Insulin Binding of Erythrocytes in Hypoinsulinemic Diabetics and its Relationship to Metabolic States

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Abstract

We studied the insulin binding of erythrocytes from 26 “well-controlled” (FBG<120 mg/dl), and 28 “poorly-controlled” (FBG<120 mg/dl) non-obese diabetic patients, and 51 age-matched normal subjects. The capacity of insulin receptors was significantly decreased in well controlled (33.7±2.2) and poorly controlled (32.4±2.3) groups compared with normal subjects (43.1±2.3 sites/cell), while fasting insulin levels of diabetics were comparable to normal subjects but their insulin response to a glucose challenge was considerably decreased. However, the “empty sites” affinity (Ke) was significantly increased in both the well controlled group (5.2±0.3) and the poorly controlled group (4.8±0.3) compared with normal subjects (4.0±0.2×10^8 M^-1). Thus, the capacity of insulin receptors to bind erythrocytes was decreased but binding affinity was increased in hypoinsulinemic diabetics. The amount of insulin bound in well-controlled diabetics was comparable to that in control subjects at physiological insulin concentrations.

The initial step in the action of insulin is binding to specific receptors on the plasma membranes of target tissue cells (Roth, 1973). The study of insulin binding to cells, therefore, is important in elucidating the mechanisms of altered carbohydrate metabolism or altered insulin sensitivity. In diabetic patients, studies on insulin receptors have been performed mainly using monocytes (Olefsky and Reaven, 1976; 1977), Recently it has been demonstrated that human circulating erythrocytes have specific insulin receptors with characteristics similar to insulin receptors of other human cell types (Gambhir et al., 1977; 1978). Although it is generally accepted that insulin does not directly affect the metabolism of mature circulating human erythrocytes, insulin receptors of erythrocytes have been shown to change in parallel with those of monocytes in certain conditions (Wachslight-Rodbard et al., 1979; Insel et al., 1980; Pedersen et al., 1980) and the latter is correlated with glucose tolerance and insulin sensitivity (Olefsky and Reaven, 1977; Beck-Nielsen and Pedersen, 1978). Furthermore, the study of erythrocyte insulin receptors requires only small volumes of blood and may therefore be a useful tool in clinical studies.

It has been recently reported that insulin binding to erythrocytes is reduced due to decreased number of receptors in diabetics (Robinson et al., 1979; De Pirro et al., 1980). However, fasting plasma insulin levels were considerably elevated in these patients (Robinson et al., 1979) and a negative correlation was found between fasting insulin levels and insulin binding (De Pirro et al., 1980). These change may therefore

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be due to so-called "down regulation" (Kahn et al., 1972; Gavin et al., 1974; Olefsky, 1976; Kasuga et al., 1978).

We have studied insulin binding to erythrocytes in non-insulin dependent diabetic patients whose fasting insulin levels were comparable to controls, but insulin response to a glucose challenge was considerably decreased.

**Materials and Methods**

**Subjects**

The study group consisted of 54 nonobese noninsulin-dependent diabetic patients (32 males and 22 females) and 51 nonobese subjects (30 males and 21 females) with normal glucose tolerance. Diagnosis of diabetes was based on an elevated fasting capillary whole blood glucose concentration of more than 120 mg/dl on at least two occasions. The patients were treated by diet alone (n=27) or sulfonylurea plus diet (n=27). All diabetic patients had a 100g OGTT on the same day as the binding studies were performed and capillary whole blood glucose and plasma immunoreactive insulin were determined before and 30, 60, 90, 120 and 180 min after glucose loading. None of the normal subjects or diabetics had anemia, liver diseases or were receiving any drugs known to affect carbohydrate or insulin metabolism except for sulfonylurea in sulfonylurea-treated patients.

**Preparation of Cells**

Blood (5-7 ml) was collected from the antecubital vein in heparinized tubes after an overnight fast. Erythrocytes were prepared by a modification of the method of Boyum (1968) as reported by Gambhir et al. (1977).

