Insulin Binding to Liver Plasma Membranes in the Obese Hyperglycemic (ob/ob) Mouse

DEMONSTRATION OF A DECREASED NUMBER OF FUNCTIONALLY NORMAL RECEPTORS

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In previous studies, the insulin resistance of the obese hyperglycemic mouse (ob/ob) was found to be associated with decreased insulin binding to liver, fat, and lymphocytes. The present study demonstrates that the insulin receptors in the liver membranes of the ob/ob mouse are decreased in number, but are indistinguishable from normal by other criteria including affinity, kinetics of association and dissociation, temperature dependence of binding, and biological specificity of the binding reaction. The receptor in liver membranes of the ob/ob mouse is also indistinguishable with respect to insulin receptor site-site interactions. Degradation of both insulin and of receptor sites was studied and did not account for differences observed in binding. We conclude that the insulin receptor from the ob/ob mouse is a functionally normal receptor and that its presence in diminished number accounts for the observed decrease in insulin binding to liver plasma membranes.

With obesity, both in rodents (1-5) and in man (6, 7), there is a correlation between insulin resistance and decreased insulin binding to its specific receptor sites on the cell surface (8). In the obese hyperglycemic mouse (ob/ob), a model of extreme insulin resistance, insulin binding to the purified plasma membrane fractions of liver and fat and to isolated hepatocytes and thymocytes has been found to be decreased (1-3, 5). In previous studies this decrease has been shown to be due to a specific alteration in the insulin receptor population with no change in other hormone receptors or in membrane structure or function, as evidenced by changes in gross morphology, enzyme activity, or protein subunit composition of the membrane (1, 2). In the present study, the detailed physicochemical properties of the insulin receptor in liver membranes of the ob/ob mouse were compared to those from its thin littermates. These studies demonstrate that, by all criteria, the insulin receptor of the ob/ob mouse is a normal receptor that is present in decreased concentration.

MATERIALS AND METHODS

C57BL/6J mice homozygous for the ob gene (ob/ob) and their thin littermates, composed of one-third normal (+/+) and two-thirds heterozygous (1/ob) animals, were purchased from The Jackson Memorial Laboratory, Bar Harbor, Maine. Mice were fed Purina rat chow ad libitum until the time of death. Age- and sex-matched controls were used for each experiment.

The fully purified plasma membrane fraction (Step 15, Ref. 9) was prepared from liver and frozen in small aliquots at -70° until use. After thawing, the membranes were diluted in Krebs-Ringer phosphate buffer, pH 7.5 (NaCl, 118 mM; KCl, 5 mM; MgSO4, 1.2 mM; KH2PO4, 1.2 mM; NaH2PO4, 10 mM) and dispersed by agitation and vigorous aspiration of the suspension into a 1-ml syringe through a 21-gauge needle. All storage and dilution was in plastic laboratory ware since the membrane particles adhere to glass, especially at room temperature. Protein concentrations were determined for each experiment by the method of Lowry et al. (10) with bovine serum albumin as a standard. 125I-insulin was prepared at a specific activity of 80 to 180 μCi/μg by a modification of the chloramine-T method (11) and separated from free I- by chromatography on a cellulose column (12). The following insulins were received as gifts: mouse insulin from Dr. J. Schlichtkrull (M 20169, Novo Research Institute, Copenhagen), guinea pig insulin from Dr. L. F. Smith (University of New Mexico, Albuquerque), and desalanine-desasparagine insulin from Dr. F. H. Carpenter (University of California, Berkeley). Porcine insulin was purchased from Eli Lilly and Co.

Several minor modifications were made in our previously described method for insulin binding (13). Equal aliquots of 125I-insulin, unlabeled insulin, and the plasma membrane fraction (each in Krebs-Ringer phosphate buffer) were pipetted directly into 0.4-ml Beckman microfuge tubes; the final incubation volume was 0.15 ml. In addition, bovine serum albumin was present at 30 mg/ml in the buffer containing the 125I-insulin. The final concentrations of 125I-insulin and bovine serum albumin were 0.1 ng/ml and 10 mg/ml, respectively, unless otherwise stated. The membrane fraction was added last and moved toward the tip by centrifugation for 1 to 2 s and then was resuspended by vigorous agitation on a Vortex mixer. The microfuge tubes were incubated in a water bath at the desired temperature and thoroughly agitated several times during the incubation. The time and...
temperature for the incubation is indicated in the figure legends. At the end of the incubation, without further dilution or transfer, the tubes were centrifuged for 1 min in a Beckman microfuge, the supernatants were aspirated and discarded, and the tips were excised and counted in a Nuclear Chicago gamma counter. Except where noted, the nonspecific binding, defined as the radioactivity associated with the pellet in the presence of 50 μg/ml of unlabeled insulin, was subtracted from the total 125I-insulin binding to yield specific binding (14).

