

Increased Insulin Binding by Hepatic Plasma Membranes from Diabetic Rats

NORMALIZATION BY INSULIN THERAPY

MAYER B. DAVIDSON and SOLOMON A. KAPLAN

From the Departments of Medicine and Pediatrics, UCLA School of Medicine, Center for the Health Sciences, Los Angeles, California 90024

ABSTRACT Hepatic plasma membranes prepared from rats rendered diabetic by streptozotocin bound approximately twice as much insulin per 50 μ g protein as control membranes. Glucagon binding of diabetic and control membranes was virtually identical. This increased insulin binding was not due to a nonspecific effect of streptozotocin, decreased degradation of insulin, slower dissociation from its receptor, or a selective higher yield of membranes prepared from the diabetic livers. Diabetic and control membranes both showed negative cooperativity. Scatchard analysis suggested that the difference in binding was due to an enhanced binding capacity of the diabetic membranes rather than increased affinity of the binding sites. Increased insulin binding of diabetic membranes was returned to normal by insulin treatment. These data are consistent with the postulate that there is an inverse relationship between circulating insulin levels and insulin binding and that insulin may modulate its own receptor. However, since it has been reported that fat, muscle, and hepatic tissue from rats made diabetic by alloxan administration are insensitive to insulin, the capacity for binding can not be the sole factor determining the response to insulin in diabetes mellitus. Therefore, sensitivity of the diabetic liver to insulin is determined, at least in part, by events subsequent to the binding of insulin to its receptor.

INTRODUCTION

Recent studies on insulin binding have revealed that in the presence of high levels of insulin, various

Part of this work has been published in abstract form (*Clin. Res.* 23: 109A, 419A, 1975) and presented at the 1975 American Diabetes Association Meetings (*Diabetes.* 24: 394, 1975).

Received for publication 6 January 1976 and in revised form 21 September 1976.

tissues manifest an impaired ability to bind the hormone. A decreased capacity for binding insulin is found in hepatic tissue from the ob/ob mouse (1-5), the db/db mouse (4, 5), the normal rat given either gold thioglucose (4) or glucocorticoids (6, 7) or harboring a tumor secreting ACTH (8), and the old obese rat compared to young lean animals (9); in monocytes removed from obese humans (10-12); in cultured lymphocytes (13, 14) incubated in vitro in the presence of insulin; in thymocytes (15) and cardiac muscle (16) from the ob/ob mouse; in skeletal muscle from old obese rats compared to young lean animals (17); in adipose tissue from obese humans (12, 18), the ob/ob mouse (19), rats given a short course of glucocorticoids (7), and the old obese rat compared to young lean animals (20). Since high levels of insulin in all of these situations are associated with a decreased capacity for binding the hormone, diminished concentrations of insulin might be expected to enhance insulin binding. The diabetic rat provided an appropriate in vivo model to test this hypothesis.

METHODS

Lewis or Sprague-Dawley rats (200-250 g) were given streptozotocin (50, 60, or 75 mg/kg) dissolved in citrate buffer (0.01 M, pH 4.0) through the tail vein. Control animals were injected with the buffer alone. Diabetic (streptozotocin) rats were not treated with insulin except for one group injected subcutaneously with 3 U of NPH insulin each morning commencing on the 2nd day after streptozotocin administration. A control group received saline injections concomitantly. The last injection was given on the day before sacrifice. One group of control rats was fasted for 48 h (with free access to water) before sacrifice in an attempt to approximate the diminished weight gain of the diabetic animals.

After 1 wk, the nonfasted animals (except the group starved for 48 h) were sacrificed by decapitation, blood was collected and allowed to clot on ice, and the liver removed. Serum glucose was measured by a glucose oxidase method (Beckman Glucose Analyzer, Beckman Instruments Inc., Fullerton, Calif.) and insulin by a double antibody method (21) using

pork insulin standards and an antiserum directed against pork insulin. Thus, although insulin concentrations may not represent true levels of rat insulin, a comparison between diabetic and control rats is valid.

