Insulin Receptors on Hepatocytes from Spontaneously Diabetic Chinese Hamsters

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Abstract

Insulin receptors on hepatocytes were studied in spontaneously diabetic Chinese hamsters, which are the animal models for insulin deficient diabetes. Insulin binding in diabetic animals increased mainly due to an increase in the number of receptors. Although binding affinity of diabetic animals was similar to that of control animals, a kinetic study revealed that both the association rate constant and the dissociation rate constant decreased in diabetic animals. Negatively cooperative interactions between receptors were demonstrated in control and diabetic animals, and both the magnitude and sensitivity of this effect was the same in both types of animals. A significant inverse correlation between insulin binding and the plasma insulin concentration was found in these animals. These results therefore suggest that there is an increase in the insulin binding in the insulin deficient diabetic state mainly due to an increase in the number of receptors with a decrease in both the association and dissociation rate constants, and these changes may be important in the altered metabolic state.

The first step in insulin action is binding to surface receptors on target cells (Czech, 1977). It seemed that the insulin receptors played an important role in the elucidation of insulin resistance in obese hyperinsulinemic diabetes, as the insulin receptors of target cells in this type diabetes generally decreased in number (Olefsky, 1976; Lerea and Livingston, 1983; Karakash and Jeanrenaud, 1983). On the other hand, in non-obese hypoinsulinemic diabetes such as streptozotocin induced diabetic rats, the number of insulin receptors did not decrease, and the measurement of insulin receptors seemed to be unimportant in the elucidation of abnormalities in this type of diabetes (Davidson and Kaplan, 1977). However, recently many reports on the kinetics, structure, internalization and recycling of insulin receptors in many experimental conditions have been published, and the study of insulin receptors has been thought to be important when considering diabetes (Okamoto et al., 1984; Gorden et al., 1982; Ronett et al., 1983).

In the Asahikawa colony of Chinese hamsters diabetes mellitus occurs at high frequency, and severe diabetic animals show various metabolic derangements, such as hyperglycemia hypoinsulinemia, and hyperlipidemia (Watanabe et al., 1983; Funaki
and Mikamo, 1983). We therefore considered that it was useful to examine the insulin receptor in spontaneously diabetic Chinese hamsters as a model of genetic insulin deficient diabetes.

**Materials and Methods**

**Animals**

Chinese hamsters were selected for the study from among the Chinese hamsters in the Asahikawa colony by the oral glucose tolerance test (OGTT). After an overnight fast (18–20 h) a zero-hour blood sample was obtained from the orbital sinus with a heparinized hematocrit tube. Immediately after zero-hour sampling OGTT (2g/kg body weight) was performed. The animals were bled 30, 60, and 120 min after glucose administration. In control group fasting plasma glucose (FPG) was less than 140 mg/dl and plasma glucose levels at 30, 60, and 120 min after glucose load were less than 180 mg/dl. In the diabetic group, FPG was more than 300 mg/dl. All animals were fed a standard diet (MF, Oriental Lobo, Tokyo) ad libitum and were maintained in an air-conditioned room illuminated from 5:00 a.m. to 7:00 p.m. They were sacrificed in non-fasting states for the experiments.

**Isolation of hepatocytes**

Hepatocytes were isolated by a modification of the method of Berry and Friend (Berry and Friend, 1969) after blood collection from a jugular vein for determination of the plasma glucose, plasma lipids, and hemoglobin A1. The method of isolating hepatocytes is briefly summarized as follows. Nontraumatic cannulation of the portal vein and preperfusion of liver using Ca²⁺-free Hanks solution (PH 7.6) with 0.19 g/l EGTA and 2.38 g/l HEPES at 37°C, were done immediately. Then the liver was perfused with the buffer including 0.5 g/l collagenase and 0.56 g/l CaCl₂ for 10 min with a peristaltic pump (10 ml/min). The liver was teased apart and the cell suspension filtered through a cell filter (150 μm and 60 μm). The cells were washed three times in ice-cold Hanks' solution (50×g, 1 min). The final cell pellets were washed three times in ice-cold Krebs Ringer bicarbonate (KRB) buffer containing 10 mg/ml bovine serum albumin. These buffers were gassed with a mixture of 95% O₂ and 5% CO₂. More than 90% of the hepatocytes were found intact as judged from their ability to exclude trypan blue.