An attempt was made to use a membrane protein concentration of 0.2 mg/ml in all experiments, but some variation in membrane concentration between experiments occurred, necessitating normalization. This correction is possible since with 125I-insulin in tracer concentration the bound to free ratio of labeled hormone is proportional to the total receptor concentration and, therefore, to the membrane protein concentration. In order to validate this correction for membrane protein concentration, the specific binding of 125I-insulin at 0.1 ng/ml was determined at membrane concentrations from 0.05 to 0.3 mg/ml for both the ob/ob and normal liver membranes. A linear correlation was evident for both the ob/ob and normal membranes, with regression coefficients of 0.96 and 0.98, respectively. The normalized bound to free ratio of labeled hormone was then simply the product of the observed bound to free ratio and the ratio of the normalized to actual membrane concentration. Since small errors in membrane concentration are amplified by this correction, precision in the Lowry determination is quite important. In addition, precautions must be exercised in working with small volumes of a membrane suspension since with repeated agitation the membranes adhere to the wall of a test tube, decreasing the concentration of membrane that is dispensed.

RESULTS

Comparison of Association and Dissociation—To assess kinetic properties of the insulin receptors of the ob/ob and normal liver membranes, association and dissociation of the 125I-insulin with both types of membranes were compared at 20°C. The time courses of association of 125I-insulin to the ob/ob and normal liver membranes when normalized to the same maximal binding were indistinguishable (Fig. 1). In both, the half-time of complex formation was 2 to 3 hours, and the maximal binding occurred at 5 to 6 hours. After 6 hours the specific binding of 125I-insulin began to decrease, suggesting degradation of the hormone, the receptor, or the hormone-receptor complex (vide infra). When the initial rates of binding to liver membranes of the ob/ob and thin mice were compared at equal receptor concentrations (a membrane concentration of 0.29 mg/ml for the ob/ob and 0.11 mg/ml for the thin), no differences were observed (data not shown).

Dissociation of 125I-insulin was initiated after 1 hour of incubation by 100-fold dilution of the incubation mixture (Fig. 2, A and B). The dissociation rates from both the normal and ob/ob liver membranes were indistinguishable, with a t½ of 60 min. Since dissociation did not follow first order kinetics, a single rate constant could not be determined.

Comparison of Insulin Receptor Site-Site Interactions Recently, DeMeyts et al. (15) have presented evidence for site-site interactions among insulin receptors of the type referred to as negative cooperativity. Negative cooperativity is said to exist when an increased fractional saturation of receptors by hormone produces a decrease in affinity of the receptor for hormone. This effect was demonstrated by showing that the rate of dissociation of 125I-insulin produced by "infinite" dilution was further increased in the presence of unlabeled insulin in the dilution medium (15). Since decreased insulin binding in the ob/ob mice could be due to increased cooperative interactions, the extent of cooperativity was compared in the ob/ob and normal liver membranes by examining dissociation of 125I-insulin receptor complexes by dilution alone and by dilution in the presence of unlabeled insulin at 1 μg/ml (Fig. 2, A and B). With the receptors from ob/ob and normal mice at equal concentration and fractional saturation, the dissociation rate was increased in the presence of unlabeled insulin to a similar extent, with the half-time of dissociation reduced to about 25 min in both groups.

The negative cooperativity exerted by insulin on its own receptor is dependent on the insulin concentration (15); this dependence was indistinguishable in the ob/ob and normal liver membranes, with maximum effect found at 1000 ng/ml (Fig. 2C). The decrease in the effect observed at higher insulin concentrations is thought to be due to dimerization of insulin, which masks the cooperative site (16). Under the conditions of these experiments, the dissociation rate and the cooperative effect were independent of membrane concentrations in the range between 0.10 and 0.40 mg/ml for the thin and 0.20 and 0.70 mg/ml for the ob/ob membranes (data not shown).

