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REDUCED INSULIN BINDING TO LIVER PLASMA MEMBRANES IN INHERENTLY OBESE DIABETIC CBA/Ca MICE

PETER V. TABERNER* and DAVID M. CONNELLY

Department of Pharmacology, University of Bristol, School of Medical Sciences, Bristol BS8 1TD, U.K.

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Abstract—Insulin binding to isolated liver plasma membranes was measured in mice from C57BL and LACG strains, and in normal and obese diabetic mice from the Bristol CBA/Ca colony. A simple and rapid three-step method for the preparation and purification of liver plasma membranes, using Percoll density gradient centrifugation was used. Both high and low affinity binding was detected in membranes from all four groups of mice. The K_d values for binding were similar in all groups, but the insulin binding capacity (B_{max}) at low and high affinity was significantly reduced in obese CBA mice compared to age-matched lean controls. It is proposed that insulin receptor down-regulation may account for the insulin resistance observed in spontaneously obese diabetic CBA mice.

Maturity onset non-insulin-dependent diabetes mellitus (NIDDM) is the most common form of diabetes and is associated with obesity, hyperinsulinaemia and insulin resistance [1]. The cause of the insulin resistance is still an open question; suggestions have included defects in the insulin receptor affecting insulin binding, defects in insulin signal transduction at the level of tyrosine kinase and the presence of circulating insulin antibodies [2].

Studies in genetically obese animal models have revealed both a decrease in insulin receptor tyrosine kinase in hyperinsulinaemic obese mice [3], and a decreased insulin binding to plasma membrane receptors [2]. This latter effect may occur in response to the high levels of circulating insulin, since it is known that the concentration of plasma membrane insulin receptors is influenced by the extracellular insulin level [4]. On the other hand, neither insulin binding nor receptor tyrosine kinase were altered in rats made diabetic by neonatal streptozotocin [5].

Although there are a number of rodent models for human NIDDM and obesity [6, 7], none of them is entirely satisfactory, either because of the greater severity of the syndrome and reduced lifespan (e.g. C57BL/KsJ with the *db* mutation, obob or KK mice), or because of the early onset of the syndrome (e.g. C57 BL/6J with the *ob* mutation). An additional problem is that the homozygotes *db/db* and *ob/ob* are infertile, so that heterozygotes have to be used for breeding.

The obese diabetic CBA/Ca mice used in this study have many of the characteristics of human NIDDM: they are hyperglycaemic, hyperinsulinaemic, hypertriglyceridaemic and insulin resistant [8]. More importantly, the obese syndrome develops relatively late in life (16–20 weeks) and is maintained throughout a normal life expectancy. Since insulin receptor binding has not previously been studied in these CBA/Ca mice, we have examined insulin binding to isolated liver plasma membranes in order to determine whether the insulin resistance in these mice can be explained in terms of reduced receptor affinity or number. Metabolically normal LacG and mildly hyperglycaemic C57BL mice have been included as additional controls for the effects on insulin binding of body weight and blood glucose levels, respectively. A preliminary account of some of these findings has already been reported [9].

MATERIALS AND METHODS

Animals. Male mice from inbred strains of C57BL/10 ScSn (C57), CBA/Ca (CBA) and outbred Lac:LCGFCFW (LacG) from the University of Bristol Medical School were used. The CBA mice included obese diabetic animals and age-matched lean normal animals as controls. The colonies were housed at 20–22° in cages of four mice with a 12-hr light–dark cycle. The mice had free access to pelleted CRM breeding diet (Labsure, Cambs., U.K.) and water.

Preparation and purification of liver plasma membranes. Preliminary experiments employed established procedures for the preparation of liver plasma membranes on sucrose density gradients [10, 11]. However, the development of more rapid procedures for preparing tissue plasma membranes using Percoll [12, 13] suggested that this would be advantageous where numerous preparations had to be made and membrane purity was important. The method finally adopted was as follows: 25-week-old mice were fasted overnight to reduce liver glycogen content, then killed by cervical dislocation. The liver was perfused *in situ* with physiological saline (0.9%, w/v) at 4°, then excised and washed with saline. Tissue was weighed, then homogenized in ice-cold 50 mM $NaHCO_3$ buffer (pH 7.4) in a Dounce homogenizer, followed by three passes in a loose-fitting teflon–glass homogenizer at 400 rpm.

* To whom correspondence should be addressed.

