Evaluation of the Method of Insulin Binding Studies in Human Erythrocytes

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Abstract

A modified erythrocyte insulin receptor assay was developed. The major advantage of our method is the smaller amount of blood necessary for the assay and consequent simple handling in the binding assay. Three ml of blood is enough for this assay, making it possible to assess insulin receptors in a group of pediatric patients. There was a close relationship between mononuclear cells and erythrocyte insulin binding assays ($r=0.900$, $p<0.05$).

With this method insulin binding studies were done on normal subjects and adult onset non-obese diabetics. Decreased insulin binding was seen in the diabetic group, and this was due to a decreased number of insulin receptors.

This erythrocyte insulin receptor assay can be a useful tool for the study of the mechanism of insulin resistance.

Since the first step in insulin action is binding to its receptor, the estimation of receptors in the diabetic state may be important in assessing the mechanism of insulin resistance, especially in patients with type A and type B insulin receptor defects (Kahn et al., 1976).

Mononuclear cells have been used for insulin receptor assay in human and the method requires over 100 ml of blood for each assay. Since Gambhir et al developed a new receptor assay method using erythrocytes (Gambhir et al., 1978), only 10 ml of blood is enough for the assay. We have partially modified their method to further decrease the amount of blood required and examined the relationship between mononuclear cells and erythrocyte binding. With this method we measured insulin binding in non-obese adult onset diabetic patients and found that this method is simple and satisfactory for the assessment of insulin resistance in man.

Materials and Methods

Materials

Purified single peak pork insulin was a gift from Shimizu Pharmaceutical Co.. The purity of this insulin was equal to that of monocomponent insulin by the electrophoretic method and biological potency was 26 U/mg. Na$^{125}$I was purchased from New England Nuclear, di-n-butyl phthalate from Nakarai Chemicals, Bovine Serum Albumin (BSA) (Fraction V) from Armour Chemical Co. and Lymphoprep® (Ficoll-Hypaque gradient solution) from Daiichi Kagaku Yakuhin.

Methods

Preparation of erythrocytes

With a heparinized syringe, 3-5 ml of blood was drawn from patients after overnight fasting. The blood was centrifuged at 2000 rpm for 10 min at 4°C to separate plasma to be used for the determination of insulin and other hormones. Buffy coat on a pellet of erythrocytes was removed with a pasteur pipette. Erythrocytes were suspended in 3 volumes...
of physiological saline, and this suspension was layered on Ficoll-Hypaque gradient solution. After centrifugation for 15 min at 1400 rpm at room temperature, supernatant fluid including a layer of mononuclear cells and the buffy coat was removed with a pipette. Erythrocytes were then suspended in a buffer consisting of HEPES 50 mM, Tris 50 mM, NaCl 50 mM, MgSO_4_ 10 mM, KCl 5 mM, CaCl_2_ 10 mM, EDTA 2 mM, glucose 10 mM and 1% of BSA, pH 8.0 and centrifuged at 1400 rmp at room temperature for 10 min. Cells were re-suspended in 3 volumes of the buffer, and this suspension was used for the receptor assay. Cells were counted with an Industrial D model Coulter Counter with an aperture of 100 μM.

Iodination of insulin

125I-insulin with a specific activity of 100–150 μCi/μg was prepared according to the method of Freychet et al. (1971).

Binding Studies

Erythrocytes were incubated with 125I-insulin (0.2 ng/ml) and unlabeled insulin at various concentrations from 0 to 100 ng/ml in a total volume of 0.5 ml in a plastic tube at 15°C, shake by hand every 60 min. Incubation was terminated by removing 100 μl aliquots from the cell suspension and immediately centrifuging the cells in plastic microcentrifuge tubes containing 100 μl of dibutyl phthalate and 200 μl of cold buffer. The cells formed a pellet under a layer of dibutyl phthalate and the radioactivity was determined. Non-specific binding was assessed by incubating cells with 100 μg/ml of unlabeled insulin and 0.2 ng/ml of labeled insulin, and the binding date were obtained by subtracting the non-specific binding. The mononuclear cell insulin binding assay method has been described elsewhere (Kobayashi et al., 1977).

Results

Time-Course of Insulin Binding.

The time-course of insulin binding to erythrocytes is shown in Fig. 1. In agreement with results reported by Gambhir et al. (1978), the binding reached a steady state at 3 hr and 30 min at 15°C. Unlike the data of Wachslicht-Rodbard (1979), 90 min, incubation was not enough time in which to reach equilibrim. Thus, we used 3 hr and 30 min incubation at 15°C for all the following experiments.

Fig. 1. Time course of 125I-insulin binding to erythrocytes at 0.2 ng/ml of insulin concentration.

Degradation of Insulin.

The degradation of 125I-insulin checked by the trichloroacetic acid precipitation method was negligible (2.3±0.6%) at the end of incubation. Unlike hepatocytes and adipocytes, degradation of the ligand is not an important factor in studies for insulin binding to erythrocytes.

Non-Specific Binding.

In order to decrease non-specific binding for accurate determination of specific binding, we examined possible factors involved in decreasing non-specific binding, such as BSA concentration and the amount of the buffer added to the microcentrifuge tubes.

A BSA concentration of 0.1% produced non-specific binding which was 28±6% of the total binding (i.e. specific binding plus nonspecific binding) whereas 1.0% of BSA decreased this to 17±4%.