Comparison of Affinity—As previously reported (2), when the percentage of 125I-insulin that is bound is plotted as a function of insulin concentration, a marked decrease in the insulin binding to the liver membranes of the ob/ob mouse as compared to the thin is observed over the range of 0.1 to 1000 ng/ml (Fig. 3A). Changes in either affinity or capacity of the insulin receptor population could account for this decrease.

When the bound to free ratio of the labeled hormone is plotted as a function of the bound hormone for the ob/ob and normal, as described by Scatchard (17), identically shaped curves are obtained (Fig. 3B). This type of curvilinear Scatchard plot reflects the continuously decreasing affinity of the insulin receptor population as the fractional saturation increases. This result would be expected with negative cooperativity (15), but may also reflect, in part, two or more discrete populations of receptors, with different affinities (14). In a model with two classes of receptor sites but without cooperativ
The enhancement of dissociation by insulin. A, dissociation by dilution was performed after the purified liver membranes from the normal mice had been incubated with $^{125}$I-insulin at 20°C for 1 hour. Duplicate 50-μl aliquots then were diluted in 5 ml of Krebs-Ringer phosphate buffer (3 mg/ml of albumin, pH 7.5) or 5 ml of buffer containing 1 μg/ml of unlabeled insulin and incubated for the indicated times at 20°C. Membrane-bound $^{125}$I-insulin was separated by 0.45 μM Millipore filters and washed with 10 ml of buffer, and the filters were counted. The total $^{125}$I-insulin binding, with the filter blank subtracted, was determined and plotted as the percentage of initial $^{125}$I-insulin binding. The data, dilution only (●) and dilution plus 1 μg/ml of unlabeled insulin (○), are the mean of duplication determinations in a representative experiment of three performed. B, dissociation of $^{125}$I-insulin from the membranes of the ob/ob mouse was treated in a similar manner as in A and plotted as dilution only (●) and dilution plus 1 μg/ml of unlabeled insulin (○). Membrane concentrations were 0.37 mg/ml for the ob/ob and 0.14 mg/ml for the thin group and were chosen so that the initial bound to free ratio (0.11 and 0.12, respectively) would be equal. C, dissociation by dilution only and dilution plus the indicated concentrations of unlabeled insulin was studied as above using a 60-min incubation at 20°C. The data (thin, ●; ob/ob, ○) are the mean of two experiments, each in duplicate, and are expressed as the percentage of the maximum cooperative effect. The maximum cooperative effect was the difference in $^{125}$I-insulin dissociated by dilution and dilution plus 1 μg/ml of unlabeled insulin.

Affinity of the liver membrane insulin receptor. A, $^{125}$I-insulin binding to liver membrane from ob/ob (●, nine experiments) and thin (●, 14 experiments) mice was determined at 20°C after 6 hours of incubation. The data are normalized to 0.2 mg/ml of membrane concentration (see "Methods and Materials"). These data also have been corrected for degradation of hormone and receptor (thin, □; and ob/ob, ○), using the rates of degradation from Figs. 7 and 9A. The $K_a$ for the insulin degrading enzyme in liver (1.7 × 10⁻¹ M) (10) was used in the Michaelis-Menten expression to correct for hormone degradation at the indicated insulin concentrations. B, $^{125}$I-insulin was incubated with liver membranes from thin (0.20 mg/ml) and ob/ob (0.22 mg/ml) mice for 6 hours at 20°C in the presence of varying amounts of insulin. Nonspecific binding has been subtracted and the data, the mean of triplicate determinations, are plotted as bound/free of the labeled hormone versus bound insulin. For both the ob/ob and thin mice, these curves may be divided into two apparent classes of receptors, a high affinity-low capacity site ($K_{app} = 1.7 × 10^{-7}$ M⁻¹) and a low affinity-high capacity site ($K_{app} = 6.2 × 10^{-1}$ M⁻¹). C, the Hill plot (20, 21) is shown for the data in B. The log $[B/B_{max} - B]$ is plotted as a function of the log-free hormone, where $B$ is the insulin bound at a given free hormone concentration. $B_{max}$ was considered to be the specific binding at 250 ng/ml since it is difficult to determine the exact contribution of nonspecific binding to total binding of $^{125}$I-insulin above this concentration. A single line fit the data for both the ob/ob mice (○) and the thin mice (●).
In the presence of cooperativity, the Hill plot (20, 21) is useful for assessing the average affinity and the extent of cooperativity. The data for the ob/ob and normal membranes superimposed on a single line (Fig. 3C) indicating that over this range both the average affinity and the extent of cooperativity were identical. The Hill coefficient (the slope of the Hill plot) was 0.75; coefficients less than 1 are consistent with negative cooperativity. It should be noted that this Hill coefficient is likely to be an overestimate since the range of concentrations considered was somewhat restricted. The average affinity constant, which is the reciprocal of the product of the Hill coefficient and the free hormone concentration that produces half-maximal saturation of the receptor, was \(1.5 \times 10^8\) M\(^{-1}\) for both the ob/ob and thin membranes.