**Iodination of Insulin**

Porcine monocomponent insulin was generously supplied by Eli Lilly Research Laboratories (Indianapolis, Ind.). $^{125}$I-Na was purchased from New England Nuclear (Boston, Mass.). Insulin was iodinated at a specific activity of about 150 µCi/g by the modification of Freychet et al. (1971) of the method of Hunter and Greenwood (1962).

**Binding Studies**

Binding studies were performed by a modification of the method described by Gambhir et al. (1977). Erythrocytes (2.4-3.2 x 10^9/ml) were incubated at 15°C with $^{125}$I-insulin (0.4 ng/ml) with or without varying amounts of unlabeled insulin in a total volume of 0.5 ml. After 210 min of incubation, duplicate to quadruplicate 100 µl samples were placed in prechilled microfuge tubes containing 200 µl of the buffer (1% BSA) and 100 µl dibutyl phthalate. Cell-bound and free insulin were separated by centrifugation in a Beckman Microfuge B for 2.5 min and the radioactivity in the cell pellets was determined. Nonspecific binding is defined as the amount of $^{125}$I-insulin that remains bound in the presence of a large excess (10^5 ng/ml) of unlabeled insulin. All data were corrected for this factor and represent specific insulin binding. Nonspecific binding was always less than 3% of total radioactivity added. The intra-assay coefficient of variation was 3.9% and interassay coefficient of variation in the same subject on different days was 9.5% for measuring specific insulin binding at tracer insulin concentration. Since the date as a function of cell concentration was linear within the above range of concentration in the incubation media, all data were normalized to 2.5 x 10^9 cells/ml for purposes of comparison.

**Statistical Analysis**

Statistical comparisons among the three groups were calculated by analysis of variance, followed by Newman-Kuels multiple range test. Person's coefficient was applied in correlation studies. Results are expressed in the figures and text as mean±SEM.

**Results**

Clinical characteristics of the study group are shown in Table 1. We divided the patients into two subgroups according to fasting capillary whole blood glucose concentration (FBG), i.e. patients whose FBG values were more than or equal to 120 mg/dl and those less than 120 mg/dl. These groups are referred to as the "poorly-controlled" group and "well-controlled" group, respectively, for the purpose of simplification. Plasma insulin levels before and 30, 60, 90, 120 and 180 min after 100 g oral glucose administration and the sum of the above six values were 9±1, 18±2, 24±3, 29±3, 33±3, 23±3 and 137±13 µU/ml in the poorly controlled group (n=28) and 9±1, 26±4, 45±7, 55±12, 48±10, 25±3 and 208±33 µU/ml in the well controlled group (n=26), respectively. Al-
Table 1. Clinical characteristics of the study group

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<th>No.</th>
<th>Age (yr)</th>
<th>Ht (cm)</th>
<th>Wt (kg)</th>
<th>Fasting blood glucose (mg/dl)</th>
<th>Fasting plasma insulin (µU/ml)</th>
<th>Blood glucose 2 h after GTT† (mg/dl)</th>
<th>Sum of insulin during GTT† (µU/ml)</th>
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§Values shown are mean±SEM.
†100 g Glucose was used for the glucose tolerance tests in diabetics, whereas 50 g glucose was loaded for the normals.

though we did not determine the insulin secretory response to a 100 g GTT in the normal subjects in this study, plasma insulin levels before and 30, 60, 90, 120 and 180 min after 100 g glucose administration and sum of them in other normal subjects whom we had already examined (age 20–63, n = 55) were 10±1, 84±4, 74±4, 63±4, 57±3, 38±2 and 323±13 µU/ml. Thus, fasting plasma insulin levels in diabetic patients were not different from normal subects but their insulin secretory response to a glucose administration was considerably weaker than that of normal subjects.

