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# DECREASED BINDING OF INSULIN TO LIVER PLASMA MEMBRANE RECEPTORS IN HEREDITARY DIABETIC MICE

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## SUMMARY

The interaction of insulin with its receptors was studied in liver plasma membranes of the young non-obese hereditary diabetic mouse (KK strain).

Under identical conditions of preparation and incubation, the membranes of the KK mouse bind only 55–70 % as much insulin per mg of protein as those of the control mouse (Swiss albino).

Scatchard analysis suggests that this decrease in binding is due to a decrease in the number of receptor sites in the membrane of the diabetic mouse.

However, the membranes of diabetic and control mice do not exhibit significant differences in hexosamine and sialic acid contents, enzyme activities, and protein and glycoprotein analysis.

The decrease in insulin receptors in the KK mouse seems to correlate with the insulin resistance which they exhibit.

## INTRODUCTION

It seems that the first step in the action of insulin and other polypeptide hormones is binding to receptor sites on the plasma membranes of target cells [1–8]. This reaction has been studied with radioactively labelled insulin and several receptor preparations [2–16].

Recently, these studies have been applied to some pathological states in which the insulin action is altered [4, 12–15].

The hereditary diabetic strain of KK mice was first characterized by Nakamura [17]. Subsequently, several authors have described the metabolic disorders of KK mice as chemical diabetes, showing mainly impaired tolerance to glucose (without hyperglycemia in the fed state) [18–21], marked elevation of plasma insulin [17–20], pancreatic islet cell hyperplasia [17], obesity [20] and diabetic-like vasculopathy [21–23].

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Resistance to endogenous and exogenous insulin has been shown [18–20].

Several of these disorders are age dependent and appear progressively after the first month of life [21–22]. In the present study, we have investigated the binding of insulin to liver membranes of young diabetic mice (4–6 weeks old). They were non-obese, exhibiting a significant but moderate increase of plasma insulin level, without hyperglycemia.

In the KK mouse liver plasma membranes, we found a decrease in insulin binding that is due to a decrease in the number of receptor sites. The composition of plasma membranes of diabetic mice, however, did not exhibit significant differences as compared to control mice.

## MATERIALS AND METHODS

Non-obese, genetically determined, spontaneously diabetic KK mice were obtained from the laboratory of Professor G. Lagrue (Unité Inserm 99, Hôpital H. Mondor Creteil) who received them directly from Hoechst Japan Limited.

In this study all KK mice were males (4–6 weeks old) raised from the $F_2$ and $F_3$ generations derived from the initial stock.

Swiss albino mice that were comparable with respect to age and sex served as controls and were kept under the same laboratory conditions. All animals were maintained at libitum on Purina rat chow.

Physiological parameters such as body weight and blood plasma insulin were determined using standard techniques [20].

Plasma membranes were prepared from livers of the diabetic and control animals (4–6 weeks old) following a 24-h fast, according to the method of Ray [24] and they were kept frozen at −20 °C until use.

Protein concentrations were determined by the method of Lowry et al. [25] using bovine serum albumin as standard.

The assay for the specific binding of $^{125}$I insulin to the membrane was a slight modification of the method of Cuatrecasas [7, 9, 26]. Briefly, 50 $\mu$l of a membrane solution (2 mg/ml), were incubated at 24 °C to equilibrium (30–40 min), in 0.5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 1 % (w/v) albumin and $^{125}$I insulin ($10^{-9}$–$10^{-11}$ M). In order to control the membrane content of each incubation sample, 4 tubes were made containing 50 $\mu$l of the same membrane solution in 0.5 ml of incubation buffer (without insulin and albumin). The variation between the amount of proteins in these control tubes and the expected content of membrane was less than 5 %. ($^{125}$I insulin (100–200 mCi/mg) was purchased from the Amersham Radiochemical Centre. The radioactivity of this product was 95 % precipitable in 20 % trichloroacetic acid.) Three ml of ice-cold Krebs-Ringer bicarbonate buffer containing 0.1 % (w/v) albumin were added and the contents passed through cellulose acetate EGWP Millipore filters under vacuum, and the filters were washed under vacuum with 10 ml of ice-cold Krebs-Ringer bicarbonate buffer containing 0.1 % (w/v) albumin

The moist filters were dried and the radioactivity was determined in a solid scintillation $\gamma$-counter [27].