A reasonable approximation of the binding capacity of the specific receptor sites for insulin can be obtained by linear extrapolations of the Scatchard plot to the abscissa. By this technique the total receptor concentration in the ob/ob mouse was found to be 33% of that in the thin (4.3 ± 0.2 versus 13.2 ± 1.2 pmol/mg of membrane protein). At tracer concentration of hormone, the bound to free ratio of labeled hormone for the ob/ob membranes is 35% of that observed in the thin mice (Fig. 3B). When the free hormone concentration is very small, the bound to free ratio of the labeled hormone approaches the product of the affinity constant of the unfilled receptors and the total receptor concentration, and thus the decrease in bound to free ratio in liver membranes of the ob/ob mouse is fully accounted for by the decrease in receptor concentration with no change in the affinity of the receptor.

Biological Specificity of Insulin Receptors—In previous studies with rat liver the biological specificity of the insulin receptor has been validated by comparing the ability of several insulin analogues to inhibit \(^{125}\)I-insulin binding (13). Consistent with these earlier studies, we have found that both the ob/ob (Fig. 4A) and normal (Fig. 4B) liver membranes bound various insulin analogues of different biological potency in a rank order proportionate to the ability of these analogues to stimulate glucose oxidation in adipose tissue (13). The finding that mouse insulin is only 20 to 30% as potent compared to the pork standard by this receptor assay could be due to higher affinity of the mouse receptor for pork insulin, or, more likely, to a decreased potency of our lot of mouse insulin due to damage in preparation or storage.

Effects of Temperature—As shown previously (14), the steady state level of binding of insulin is inversely related to temperature (Fig. 5). For both the ob/ob and thin mice, the \(^{125}\)I-insulin binding was higher at lower temperatures (Fig. 6); at all temperatures the relative decrease in insulin binding to the ob/ob membranes was constant. Multiple factors are responsible for the lower level of binding observed at higher temperatures, including decreased affinity, decreased number of receptors (14), and enhanced degradation of insulin and receptors.

Degradation of Insulin—It has been shown with liver membranes (10) that binding to specific receptors and degradation of insulin are separate processes. Degradation of insulin is a function of time, temperature, and membrane concentration. As has been previously reported from studies at 30° (2), degradation of hormone at 20° was slower in the ob/ob membrane than in the thin membranes when similar membrane concentrations were used (Fig. 7). The reason for the slower rate of hormone degradation in the ob/ob is unclear. The difference in rates of hormone degradation were much less pronounced than the differences in binding and the decreased rate of degradation would, if anything, have made the decrease in insulin binding in the ob/ob mouse less apparent (vide infra).

Degradation of Receptor—The degradation of the insulin receptor in the ob/ob and normal membranes was similar when compared at equal receptor concentrations. Receptor degradation in membranes of the thin mouse was dependent on time.
The variants of hemoglobin illustrate several of the examples of genetic defects altering protein structure and response to the metabolic alterations of obesity. There are many possible mechanisms, including an altered affinity for ligand, impaired cooperative interactions between subunits, decreased temperature, and membrane concentration. Receptor degradation was rapid at higher temperatures, with 15% of the initial receptor population degraded per hour at 37°C (membrane concentration of 0.28 mg/ml) (Fig. 8). At 20°C, receptor degradation was much slower; however, significant degradation did occur during the 6-hour incubation period. When compared at equal membrane concentrations, receptor degradation in the ob/ob mouse was somewhat less than that in the thin mouse (Fig. 9A). However, when receptor degradation was related to receptor concentration, no difference between the ob/ob and normal membranes was evident (Fig. 9B). Determination of the rates of hormone and receptor degradation as a function of liver membrane concentration allowed correction of the binding data for these processes. These corrections (Fig. 9A) resulted in only minor quantitative differences which, if anything, increase the apparent deficiency of insulin binding to the ob/ob membranes.