Fig. 1 shows the ability of erythrocytes from normal subjects (●), poorly controlled diabetics (○) and well controlled diabetics (△) to specifically bind insulin. Cells (2.4–3.2×10⁷ cells/ml) were incubated for 210 min at 15°C with ¹²⁵I-labeled insulin (0.4 ng/ml) plus unlabeled insulin to give the indicated total insulin concentrations. All data are corrected for nonspecific binding as indicated in the text and normalized to 2.5×10⁹ cells/ml. Data represent mean±SEM. *p<0.05, **p<0.01 compared with normal subjects. *p<0.05 poorly controlled diabetics vs well controlled diabetics.
ficant. However, the specific binding was significantly decreased at 1.4 ng/ml or greater insulin concentrations in the poorly controlled group and decreased at 100 ng/ml in the well controlled group when compared with normal subjects. Significant differences in insulin binding were also found between the well controlled group and the poorly controlled group at insulin concentrations of 5.4, 10.4, and 25.4 ng/ml. The concentration of insulin required to inhibit 50% of tracer insulin binding (ID$_{50}$) was lower in diabetics (4.3 ± 0.2 and 4.8 ± 0.3 ng/ml in the poorly controlled and well controlled groups, respectively) than in normal subjects (6.2 ± 0.3 ng/ml, p < 0.01). Fig. 2 represents the Scatchard plots (Scatchard, 1949) derived from the data in Fig. 1. The intercept of the terminal slope of the plots with the abscissa determines the maximum binding capacity. Actually, we drew a least squares straight line to fit the data at insulin concentrations of 25, 50 and 100 ng/ml according to Kahn (1974) and calculated maximum binding capacity from the x-intercept of the line in each individual. Maximum binding capacity was significantly lower in the diabetic groups (134 ± 10 and 140 ± 9 fmol/2.5 × 10$^9$ cells/ml in the poorly and well controlled groups, respectively) than in the normal subjects (179 ± 9 fmol/2.5 × 10$^9$ cells/ml, p < 0.01). The total number of receptor sites was obtained by multiplying these figures by Avogadro's number: 32.4 ± 2.3, 33.7 ± 2.2 and 43.1 ± 2.3 sites per cell in poorly controlled, well controlled groups and normal subjects, respectively. Insulin binding affinity was analyzed by the average affinity profile plot method (De Meyts and Roth, 1975) (Fig. 3). The "empty sites" affinity (Kc), which represents the average affinity obtained at an insulin concentration of 0.4 ng/ml for comparative purposes in this study, was significantly
increased in both the well controlled group (5.2 ± 0.3) and poorly controlled group (4.8 ± 0.3) compared with normal subjects (4.0 ± 0.2 × 10^8 M⁻¹).

Since the mean value of specific binding at tracer insulin concentration was higher in the well controlled group than in the poorly controlled group, we examined the correlation between fasting blood glucose levels and specific binding at tracer insulin concentration in diabetics and found a slight inverse correlation between these parameters (n=54, r = -0.30, p<0.02). However, there was no correlation between fasting blood glucose levels and receptor number or fasting plasma insulin levels and receptor number. We subdivided the diabetics according to the type of treatment (i.e. diet-treated group and sulfonylurea-treated group) and compared the results of the binding studies. However, we could not find any significant differences between these two groups.

We found some of the results were affected by aging of ¹²⁵I-insulin (data not shown). We performed, therefore, the experiments both in normal subjects and in diabetics almost on the same day using the same label (approximately 13 days after the iodination).

