Biochimica et Biophysica Acta, 512 (1978) 29—40

BBA 78117

INVOLVEMENT OF GLYCOCONJUGATES IN INSULIN-RECEPTOR INTERACTIONS

STUDIES IN LIVER PLASMA MEMBRANES OF CONTROL AND DIABETIC MICE

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(Received December 1st, 1977)

Summary

The involvement of glycoconjugates in the insulin-receptor interactions in mouse liver is tested by digestions of membranes with various enzymes. Trypsin decreased the binding of [^{125}I]insulin to liver membranes. After digestion with β-galactosidase no "high affinity" receptor sites could be detected. The effects observed with plant lectins confirm the involvement of galactoconjugates in the insulin binding process. *Sophora japonica* and *Ricinus communis* lectins (with galactose specificity) and concanavalin A largely inhibit the binding process of insulin and those effects concern the "high affinity" receptor sites. Other lectins (wheat germ agglutinin, *Dolichos*) and enzymes (α-L-fucosidase, β-*N*-acetyl-hexosaminidase and neuraminidase) are without effect on insulin binding.

Comparative studies performed on diabetic mouse liver membrane (KK mice), previously characterized by decreased number of insulin receptors, are in good agreement with qualitatively similar receptor sites in both non-diabetic (control) and diabetic mice. Effects of enzymes and lectins yielded same results as compared to control membranes. Plasma membrane proteins and glycoproteins in both types of mouse are indistinguishable with respect to enzymic and chemical analysis. Sodium dodecyl sulphate acrylamide gel electrophoresis shows identical patterns. Moreover, the decrease in the number of insulin receptors is easily reversed with diet restriction. These data are consistent with the similarity of receptor sites in control and diabetic liver membrane.

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Introduction

On the basis of results obtained in our previous studies [1,2] concerning the insulin receptors of liver plasma membranes, the present work was initiated to investigate the chemical nature of insulin receptors. Reports from different laboratories lead to the postulation that membrane glycoproteins might play a role in the binding process of insulin [3—6] and other hormones [7,8] to cell surface. Those observations, as well as the increasing evidence that cell surface glycoconjugates are involved in a variety of surface-mediated processes (reviewed in refs. 7—9), prompted us to investigate, using enzymic and lectins pribes with high carbohydrate specificity, whether some of those molecules are implicated in insulin binding. In particular, we have studied the possibility of a role played by galactose residues, galactoglycoproteins, being characterized in various cell surface processes [10,11]. The effect of some proteases (trypsin) and glycosidases (β-galactosidase, neuraminidase, α-L-fucosidase and β-*N*-acetyl-hexosaminidase) on insulin binding by liver plasma membranes was investigates. To obtain additional controls, studies were performed using different plant lectins which bind specifically either to galactose (*Sophora japonica* and *Ricinus communis*), to mannose (concanavalin A) or to hexosamines (wheat germ agglutinin, *Dolichos*) [9—12]. To investigate the possibility that some receptor sites would be preferentially modified by enzyme and lactin treatment, binding experiments were conducted over a wide range of insulin concentrations.

As previously shown [1] insulin receptors of spontaneously diabetic KK mice exhibit a decrease in the number of "high" and "low affinity" receptor sites without significant alteration in their criteria of affinity. To obtain further information on insulin receptors in the diabetic state, similar studies were performed with diabetic KK mice. Membrane glycoproteins in both types of mouse were also comparatively analyzed [13]. Moreover, we investigated the effect of diet restrictions as previously studied with other strains of diabetic mice [14].

In the present report we demonstrate that "high affinity" receptor sites for insulin involved galactosyl residues. Evidence is accumulated in support of a similar nature of receptor sites in diabetic and control mice.

Materials and Methods

Animals and liver membrane preparation. Diabetic KK mice were obtained from the laboratory of Professor J.P. Levy (Service d'Hématologie. Hopital Cochin), who received them directly from Hoechst Japan Limited. Swiss Albino mice that were comparable with respect to age and sex (5—7 weeks old, male) served as control and were kept under the same laboratory conditions. Mice were fed ad libitum until the time of death, unless otherwise noted. Physiological parameters, such as body weight and blood plasma insulin were determined using standard techniques [15]. Plasma membranes were prepared from livers of the diabetic and control animals as previously described [1] according to the method of Ray [16] and they were kept frozen at −20°C until use. 5′ Nucleotidase assays were carried out at 37°C according to the method of

De Duve [17]; glucose-6-phosphatase assays, to determine microsomal contamination, were performed by the method of Harper [18]. Protein concentrations were determined for each experiment by the method of Lowry et al. [19] with bovine serum albumin as standard.

