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IMPROVED INSULIN RECEPTOR ASSAY: EFFECTS OF AN ANTI-DIABETIC SULPHONYLUREA ON LIVER MEMBRANE INSULIN RECEPTORS FROM OBESE HYPERGLYCAEMIC MICE

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1 A simple assay of liver membrane insulin receptors is described, under conditions which apparently stabilized the insulin-receptor complex. The receptor population was measured in the presence of physiological doses of radioactive hormone under conditions which minimized negative co-operativity effects.

2 Equilibrium between receptor and hormone was attained *in vitro* within 60 min of incubation time at 13°C with no apparent loss of binding sites after 3 h of incubation.

3 Liver membranes from lean mice possessed 8.8 times the receptor number per mg membrane protein measured in membranes from obese hyperglycaemic (ob/ob) littermates. After treatment of ob/ob mice with ARDF-26 (Gliquidon; Boehringer Ingelheim Ltd) the concentration of liver membrane insulin receptors was increased 5 fold.

Introduction

Membranes prepared from biological tissues possess components which bind [^{125}I]-insulin with high affinity and specificity. It is believed that the high-affinity components may represent insulin receptors through which the actions of the hormone may be mediated. In published studies, attempts to measure the parameters of high-affinity binding were hampered by the instability of the hormone–receptor complex *in vitro*. Thus, binding of [^{125}I]-insulin to human adipocytes declined within 1 h of incubation (Olefsky, Jen & Reaven, 1974), as did binding of the hormone to liver membranes of ob/ob mice (Kahn, Neville, Gorden, Freychet & Roth, 1972).

The construction of binding isotherms is generally achieved by use of a fixed tracer amount of radioactive insulin and varying amounts of unlabelled hormone. This procedure yields transformations of binding isotherms which are not linear, and the contribution of high-affinity components to, for example, a Scatchard plot (Scatchard, 1949) must be derived mathematically (for example, see Rosenthal, 1967). Furthermore, the inclusion of large, supraphysiological doses of unlabelled insulin (up to 1000 ng/ml) in the incubate increases the risk of negative co-operative effects which are known to occur in the presence of high insulin concentrations (de Meyts, Roth, Neville, Gavin & Lesniak, 1973).

In this paper, conditions are described for the accurate measurement of high affinity [^{125}I]-insulin binding to isolated liver membranes by direct observation of suppressible binding activity in the presence of physiological doses of insulin. The method has been used to measure insulin receptor sites on liver membranes of ob/ob mice after treatment with an anti-diabetic sulphonylurea derivative, 1-cyclohexyl-3-[[*p*-[2-(3,4-dihydro-7-methoxy-4,4-dimethyl-1,3-dioxo-2(1H)-isoquinolyl)ethyl]phenyl]sulphonyl]urea (ARDF-26; Boehringer Ingelheim Ltd). It has been reported elsewhere that insulin receptors on mononuclear leukocytes of diabetic patients treated with another sulphonylurea derivative, chlorpropamide, were increased (Olefsky & Reaven, 1976).

Methods

Animals

Six-week-old male C57 BL/6J obese, hyperglycaemic (ob/ob) mice and their lean littermates were purchased from the Medical Research Council Laboratory Animals Centre, Carshalton, Surrey. Animals were housed 5 per cage under regulated lighting conditions (lights on 05h 00min: lights off 19h 00min) at 22 to 24°C and fed Dixon's feed 41B and tap water which were available at all times. Obese mice weighed 28 to 30 g and lean mice weighed 18 to 21 g.