Every determination of binding was performed in triplicate and for every such determination in parallel, duplicate samples were made in the presence of native

insulin (20–40 $\mu$g/ml) to determine and correct for the "non-specific" binding of insulin [7, 9, 26].

Using these experimental conditions, the subtracted "non-specific" binding represented only about 10–20 % of the total binding for membranes of control and diabetic mice.

Degradation of the unbound insulin present in the filtrate was measured by the loss of ability to bind to fresh aliquots of membranes [16]. After the incubation of 4 ng $^{125}$I insulin with 100 $\mu$g of membranes in 0.5 ml of Krebs-Ringer bicarbonate buffer (20 min, 24 °C), the medium was filtered and the filter rinsed with 1.5 ml of Krebs-Ringer bicarbonate buffer at 24 °C.

Aliquots of the filtrate (0.5 ml containing approximately 1 ng $^{125}$I insulin) were incubated with fresh membranes (100 $\mu$g) for an additional 20 min and the specific insulin binding radioactivity, determined as described above, was compared with the results obtained in parallel incubations of 1 ng fresh $^{125}$I insulin with 100 $\mu$g of membrane proteins.

The results of the binding assays were expressed according to the Scatchard representation [28], as modified by Kahn et al. [16].

For each determination, the dissociation constants ($K_d$) and the number of receptor sites per mg of membranes ($N$) were calculated.

12 independent determinations were made for control mice (8 membrane preparations). 8 independent experiments were performed with 6 membrane preparations of diabetic mice.

The values of the binding constants are the mean of these determinations $\pm$ standard error (S.E.).

Statistical analysis of the differences between normal and diabetic membrane constants were made using the Students t-test.

5'-Nucleotidase assays were carried out at 37 °C according to the method of De Duve [29].

Glucose-6-phosphatase assays, to determine microsomal contamination, were performed by the method of Harper [30].

The hexosamine content of the plasma membranes was determined by the modified Elson-Morgan reaction as described previously [31, 32]. Sialic acid determinations were performed by the method of Warren [32, 33].

Gel electrophoresis of membrane proteins in the presence of sodium dodecylsulphate was conducted in a discontinuous buffer system as described by Neville et al. [34].

Some membranes of control and diabetic mice (5–10 mg/ml), solubilized with 1 % Triton X-100 according to Cuatrecasas [10, 11], were also analysed by acrylamide gel electrophoresis [34].

Protein bands were identified by staining with coomassie blue and glycoproteins were characterised by periodate-Schiff stain coloration [35].

## RESULTS

### *Physiological parameters*

The young KK mice studied (4–6 weeks old) are slightly, but not significantly, heavier than normal mice. They do not exhibit increased blood sugar levels. They

TABLE I

BLOOD SUGAR AND PLASMA INSULIN IN 4-WEEK-OLD KK AND SWISS ALBINO MICE

(non fasted) (KK mice of $F_2$ generation) (n.s.: non significant difference)

| Type mouse | No. | Blood sugar (mg %) | Plasma insulin ($\mu$U/ml) | Body weight (g) |
|---|---|---|---|---|
| Swiss albino | 12 | 150±10 | 14±1 | 17±1 |
| KK | 12 | 170±10 | 26±3 | 19±2 |
| *P* | | n.s. | <0.01 | n.s. |

do, however, show a moderate but significant elevation of plasma insulin concentration (Table I).

KK mice are known to be insulin resistant at this age [18–20].