Binding essays. [^{125}I]Insulin (100—200 mCi/mg) was purchased from the Amersham Radiochemical Center. The radioactivity of this product was 95% precipitable in 20% trichloracetic acid. Porcine insulin was purchased from Schwarz Mann Laboratory. The procedure used for measuring the specific binding of iodoinsulin to membranes has been described earlier [1]. The procedure basically consists in incubating liver membrane proteins (2 mg/ml) for 60 min at 22°C in 0.5 ml Krebs-Ringer phosphate buffer, pH 7.6, (NaCl 118 mM/KCl 5 mM/$MgSO_4$ 1.2 mM/KH_2PO_4 1.2 mM/$CaCl_2$ 1.3 mM) containing 1.5% (w/v) albumin, with [^{125}I]insulin (10^{-11}—10^{-8} M). Time and pH of incubation have been previously determined for each type of membranes [20]. The tubes were thoroughly agitated several times and at the end of the incubation the membrane-bound hormone was separated by filtration of duplicate or triplicate samples through cellulose acetate EGWP Millipore Filters (0.2 μm) under vacuum and the filters were washed with 10 ml ice-cold Krebs-Ringer phosphate buffer containing 0.1% (w/v) albumin. The filters were dried and counted in a Nuclear Chicago Gamma counter.

The non-specific binding of [^{125}I]insulin was determined for every experimental point by including samples in which native insulin (50 μg/ml) was added to the membrane proteins 30 min before [^{125}I]insulin. Under those conditions the "non-specific" binding of insulin to liver membranes represents only 10—15% of the total binding for purified membranes of the control and diabetic mice. In all binding studies the non-specific binding was substracted from the total [^{125}I]insulin binding. Degradation of the unbound insulin in the supernatant was measured by loss of ability to bind to fresh aliquots of membranes as previously described [1]. An estimation of degradation revealed that, under our experimental conditions, there was no significant degradation of unbound [^{125}I]insulin in either the control or diabetic membrane preparations (less than 5%).

Enzymic digestions. Clostridium perfringens neuraminidase (EC 3.2.1.18) was obtained from Worthington. Neuraminidase activity was verified by incubating 5—100 munits of enzyme in Krebs-Ringer phosphate buffer/1.5% albumin, pH 7.6, containing 1 mg of membrane proteins.

After 15 min at 37°C the sialic acid content was determined by the thiobarbituric acid procedure of Warren [21]. Trypsin (beef pancreas, EC 3.4.21.4) was obtained from Miles Laboratories. Highly purified *Charonia lampas* β-galactosidase (EC 3.2.1.23) and β-*N*-acetyl-hexosaminidase (EC 3.2.1.52) were obtained from Seikagu. α-L-Fucosidase (EC 3.2.1.51) was obtained from Sigma. β-Galactosidase and α-L-fucosidase activities were verified by incubating $2 \cdot 10^{-7}$ M methyl-umbelliferyl-β-D-galactose and $0.5 \cdot 10^{-7}$ M methyl-umbelliferyl-α-L-fucose in Krebs-Ringer buffer/1.5% albumin containing 5—100 munits of each enzyme. After 15 min at 37°C the fluorescent product is evaluated by fluorimetric techniques. β-*N*-Acetyl-hexosaminidase activity was verified by incubating $1.5 \cdot 10^{-7}$ M methyl-umbelliferyl-β-*N*-acetyl-glucosamine in 0.05 M citrate buffer, pH 5.0, containing 5—50 munits enzyme. The specific proce-

dures used for digestion of membrane proteins with enzymes are described in the appropriate figure legends. Generally, unless otherwise noted, the membrane proteins were incubated with the enzyme in Krebs-Ringer phosphate buffer, pH 7.6, containing 1.5% (w/v) albumin, for 15 min at 37°C, then washed with large volumes of buffer before examination of their capacity for [^{125}I]insulin binding.