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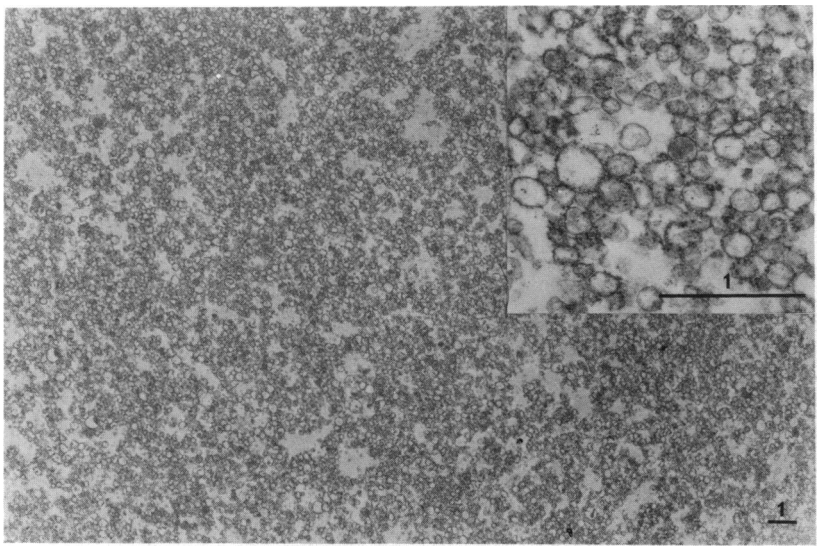

Figure 1 Electron micrographs of sections through 40,000 *g* pellets after purification of mouse liver membranes as described in the text. The scale is indicated on the photograph (1 = 1 μm). Inset: a further magnification of part of the larger photograph.

Preparation of microsomal fractions

Purified liver membranes were prepared essentially as described by Cuatrecasas (1972). Livers were dissected and minced with scissors in ice-cold 0.25 M sucrose solution (0.5 g/ml) and homogenized mechanically with a ground-glass pestle and a Griffin & George Stirrer set at 30% of maximum speed for 1 min. Tissues from different animals were not pooled. The homogenate was centrifuged at 600 *g* for 10 min and the supernatant centrifuged at 12,000 *g* for 30 min. These steps were each repeated once. The resulting supernatant was centrifuged at 40,000 *g* (RAV) for 40 min in an MSE Superspeed 65 ultracentrifuge. All steps were carried out at 4°C, or less. The pellet was homogenized manually in a buffer consisting of Tris–HCl 50 mM, $MgCl_2$ 10 mM, NH_4Cl 5mM and bovine serum albumin (Sigma London) 0.1% (w/v), pH 7.9 (hereafter referred to as 'assay buffer'). The protein concentrations of the membrane suspensions, determined by the method of Lowry, Rosebrough, Farr & Randall (1951) were 1.9 ± 0.06 mg/ml; $n = 9$ (mean ± s.d.) for obese mice, and 1.38 ± 0.04 mg/ml; $n = 9$ for lean mice. The homogeneity of the microsomal preparation thus prepared was verified by electron microscopy (Figure 1).

Drugs

[^{125}I]-insulin (100 ng/ml; >50 μCi per μg) was purchased from the Radiochemical Centre, Amersham (IM.38) and stored at 2 to 4°C. Radioinert bovine pancreatic insulin was obtained from Sigma, London. ARDF-26 was donated by Boehringer Ingelheim Ltd. All reagents were at least of Analar grade.

Incubation and assay procedures

In preliminary experiments, 0.1 ml aliquots of membrane suspensions from obese or lean mice were incubated with a fixed concentration (1.5nM) of [^{125}I]-insulin in plastic tubes. Parallel sets of incubates contained in addition a 500 fold excess of unlabelled insulin. All incubations were performed at least in duplicate. After incubation, incubates were layered over 0.4 ml of assay buffer containing 0.3 M sucrose in Beckman microfuge tubes and centrifuged in a Beckman microfuge. The tip of the tube was cut off after aspirating the supernatant and the radioactivity in the pellet was measured in a Packard Tri-Carb scintillation counter. The efficiency of the counting was 72%. The suppressible precipitated radioactivity was measured by subtracting the ct/min measured in the presence of excess unlabelled insulin.

For the assay, 0.1 ml aliquots of membrane suspensions were incubated with 0.1 ml of a solution in assay buffer of one of a range of concentrations of [^{125}I]-insulin from 0.03 to 1.5 × 10^{-9} M. A parallel set of tubes contained in addition a 500 fold excess of unlabelled insulin. After incubation at 13°C for 90 min (see Figure 3a), incubates were centrifuged as described above, and the precipitated radioactivity