Monoiodo [^{125}I]insulin with a sp act of 50–200 Ci/ μg was prepared according to the method of Freychet et al. (22) except that rabbit serum was used instead of bovine serum albumin. Livers from three to five rats of each group were pooled and hepatic plasma membranes were prepared according to the method of Neville (23). Partially purified membranes (Step II) were used because further purification did not materially affect insulin binding and markedly decreased the yield. Binding of [^{125}I]insulin to these membranes was performed according to the method of Kahn et al. (2) with the following modifications. Incubations were carried out at 23°C for 150 min in a final volume of 150 μl . Rabbit serum at a final concentration of 7.5% was used instead of bovine serum albumin since certain lots of the latter inhibited binding. Bound and free insulin were separated by filtering and washing under reduced pressure on cellulose acetate EGWP Millipore filters (Millipore Corp., Bedford, Mass.) as described by Cuatrecasas (24). Approximately 28,000 cpm representing 0.4–0.5 ng [^{125}I]insulin was added to tubes containing for the most part between 30 and 90 μg membrane protein which was measured by the method of Lowry et al. (25). Insulin binding and membrane proteins were linearly related between 30 and 150 μg . Each tube was corrected for nonspecific binding (average 2.2% of added counts) which was the amount of radioactivity remaining in the bound fractions in the presence of 5 μg of unlabeled insulin. In the absence of unlabeled insulin, the binding of [^{125}I]insulin to control membranes prepared from Lewis rats in 14 assays was $6.4 \pm 1.1\%/50 \mu\text{g protein} \pm \text{SE}$. Under the same conditions in 38 assays on control membranes from Sprague-Dawley rats, the value was $11.8 \pm 0.6\%/50 \mu\text{g protein} \pm \text{SE}$ which was significantly higher ($P < 0.001$) than in Lewis rats.

Degradation of insulin was measured by incubating [^{125}I]insulin with plasma membranes as described above. At the end of the incubation, a 50- μl aliquot was removed, added to 0.5 ml of 0.1% bovine serum albumin, and precipitated with 0.5 ml of 10% TCA. The mixture was centrifuged and the pellet washed with cold TCA. The pellet was then dissolved in 0.5 ml of 0.5 N NaOH for counting in a gamma well counter. Another 50- μl aliquot of the original media was incubated overnight with excess anti-insulin antibody and the immuno-precipitated counts determined after addition of normal guinea pig serum and antibody directed against this serum.

Negative cooperativity was assessed in control and diabetic membranes by a modification of the method of De Meyts et al. (26). After the 150-min incubation period as described under the binding studies, both sets of membranes were each diluted 100-fold with either buffer alone or containing insulin (1 $\mu\text{g}/\text{ml}$) at 4°C. Zero time aliquots were processed and the membrane preparations were placed in a shaking water bath maintained at 23°C. Aliquots were removed at appropriate times for filtering and counting.

Glucose-6-phosphatase (27) and 5'-nucleotidase (28) were measured in plasma membrane preparations. There was a 10-fold increase in the activity of the latter enzyme in membrane preparations compared to the homogenates from which they were prepared.

Binding of ^{125}I -glucagon to hepatic plasma membranes was carried out exactly as described by Rodbell et al. (29) except that the pellets were washed with buffer instead of a sucrose solution.

Because of interassay variability of insulin binding by hepatic plasma membranes, only values obtained in the same

TABLE I
Glucose and Insulin Concentrations at Sacrifice and Weight Changes Over the Preceding Week

Conditions	Glucose	Insulin	Weight change
	mg/100 ml \pm SE	$\mu\text{U}/\text{ml} \pm \text{SE}$	g \pm SE
1. Control	145.4 \pm 2.3 (n = 45)	26.3 \pm 1.8 (n = 45)	+43.1 \pm 3.7 (n = 17)
2. Streptozotocin, 75 mg/kg	514.6 \pm 19.7 (n = 37)	6.7 \pm 1.1 (n = 37)	-15.2 \pm 3.9 (n = 9)
3. Streptozotocin, 60 mg/kg	442.0 \pm 11.7 (n = 13)	16.1 \pm 2.0 (n = 13)	-7.8 \pm 4.8 (n = 13)
4. Streptozotocin, 50 mg/kg	419.4 \pm 22.0 (n = 5)	29.4 \pm 4.4 (n = 5)	+36.4 \pm 3.9 (n = 5)
5. Streptozotocin, 75 mg/kg + insulin, 3 U NPH per day	520.8 \pm 27.4 (n = 4)	70.8 \pm 29.4 (n = 4)	+50.0 \pm 3.1 (n = 4)

assay were compared. To that end, a two-tailed paired *t* test at each insulin concentration was utilized. In experiments in which binding of hepatic membranes from more than two groups of rats was compared, an analysis of variance was used (30). Significance for all statistical tests was accepted at the 5% level.