Effect of lectins on binding of insulin. Concanavalin A, twice crystallized, was obtained from Worthington, methyl-α-D-mannopyranoside from Sigma. Wheat germ agglutinin was purchased from Miles Laboratories, (WG27 A); it has been purified by affinity chromatography. Plant lectin (*Sophora japonica*) with specificity for D-galactose, was purified following the procedure described by Font and Bourrillon [22]. *Ricinus communis* plant lectin, with specificity for D-galactose, and *Dolichos biflorus*, with specificity for *N*-acetyl-galactosamine, were a gift from Professeur Bourrillon (Faculty of Medicine, Paris). The lectins' specificities were verified by agglutinin essays using type A and B erytohrocytes. The specific procedures used for incubation of membrane proteins with plant lectins are described in the appropriate figure legends. Generally, membrane proteins were incubated with lectins in Krebs-Ringer phosphate buffer, pH 7.6, containing 1.5% albumin for 50 min at 22°C. Specific insulin binding is measured under the conditions described earlier. The plant lectins do not alter the non-specific binding of [^{125}I]insulin to membranes or filters under any of the conditions used in the present studies.

Polyacrylamide gel electrophoresis. Gel electrophoresis of membrane proteins in the presence of sodium dodecyl sulfate (SDS) was conducted in a discontinous buffer system as described by Neville et al. [23]. Suspension of membranes (5 mg/ml) in Tris · HCl 0.05 mM/EDTA 0.5 mM/SDS 0.1% were clarified by addition of 1% (v/v) β-mercaptoethanol and 1 mg/mg SDS, and incubated for 20 min at 37°C. Protein bands were identified by staining with Coomassie Blue and glycoproteins were characterized by the periodic-acid Schiff stain [24]. Liver membrane glycoproteins were identified by in vivo administration of [^{14}C]glucosamine. [^{14}C]Glucosamine (6 μCi) (Amersham Radiochemical Centre, 55 mCi/mM) were injected intraperitoneally into control and diabetic mice, 90 min later, mice were decapitated and liver membranes prepared as described above. Labelled materials were counted in liquid scintillation systems using the Bray liquid. Labelled membranes were solubilized with SDS and electrophoresed as previously reported. Gels were sliced into 30 segments of 1.5 mm and deposited into liquid scintillation vials containing 0.3 ml of H_2O_2. After incubation at 50°C for 5 h, 10 ml of Bray scintillation liquid were added to each vial. The vials were subsequently counted for radioactivity in a Intertechnic liquid scintillation counter, and quenching was monitored by the external standard ratio method.

Results

Effect of various concentrations of enzymes and lectins on insulin binding

All binding assays used in membrane alteration experiments were performed with non saturating concentrations of hormone; under those conditions even

small changes in the affinity of the receptors for insulin would have been readily detected. Using 0.02 nM [^{125}I]insulin, 40% of the bound hormone is bound on the "high affinity" site fraction and 60% on the "low affinity" receptors sites. These high and low affinity receptor sites are observed as previously reported [1].

Effect of digesting liver membranes with enzymes

Treatment with trypsin. The specific binding of [^{125}I]insulin to liver membranes largely decreased after digestion with trypsin (Fig. 1A). After digestion with 5 units of mg of membrane proteins no specific [^{125}I]insulin binding could be detected under the conditions described.

Treatment with neuraminidase. Digestion of liver membranes with neuraminidase did not affect the specific binding of [^{125}]insulin to those membranes. Even after treatment with enzyme concentration as high as 100 munits per mg, which resulted in the release of 40% of the *N*-acetyl-neuraminic acid content (6 μg/mg proteins), the binding of insulin to membranes was not appreciably affected (Fig. 1B).

Treatment with β-galactosidase. Digestion of liver membranes with concentrations of β-galactosidase, under the conditions described in Fig. 1B, resulted in an inhibition of the specific [^{125}I]insulin binding. This inhibition reached a maximum at 50 munits per mg of membrane proteins (Fig. 1B). For higher enzyme concentrations this inhibition exhibited a plateau (60—70% of the total specific binding). Studies of sequential digestion of liver membranes with those two enzymes showed the same alteration that with β-galactosidase alone. Those results suggest that neuraminidase does not uncover new galactose residues which are involved in the recognition function of the receptor.

