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Abstract

125I-labeled insulin binding to peripheral human erythrocytes was studied in patients with chronic liver disease. The maximum specific 125I-labeled insulin binding was 12.10 +/- 1.13 %/4 x 10(9) cells (mean +/- SD, n = 10) in normal subjects, and significantly higher in cirrhotic patients (15.32 +/- 1.73 %, n = 11, P less than 0.01) but not in patients with acute and chronic hepatitis (11.44 +/- 2.10 %, n = 3 and 13.2 +/- 1.87 %, n = 7 respectively). The complication of diabetes mellitus significantly increased (P less than 0.05) the maximum insulin binding in chronic hepatitis. Scatchard analysis and average affinity analysis of the binding data suggest that increased insulin binding in cirrhotic patients is due to an increase in the number of insulin binding sites per erythrocytes. The complication of diabetes in chronic liver diseases results in an increase in affinity of insulin binding sites.

KEYWORDS: insulin binding, erythrocyte, liver disease.
INCREASED INSULIN BINDING TO ERYTHROCYTES IN CHRONIC LIVER DISEASE

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Abstract. $^{125}$I-labeled insulin binding to peripheral human erythrocytes was studied in patients with chronic liver disease. The maximum specific $^{125}$I-labeled insulin binding was $12.10 \pm 1.13 \% / 4 \times 10^9$ cells (mean $\pm$ SD, $n = 10$) in normal subjects, and significantly higher in cirrhotic patients ($15.32 \pm 1.73 \%, n = 11, P < 0.01$) but not in patients with acute and chronic hepatitis ($11.44 \pm 2.10 \%, n = 3$ and $13.2 \pm 1.87 \%, n = 7$ respectively). The complication of diabetes mellitus significantly increased ($P < 0.05$) the maximum insulin binding in chronic hepatitis. Scatchard analysis and average affinity analysis of the binding data suggest that increased insulin binding in cirrhotic patients is due to an increase in the number of insulin binding sites per erythrocytes. The complication of diabetes in chronic liver diseases results in an increase in affinity of insulin binding sites.

Key words: insulin binding, erythrocyte, liver disease.

Insulin resistance and impaired glucose utilization are observed in some pathological states such as obesity and non-insulin dependent diabetes mellitus. On the contrary, increased insulin sensitivity is seen in anorexia nervosa and growth hormone or glucocorticoid deficiency. The altered insulin binding in these pathological states is considered to contribute to the abnormal insulin sensitivity (1-3).

In chronic liver disease, insulin resistance with elevated peripheral insulin levels, an increased prevalence of glucose intolerance and diabetes mellitus have been documented (4-6). There is considerable interest in the insulin binding state in chronic liver disease in the insulin sensitive tissues like liver, muscle and adipose tissue as a means of understanding the abnormal carbohydrate metabolism of this condition. However no such a direct study has been done probably because of difficulties in obtaining enough materials in patients with chronic liver disease.

Recently it was shown that insulin binding sites on peripheral human erythrocytes were indistinguishable from insulin receptors in the insulin sensitive tissues (7,8) and might mirror their status (3,9). I therefore studied insulin binding by peripheral erythrocytes in patients with chronic liver disease.
MATERIALS AND METHODS

The studies were performed on 10 healthy subjects and 34 patients with liver disease who were admitted to the First Department of Internal Medicine, Okayama University Medical School. The clinical diagnoses of liver diseases were confirmed by peritoneoscopy and/or liver biopsy. Patients with fasting blood glucose levels higher than 140 mg/ml or who were being treated with insulin by injection were considered to have the complication of diabetes mellitus. Eight of 19 cirrhotic patients and three patients with chronic hepatitis had diabetes mellitus. Three patients with acute hepatitis and two patients with extrahepatic obstructive jaundice were also studied.

Detailed experimental procedures for insulin binding to human erythrocytes are described elsewhere (8). $^{125}$I-labeled porcine insulin with a specific activity of 100 to 150 mCi/mg was prepared in the modified method of Hunter and Greenwood (10). Six ml of venous blood were drawn into 1/20 vols of heparin as anticoagulant after overnight fasting. Erythrocytes were washed and purified by three successive centrifugations in the assay buffer (50 mM Hepes, 50 mM Tris, 10 mM MgCl$_2$, 10 mM CaCl$_2$, 2 mM EDTA, 5 mM KCl, 10 mM Glucose, 50 mM NaCl and 0.5 % bovine serum albumin, pH 8.0) and finally made up to about $4 \times 10^9$ erythrocytes/ml in the same buffer. Leukocyte contamination was less than one leukocyte per $10^5$ erythrocytes and considered to have a negligible influence on the insulin binding assay of erythrocytes.