**Binding study**

¹²⁵I-insulin was prepared by the chloramine T-method with a specific activity of 200 μCi/g. The hepatocytes were suspended to give a final concentration of 2.5×10⁶/ml in the KRB buffer, and were incubated with ¹²⁵I-insulin (0.2 ng/ml) and unlabeled porcine insulin over a range of concentrations from 0.1 to 2000 ng/ml for 2 h at 20°C. After incubation, aliquots of the mixture (250 μl) were added to 1 ml of ice-cold KRB buffer, centrifuged at 2000 rpm and 4°C for 1 min. The cells were washed twice, and radioactivity in the pellets was determined. Non specific binding, defined as radioactivity in the presence of 2000 ng/ml of unlabeled insulin, was less than 25% of the total binding. Degradation of ¹²⁵I-insulin during the binding assay determined by the TCA precipitation method was less than 10% of total radioactivity. The number of receptors and “empty site” affinity constant were calculated from Scatchard plots (Scatchard, 1949) by the method of DeMeyts et al. (1975).

**Association study**

Isolated hepatocytes were incubated in the KRB buffer containing 0.6 ng/ml of ¹²⁵I-insulin at 20°C. At the indicated time, 1 ml of ice-cold KRB buffer was added and centrifuged at 2000 rpm and 4°C 1 min. Radioactivity in the cell pellets was then counted. Non specific binding was determined as the binding in the presence of 2000 ng/ml of unlabeled insulin.

**Dissociation study**

All dissociation studies were performed at 20°C by a modification of the method of Okamoto et al. (1984). Hepatocytes were preincubated with ¹²⁵I-insulin (1 ng/ml) in a total volume of 3 ml. After steady state binding was achieved by incubation for 120 min, the cells were centrifuged at 500 rpm for 2 min. The buffer was removed and replaced with an equal amount of insulin-free media at 20°C. Aliquots of the cell suspension (100 μl) were distributed into tubes containing 1.9 ml of the buffer with (dilution plus insulin) or without (dilution only) unlabeled insulin (1000 ng/ml) at 20°C. At the indicated time, the cells in these tubes were centrifuged at 2000 rpm for 1 min. Radioactivity in the cell pellets was counted, and non specific binding was subtracted from total binding.
Assays
Plasma glucose was measured by the glucose oxidase method. The plasma immunoreactive insulin concentration was determined by the enzyme immunoassay method (Yoshioka et al., 1978) using porcine insulin as a standard. Plasma total cholesterol (TC) and triglyceride (TG) were measured enzymatically. Hemoglobin A1 (HbA1) was measured by the affinity chromatography method.

Statistics
Results were expressed as mean±SEM. Statistical analyses were performed by Student’s t-test after examining distribution with the F-value.

Results
Animals
Characteristics of the experimental animals are summarized in Table 1. Plasma glucose levels were markedly increased, and insulin concentrations were decreased in the spontaneously diabetic Chinese hamsters. In diabetic animals, plasma lipids and HbA1 were also increased. The duration of diabetes was at least 3 months.

Table 1. Characteristics of Experimental Animals. Values are mean±S.D. Chinese hamsters were sacrificed in non-fasting. Diabetic, spontaneously diabetic Chinese hamsters in the Asahikawa colony.

<table>
<thead>
<tr>
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<th>Control</th>
<th>Diabetic</th>
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<tbody>
<tr>
<td>Age (months)</td>
<td>9.3±4.9</td>
<td>7.1±2.0</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>39.9±5.5</td>
<td>37.4±6.3</td>
</tr>
<tr>
<td>Plasma Glucose (mg/dl)</td>
<td>138±25</td>
<td>510±93**</td>
</tr>
<tr>
<td>Plasma Insulin (µU/ml)</td>
<td>110±51</td>
<td>19±11</td>
</tr>
<tr>
<td>Hemoglobin A1 (%)</td>
<td>7.3±1.4</td>
<td>20.9±3.9**</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>132±31</td>
<td>249±152</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>133±86</td>
<td>439±462</td>
</tr>
</tbody>
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** p<0.01

Binding study
Insulin binding to hepatocytes is shown in Fig. 1-a. Specific insulin binding at a tracer concentration of insulin (0.2 ng/ml) was 6.68±1.10% in the control and 11.30±2.72% in diabetic animals. The receptor concentration and affinity constant obtained