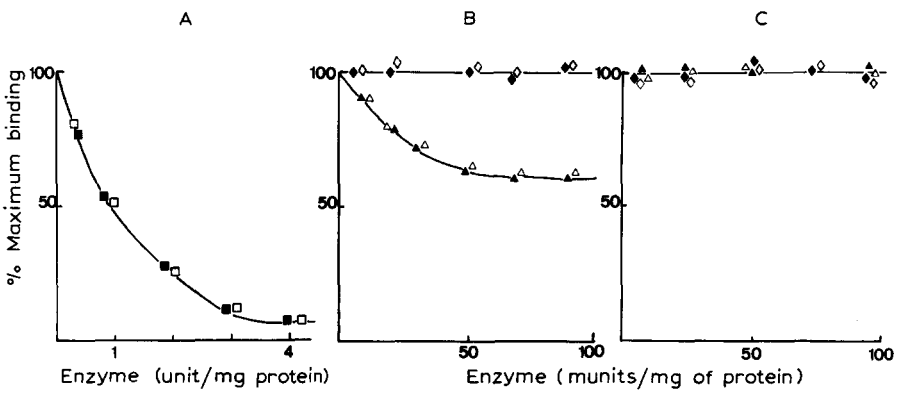

Fig. 1. Effect of various enzymes on the specific insulin binding to liver plasma membranes of control (■, ▲, ♦) and diabetic (□, △, ◇) mice. Membranes (1 mg/ml) are incubated with various concentrations of enzymes as indicated in the text. They are then washed and incubated at the concentration of 0.2 mg/ml for 20 min at 22°C in the presence of $3 \cdot 10^{-11}$ M [^{125}I]insulin. Results are expressed as percentage of the maximal binding (60 pg/mg) obtained without enzymic digestion. Each point is the mean of three determinations on three different membranes preparations. (A) Effect of trypsin (■), 0.2—5 units per mg membrane protein. The same results are obtained when the specific binding is performed in the presence of soybean trypsin inhibitor (1 μg/units trypsin). In those cases the washing step is omitted. (B) Effect of neuraminidase (♦) and β-galactosidase (▲) (5—100 munits/mg membrane protein). The same results are obtained when the washing step is omitted. (C) Effect of α-L-fucosidase (▲) and β-*N*-acetyl-hexosaminidase (♦). With β-*N*-acetyl-hexosaminidase the digestion is performed in citrate 0.05 M buffer, pH 5.0.

Treatment with α-L-fucosidase and β-N-acetyl-hexosaminidase. Treatment with α-L-fucosidase and β-*N*-acetyl-hexosaminidase did not cause inhibition in [^{125}I]insulin binding (Fig. 1C). All enzymes were tested for their capacity to hydrolyze synthetic substrates in the experimental conditions used. Liver membrane proteins were equally efficient in their capacity to bind [^{125}I]insulin even after a preincubation of 15 min at 37°C in the citrate buffer, pH 5.0, as used for digestion with β-*N*-acetyl-hexosaminidase.

Those data provide direct evidence about the involvement of galactose residues in the binding process of insulin, whereas terminal fucosyl and hexosaminyl residues are not implicated in this process.

Interaction of lectins with the insulin receptors of liver membranes

Effect of concanavalin A on binding of insulin. Concanavalin A markedly inhibits the binding of insulin to liver membranes (Fig. 2A) provided the lectin is preincubated with the membranes for a time long enough to achieve binding equilibrium of the lectin [9,12]. The effects of concanavalin A can be partially reduced (by about 70%) by adding the specific monosaccharide methyl-α-D-mannopyranoside.

Effect of wheat germ agglutinin and Dolichos biflorus. Wheat germ agglutinin and *Dolichos*, which have specificity β-*N*-acetyl-glucosamine and β-*N*-acetyl-galactosamine respectively [9,12] did not affect the specific binding of [^{125}I]insulin to the liver membranes, even at concentrations of 100 μg/mg membrane proteins (Fig. 2B).

Effect of S. japonica and R. communis. Those lectins, the specificity of which is D-galactose [9,12] caused a decrease of about 40% in the specific binding of [^{125}I]insulin at a concentration of 250 μg per mg of membrane proteins (Fig. 2C). Even with higher concentrations of lectins the maximum inhibi-