About $3.2 \times 10^9$ cells/ml in the assay buffer were incubated with 0.8 ng/ml $^{125}$I-labeled insulin in the presence of native insulin ranging from 0 to $10^4$ ng/ml at 15 °C for 150 min in a final vol of 0.5 ml. At the end of the incubation, 0.2 ml duplicate aliquots were transferred to prechilled polystyrene microfuge tubes (Fisher Scientific) containing 0.3 ml dibutyl phthalate and 0.3 ml assay buffer. After centrifugation at 4,000 × g for 5 min at 4 °C in a Fisher Model 50 Microfuge and counting of total radioactivity in a well type gamma-counter, the supernatant was aspirated and discarded. The cell pellet was excised and bound radioactivity was counted. Per cent $^{125}$I-labeled insulin binding was calculated as (bound radioactivity/total radioactivity) × 100. Nonspecific binding was defined as bound radioactivity in the presence of $10^4$ ng/ml native insulin and always less than three per cent of available radioactivity. Specific binding was obtained by subtracting nonspecific binding from total insulin binding at each insulin concentration. Results were normalized to per cent specific $^{125}$I-labeled insulin binding/4 × $10^9$ cells. Degradation of radiolabeled insulin determined by 10 % trichloroacetic acid solubility was negligible during incubation.

Scatchard analysis (11) and average affinity analysis (12) were employed for data analysis.

Plasma insulin levels were determined by radioimmunoassay using Insulin Riakit (Dinabot Radio Isotope Research Lab). The results are expressed as a mean ± SD not indicated specifically and the statistical significance of the difference between two means was tested employing the unpaired t-test (13).

RESULTS

The maximum per cent $^{125}$I-labeled insulin binding to erythrocytes in chronic liver disease is given in Fig. 1 and Table I. When the “mean ± 2 × SD” obtained with ten normal subjects is taken as normal, eight out of 11 liver cirrhotic patients, five out of eight cirrhotic patients with diabetes, and all of three...
patients with chronic hepatitis and diabetes showed abnormally high values. The mean values for these pathological conditions were 15.32 ± 1.73 %, 17.21 ± 3.32 % and 16.97 ± 1.84 % respectively and significantly higher than those for normal subjects (Table I). There was no significant difference in the maximum per cent binding of chronic hepatitis, acute hepatitis, extrahepatic obstructive jaundice and normal control. At insulin concentrations higher than the physiological range, no significant difference in per cent insulin binding was observed except at 100.8 ng/ml insulin.

Fig. 1. Per cent 125I-labeled insulin binding to peripheral erythrocytes in liver cirrhosis and chronic hepatitis. Insulin binding assay was performed at 0.8 ng/ml 125I-labeled insulin as described in the text. Results are expressed as per cent specific 125I-labeled insulin binding/4 × 10^9 erythrocytes. The shaded area represents the normal range (12.10±1.13%, mean ± 2 × SD).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of patients</th>
<th>Insulin concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>Normal subjects</td>
<td>10</td>
<td>12.10±1.13</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>11</td>
<td>15.32±1.73</td>
</tr>
<tr>
<td>Liver cirrhosis with diabetes mellitus</td>
<td>8</td>
<td>17.21±3.32</td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td>7</td>
<td>13.29±1.87</td>
</tr>
<tr>
<td>Chronic hepatitis with diabetes mellitus</td>
<td>3</td>
<td>16.97±1.84</td>
</tr>
</tbody>
</table>

a, Values given as "mean ±SD"; b, p<0.001; c, p<0.01; d, p<0.02; e, p<0.05 compared with value for normal subjects. f, p<0.05 compared with values for patients with chronic hepatitis and diabetes mellitus.
There was no significant correlation between per cent insulin binding and plasma immunoreactive insulin levels, peripheral reticulocyte counts, serum aspartate and alanine aminotransferase activities, cholinesterase activities, serum cholesterol levels and disappearing rates of indocyanine green (data not shown).