RESULTS

Random plasma glucose and insulin concentrations and weight changes in the control and various diabetic groups of rats studied are shown in Table I. The results from Lewis and Sprague-Dawley animals are combined. Rats were initially rendered diabetic with 75 mg/kg of streptozotocin and compared to control animals (condition 1 vs. 2, Table I). Control rats gained weight while diabetic animals lost. Glucose concentrations at sacrifice were markedly elevated in the diabetic rats while insulin levels were inappropriately low.

The number of counts of [^{125}I]insulin bound by hepatic plasma membranes prepared from control and diabetic rats is shown in Fig. 1. Since the fraction of insulin bound differed between the two strains of animals, values for control and diabetic membranes in each strain are compared separately. In 11 separate paired comparisons in four pools (a pool represents the livers of three to five animals) of Lewis rats, diabetic membranes bound significantly more counts at all eight insulin concentrations. Similarly, diabetic membranes from Sprague-Dawley rats (two pools) bound significantly more counts in seven separate paired experiments at all concentrations of insulin except the extremely high level of 100 ng/tube (even though over twice as many counts were bound by the diabetic membranes at this insulin concentration also).

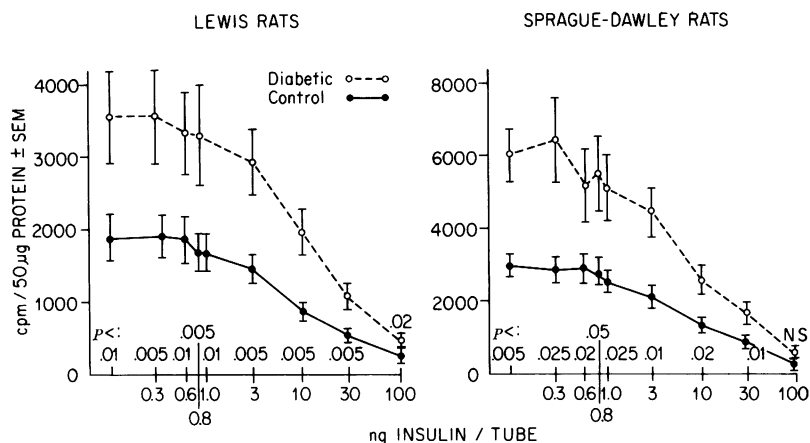


FIGURE 1 Specific binding of radioactive insulin by hepatic plasma membranes from diabetic and control rats. Note the difference in the numbers on the ordinate between the Lewis and Sprague-Dawley animals. 11 paired experiments were performed on membranes from four pools of Lewis rats and 7 paired experiments were performed on membranes from two pools of Sprague-Dawley rats.

Scatchard analysis (31) of these same data is depicted in Fig. 2. The diabetic and control curves are curvilinear and essentially parallel to each other for both the Lewis and Sprague-Dawley rats. This implies that the binding affinities for insulin of the diabetic and control hepatic plasma membranes are similar and that the differences in binding are due to an increased number of insulin receptors in the diabetic liver. Curvilinear Scatchard plots were initially interpreted as representing two classes of insulin receptors (2). Recent analysis by De Meyts et al. (26), however, has shown that site to site interactions occur when insulin binds to plasma membranes and the curvilinear shape of the plot is best explained by a negative cooperative effect on one class of binding sites (26). The diabetic and control hepatic membranes also show negative cooperativity (Fig. 3). Dissociation of insulin from these hepatic plasma membranes is slower than from

cultured lymphocytes (26). This is consistent with a recent study which demonstrated that [125 I]insulin dissociates from its receptors on cultured lymphocytes more quickly than it does from its receptors on membranes (32). It is important to emphasize that differing rates of dissociation cannot account for the enhancement of insulin binding by the diabetic membranes since the control and diabetic curves (Fig. 3) are similar when compared under the same conditions (dilution only or dilution + insulin).