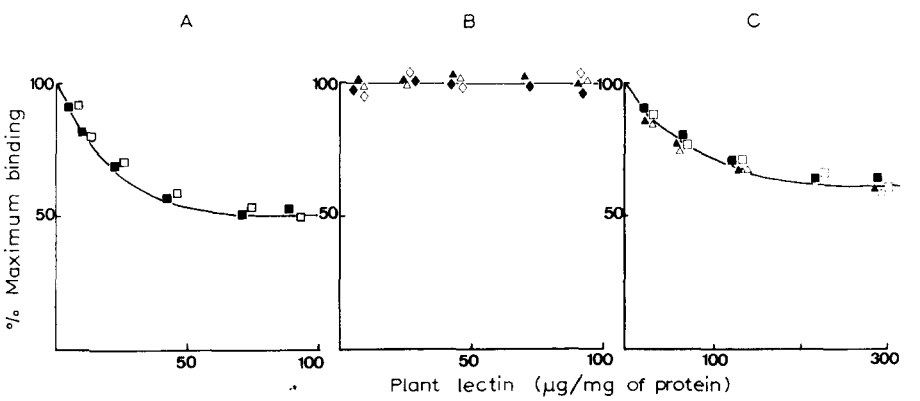

Fig. 2. Effect of various plant lectins on the specific binding of insulin to liver plasma membranes of control (■, ▲, ♦) and diabetic (□, △, ◇) mice. Liver membranes (0.3 ml containing 600 μg proteins) were incubated for 50 min at 22°C with various concentrations of lectins. After incubation, [^{125}I]insulin, $3 \cdot 10^{-11}$ M, and 25 μg of native insulin (for control tubes only) were added as indicated in Materials and Methods. (A) Effect on concanavalin A (■): in some cases the concanavalin A had been preincubated for 20 min at 22°C with 0.1 M methyl-mannopyranoside before addition of liver membranes. (B) Effect of wheat germ agglutinin (♦) and *Dolichos* lectins (▲). (C) Effect of *S. japonica* (■) and *R. communis* (▲) lectins. Under conditions described, the lectin binding to liver membranes (50 min at 22°C) approaches equilibrium. Plant lectins do not affect the non-specific binding of [^{125}I]insulin.

tions obtained were about 40%. With all lectins and enzymes tested, the non-specific binding was similar to that obtained with untreated membranes.

Inhibitions caused by plant lectins with galactose specificity implicate the participation of galactoglycoconjugates in the binding process of insulin. These results validate the previous data obtained with enzymic alterations of liver plasma membrane.

Binding properties of the insulin receptors pretreated with enzymes and lectins

As previously reported [1] Scatchard plots [25,26] for the diabetic and normal mice resulted in identically shaped curves. This result is concordant with two discrete populations of receptors with different affinities but it may also reflect negative cooperativity as described by De Meyts [27]. We studied the effect of various enzymes and lectins on Scatchard plots of [^{125}I]insulin binding to liver membranes of control and diabetic mice. Enzymes and lectins were used at a concentration such that the maximum effect be obtained. As shown in Fig. 3, neuraminidase did not affect insulin binding in a wide range of hormone concentrations. Both "high" and "low affinity" sites were intact. After treatment with concanavalin A and β-galactosidase we did not observe the high affinity sites. The number of low affinity sites were close to that observed with untreated control membranes. Treatment with *R. communis* lectin yielded similar results. Nevertheless, we observed a slight decrease by about 10—20% in the number of low affinity sites when liver membranes were preincubated with concanavalin A (Table I). In all cases studied non-specific binding was of the same order of magnitude as compared to control membranes.

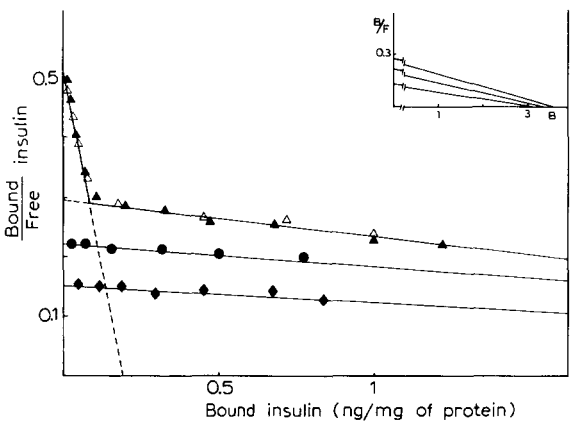

Fig. 3. Scatchard analysis of the effect of various enzymes and lectins on receptor-insulin interactions in control mouse liver membranes. Concentration of membrane is 0.2 mg/ml; [^{125}I]insulin concentration varies between $8 \cdot 10^{-12}$ and 10^{-8} M. Hormone bound to membranes was expressed as ng/mg proteins. Free hormone was expressed as ng/ml. For each concentration three identical incubations were performed. Each point is the mean of the results obtained. Data are corrected for non-specific binding. Digestion with neuraminidase (▲) (100 munits/mg) and β-galactosidase (●) (100 munits/mg) were performed for 15 min at 37°C. The washing step is omitted. The results are the mean of three determinations on three different membrane preparations. Membranes are incubated with 50 μg concanavalin A (◆) for 50 min at 22°C, and the results are the mean of five determinations on five different membrane preparations. Insert: extrapolation of low affinity plot for high hormone concentration.