Scatchard analysis (11) and the average affinity analysis (12) of binding data are shown in Figs. 2 and 3. In liver cirrhosis, increased maximum binding capacity with little change in binding site affinity in the physiological range of insulin concentration was observed (Table 2). The number of binding sites calculated from the intercept of the terminal portion of the Scatchard plots with

![Graphs showing insulin binding data](image)

Fig. 2. Comparison of $^{125}$I-labeled insulin binding to erythrocytes in liver cirrhosis (▲▲), liver cirrhosis complicated with diabetes mellitus (■■) and normal control (●●). Panel a: Competition curves plot per cent specific $^{125}$I-labeled insulin binding/4 x 10$^9$ erythrocytes as a function of insulin concentration. Each point is the mean of n = 11 for liver cirrhosis and n = 8 for liver cirrhosis complicated by diabetes mellitus. The shaded area represents the values for normal subjects (mean ± SD, n = 10).
Panel b: Scatchard plots (11) were derived from the same data. The bound/free insulin (B/F) is plotted as a function of bound insulin (B). The intercept of the curve with the abscissa gives the maximum binding capacity (Ro).
Panel c: The same data are replotted as an affinity profile according to De Meys et al. (12). Average affinity ($\bar{K}$) is plotted as a function of binding site occupancy ($\bar{Y}$), where $\bar{K} = (B/F)/(Ro - B)$ and $Y = (B/Ro) \times 100$. 

http://escholarship.lib.okayama-u.ac.jp/amo/vol35/iss3/1
the abscissa was 230 binding sites/erythrocyte for normal subjects and 290 binding sites/erythrocyte for liver cirrhotic patients (Table 2). In chronic hepatitis, decreased binding site affinity cancelled the effect of the increased binding capacity on maximum insulin binding, which resulted in normal per cent $^{125}$I-labeled insulin binding. The complication of diabetes mellitus in both liver cirrhosis and chronic hepatitis seems to increase binding site affinity at empty site from $1.56 \times 10^8$ M$^{-1}$ to $2.00 \times 10^8$ M$^{-1}$ in the former and $1.19 \times 10^8$ M$^{-1}$ to $1.63 \times 10^8$ M$^{-1}$ in the latter without significant changes in binding site affinity at filled sites and maximum binding capacity (Table 2). As a result, the maximum per cent $^{125}$I-labeled insulin binding in patients with chronic hepatitis complicated with diabetes was significantly ($P < 0.05$) higher than that in patients with chronic hepatitis without diabetes.

Fig. 3. Comparison of $^{125}$I-labeled insulin binding to erythrocytes in chronic hepatitis (△), Chronic hepatitis complicated with diabetes mellitus (■) and normal control (○).

Panel a: Competition curves. Each point is the mean of $n = 7$ for chronic hepatitis and $n = 3$ for chronic hepatitis complicated with diabetes mellitus. The shaded area shows the mean ± SD for ten normal subjects.

Panel b: Scatchard plots derived from the same data.

Panel c: Average affinity profiles from the same data.
Though the number of patients was limited, there seemed no significant alterations in maximum binding capacity and binding site affinity in acute hepatitis and extrahepatic obstructive jaundice (Table 2).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Binding site Concentration</th>
<th>Binding site affinity (×10⁶M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>230</td>
<td>1.50</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>290</td>
<td>1.56</td>
</tr>
<tr>
<td>Liver cirrhosis with diabetes mellitus</td>
<td>260</td>
<td>2.00</td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td>320</td>
<td>1.19</td>
</tr>
<tr>
<td>Chronic hepatitis with diabetes mellitus</td>
<td>320</td>
<td>1.63</td>
</tr>
<tr>
<td>Acute hepatitis</td>
<td>230</td>
<td>1.41</td>
</tr>
<tr>
<td>Extrahepatic obstructive jaundice</td>
<td>240</td>
<td>1.34</td>
</tr>
</tbody>
</table>

\(a\), Values are calculated using mean values given in Table 1, according to Scatchard (12) and De Meyts et al. (13); \(b\), Values are number of binding sites/erythrocyte. \(c\), \(K_e\) and \(K_f\) represent average affinity at empty sites and filled sites respectively.

DISCUSSION

Increase in the number of insulin binding sites on peripheral erythrocytes with little change in their binding affinity was shown in liver cirrhosis, which resulted in overall increase in insulin binding in the physiological range of insulin concentration. On the other hand, parallel decrease in the binding affinity cancelled the effect of the increased binding capacity in chronic hepatitis. The complication of diabetes mellitus in chronic liver disease was accompanied by increased binding affinity without appreciable change in binding site concentration.

