0.05 mol/l, pH 7.4). Anti-receptor serum (5 μl , diluted with normal human serum) was added and the incubation continued for a further 1 h at 20°C. Rabbit anti-human IgG (0.2 ml) was added and the mixture incubated for 12 h at 4°C and centrifuged. The radioactivity of the precipitated pellet is plotted as a percentage of the count of the ^{125}I -insulin: receptor complex formed as above in the absence of anti-receptor serum but precipitated with poly ethylene glycol 6000 (final concentration 12.5%) in place of anti-human IgG. Each point represents the mean of duplicate determinations

Results and Discussion

When ^{125}I -insulin bound membranes were treated with 1 mmol/l DSS for 20 min at 4°C, 50%–60% of the bound ^{125}I -insulin was covalently linked to the receptor, based on the dissociation of ^{125}I -insulin from the membranes after treatment with 0.1 N HCl. The ^{125}I -insulin crosslinked membranes were solubilized with Triton X-100 and subjected to gel-filtration on a column of Sepharose 6B. ^{125}I -insulin: receptor complex eluted as a molecule of apparent molecular weight 300,000 (data not shown), which is comparable to that of the non-crosslinked receptor of human placenta [11]. Figure 1 shows the extent of precipitation of three anti-receptor sera (A, B, C) after incubation with ^{125}I -insulin: receptor complex (300,000 species) and addition of anti-human IgG serum. Serum A was the most potent, with maximum precipitation occurring at a serum dilution of 1:50. We observed 58%–84% precipitation of the ^{125}I -insulin:receptor complex using serum A at this dilution in several experiments and increasing amounts of anti-serum did not improve the efficiency of precipitation. Thus, treatment with DSS may alter some of the receptors so that anti-receptor antibodies cannot bind them.

The three anti-receptor sera were tested also for their ability to precipitate the insulin receptor using the method described by Harrison et al. [5]. Interestingly, serum B was the most potent in this assay with maximum precipitation at a dilution of 1:500 (Fig. 2). Possibly, serum B contains a subpopulation of antibodies

Fig. 3. Effect of anti-insulin antibody on immunoprecipitation. Solubilized ^{125}I -insulin crosslinked receptor (3000 cpm) was incubated with guinea pig anti-porcine insulin serum (10 μl) for 4 h at 24°C in the presence (○) or absence (●) of unlabelled insulin (10 $\mu\text{g}/\text{ml}$) in Tris-HCl buffer (0.5 ml, 0.05 mol/l, pH 7.4) containing 0.5% BSA and 0.05% Triton X-100. After further incubation with protein-A Sepharose (10 mg) for 2 h at 4°C and centrifugation, the precipitated pellet was washed twice and counted. Radioactivity is plotted as a percentage of the total (mean of duplicate determinations)

recognizing the determinants which are sensitive to treatment with DSS, which would account for its decreased immunoprecipitation with ^{125}I -insulin crosslinked receptor. In a number of experiments, neither serum A nor B precipitated >90% of the non-covalently bound ^{125}I -insulin: receptor. Failure of the anti-sera to precipitate the complex completely is due to a partial dissociation of ^{125}I -insulin by the anti-receptor sera (data not shown). When corrected for this dissociation, almost all the ^{125}I -insulin: receptor complexes were precipitated by either serum A or B at 1:50 dilution.

A difference between the two assays was noted also in the effect of unlabelled insulin on precipitation of the receptor. Unlabelled insulin is known to reduce the immunoprecipitation of insulin receptors to which ^{125}I -insulin is non-covalently [5] or covalently bound [8]. By contrast, unlabelled insulin at concentrations $\leq 10 \mu\text{g}/\text{ml}$ did not impair the precipitation of the ^{125}I -insulin crosslinked receptor of human placenta (data not shown). Recently, it has been reported that immunoprecipitation of insulin receptors labelled with [^3H]-sugars or [^{35}S]-methionine is reduced by preincubation with high concentrations of unlabelled insulin [12, 13]. Therefore, binding of insulin appears to alter the conformation of the receptor such that the affinity for anti-receptor antibodies is reduced. We found that treatment of placental membranes with DSS (0.5–2.0 mmol/l) caused a significant loss in the insulin binding activity of the sites not occupied by ^{125}I -insulin, which may explain the failure of unlabelled insulin to impair the precipitation of the ^{125}I -insulin crosslinked receptor complex. Immunoprecipitation of the complex in the presence of excess insulin suggests that a subpopulation of anti-receptor antibodies recognize determi-

nants outside the insulin binding sites of the receptor molecule.

Previously, Heinrich et al. [14] have reported that the ^{125}I -insulin crosslinked receptor of rat adipocytes can be precipitated by anti-insulin antibodies. Confirming their observations, ^{125}I -insulin: receptor complex was precipitated dose-dependently by an antibody to porcine insulin (Fig. 3). In this instance, unlabelled insulin completely inhibited the precipitation. Thus, it is evident that anti-insulin antibodies (at least in part) can bind ^{125}I -insulin crosslinked to the receptor. Two further batches of guinea pig anti-porcine insulin sera confirmed these results, but neither batch precipitated >70% of the crosslinked complexes. Random crosslinking on the receptor molecule may inhibit access of the antibody to receptor bound ^{125}I -insulin. Binding of insulin antibody to receptor bound insulin may be clinically of some importance. Kahn et al. [15] have shown that when adipocytes are exposed to submaximal doses of insulin, addition of anti-insulin antibody at low concentrations enhances the biological activity of insulin.

In conclusion, we have shown that ^{125}I -insulin crosslinked receptor can be used for detection of anti-receptor antibodies. In this assay, unlabelled insulin does not affect the precipitation of the receptor, so the effect of anti-insulin antibodies can be eliminated by the addition of unlabelled insulin. The sensitivity of the method is comparable to that of a method reported previously [5]. Thus, the present immunoprecipitation assay will be useful for detecting patients with anti-receptor antibodies to the insulin receptor.

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