Since negative cooperativity is a likely explanation for the curvilinearity of the Scatchard plots, we have not calculated "affinity constants" or "total binding capacities." Instead, we have compared insulin binding at various concentrations of insulin in experimental and control animals. Hepatic plasma membranes from both diabetic Lewis and Sprague-Dawley rats bound approximately twice as much hormone at each concentration as their respective controls (Fig. 4). Note that the amount of insulin bound is plotted on a log scale which reduces the apparent difference between the two curves. As expected from the data in Fig. 1, this difference is statistically significant at all eight insulin concentrations in the Lewis strain and in all but the highest insulin concentration in the Sprague-Dawley strain.

Since these results are expressed per 50 μ g of total membrane protein, increased binding could be observed if the plasma membrane preparations of the diabetic animals were less contaminated with extraneous cellular components other than plasma membranes. The data in Table II, however, make this possibility unlikely since the control membranes have higher activities of 5'-nucleotidase, a plasma membrane marker, than the diabetic membranes. Conversely, the

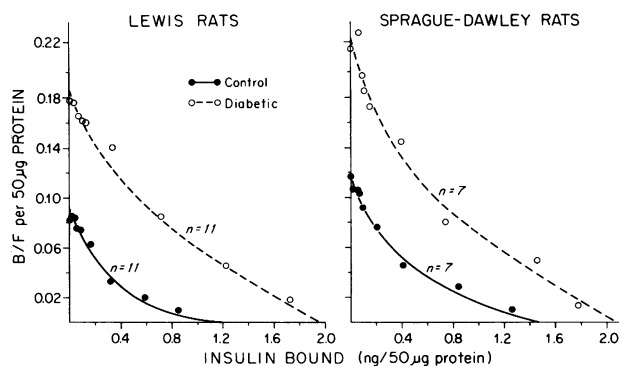


FIGURE 2 Scatchard analysis of insulin binding to diabetic and control hepatic plasma membranes from Lewis and Sprague-Dawley rats. (Data from Fig. 1).

diabetic membranes have increased activities of glucose-6-phosphatase, a microsomal membrane marker, than the control membranes. If insulin binding were expressed per unit of 5'-nucleotidase as advocated by some (33), there would be a fourfold increase of hormonal binding by diabetic membranes instead of a twofold enhancement when expressed per 50 μ g of membrane protein. The same pattern of decreased activities of plasma membrane enzymes and increased amounts of microsomal membrane enzymes has also been noted in plasma membranes of adipocytes from diabetic rats (34). Equal activities of 5'-nucleotidase were recovered, however, in fully purified hepatic plasma membranes prepared from diabetic and control Chinese hamsters (35).

The increased binding of insulin by diabetic membranes is also not explained by a difference in insulin degradation. Intact [¹²⁵I]insulin was measured by both TCA and antibody precipitability after 2.5 h of incubation in the absence and presence of membranes (Table III). The results are expressed as the percentage of intact insulin recovered in tubes containing membranes compared to the amount measured in a paired tube without membranes. There is no significant difference in insulin degradation between control and diabetic membranes using either TCA or antibody precipitability to assess the presence of intact insulin.

The specificity of the increased insulin binding by diabetic membranes was evaluated by measuring the binding of ¹²⁵I-glucagon to these same membrane preparations (Fig. 5). Glucagon binding was virtually

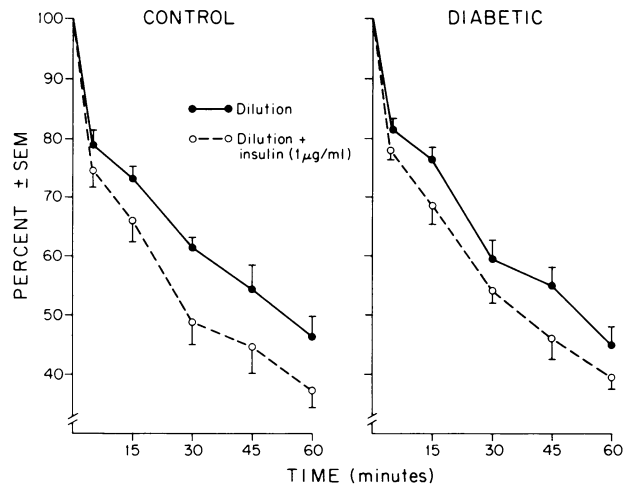


FIGURE 3 Dissociation of insulin from control and diabetic membranes. The points at 15, 30, and 60 min represent the mean of six separate experiments while the points at 5 and 45 min represent the mean of four separate experiments.

identical in hepatic plasma membranes prepared from diabetic and control Sprague-Dawley rats.