TABLE I

EFFECT OF β-GALACTOSIDASE AND CONCANAVALIN A ON THE INSULIN-BINDING CHARACTERISTICS IN CONTROL MICE

Data were corrected for non-specific binding and constants for the two types of mouse were expressed according to the Scatchard representation modified by Kahn et al. [35] according to Klotz [25]. For treated membranes no correction has been made because a single type of site. All values were the mean ±S.E. of 20 determinations of controls and 4 determinations on treated membranes. n.d., not detectable. K_D is expressed as M/l, N is expressed as pM × 10^{-2}/mg protein. Concanavalin A: 50 μg/mg protein, 50 min, 22°C. β-Galactosiase: 100 munits/mg, 15 min, 37°C.

	High affinity, low capacity sites		Low affinity, high capacity sites	
	K_D 10^{-11}	N	K_D 10^{-9}	N
Control	5.7 ± 1.5	3.1 ± 0.5	3.1 ± 0.4	58.9 ± 5
Concanavalin A	n.d.	n.d.	3.4 ± 0.3	50.4 ± 8
β-Galactosidase	n.d.	n.d.	2.6 ± 0.4	58.2 ± 5

Comparative analysis in diabetic insulin receptors

In previous studies [1] the diabetic syndrome of the KK mice has been characterized by a decrease in the number of insulin receptors without change in their criteria of affinity.

As shown in Fig. 1, A—C and Fig. 2, A—C (blank symbols) sensibility of diabetic insulin receptors to enzymes and lectins were similar. In particular, the effect of β-galactosidase, the action of concanavalin A, *S. japonica* and *R. communis* lectins were identical in control and diabetic mice.

Moreover, as compared to control membranes, similar results were observed when Scatchard analysis of insulin binding were performed on diabetic treated membranes. Neuraminidase caused no effect. No high affinity sites were detected after treatment with β-galactosidase and concanavalin A; *R. communis* lectin yielded similar results. The number of low affinity sites slightly decreased after concanavalin A treatment. However, these differences were statistically non significant (Table II).

Insulin receptors were studied in diabetic KK mice before and after 16—20 h fasting. A significant increase in the number of insulin receptors was observed together with a simultaneous decrease in plasma insulin to normal rates (Table III).

TABLE II

EFFECT OF β-GALACTOSIDASE AND CONCANAVALIN A ON THE INSULIN-BINDING CHARACTERISTICS IN DIABETIC MICE

Binding characteristics are determined and expressed as indicated in Table I.

	High affinity, low capacity sites		Low affinity, high capacity sites	
	K_D 10^{-11}	N	K_D 10^{-9}	N
Diabetic membrane	5.5 ± 0.8	1.7 ± 0.3	3.7 ± 0.3	38.1 ± 3
Concanavalin A	n.d.	n.d.	3.7 ± 0.3	31.3 ± 4
β-Galactosidase	n.d.	n.d.	2.5 ± 0.5	34.5 ± 5

TABLE III

EFFECT OF FASTING ON PLASMA INSULIN AND INSULIN RECEPTORS IN DIABETIC MICE

Swiss albino, mice not fasted; KK, mice of the F_2 generation (male, 5—7 weeks); KK fasted, KK mice fasted for 16—20 h. The results are the mean ± S.E. of 20 determinations (Swiss albino), 10 determinations (KK mice) and 4 determinations (fasted KK mice).

Type of mice	Plasma insulin *	Binding capacity **
Swiss albino	15 ± 1	58.9 ± 5
KK	26 ± 3	38.15 ± 3
KK fasted	18 ± 2	58.4 ± 5

* μunits/ml determined by radioimmunological assay [15].
** pM X 10^{-2} /mg protein determined by Scatchard analysis.