It is of moderate interest whether the observed changes in insulin binding to erythrocytes in chronic liver disease correlate with those in insulin receptors in insulin sensitive tissues. Though circulating monocytes are believed to mirror the status of insulin receptors in insulin sensitive tissues like adipose tissue (14,15), it is controversial whether insulin binding sites in peripheral erythrocytes correlate well with those in the target tissues for insulin. Several authors report a significant decrease in insulin binding to erythrocytes in adult onset diabetes mellitus compared with the normal control (9,16) which was similar to that observed with circulating monocytes (17). Others, however, found no such correlation (18,19). Down regulation, in which ambient insulin concentration regulates insulin receptor concentration, is generally observed in insulin sensitive tissues and
monocytes (20), but not in peripheral human erythrocytes (18,19,21). In the present study, down regulation in erythrocytes from patients with chronic liver disease was not found. In addition, erythrocytes from normal subjects were incubated with patient’s own plasma and with plasma from patients with liver cirrhosis who showed elevated insulin binding for up to five hours at 37 °C. No difference in maximum per cent insulin binding was observed between cells incubated with normal plasma and with plasma from the patient with liver cirrhosis. On the other hand, it was suggested that insulin receptors are regulated independently of plasma insulin levels in liver disease (22). Primary alteration in the number of hepatic insulin receptors, which subsequently led to insulin resistance and hyperinsulinemia was documented in D-galactosamine induced acute hepatic injury of rats (22) in contrast to down regulation confirmed in hepatic plasma membrane of the rats made hyperinsulinemic or hypoinsulinemic (23,24). Therefore, neither plasma insulin nor other plasma components seem to regulate insulin binding on peripheral erythrocytes in chronic liver disease. However, one must be careful in assuming that insulin binding sites in erythrocytes directly mirror insulin receptors in the target tissues for insulin.

Increase in the insulin receptor number accompanying cell differentiation was observed on 3T3-L1 preadipocytes (25), but Friend erythroleukemia cells show decrease in insulin receptors during cell differentiation (26). These observations suggest that the mean age of an erythrocyte preparation used for binding assay may influence insulin binding. In fact, the levels of specific insulin binding correlate well with the number of reticulocytes (27,28). In the present study, we failed to demonstrate any direct correlation between the per cent $^{125}$I-labeled insulin binding and the number of reticulocytes in peripheral blood. However, as a reticulocyte count does not always mean the average age of a erythrocyte preparation and as shortened erythrocyte survival time in chronic liver disease was confirmed (29), it is still possible that increased insulin binding in chronic liver disease reflects the relatively younger population of erythrocytes in chronic liver disease.

Interestingly, increased binding capacity with lowered binding affinity in erythrocytes from children (30) and increased binding capacity with normal or increased binding affinity in cord blood erythrocytes (30,31) are similar to the changes observed in chronic hepatitis and liver cirrhosis in the present study. These alterations in insulin binding in the cord blood erythrocytes are also not correlated with plasma insulin levels (30,31) and may not be caused by short exposure of cells to the cord plasma (32). Chronic exposure of erythrocytes to abnormal levels of multiple plasma components in vivo, which may be seen in both chronic liver disease and in the gestational mother or fetus, may explain the alterations in the insulin binding data in the present study.

The reason for the change in the affinity of the insulin receptors is unknown (20). An increase in the affinity of the empty insulin receptor was observed in
obese patients subjected to fasting (33), in some patients with insulinoma (34), in normal subjects given glucose (35) and in patients with acromegaly (36). In addition, an increase in affinity was also seen in turkey erythrocytes exposed to insulin (36). Circulating factors such as insulin and growth hormone may be responsible for these alterations in insulin receptor affinity and may induce an increase in the binding affinity of insulin binding sites in erythrocytes in chronic liver disease complicated by diabetes mellitus.

In summary, insulin binding to circulating erythrocytes at physiological insulin concentrations was shown to increase in chronic liver disease due to increase in the number of binding sites. The complication of diabetes mellitus in chronic liver disease causes an increase in binding affinity at the empty sites. The relation of these changes to the pathogenesis of insulin resistance in chronic liver disease is unclear.

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Insulin Binding in Chronic Liver Disease


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