*$^{125}I$ insulin binding*

Under identical conditions, $^{125}$I insulin binding by the liver plasma membranes of the diabetic mice was markedly decreased as compared to that obtained for an identical membrane preparation of the control mice (Fig. 1).

For example, it was necessary to raise the insulin concentration by 50–75 % to obtain values of the hormone bound to the diabetic membranes of an order comparable to that obtained with the control membranes (Fig. 1).

Scatchard analysis suggested that the receptor population of both membrane preparations is heterogeneous with respect to equilibrium constants (Fig. 2).

Two types of sites were observed: a high affinity, low capacity site (with a dissociation constant, $K_{d_1}$, near $5 \cdot 10^{-11}$ M and a binding capacity, $N_1$, of about

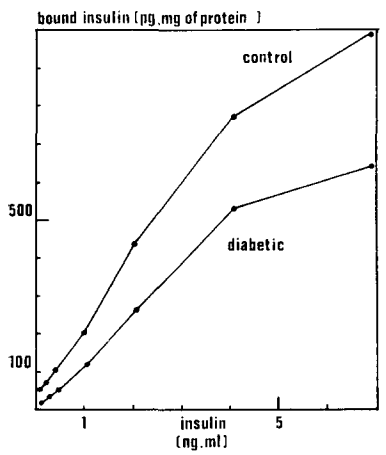


Fig. 1. Specific binding of $^{125}$I insulin to liver membranes of diabetic and control mice. Membranes (0.15 mg/ml) were incubated for 30 min at 24 °C with the indicated amount of $^{125}$I insulin. Specific binding was determined as described in Materials and Methods. Each point is the mean of two separate experiments with one membrane preparation performed in triplicate. The standard deviation for all points was less than 5 %. Similar experiments were performed with each membrane preparation (12 for control mice and 8 for diabetic mice).

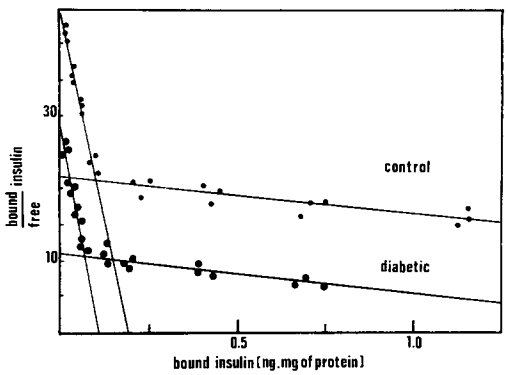


Fig. 2. Scatchard analysis of receptor-insulin interaction in diabetic and control mouse liver membranes. A characteristic determination of kinetic constants in mouse liver, according to the Scatchard representation [28] is shown. The concentration of both types of membranes was 0.2 mg/ml; the insulin concentration varies between $8 \cdot 10^{-12}$ and $1.5 \cdot 10^{-9}$ M. Hormone bound to membranes was expressed as ng/mg of protein. Free hormone was expressed as ng/ml. The bound to free insulin ratio values are off by $10^{-2}$. For each concentration three identical incubations were performed. Each point represents one incubation. Data are corrected for non-specific binding. For further details on experimental procedure see Materials and Methods. Similar determinations were performed with each membrane preparation (12 for control mice and 8 for diabetic mice).

$3 \cdot 10^{-14}$ mol of hormone per mg of membrane proteins for the control mice), and a low affinity, high capacity site (with a $K_{d_2}$ of about $3 \cdot 10^{-9}$ M and a binding capacity $N_2$, of $70 \cdot 10^{-14}$ mol/mg for the control mice) (Table II).

The difference observed in the amount of insulin binding by the membranes of diabetic mouse liver appears to be due to a decrease in the number of receptor sites of these membranes.

Statistical analysis of the results indicate that the decrease of 41 % and 48 % in the number of high affinity ($N_1$) and low affinity ($N_2$) sites, in diabetic membranes are significant ($P < 0.05$) and highly significant ($P < 0.01$), respectively (Table II).