Comparative analysis of liver membrane proteins and glycoproteins. The protein subunit patterns of plasma membranes, as determined by discontinous SDS electrophoresis in acrylamide gels, were virtually indistinguishable (Fig. 4). Both membranes showed identical patterns with about 35 resolvable protein subunits. No bands of non-identical relative mobility could be found when comparing the two preparations. Five glycoproteins were detected by the periodic acid- Schiff staining procedure in both strains of mice. A significant band was seen which migrated slightly ahead or just at the dye front and may represent glycolipid material [24]. The liver membrane glycoproteins were also observed by in vivo incorporation of [^{14}C]glucosamine. SDS acrylamide patterns showed the five main glycoproteins. The radioactivity incorporated in the diabetic membrane glycoproteins (expressed as [^{14}C]glucosamine cpm per mg

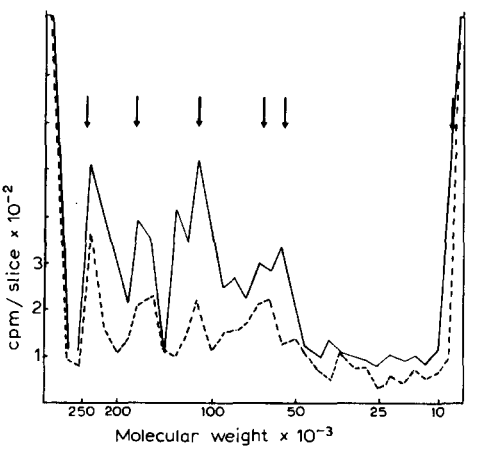

Fig. 4. Comparison of the radioactivity profiles of sodium dodecyl sulphate polyacrylamide gels of plasma membranes labelled in vivo with [^{14}C]glucosamine from control (———) and diabetic (- - - - -) mice. Radioactivity incorporated: (a) in control plasma membranes, 25950 cpm per mg proteins; (b) in diabetic plasma membranes, 12300 cpm per mg proteins. (Numbers are representative of one experiment. The same profile of glucosamine label has been observed in a variety of independant experiments (3 for each type of mice).) Detail of preparation of samples and conditions of electrophoresis were given in the text. Arrows indicate the position of periodic acid-Schiff's reagent-positive components. The band migrating at the dye front may represent glycolipid material. Materials whose molecular weight is higher than 250000 stay in the stacking gel.

protein) was half that obtained for the control membranes treated in the same experimental conditions (Fig. 4).

Based on the present results it seems unlikely that diabetic mouse insulin receptors are structurally modified in their carbohydrate complex composition, as compared to control insulin receptors.

Discussion

Many attempts have been made at examining the participation of glycoconjugates in the binding process of insulin to various cells [3,5,28]. However, it seemed of great interest to investigate in detail the involvement of some particular sugar residues in the insulin binding process. Accordingly, in the present work, we studied the effect of various high specificity enzymes and lectins, on the insulin binding. Effects observed with trypsin [4,29,30] and neuraminidase [3] were in good agreement with the data reported in previous works. Trypsin decreased hormone binding with evidence of membrane protein alterations. Neuraminidase did not affect insulin binding while 50% of the *N*-acetyl-neuraminic content had been liberated in medium under our experimental conditions. Treatment with β-galactosidase prior to insulin binding led to decreased insulin specific binding. This process occurred with low enzyme concentrations and reached a plateau, suggesting that all the residues involved in the binding process were hydrolyzed. Sequential digestion with neuraminidase plus β-galactosidase showed the same alteration. Treatment with α-L-fucosidase and β-*N*-acetyl-hexosaminidase modified neither the specific insulin binding nor the non-specific binding. In all cases studied, digestion periods were short enough to maintain the biological functions of the membranes and controls were made to confirm enzymic action by digesting the specific synthetic substrates.

The various results are consistent with the involvement of glycoconjugates with terminal galactose residues in the recognition of insulin by the receptor. The participation of terminal fucosyl, *N*-acetyl-neuraminyl and hexosaminyl residues seems unlikely.

Preincubations of membranes with plant lectins with different carbohydrate specificities are in good agreement with the preceding results. In all cases insulin binding studies were performed under conditions to allow lectin binding to approach equilibrium. Non-specific binding was not increased by lectin preincubations. The effect of concanavalin A, which caused 40—50% inhibition in insulin binding seems to implicate the presence of mannose residues in the hormonal receptor site. Inhibition induced by that lectin was partially prevented by methyl-α-D-mannopyranoside. Observations made by Cuatrecasas [28] that plant lectin concanavalin A had insulin-like effects, emphasize the specificity of concanavalin A for insulin receptor structure. However, the ability of concanavalin A to block insulin binding may only be due to the close association of the two receptors and the large size of concanavalin A [31] and/or its charge or induced conformational change in the glycocalix [12,32].