Fig. 1-a. Specific 125I-insulin binding to hepatocytes from control (●) and spontaneously diabetic Chinese hamsters (○). Data represent mean±S.E.M. * p<0.05; ** p<0.01.
from the Scatchard plot (Fig. 1-b) were $1.79 \times 10^6$ cell$^{-1}$ and $9.00 \times 10^7$ M$^{-1}$ in the control and $4.01 \times 10^6$ cell$^{-1}$ and $6.80 \times 10^7$ M$^{-1}$ in the diabetic. Thus insulin binding in the diabetic increased mainly due to an increase in the number of receptors. To analyze the change in affinity more precisely, these data were plotted on an average affinity profile (Fig. 1-c) as described by DeMeyts and Roth (1975). With this analysis, the average affinity of the receptor is plotted as a function of fractional receptor occupancy. As can be seen in Fig. 1-c, the average affinity ($K$) falls as a function of receptor occupancy (negative cooperativity) until the lowest observed affinity ($K_f$) is reached. $K_e$ represents the highest observed affinity of the receptors and is exhibited in the native or "empty site" state. 50% of the decrease in $K$ is reached at a fractional receptor occupancy of 2.1% and 2.4% in cells from control and diabetic animals, respectively. The $K_f/K_e$ ratio represents the potency of the cooperative effect. This ratio is 0.7 and 0.67 in cells from control and diabetic animals, respectively. Then negatively cooperative interactions between receptors were readily demonstrated in control and diabetic animals, and the magnitude and sensitivity of this effect was the same in both groups of animals. To make clear the changes in the affinity constant and the cooperative effect in these animals, the association and dissociation rate were measured.

**Association study**

Fig. 2-a shows the initial part of the time course of insulin binding. The actual amount bound to hepatocytes from diabetic Chinese hamsters was significantly higher than control cells. To compare the rate of association on a uniform basis, we calculated the ratio of bound insulin to the number of receptors according to the method of Okamoto et al. (1984). With this analysis, it was suggested that the association rate constant in diabetic animals was lower than in control (Fig. 2-b). The association rate constants calculated from the initial 6 minutes were $1.54 \times 10^5$ M$^{-1}$ S$^{-1}$ in the control and $1.25 \times 10^5$ M$^{-1}$ S$^{-1}$ in the diabetic animals.
**Dissociation study**

The results of the dissociation study are summarized in Fig. 3. The dissociation rate constant estimated from the "dilution only" study was decreased in diabetic animals. $^{125}$I-insulin dissociation from hepatocytes of both control and diabetic animals was faster in the "dilution plus insulin" group than in the "dilution only" group. The magnitude of this effect was the same in all these animals. These results agree with the observation of negative cooperativity in a binding study.
Fig. 3. Dissociation of $^{125}$I-insulin from hepatocytes in the absence of insulin (dilution only; —) or in the presence of insulin (dilution plus insulin; - - -). Control, ●; spontaneously diabetic Chinese hamsters, ○; data represent mean ± S.E.M. * $p<0.05$; **$p<0.01$.

Fig. 4. Correlation between specific $^{125}$I-insulin binding to hepatocytes and plasma insulin concentration in jugular vein. All animals were fed ad libitum. An inverse correlation was found as the correlation ratio was $-0.66$ ($p<0.01$). Control, ●; spontaneously diabetic Chinese hamsters, ○.
Correlation between specific $^{125}$I-insulin binding to hepatocytes and plasma substances

A significant inverse correlation was found between the binding to hepatocytes and the plasma insulin concentration in the jugular vein at the time of sacrifice (Fig. 4). The correlation ratios were $-0.66$ between the binding and the plasma insulin concentration ($p<0.01$), $0.77$ between the binding and the plasma glucose concentration ($p<0.01$), $0.72$ between the binding and Hb Al ($p<0.01$), $0.58$ between the binding and TC ($p<0.05$), and $0.46$ between the binding and TG (no significance).

Discussion

In the present study, we have explored insulin receptor kinetics on hepatocytes from control and spontaneously diabetic Chinese hamsters. From one to three months after birth, these diabetic animals show various metabolic derangements such as hyperglycemia, hypoinsulinemia, and hyperlipidemia, as shown in Table 1 (Watanabe et al., 1983; Funaki and Mikamo, 1983; Iwashima et al., 1985). We think that the plasma insulin levels in this study may be slightly high because of both the non-fasting butchery and the insulin assay with antiporcine insulin antibody as an insulin antibody and porcine insulin as a standard insulin. The animals used in this experiment had never received insulin injections. The plasma insulin levels of non-fasting Chinese hamsters in Gerritsen and Dulin's report (1967) are similar to our data. They reported that the plasma insulin levels of the non-diabetic and the mildly diabetic were significantly increased by feeding.