TABLE II

KINETIC CONSTANTS IN LIVER MEMBRANES OF DIABETIC AND CONTROL MICE

Data were corrected for non-specific binding and the constants for the two sites were determined as described in Materials and Methods. Site I is the high affinity, low capacity site, Site II is the low affinity, high capacity site. The numbers represent the mean ± S.E. of 12 determinations (control mice) and 8 determinations (diabetic mice), each determination was performed as indicated in Fig. 2.

| Kinetic constant | Site | Control membrane | Diabetic membrane | % of variation | Statistical analysis |
|---|---|---|---|---|---|
| Dissociation constant (mol/l) | I | $(5.5 \pm 1.4) \cdot 10^{-11}$ | $(4.6 \pm 1.7) \cdot 10^{-11}$ | −16 % | Non significant |
| | II | $(2.9 \pm 0.3) \cdot 10^{-9}$ | $(3.2 \pm 0.4) \cdot 10^{-9}$ | +10 % | Non significant |
| Binding capacity (mol/mg protein) | I | $(2.9 \pm 0.3) \cdot 10^{-14}$ | $(1.7 \pm 0.3) \cdot 10^{-14}$ | −41 % | $P < 0.05$ |
| | II | $(67 \pm 3) \cdot 10^{-14}$ | $(35 \pm 2) \cdot 10^{-14}$ | −48 % | $P < 0.01$ |

TABLE III

DEGRADATION OF $^{125}$I INSULIN BY CONTROL AND DIABETIC MEMBRANES

The values are the mean ±SD. For details in experimental procedure see Materials and Methods.

| Type of membrane preparations | No. | Specific binding (pg of $^{125}$I insulin per mg of membrane proteins) | |
|---|---|---|---|
| | | "Fresh" $^{125}$I insulin without preincubation (A) | $^{125}$I insulin preincubated with membranes and reincubated as in (A) (B) |
| Control | 4 | 410±20 | 400±30 |
| Diabetic | 3 | 230±15 | 210±20 |

No significant differences were observed between the dissociation constants of the two mouse strains. In addition to a true alteration in the insulin-receptor interaction, a possible cause of the apparent reduction in insulin binding in the diabetic mouse is an increased insulin degradation within these membranes. An estimation of degradation revealed that, under our experimental conditions, i.e. short incubation time (30 min) and low membrane concentration (0.1–0.2 mg/ml), there was no significant degradation of unbound $^{125}$I insulin in either the control or diabetic membrane preparations (less than 5 %) (Table III).

*Membrane purification and composition*

Comparisons of some characteristic parameters of the two types of membranes are presented in Table IV. No significant differences are seen in the protein yield and the activity of membrane-bound enzyme 5'-nucleotidase. The index of microsomal contamination, i.e. glucose-6-phosphatase activity, has approximately the same value in diabetic and control membrane preparations. The amounts of sialic acid

TABLE IV

CHEMICAL AND ENZYMIC COMPOSITIONS OF CONTROL AND DIABETIC MICE LIVER PLASMA MEMBRANES

Values are given ±SD with the numbers of the determination in parentheses. For details on the methods used see Materials and Methods.

| | Control mice | Diabetic mice |
|---|---|---|
| mg of membrane proteins per g of liver (wet weight) | 1.1±0.2 (10) | 1.3±0.2 (6) |
| 5'-Nucleotidase ($\mu$mol $P_i$ liberated per mg proteins per h) | 27±6 (10) | 31±7 (6) |
| Glucose-6-phosphatase ($\mu$mol $P_i$ liberated per mg proteins per h) | 2±1 (10) | 3±1 (6) |
| Sialic acid ($\mu$g per mg of proteins) | 16±3 (4) | 13±1 (3) |
| Hexosamines ($\mu$g per mg of proteins) | 9±1 (4) | 7±1 (3) |

and hexosamines are slightly lower in diabetic liver membrane but these variations are not significant.