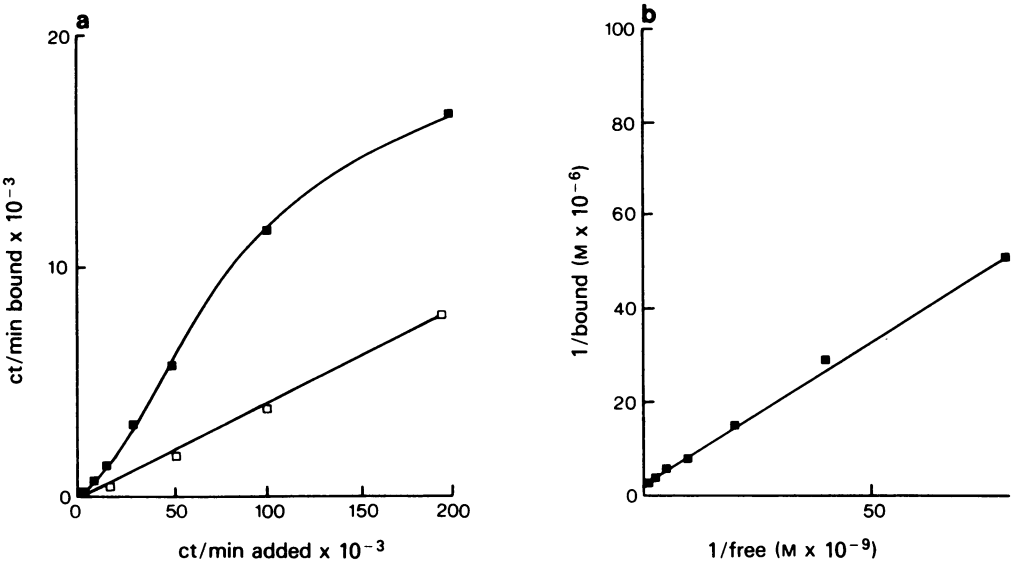

Figure 2 (a) Binding isotherm of the reaction between purified liver membranes and [^{125}I]-insulin alone (■) or in the presence of excess unlabelled insulin (□). Details of the procedure are given in the text. (b) Lineweaver-Burk plot of the saturable bound [^{125}I]-insulin shown in (a).

measured. The following formula can be used to calculate the free and also the suppressible bound radioactivity:

$$f_i = T_i(\text{ct/min}) - \left[b_i - \left(\frac{T_i}{T_{ns}} \times b_{ns}\right)\right] \quad (1)$$

where f_i = free [^{125}I]-insulin (ct/min) in incubate i in the absence of excess unlabelled insulin; T_i = total radioactivity (ct/min) in incubate i, giving b_i ct/min bound in the absence of unlabelled insulin; T_{ns} = total radioactivity (ct/min) in incubate ns giving b_{ns} ct/min bound in the presence of excess unlabelled insulin (ns = non-saturable).

It will be shown in Results (Figure 2a) that the bound ct/min in the presence of excess unlabelled hormone are a linear function of the added ct/min. Furthermore, this isotherm intersects the abscissa at the origin. Therefore values for b_{ns} and T_{ns} can be inserted into formula (1) as constants while varying values for T_i and b_i.

Administration of ARDF-26

Four weeks after arrival the animals were divided into four groups. One group of lean mice received no treatment; one group of obese mice were injected twice daily for 7 days subcutaneously with 0.3 mg/kg of ARDF-26 in not more than 0.1 ml of a solution of 10% Lactomide; one group of obese mice received 0.1 ml of the solvent alone; one group of obese mice received no treatment. Weights were recorded daily. On the morning of the 8th day the animals were injected and killed. Membranes were prepared and assayed on the day of preparation without freezing and thawing the suspension.

Results

Adsorption of [^{125}I]-insulin to vessel walls

In preliminary experiments it was found that although little radioactivity adhered to the walls of the plastic vials used, the microfuge tubes adsorbed unacceptable levels of [^{125}I]-insulin, and the insulin could not be removed, even after rinsing the walls of the tubes with glacial acetic acid. Only after the tips were cut was the blank value reduced to the background 'noise' of the scintillation counter (about 60 ct/min).

Attainment of binding equilibrium

Equilibrium between the hormone and suppressible binding was attained within 60 min at 13°C (Figure 3a). There was no apparent loss of suppressible binding activity after 3 h of incubation. Similar results were obtained with membranes from both obese and lean mice. In subsequent studies, incubations were carried out at 13°C, for 90 min.