Discussion

The insulin receptor concentration in erythrocytes calculated from the Scatchard plots was significantly lower in diabetics than in age-matched healthy subjects. Although it has not been concluded whether the curvilinearity of the Scatchard plots is due to negative cooperativity among binding sites (De Meyts and Roth, 1975) or the existence of two or more classes of binding sites with different capacities and affinities (Pollet et al. 1977; Olefsky and Chang, 1979) or both of these factors, the
The total receptor number is the same whichever interpretation is preferred. Olefsky and Reaven (1976; 1977) have reported decreased insulin receptors on monocytes in patients with fasting hyperglycemia who have fasting hyperinsulinemia as a group but decreased insulin response to a glucose challenge. In their report, diabetic patients with normal fasting insulin levels had normal insulin binding. Also in previous reports of erythrocyte insulin receptors, fasting insulin levels of the patients were considerably increased (Robinson et al., 1979) or a negative correlation was found between fasting insulin levels and insulin binding (De Pirro et al., 1980). Therefore, it is suggested that insulin receptors are decreased due to “down regulation”. However, in our study of fasting insulin levels of nonobese diabetics were not different from controls but their insulin response to a glucose challenge was considerably decreased, as we have already reported (Kosaka et al., 1974; 1980). These data suggest that the 24-h insulin levels in the diabetic patients in this study were lower than in control subjects. Thus, our results show that decreased insulin receptors on erythrocytes in diabetics cannot be explained by plasma insulin levels. The insulin receptor number in erythrocyte was never increased in spite of hypoinsulinemia. Our results on receptor number in normal subjects were comparable to those of some other reports (Kappy and Plotnick, 1979; De Pirro, 1980), but smaller than those of others (Gambhir et al., 1977; 1978; Wachslicht-Rodbard et al., 1979). However, this discrepancy is mainly due to differences in interpretation rather than differences in data. We used an insulin concentration of 100 ng/ml as a maximum insulin concentration in our Scatchard plots and this is the reason why we obtained somewhat smaller values than others, as stated by Kappy and Plotnick (1979).

On the other hand, specific binding at tracer insulin concentration was comparable in diabetics and controls, and this is due to an increased affinity in spite of a decreased number of receptors in diabetics. Our results are not compatible with previous reports (Robinson et al., 1979; De Pirro et al., 1980; Kobayashi et al., 1980), which have shown little or no change in affinity in diabetics. This difference may be explained by a difference in the diabetic states of the patients because the mean value of fasting blood glucose levels of the previous reports was more than 180 mg/dl and greater than that of our patients. That is, we found that there was an inverse correlation between specific binding at tracer insulin concentration and fasting blood glucose levels, but no correlation was found between receptor number and fasting blood glucose levels. Therefore, our present study suggests that the specific binding may be decreased in a poorly controlled diabetic state by a decrease in affinity which is elevated in relatively well-controlled conditions, and not by a change in the receptor number. The receptor number of erythrocytes is decreased throughout the derangement of metabolic conditions when compared to controls. The above explanation seems to be supported by the recent report that hyperglycemia for more than 3 h in vivo decreased the affinity of insulin receptors for erythrocytes or monocytes (Insei et al., 1980). The amount of insulin bound in the well controlled group was comparable to normal subjects at physiological insulin concentrations because of increased affinity but it is still decreased in poorly controlled group at 1.4 ng/ml or more insulin concentrations. In the well controlled group, the increased affinity and comparable amount of insulin bound might play a contributory role in improving the diabetic states, if the same change occurs in the major target tissues. However, it should be noted that we cannot tell which is the cause and which is the effect.
Sulfonylurea therapy has been reported to be associated with a diminution in hyperglycemia and a concomitant return toward normal insulin binding of monocytes (Olefsky and Reaven, 1976). It has been suspected that an increase in insulin binding might be related specifically with sulfonylurea therapy. However, in our study of erythrocytes, no difference was found between diet-treated and sulfonylurea-treated patients. Therefore, an increase in insulin binding might be associated with an improvement in the diabetic state and not specifically associated with sulfonylurea therapy.

Even in the well controlled group, the metabolism is not perfectly corrected as shown by elevated fasting and postprandial blood glucose levels, for example. Therefore, we suspected that deranged metabolism decreased insulin receptors in diabetics. However, we cannot find significant differences in receptor number between the well and the poorly controlled groups. Since circulating erythrocytes have no nuclei and their life span is about 120 days, it is very probable that the number of erythrocyte insulin receptors was affected by the metabolic state several months before the binding studies. However, almost all of the patients (n=24) in the well controlled group (n=26) were seen regularly at our outpatient clinic and their fasting blood glucose levels three and six months before the binding studies were 112±5 and 110±3 mg/dl, respectively. Therefore, we can rule out the possibility that the decrease in the amount of insulin receptors in the well controlled group was due to poor control of the diabetic state several months previously. It is also unlikely that fasting hyperinsulinemia was present several months before in our hypoinsulinemic patients. The mechanisms of decreased insulin receptors in hypoinsulinemic diabetic patients remain to be determined in future studies.

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References