The effect of varying doses of streptozotocin are shown in fig. 6. By an analysis of variance, control membranes bound significantly less insulin than membranes prepared from rats treated with 75 mg/kg ($P < 0.001$) or 60 mg/kg ($P < 0.001$) of the diabetogenic agent. Insulin binding by hepatic membranes of animals treated with 50 mg/kg was more than control membranes but less than membranes of rats given

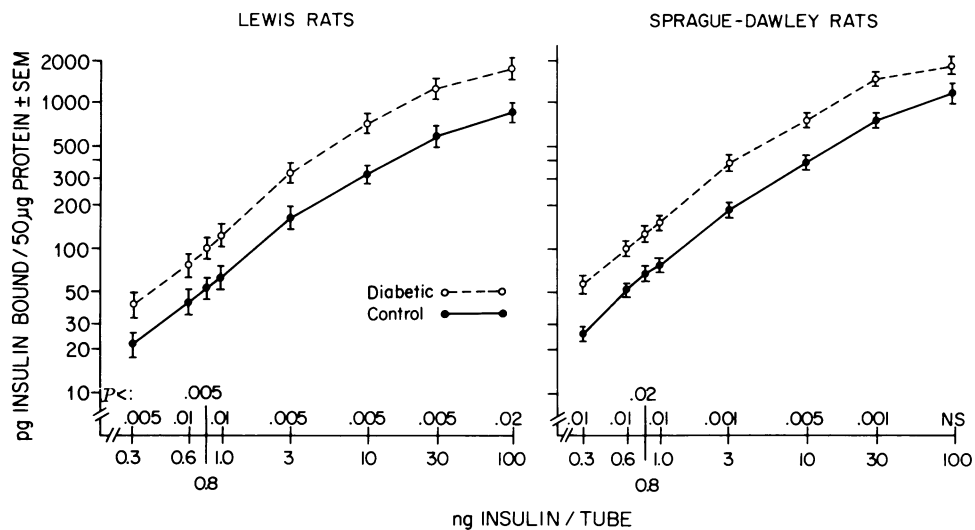


FIGURE 4 Amount of insulin bound by hepatic plasma membranes from diabetic and control rats. See legend to Fig. 1 for the number of paired experiments. Note that the scale of the ordinate is logarithmic and that the binding of insulin to the diabetic membranes is increased approximately twofold. For instance, at 10 ng/tube in the Lewis rats, diabetic membranes bound 715 pg insulin/50 μ g protein compared to 320 pg insulin/50 μ g protein by the control membranes.

TABLE II
Enzymatic Activity of Hepatic Plasma Membranes Prepared from Eight Separate Pools of Rats in Each Group. Each Pool Represents from Three to Five Livers

	5'-Nucleotidase	Glucose-6-phosphatase
	$\mu\text{g P/mg/min} \pm \text{SE}$	$\mu\text{g P/mg/min} \pm \text{SE}$
Control	140.8 \pm 32.6	9.8 \pm 1.4
Diabetic	62.4 \pm 13.6	14.3 \pm 1.6

larger doses of streptozotocin, and hence was not significantly different from any of them. Although plasma glucose levels at sacrifice were similar in all groups given streptozotocin, insulin concentrations were inversely related to the amount of drug (Table I, conditions 2–4). The animals given 50 mg/kg demonstrated similar weight gain and insulin levels as the controls (Table I, conditions 1 and 4). It has previously been shown that between 45 and 55 mg/kg of streptozotocin is a critical dose for the severity of the resultant diabetic state in rats (36). The lower amount was associated with significant weight gain after 1 wk whereas rats given the higher dose actually lost weight. The response to 50 mg/kg dose in these five rats resembled the smaller dose (36).