Other lectins with higher specificities were tested. *R. communis* and *S. japonica* (that are galactose specific) mimicked the effect of β-galactosidase, i.e., a decrease in insulin-specific binding up to 30—40%. In addition, other plant lectins, wheat germ agglutinin and *D. biflorus* lectin (*N*-acetyl-glucosamine- and

N-acetyl-galactosamine specific) do not modify the insulin binding. Those results are consistent with those obtained after β-*N*-acetyl-hexosaminidase digestion. However, we do not observe with wheat germ agglutinin, the biphasic effect shown by Cuatrecasas [28] on liver plasma membranes; perhaps in relation to the quite different membrane preparation used by that author.

All those results strongly suggest the role of galactosyl residues in the receptor site without excluding the participation of non-terminal hexosaminyl and mannosyl residues.

The partial inhibitory effect (up to 40%) of β-galactosidase, *S. japonica* and *R. communis* lectins lead us to investigate whether among the heterogeneous population of receptor sites some would be preferentially inhibited. Consequently, we have studied by Scatchard analysis the insulin-binding properties of pretreated membranes. As previously reported [1] control membranes exhibit two discrete populations of receptors, the one with "high affinity", "low capacity" and the other with "low affinity", "high capacity". Scatchard analysis of those data showed that β-galactosidase, as well as a lectin with galactose specificity (*R. communis*) (data not shown), provoke the disappearance of high affinity sites without significant alteration of low affinity sites. With concanavalin A high affinity sites are not detected, while the number of low affinity sites is slightly decreased. But the different limitations reported above concerning action of concanavalin A on cell surface made interpretations uncertain [11,28,32]. Those data are concordant with the existence of two distinct populations of receptors [1], one of them (the high affinity sites) would involve galactose residues necessary to the specific binding of insulin. However, the hypothesis of a single homogeneous population of receptors with inter-site interaction, as described by De Meyts et al. [27], cannot be excluded.

Whatever hypothesis is assumed, the disappearance of the high affinity sites fraction, which play the prime physiological role [33] allow us to emphasize the biological importance of glycosidic residues, and particularly galactose residues, in the specific binding process of insulin.

Insulin receptor sensibility to enzymes and lectins was studied in the diabetic syndrome to obtain further information on the decrease in insulin binding observed in diabetic mice. All inhibition experiments resulted in curves similar to control ones. Chemical and analytical data of membrane glycoproteins did not show significant differences in both mouse strains. Comparative SDS-acrylamide gel electrophoresis showed the same patterns. [^{14}C]Glucosamine incorporation studies give no more information about the modifications occurring in the glycoprotein content of KK mouse liver membranes. The reduction observed in the incorporation rate might reflect more metabolic disorders than structural alteration of diabetic membranes glycoproteins. Based on the present results it seems unlikely that insulin receptors exhibit structural modifications in their glycosylated components.

The physiological regulation in the receptor number in KK mice remains unclear. At present, we do not know whether the receptors are structurally normal but unfunctional or whether protein synthesis is necessary to increase their number in relation to the decrease in plasma insulin. However, we and others [13], have observed an increase in receptor number after diet restriction. Based on those various studies it seems unlikely that insulin-receptor deficiency

is a primary genetic lesion in diabetic liver plasma. Studies of Soll et al. [37] in the ob/ob mice argue in favor of this hypothesis. Nevertheless, the absence of a genetic defect in the insulin-receptor function in liver membrane does not discard the possibility of a receptor misfunction in vivo.

Our work provides new information on the involvement of cell-surface glycoconjugates in membrane recognition processes. In the diabetic syndrome of KK mice insulin-receptor interactions are disturbed without any structural alteration of receptor sites.

Acknowledgments

This work had been supported by grants from I.N.S.E.R.M., F.R.A. 19: (ATP no. 49.77.81 and C.A. 405 no. 77.1.191.7), from C.N.R.S., E.R.A. 691 and from "Fondation pour la Recherche Médicale Française". The authors wish to thank Doctor J. Capeau for her kind and valuable help in reading the manuscript.

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