Sodium dodecylsulphate acrylamide gel electrophoretic patterns of protein and glycoprotein were virtually the same in both types of membranes. Spectrophotometric scanning of protein and glycoprotein subunits gave very similar results in both cases.

Sodium dodecylsulphate acrylamide gel experiments performed on the Triton X-100-soluble extracts of the control and diabetic membranes which seem to contain 90 % of the membrane insulin receptors as reported by Cuatrecasas [10, 11], did not indicate any significant difference between the two extracts.

## DISCUSSION

The spontaneously diabetic KK mice have been found to be a representative model of the genetic, metabolic and vascular aspects of this disease [17–23]. In the young KK mice, early metabolic changes are characterized by a moderate increase of plasma insulin [30], a decreased sensitivity of tissue to endogenous and exogenous insulin [17–20] and an impairment of the tolerance to glucose [19–21]. Hyperglycemia is mild and intermittent [19–21]. Our data suggest that in this syndrome there is an impairment of the insulin-receptor interaction in the liver plasma membranes, due to a decrease in the number of receptors. This decrease concerns the high affinity sites as well as the low affinity sites.

With reference to binding data for insulin-receptor interactions, possible inter-site interactions have been suggested [36]. However, several analyses of such data by others do not take into account this hypothesis in the calculation of binding constants [14–16].

In the present study, it was necessary to raise the insulin concentration by 50–75 % in the diabetic mouse membranes to obtain values of hormone bound per mg of membrane protein similar to those found with control mouse membranes. Experimental controls suggest that this alteration is not due to an increased insulin degrading activity. Characterization studies of other parameters of the membrane reveal a general similarity between the membrane preparations of diabetic KK mice and control mice. Furthermore, they indicate a good reproducibility of the characteristic values obtained with each membrane preparation.

The activity of the membrane-bound enzyme 5′-nucleotidase and the activity of glucose-6-phosphatase are similar in both preparations. (Similar activities were reported by Evans [37] in mouse liver plasma membranes.) Thus the decrease of the insulin binding capacity observed in diabetic mouse liver, is noted whether the data are expressed per mg of membrane protein or per unit of 5′-nucleotidase. The insulin receptor structurally may be a glycoprotein [9, 38]. Even though the difference of the estimated values of membrane glycoprotein content (sialic acid and hexosamines) between the two groups was not significant, the estimations were not of sufficient accuracy to exclude the possibility of a very small difference in the amount of insulin receptor glycoprotein on the plasma membranes.

Similar binding data have been obtained with the obese hyperglycemic mouse (Ob/Ob) [14]. The Ob/Ob mouse is also a genetic mutant characterized in part by hyperinsulinemia, resistance to both exogenous and endogenous insulin [14]. Despite

methodological differences between this work and our study, which is performed according to the method of Cuatrecasas [7, 9, 26], there is a parallel decrease in the insulin binding capacity of the liver membrane in the two strains of mouse (KK and Ob/Ob). The values of the kinetic constant for the control mouse obtained in our study and those of Kahn et al. [14] are of the same order of magnitude as those reported in a recent publication [16].

The most important point is that these studies demonstrate a decrease of the insulin binding capacity in two strains of mouse with hereditary alteration of insulin metabolism. In contrast to these findings, it has been found with a methodology similar to our experimental procedure, that in streptozotocyn-induced diabetic rats the insulin binding by isolated fat cells is not altered [13]. This difference may represent differences in the mechanism of insulin resistance in experimentally induced diabetes as compared to hereditary diabetes [23–29]. It is known that streptozotocyn-induced diabetes has not the same pathogenic evolution as that found in genetic diabetes, particularly concerning the diabetic vasculopathy [29].

In summary, the significance of these findings is that in young diabetic mice the insulin-resistant state and the moderate hyperinsulinemia correlate well with the decrease in insulin binding capacity observed in the liver.

Further studies of the KK mice and similar animal models may yield information regarding the regulation and the role of the insulin receptor in pathological states.

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