When diabetic rats were treated with 3 U of NPH insulin per day (Fig. 7), insulin binding by their hepatic plasma membranes returned to normal and was significantly less than untreated animals ($P < 0.02$). Glucose concentrations were not diminished by insulin treatment (Table I, condition 5), but these animals had been injected 24 h before sacrifice. Glucose levels are significantly decreased in diabetic rats 8–12 h after NPH insulin.¹ Insulin concentrations at sacrifice varied widely in this group of treated animals (19–142 $\mu\text{U/ml}$), but are true measurements of immunoreactive insulin as no insulin antibodies could be detected in the plasma (charcoal-dextran separation of bound and free fractions).

To ascertain that the increased insulin binding by hepatic plasma membranes prepared from these diabetic rats was not secondary to the associated catabolism, four control animals were fasted with free access to water for 48 h before sacrifice. After the fast, these starved rats were 9.8 g less than their original weight. This change is comparable to the 8–15-g weight loss of the severely diabetic rats (Table I, conditions 2 and 3) and markedly different than the 43-g weight gain of nonfasted controls (Table I, condition 1). Six comparisons were performed among membranes prepared from one pool each of control animals, rats given 60 mg/kg of streptozotocin, and the starved controls. Insulin binding was similar in the two nondiabetic groups but

¹ Molnar, G., and J. Brown. Personal communication.

significantly higher ($P < 0.01$) in the diabetic membranes compared to fasted controls (data not shown). Thus, the enhanced insulin binding by diabetic hepatic plasma membranes is probably not due to the catabolism associated with insulin lack.

DISCUSSION

These data indicate that binding of insulin to hepatic plasma membranes of diabetic rats is increased. Approximately twice as much insulin was bound per unit of hepatic plasma membrane protein in two strains of rats rendered diabetic by streptozotocin (60–75 mg/kg) compared to appropriate controls (Figs. 4 and 6). This increased binding of insulin to diabetic membranes was not due to less contamination with other cellular components (Table II), impaired insulin degradation (Table III), or slower dissociation of insulin from the membranes (Fig. 3). Enhanced binding would also not seem to be a nonspecific effect of streptozotocin since insulin binding by rats given 50 mg/kg was not significantly different from controls (Fig. 6). Furthermore, the catabolic effect of uncontrolled diabetes mellitus is probably not responsible for the difference since control rats starved for 2 days before sacrifice had a similar overall weight loss but significantly less insulin binding than the diabetic animals. Finally, increased binding by diabetic membranes is specific for insulin since glucagon binding was unaltered (Fig. 5).

Insulin binding in diabetes mellitus has been assessed in several other studies. In one, no difference was demonstrated between binding of insulin to adipocytes from control and streptozotocin diabetic rats (37). However, no difference could be demonstrated between controls and prednisone-treated animals (37) either. Since adipocytes as well as hepatocytes from dexamethasone-treated rats show decreased insulin binding (6, 7), these negative results must be interpreted with caution. In another study, monocytes harvested from diabetics bound less insulin than cells from either normals or patients with hyperglycemia secondary to pancreatitis (38). However, fasting insulin

TABLE III
Degradation of [¹²⁵I]Insulin by Hepatic Plasma Membranes

Precipitant	Control (n = 5)	Diabetic (n = 5)
	%	%
TCA	83.2 \pm 1.9	80.4 \pm 1.1
Antibody	92.1 \pm 3.9	87.0 \pm 7.2

Values are expressed as percent of counts (\pm SEM) precipitated compared to control tubes in which membranes were omitted.