Assay

A typical binding isotherm of the reaction between [^{125}I]-insulin and membrane components appears in Figure 2a. In the absence of added unlabelled insulin

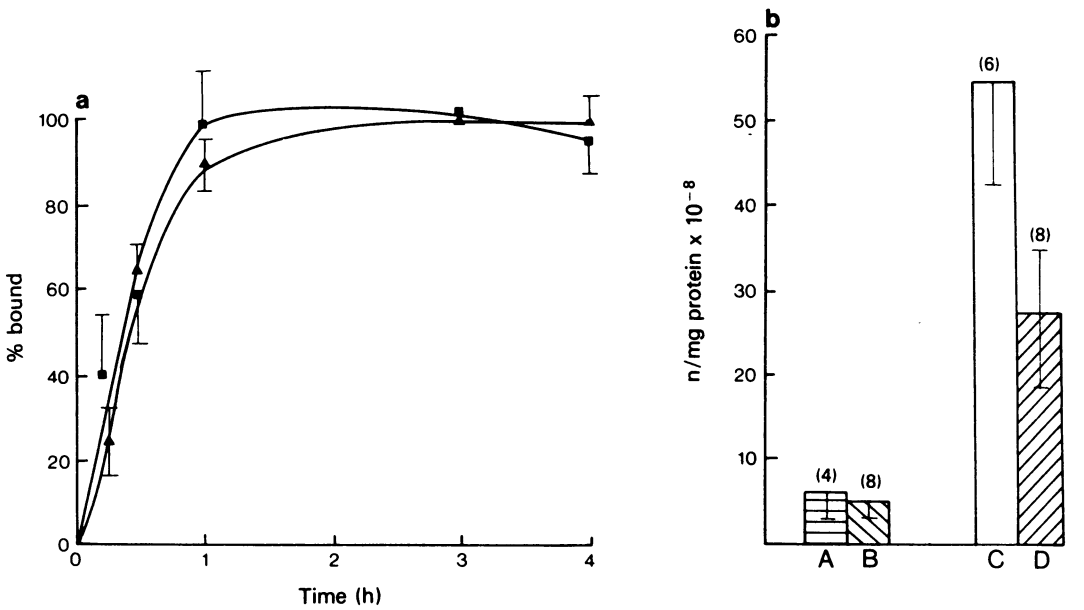

Figure 3 (a) Attainment of equilibrium between [^{125}I]-insulin and saturable binding components on purified membranes from livers of lean (▲) or obese (■) mice at 13°C. Results are expressed as the saturable ct/min bound as a percentage of those bound at 3 h. Vertical lines show s.e. mean. (b) (A) Insulin receptors in untreated ob/ob mice; (B) control injected ob/ob mice; (C) untreated lean mice; (D) ARDF-26 treated ob/ob mice. B vs. D, $P < 0.001$, Student's t test. The receptor number was obtained by multiplying the molar binding capacity per mg protein by Avogadro's number. Numbers in parentheses are number of observations; vertical lines show s.e. mean.

the isotherms tended towards saturation, while in the presence of unlabelled hormone the ct/min bound were a function only of the added ct/min. Therefore in routine assays the numbers of tubes containing unlabelled insulin could be reduced for convenience and economy.

Using formula (1) and the double reciprocal Lineweaver-Burk plot, the molar dissociation constants (K_d) and the binding capacity were calculated for the reaction between [^{125}I]-insulin and suppressible binding components of liver membranes. The double reciprocal plots were consistently linear (Figure 2b, $r = 0.998 \pm 0.01$; mean ± s.d. of 25 measurements). The K_d of the binding reaction was low (Table 1) indicating a high affinity and similar values were obtained from liver membranes of lean and obese mice. But membranes from lean mice possessed, per mg protein, approximately 8.8 times the numbers of high affinity binding sites from liver membranes of ob/ob mice ($P < 0.001$, Student's t test; Figure 3b).

ARDF-26 treatment

After injection of ARDF-26, liver membranes from ob/ob mice possessed approximately 5 times the numbers of insulin binding sites measured on membranes from ob/ob mice injected with the solvent alone (Figure 3b; $P < 0.001$) and the mean K_d of the reaction was significantly higher in ARDF-26-treated mice (Table 1; $P < 0.001$). Although not shown, the weights of the animals were unaffected during either course of treatment.