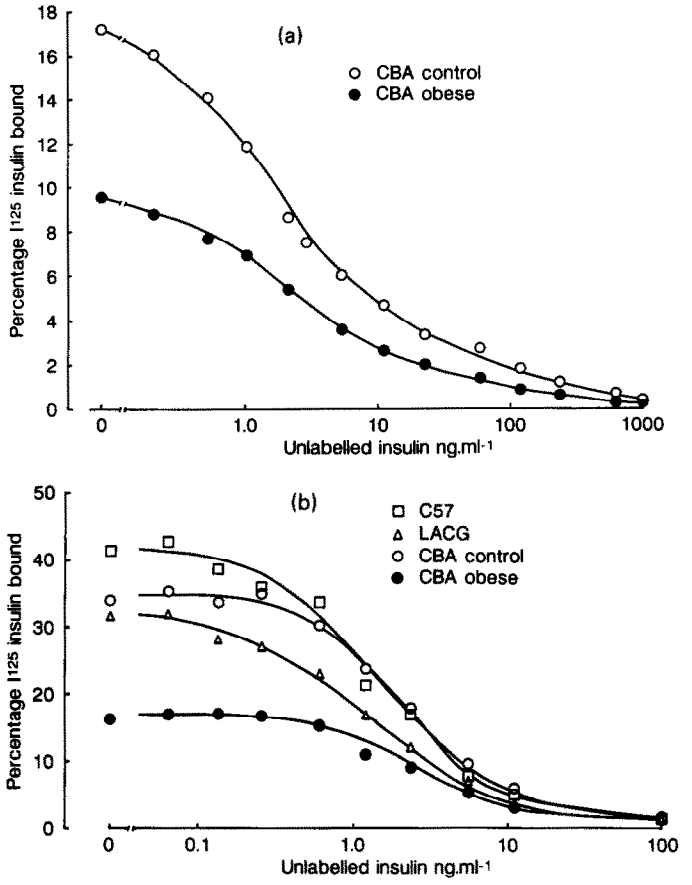

Fig. 1. (a) Displacement of the low affinity of A14-I^{125}insulin (0.06 ng) to liver plasma membranes (0.15 mg protein) by unlabelled insulin. Data points are the means of three independent experiments. (b) Displacement of the high affinity binding of A14-I^{125}insulin (0.06 ng) to liver plasma membranes (0.33 mg protein) by unlabelled insulin. Data points are the means of duplicates.

Table 1. Body weights and blood chemistry of the mouse strains

Mouse	Age (weeks)	Body weight (g)	Plasma glucose (mmol/L)	Plasma insulin (μg/mL)
C57	8	25.8 ± 0.4 (8)	11.40 ± 1.10 (6)	0.759 ± 0.174 (6)
	25	32.4 ± 0.9 (12)	10.70 ± 0.41 (12)	0.713 ± 0.185 (8)
LACG	8	33.1 ± 0.4 (8)‡	9.42 ± 0.40 (6)	0.962 ± 0.206 (8)
	25	42.2 ± 1.2 (12)	8.47 ± 0.27 (12)	0.898 ± 0.200 (16)
CBA	8	26.5 ± 1.0 (12)	10.20 ± 0.63 (12)	0.763 ± 0.135 (8)
CBA lean	25	34.1 ± 0.8 (12)	9.36 ± 0.33 (9)	1.230 ± 0.231 (8)
CBA obese	25	42.8 ± 0.8 (12)†	13.7 ± 1.6 (12)*	4.45 ± 0.43 (10)†

Results are means ± SE of N observations (shown in parentheses).
Obese > lean at the same age (t-test). *$P < 0.05$; †$P < 0.001$
LACG > CBA at the same age (t-test); ‡$P < 0.01$.

Homogenates were diluted to 3% (wet w/v) with $NaHCO_3$ buffer and filtered through nylon mesh. Aliquots of 50 mL filtrate were spun at 600 *g* for 3 min; the pellets were then resuspended in 7 mL of 50 mM Tris–HCl buffer (pH 7.6) containing 0.25 M sucrose. The resuspended pellet was applied to a 10 mL tube of Percoll (Pharmacia) prepared to a final density of 1.060 g/cm^3. The plasma membrane preparations were all assayed for alkaline phosphatase, EC 3.1.4.1 [14], succinate dehydrogenase, EC 1.3.99.1 [15], and total protein, which was assayed using essentially the method of Lowry *et al.*

[16]. The optimum centrifugation conditions were 12,000 g_{av} for 20 min in a swing-out rotor, using an MSE 65 centrifuge. The plasma membrane-rich band (usually about 2 mL volume) lay at 0.5–1 cm below the lipid layer at the top of the column, and was aspirated off with a Pasteur pipette and diluted × 4 with Ca^{2+}-free Krebs–Ringer bicarbonate buffer (pH 7.8). The Percoll was removed by centrifugation at 3000 g for 10 min to precipitate the plasma membranes. These were then gently resuspended in 8 mL Krebs bicarbonate buffer and respun under the same conditions. After the two washings, the final pellets were resuspended in 5 mL Krebs bicarbonate buffer, divided into 1.25 mL aliquots, and frozen at −85° until required.