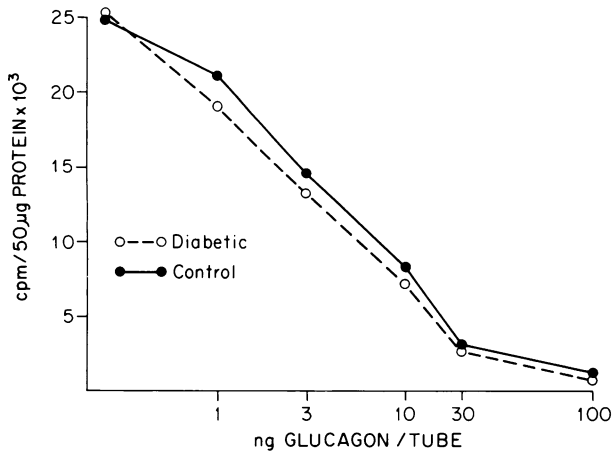


FIGURE 5 Specific binding of radioactive glucagon by hepatic plasma membranes from diabetic and control Sprague-Dawley rats. Five paired experiments were performed on membranes prepared from one pool of animals in each group. These diabetic membranes also manifested increased insulin binding (data not shown).

concentrations in the diabetics were twice as high as those in both the normal controls and patients with pancreatitis. Thus, increased insulin concentrations probably account for the decreased hormonal binding (1-20) in these patients. However, purified hepatic plasma membranes from Chinese hamsters (35) and rats² rendered diabetic with streptozotocin bound approximately twice as much insulin as control membranes, a result in close agreement with our data.

Previous studies in which elevated insulin levels are associated with decreased hormonal binding under a variety of conditions (1-20) have suggested that the prevailing insulin concentration may itself modulate the number of insulin receptors (13). The present study supports this postulate. Although the numbers in some of the groups are small, insulin binding seems to correlate inversely with insulin concentrations (Table I). For instance, rats given 60 and 75 mg/kg of streptozotocin had significantly lower insulin concentrations at sacrifice (Table I, conditions 2 and 3) than controls and bound significantly more insulin (Figs. 4 and 6). Rats given 75 mg/kg of streptozotocin and treated with insulin (Fig. 7) or given 50 mg/kg of streptozotocin (Fig. 6) had no decrease in insulin concentrations (Table I, conditions 4 and 5) and no change in insulin binding compared to controls (Figs. 6 and 7).

In the present study, increased insulin binding associated with the insulinoprivic state of diabetes mellitus was reversed by insulin therapy (Fig. 7). The opposite manipulation of insulin levels and binding has also been demonstrated. Food restriction reduces the hyperinsulinemia and restores toward normal

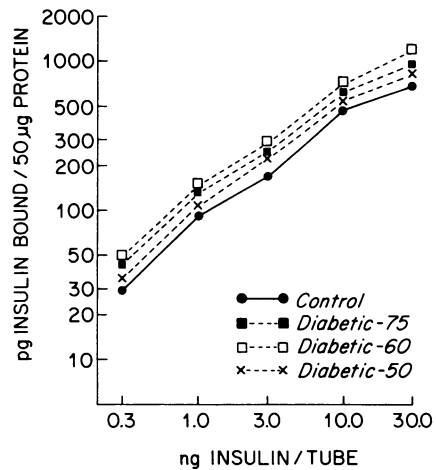


FIGURE 6 Effect of varying doses of streptozotocin (75, 60, or 50 mg/kg) on insulin binding by hepatic plasma membranes. 12 separate comparisons were made among control and diabetic rats treated with either 60 or 75 mg/kg on membranes prepared from two pools of livers in each group. Membranes prepared from one pool of diabetic rats given 50 mg/kg were also included in six of these comparisons.

insulin binding by hepatic plasma membranes from the ob/ob mouse (4, 39), the db/db mouse (4), and the gold thioglucose rat (4), by adipocytes from the ob/ob mouse (39) and by monocytes from obese human subjects (11). The enhancement of insulin binding by caloric restriction in the ob/ob mouse can be prevented by administering exogenous insulin during this period (4). Treatment of the ob/ob mouse with oxytetracycline also decreases insulin levels and increases