Specific $^{125}$I-insulin binding to hepatocytes from spontaneously diabetic Chinese hamsters increased mainly due to an increase in the number of insulin receptors (Fig. 1). This result agrees with the Hepp's report on the use of liver plasma membrane from spontaneously diabetic Chinese hamsters in an Upjohn colony (Hepp et al., 1975). As we found a significant inverse correlation between the insulin binding and the plasma insulin concentration (Fig. 4), hypoinsulinemia may seem to cause the up-regulation of the insulin receptors in these insulin deficient animals.

We found a decrease in both the association and dissociation rate constant of $^{125}$I-insulin to hepatocytes from spontaneously diabetic Chinese hamsters. This is a new finding. Affinity did not increase because of a decrease of the association and dissociation rate constant, since the affinity constant of the insulin binding reaction represents the ratio of the rate constants for association and dissociation.

The metabolic abnormalities, such as hyperglycemia, hypoinsulinemia, and hyperlipidemia, seen in these experimental animals might give rise to a decrease in both the dissociation and association rate in hepatic insulin receptors. In general, it proved that the hepatic insulin receptors caused internalization or recycling (Marshall et al., 1981; Gorden et al., 1982). If the insulin receptors in spontaneously diabetic Chinese hamsters show internalization or recycling as seen in other animals, the change in the plasma membrane content probably due to chronic hyperglycemia or hyperlipidemia may influence this process and then the dissociation or association rate constant of insulin receptor may decrease. On the other hand, it was reported that the diabetic state changed the lipid content or fluidity in plasma membranes and the membrane lipid environment could influence the binding properties of the insulin receptor (Ginsberg et al., 1981; Gould et al., 1982). We may suppose that the modification of the insulin receptor itself may be caused by metabolic abnormalities and the abnormal insulin binding may cause the decrease in
both the association and dissociation rate constants. From this time on, we shall endeavour to elucidate the mechanism of these changes.

There were few reports on the study of insulin receptors in spontaneously insulin deficient diabetic animals. Okamoto et al. studied the insulin receptors on hepatocytes from streptozotocin-induced diabetic rats (Okamoto et al., 1984). They reported that in diabetic rats the number of receptors and affinity constant were increased with a decrease in the dissociation rate constant. The association rate constant in their report, which was $1.0 \times 10^5 \text{ M}^{-1} \text{S}^{-1}$ in rats, was comparable with ours, which was $1.25$ or $1.54 \times 10^5 \text{ M}^{-1} \text{S}^{-1}$ in Chinese hamsters. Olefsky and Kobayashi (1978) reported the fasting-induced increase in insulin binding with a decrease in the dissociation rate constant. They suggested the possibility that the fasting-induced change might be related to some intracellular or intramembranous chemical changes. This is very interesting evidence of a decrease in the dissociation rate constant in our diabetic animals, and it may occur partially through the same mechanism in those conditions. The preliminary study using the isolated hepatocytes from Sprague-Dawley rats showed the same association (normal: $1.01 \times 10^5 \text{ M}^{-1} \text{S}^{-1}$; streptozotocin-induced diabetic: $0.96 \times 10^5 \text{M}^{-1} \text{S}^{-1}$) and dissociation (normal: $4.33 \times 10^{-4} \text{S}^{-1}$; diabetic: $2.67 \times 10^{-4} \text{S}^{-1}$) rate constants as in Okamoto's (Okamoto et al., 1984). Therefore, it was suggested that the dissociation rate constant of hepatic insulin receptor in Chinese hamsters would be lower than in rats. As we did not use the insulin from Chinese hamster but porcine insulin in the binding study and the dissociation study, we might get different results from those for rats.

The affinity profile and the dissociation study revealed the negatively cooperative interactions between receptors in both control and diabetic (Fig. 1-c and Fig. 3). The $K_f/K_e$ ratio, which was defined as representing the potency of the cooperative effect, and the fractional occupancy at half of the decrease in $K$, which was defined as representing the sensitivity of the cooperative effect (DeMeyts et al., 1973), were almost the same in the hepatocytes from both control and diabetic animals.

In conclusion, we found an increase in the number of insulin receptors on hepatocytes from spontaneously diabetic Chinese hamsters with a decrease in both the association rate constant and the dissociation rate constant. The putative mechanism of these changes remains to be identified. As these changes may be important in the altered metabolic state in insulin deficient diabetes, we have to do a more precise study with these animals. We consider that the spontaneously diabetic Chinese hamsters are useful to use in studying insulin receptor kinetics in the insulin deficient state.

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**References**