**DISCUSSION**

The obese hyperglycemic syndrome in mice (ob/ob) is characterized by hyperphagia, marked obesity, and insulin resistance, manifest by hyperglycemia in the face of abnormally high circulating levels of biologically active insulin (23). In these ob/ob mice decreased insulin binding to specific receptors on the surface membranes of liver, fat, and thymic lymphocytes (1-3, 5) has been found as a biochemical correlate to their insulin resistance. In previous studies using purified liver membranes, this decreased insulin binding was shown to be a specific alteration in the membrane receptors for insulin; other hormone receptors, protein subunit composition, membrane marker enzymes, and gross membrane morphology were unchanged in the ob/ob mouse compared to normal (1, 2). Although these studies were strong evidence for a specific defect in insulin binding to liver membranes, they did not answer the question of whether the insulin receptor in the ob/ob mouse was a defective receptor (product of an abnormal structural gene) or a normal receptor present in decreased concentration as a result of deranged regulation of receptor concentration, decreased stability of the receptor, or in response to the metabolic alterations of obesity. There are many examples of genetic defects altering protein structure and function. The variants of hemoglobin illustrate several of the possible mechanisms, including an altered affinity for ligand, impaired cooperative interactions between subunits, decreased stability leading to more rapid degradation (24), and disordered regulation of synthesis (25). In the present study, by means of defining the functional characteristics of the insulin receptor, we sought to determine whether the impaired insulin binding in the ob/ob mouse was itself a direct consequence of the genetic defect. Studies of the kinetic properties of the insulin receptor of the ob/ob and normal mice revealed that the initial rates of association and the half-time of dissociation by dilution were indistinguishable. Insulin receptor site-site interactions as evidenced by the insulin-induced acceleration of dissociation rate were also comparable. In the Hill analysis, the average affinity constants were equal. Comparison of the Scatchard curves at the same degree of fractional saturation indicated that the apparent affinities were the same, with the major alteration being the decrease in receptor concentration. The insulin receptor from the ob/ob mouse had an unaltered biological specificity for insulin. In addition the shift in insulin binding with temperature was parallel in the membranes of both groups of mice. Therefore the decreased insulin binding observed in the liver membranes of the ob/ob mouse is fully accounted for by the decreased concentration of an insulin receptor which by the above functional criteria is otherwise normal.

Factors involved in the regulation of the insulin receptor are in large part undefined, and a genetic mutation in regulation of the insulin receptor concentration also could lead to a decrease in insulin receptors by either impaired synthesis or increased degradation of the receptor. The available data, however, indicate that this is not the case and that the insulin receptor in the ob/ob mouse responds to the regulatory influences of insulin concentration in a manner similar to that observed in normal animals and man. Thus, normal mice made obese and hyperinsulinemic by administration of gold thioglucose (26, 27) and thin, hyperinsulinemic diabetics (28) demonstrate decreased insulin binding; both obese and normal mice show an increase in insulin receptors after diet restriction (26, 27); and sustained hyperinsulinemia both in obese mice and in *in vitro* tissue culture can induce or maintain a decrease in insulin receptors (26, 27, 29).

\[\text{A. H. Soll, C. R. Kahn, D. M. Neville, Jr., and J. Roth, submitted for publication.}\]
Since the insulin receptor is defined only by its functional characteristics, we cannot exclude an alteration in the receptor molecule at sites other than those responsible for the binding of hormone and for cooperative interactions. A decreased insulin receptor concentration might occur with such an alteration if receptor stability was greatly impaired. The in vitro studies with liver membranes reported here, however, show no appreciable difference in receptor degradation.

The pathophysiological mechanism by which the ob mutation results in the obese hyperglycemic syndrome remains unclear; based on the present results it seems unlikely that the insulin receptor deficiency is the primary defect. Rather, the insulin receptor in the ob/ob mouse is functionally normal. As in other forms of obesity there are fewer receptors per cell accounting for the decreased insulin binding. The insulin resistance displayed by these mice appears to be a result of this decreased insulin receptor concentration. Since the filling of some of the receptor sites by insulin will reduce the affinity of all the receptors, the finding of normal affinity and of cooperative interactions where insulin binding is found to be decreased, the alteration accounting for the diminished binding may be a decrease in the insulin receptor concentration, or a change in receptor affinity, or cooperative interactions. Obviously, each of these will require further study.

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*J. Biol. Chem.* 1975, 250:4702-4707.