Table 1 Molar dissociation constants (K_d) of the reaction between [^{125}I]-insulin and high-affinity binding components on purified liver membranes from lean, obese, and obese mice injected with ARDF-26 (M × 10^{10}; mean ± s.e. mean)

	Lean	*ob/ob*
Untreated	3.94 ± 0.6 (6)	2.22 ± 0.6 (4)
ARDF-26	—	8.88 ± 3.6 (6)
Control	—	2.68 ± 0.5 (8)

Numbers in parentheses refer to numbers of animals.

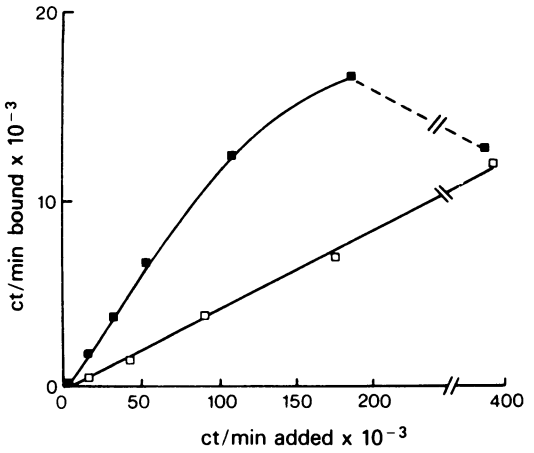

Figure 4 Evidence that negative co-operative effects occur in the assay system. In the presence of [^{125}I]-insulin in excess of 200,000 ct/min (1.5 nM) per incubate, the saturable binding (■) of the radioactive hormone was depressed to values observed in the presence of excess unlabelled hormone (□).

Negative co-operativity

From Figure 4 it can be seen that if the concentration of [^{125}I]-insulin was raised above 1.5 nM (200,000 ct /min), there was an apparent loss of high affinity binding. Therefore it is recommended that in this assay system the concentrations of [^{125}I]-insulin be not higher than 1.5 nM.

Discussion

A simple method is described for the reliable measurement of high affinity insulin binding on purified membrane fractions under conditions which optimize receptor stability and minimize negative co-operativity effects.

The labile nature of the reaction between [^{125}I]-insulin and the high-affinity binding sites is well documented (Kahn *et al.*, 1972; Olefsky *et al.*, 1974). In the present assay system the use of an incubation temperature of not more than 13°C apparently stabilized the binding reaction. The negative co-operativity phenomenon described by de Meyts *et al.* (1973) was observed if concentrations of [^{125}I]-insulin exceeded 1.5 nmol/l, and it is recommended that this concentration should not be exceeded. While negative co-operativity may represent a physiologically important mechanism, its occurrence in the assay system will result in a gross underestimate of the binding capacity.

The measurement of greatly reduced numbers of insulin receptors on liver membranes of ob/ob mice confirms previous observations (Kahn *et al.*, 1972; Kahn, Neville & Roth, 1973; Soll, Goldfine, Roth & Kahn, 1977). The apparent increase in insulin receptors on liver membranes after ARDF-26 treatment is consistent with the effects of another sulphonylurea derivative, chlorpropamide, on insulin receptors on mononuclear leukocytes from human subjects (Olefsky & Reaven, 1976). It may be relevant that in both studies the drug was unable completely to 'restore' receptor numbers to normal values. However, it remains to be seen whether the effect of ARDF-26 is a direct one, or occurs as a consequence of, for example, altered serum insulin concentrations. Serum insulin concentrations appear to determine receptor numbers *in vitro* (Gavin, Roth, Neville, de Meyts & Buell, 1974).

Furthermore, several agents appear capable of altering insulin receptor concentrations; for example, cyclic adenosine 3',5'-monophosphate (cyclic AMP, Thomopoulos, Kosmakos, Pastan & Lovelace, 1977), and cyproterone acetate and clomegestone (Krauth & Schillinger, 1977) were shown to alter insulin receptor levels. It also remains to be seen whether the enhancement of insulin receptor concentrations by antidiabetic and other substances is not simply due to an effect on plasma membrane glycoproteins generally. Chang, Huang & Cuatrecasas (1975) proposed that in ob/ob mice there are generalized changes in membrane glycoproteins.

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