As for the buffer volume, 200 μl in microcentrifuge tubes gave non-specific binding of 14% and 100 μl gave 19%. Thus, an increased volume of washing buffer in the microcentrifuge tubes decreased non-specific
insulin binding. Therefore, we used 200 µl of the buffer containing 1.0% of BSA in microcentrifuge tubes.

Effect of Storage of Erythrocytes on Insulin Binding.
For various reasons, blood samples from patients may not be immediately assayed for insulin binding. To see the effect of the storage of cells on the later insulin binding assay, we examined insulin binding to the cells stored for various periods at 4°C in the buffer. As shown in Fig. 2, three hr and six hr storage at 4°C did not affect insulin binding. However, decreased insulin binding was noted when the cells were stored for 18 hr at 4°C. Thus, if cells are stored at 4°C in the buffer, a delay of up to three to six hr does not change any binding results.

Relationship between Mononuclear Cell Binding and Erythrocyte Binding.
Fig. 3 demonstrates the relationship between mononuclear cell and erythrocyte insulin binding studies. There was a close

\[ y = 0.68x + 0.47 \]
\[ r = 0.903 \] (p = 0.01)
Table 1. Characterization of subjects

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>FBS* (±SEM)</th>
<th>IRI† (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normals (n=17)</td>
<td>24-44</td>
<td>83± 4</td>
<td>10±1</td>
</tr>
<tr>
<td>Diabetics (n=35)</td>
<td>18-62</td>
<td>183±21</td>
<td>9±1</td>
</tr>
</tbody>
</table>

* FBS: Fasting blood sugar. The data indicate mean±standard error of mean.
† IRI: Immunoreactive insulin after overnight fasting.
P value indicates a statistical difference between normal and diabetic subjects. NS: not significant. Diabetics are all less than 110% of ideal weight.

relationship between the two methods (r=0.900, p>0.05). Erythrocytes seem to behave in the same manner as mononuclear cells in regard to insulin binding.

Precision of the assay
To establish the precision of this assay in erythrocytes, we have determined the coefficient of variation for percent binding at 0.2 ng/ml of the insulin concentration. The coefficient of variation for a single assay (5 duplicates) was 2.7% (mean 5.21%, SD 0.14%). To determine the intersay coefficient of variation we performed insulin binding studies on the same patient on four separate occasions, provided the person was in the same condition for these occasions in regard to insulin binding. The intersay coefficient of variation was 4.8% (mean 5.62%, SD 0.27%).

Insulin Binding in Normal and Diabetic Subjects.
Using this method, we examined insulin binding in normal and adult onset non-obese subjects. The characterization of patients is shown in Table 1. Decreased insulin binding is seen in these patients, as shown in Fig. 4. a. Scatchard analysis showed that this decreased insulin binding is due to a decrease in the number of insulin receptors rather than decreased affinity for insulin receptors (Fig. 4. b.). The number of insulin receptors per cell is 60 for control and 40 for patients.

Discussion
We have reported a modified method for insulin binding studies in human erythrocytes. The major advantage of our method is the simple handling in the binding assay after incubation, i.e. simply cutting through a dibutyl phthalate layer without an aspiration step for separation of bound insulin from unbound since the microcentrifuge tubes are smaller than those used by Gambhir et al... Since 3 ml of blood is enough for our insulin binding study method, even a group of pediatric patients can be assessed for insulin receptors. We have confirmed that erythrocytes can be used for the insulin binding assay instead of mononuclear cells (Fig. 3). However, since erythrocytes do not have nuclei and are structurally simple, they may be unable to acutely regulate the number of insulin receptors in response to environmental change, such as a change in circulating insulin concentration. Recently, insulin-receptor complex was reported to be internalized and degraded at the lysosomes, and this is a possible mechanism in decreasing insulin receptor by hyperinsulinemia, i.e. down regulation (Gorden et al., 1978; Schlessinger et al., 1978). Thus, the number of insulin receptors of erythrocytes may not be able to change in response to circulating insulin levels since these cells do not show much lysosomal activity. However, receptors on erythrocytes can show acute change in affinity in response to fasting (Kobayashi et al., 1980).

The results of insulin binding in normal subjects are comparable to these of Gambhir et al., (1978). However, the receptor number of our control is significantly lower than that of his group. This discrepancy
resulted from the fact that they used a higher insulin concentration in the binding assay which caused the intersection of their Scatchard plot curves with the horizontal axis to shift to the right because the curve was concave. In our assay system where the highest concentration is 100 ng/ml, the intersection of the curve with the horizontal axis is shifted somewhat to the left compared to their curves, giving a smaller number of insulin receptors.

In agreement with our results, Robinson et al reported decreased number of insulin receptors in non-obese diabetic subjects (Robinson et al., 1979). Circulating insulin levels regulate the number of insulin receptors in vivo (Bar et al., 1976; Kobayashi and Olefsky, 1978) and decreased plasma insulin levels in streptozotocin-treated diabetic rats results in an increased
receptor number (Hepp et al., 1975; Kasuga et al., 1978; Kobayashi and Olefsky, 1979). Since the patients mentioned in this report do not show signs of hyperinsulinemia, the mechanism of decrease in the insulin receptor is not known. Currently, we are investigating this important aspect by examining an early phase of diabetes.

We believe that erythrocytes insulin-receptor assay can be a useful tool in studying the mechanism of insulin resistance in the various diabetic states so long as one recognized the limitations of this assay. Furthermore, since the assay requires only a small amount of blood and is rather simple, the time sequence of an acute change in receptor affinity can be studied with it in various clinical conditions.

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References