Insulin binding assay. Insulin binding to plasma membranes was measured by standard procedures [17] using displacement of labelled insulin (human A14-I^{125}insulin, Amersham IMI66) by varying concentrations of cold insulin (Human Actrapid, Novo Industri, Denmark). Plasma membranes were thawed and resuspended in Ca^{2+}-free Krebs bicarbonate buffer (pH 7.8) containing 30 mM Tris–HCl to a final protein concentration of about 0.45 mg/mL. The labelled and unlabelled insulin solutions were made up in the same buffer, but containing 10 or 30 mg/mL bovine serum albumin (RIA grade, Sigma), respectively. The final assay volume was 0.3 mL and contained 1 mM *N*-ethyl maleimide to prevent insulin degradation by non-specific proteases [18]. Non-specific binding was determined by the addition of 0.1 mg/mL cold insulin to replicate tubes.

A pre-incubation of 8 hr in sealed plastic Eppendorff tubes at 4° preceded the addition of labelled insulin, after which the tubes were vortexed and incubated for a further 16 hr at 4°. Incubations (in duplicate) were terminated by centrifugation at 5000 g for 150 sec at 0°. The pellet was washed once with buffer and respun, then the base of the tube was guillotined and placed in a sample tube for gamma counting. All the assays were performed in duplicate and the amount of insulin bound expressed per mg of plasma membrane protein.

The kinetic parameters for insulin binding were derived from plots of the fractional binding of labelled insulin against the concentration of cold insulin on a log scale. The data did not conform to the Law of Mass Action, so the dissociation constants (K_d) and maxima (B_{max}) were estimated using the two-site inhibition program described by Barlow [19] and solved by the line-fitting method of least squares.

Plasma glucose levels were measured using a Beckman Glucose Analyzer 2; plasma immuno-reactive insulin was measured by radio-immunoassay as described previously [8].

RESULTS

The C57, LACG and normal and obese CBA mice used for the preparation of liver plasma membranes were monitored in terms of body weight, plasma glucose and plasma insulin. The results are summarized in Table 1. In the 25-week-old CBA mice obesity was defined as >40 g body weight, and diabetes as plasma glucose >10 mM. Control (lean) CBA mice weighed less than 36 g and their plasma glucose was below 9 mM.

The LACG, C57 and control CBA mice showed a similar proportional weight gain between 8 and 25 weeks of age. Although the 25 week LACG mice were of similar body weight to the obese CBA mice, they are a much larger strain and were already significantly heavier ($P < 0.01$) at 8 weeks than either C57 or CBA mice (see Table 1). The CBA mice showed higher plasma insulin levels than C57 or CBA mice at 25 weeks; in the case of the obese CBA mice the increase was highly significant ($P < 0.001$).

Liver plasma membranes

After centrifugation the Percoll gradient yielded a surface lipid layer, a well-defined plasma membrane-rich layer, and a bottom layer containing mitochondria, nuclear material and a Percoll pellet. The density gradient centrifugation and washing increased the relative recovery of alkaline phosphatase by a factor of × 15–20 from the original tissue homogenate (103.7 mUnits/mg protein compared to 6.7 mUnits/mg protein). There were no significant differences in the absolute specific activities of either alkaline phosphatase or succinate dehydrogenase in the membrane fractions from the different mouse strains, although it was observed that the lipid layer on the Percoll gradient was larger in the homogenate prepared from livers of obese CBA mice.

Insulin binding assays

Insulin degradation during the incubation period was found to be negligible. Degradation of the insulin receptor was minimized by the low temperature of the incubation. Preliminary experiments revealed that the binding sites were stable for up to 48 hr at 4°. Scatchard plots of the binding data were curvilinear, implying the presence of multiple binding sites, but direct plots of bound insulin versus the total insulin concentration indicated that saturation of specific binding of the low affinity site had not occurred. Thus, the kinetic parameters for binding could not be validly estimated from the Scatchard plot, and a displacement binding assay was used.