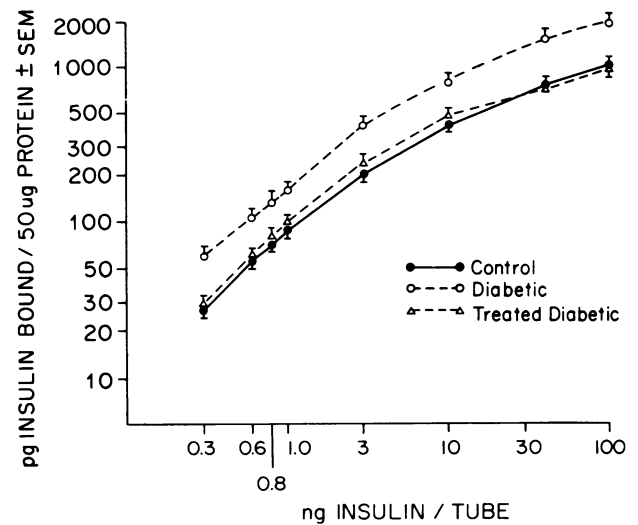


FIGURE 7 Effect of insulin treatment (3 U NPH per day) on insulin binding by hepatic plasma membranes of diabetic rats. Five comparisons were made on membranes prepared from one pool of animals in each group.

² Carter, J., and V. Chandramouli. Personal communication.

insulin binding by both hepatic plasma membranes and adipocytes (39). New Zealand obese mice have increased insulin concentrations and decreased binding by hepatic plasma membranes (33). Implantation of normal islets of Langerhans into these animals results in normalization of both circulating insulin levels and hormonal binding (33). Removal of the transplant causes reversal to the original situation, i.e., increased insulin concentrations and decreased binding (33). Finally, an inverse correlation between insulin concentrations and binding also exists in another insulinoprivic state. Hepatic plasma membranes (40) and thymocytes (41) from hypophysectomized rats (which have decreased plasma insulin levels) demonstrate enhanced insulin binding. Thus, the evidence that insulin can modulate its own receptor seems substantial. Hormone modulation of its specific receptor may be a general phenomenon since high levels of growth hormone (42), beta-adrenergic catecholamines (43), and thyrotropin releasing hormone (44) are associated with decreased binding of the respective hormone while conversely the absence of prolactin seems to inhibit activity of its receptor (40).

The relationship between insulin binding and insulin action seems to be more complex. When both are compared directly on the same tissue, only a small percentage of insulin receptors needs to be occupied to exert a maximal hormonal effect (45–48). In addition, there are many situations in which insulin binding and insulin action do not change in parallel. For instance, insulin action is impaired on fat (49), muscle (50), and liver (51) of diabetic rats but as demonstrated in the present study, insulin binding (at least in the liver) is increased in these animals. Similarly, in caloric deprivation, insulin action is impaired (52–54) while insulin binding is enhanced (4, 53, 54). In suckling mice (41) and rats fed a high carbohydrate diet (55), insulin activity is increased with no changes in insulin binding. In starved or streptozotocin-treated ob/ob mice, insulin binding increases but the insulin effect on liver and adipose tissue remains markedly diminished (56).

Therefore, diverse relationships can exist between insulin binding and action: both are decreased in hyperinsulinemic conditions of insulin resistance; both are increased in hypophysectomy; insulin binding is increased while insulin action is impaired in the insulinoprivic states of diabetes mellitus and caloric deprivation; and insulin action is enhanced with no change in insulin binding in both suckling mice and in rats fed a high carbohydrate diet. This would imply that while insulin binding is necessary for the hormonal effect, other factors subsequent to binding may significantly modify insulin action in certain circumstances. Rate-limiting intracellular events probably exist in insulinoprivic diabetes mellitus since insulin

binding is increased but insulin action is impaired. This same conclusion was recently reached after studying insulin binding, glucose transport, and glucose oxidation in the large adipocyte of the obese rat; i.e., intracellular events rather than hormonal binding are the rate-limiting factors determining the metabolism of glucose by the fat cell in obesity (57).

Note added in proof. These results have recently been confirmed. Hepatic plasma membranes from Chinese hamsters and mice rendered diabetic with streptozotocin bound significantly more insulin than control membranes (58). This enhanced binding returned to control levels in both species after 5 days of insulin therapy (58).

ACKNOWLEDGMENTS

We thank Marilyn Scott and Robert G. Karjala for their excellent technical assistance. Dr. W. E. Dulin of The Upjohn Co., Kalamazoo, Mich. kindly supplied the streptozotocin. Computing assistance was obtained from the Health Sciences Computing Facility, UCLA, sponsored by the National Institutes of Health Special Research Resources grant RR-3.

Supported by grants AM 15833 and 14702 from National Institute of Arthritis and Metabolic Diseases, The Kroc Foundation and the Diabetes Association of Southern California.

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