The binding curves from normal and obese CBA mice, from which high and low affinity binding parameters were derived, are shown in Fig. 1a and b, respectively. In the high affinity binding assays the membrane protein concentration was increased to 0.33 mg/assay from the 0.15 mg used for low affinity binding. The kinetic parameters are set out in Table 2. The results show no statistically significant differences between the high or low affinity dissociation constants for the four groups of mice. The receptor density (B_{max}) was also very similar between C57 and LACG mice. However, both low and high affinity B_{max} values were significantly decreased in obese CBAs compared to normal lean CBAs. It appeared that high affinity binding capacity was greater in the lean CBA mice compared to both C57 and LACG mice, although the differences were not statistically significant.

Table 2. Kinetic parameters for insulin binding to liver plasma membranes

	High affinity		Low affinity	
	K_d	B_{max}	K_d	B_{max}
C57	1.44 ± 0.26	1.16 ± 0.11	119 ± 25.2	21.3 ± 2.4
LACG	1.35 ± 0.23	1.04 ± 0.10	163 ± 21.2	23.4 ± 2.3
CBA lean	1.36 ± 0.19	1.47 ± 0.16	137 ± 23	22.8 ± 2.9
CBA obese	1.67 ± 0.26	0.96 ± 0.06*	154 ± 22	13.7 ± 1.9*

Units: K_d: ng/mL; B_{max}: ng/mg protein.

The experimental data were fitted to the expression: $y = \frac{m_1 \cdot k_1}{x + k_1} + \frac{m_2 \cdot k_2}{x + k_2}$ where y = concentration of insulin bound, x = total insulin concentration, m = the maximum concentration of cold insulin bound, and k = the affinity constant for binding. The binding parameters K_d and B_{max} for high and low affinity were readily calculable from the derived values of k_1, k_2 and m_1, m_2, respectively (see Ref. 19).

The results are means ± SE based on N = 24 (three independent experiments, each with eight data points). Individual data points were the medians of triplicate assays. The statistical comparisons were made using the independent estimates of K_d and B_{max}.

* Obese < lean (Fisher exact test): $P < 0.02$.

DISCUSSION

The obese CBA mice exhibit many of the features of human NIDDM, including mild obesity, hyperinsulinaemia, islet hyperplasia and insulin resistance [8, 20]. In this study they have been compared with normal (lean) littermates, and the large (but non-obese) LACG strain, and a mildly hyperglycaemic, insulin sensitive C57 strain [8]. The presence of both high and low affinity insulin binding sites has been confirmed in liver plasma membranes from the C57, LACG, and normal and obese CBA mice. The high affinity site is thought to be the physiologically important receptor [21]. Results from the two series of insulin binding studies over both the extended insulin concentration range (0–1 mg/mL), and the high affinity site concentration range (0–0.1 mg/mL), clearly show that CBA obese mice have a decreased ability to bind insulin compared to lean control CBA, and C57 and LACG mice. This appears to be due to a decrease in receptor number rather than a decrease in binding affinity. It cannot be attributed to increased receptor degradation in the plasma membranes from obese mice, since this was found to be negligible.

In contrast, the mildly hyperglycaemic C57 mice showed no impairment of insulin binding compared to the normoglycaemic LACG mice, although the high affinity B_{max} was somewhat lower than that measured in the lean CBA mice. The LACG mice appeared normal both in terms of plasma levels of glucose and insulin, and insulin-receptor binding.

The binding of insulin to its receptor has been studied in a number of animal models of obesity [22–24], as well as in tissue obtained from obese patients with or without NIDDM [25, 26]. A direct relationship appears to exist between the increase in circulating insulin level and the reduction in receptor number [2]. Indeed, mild type II NIDDM can be adequately explained in terms of insulin resistance due to down-regulation of the receptor, although the insulin resistance in the more severe hyperglycaemias (not necessarily associated with obesity) probably involves a post receptor binding defect [27]. On the other hand, the maximum biological response to insulin can be observed with a low receptor occupancy, so a reduction in B_{max} will not necessarily reflect a reduced biological response *in vivo*.

The diabetes–obesity syndrome in the inbred male CBA mouse shares many of the characteristics of that recently reported to occur in male C3H.SW/SnJ mice [28]. However, the particular advantage of the CBA mouse as a model for human Type II maturity onset NIDDM, is that the syndrome is not environmentally dependent, and does not fully manifest itself until the mice are mature adults at 25 weeks of age. For this reason, the aetiology of this syndrome merits further study as a model for the human disease.

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