HETEROGENEITY OF INSULIN RECEPTORS ON FAT CELL MEMBRANES

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SUMMARY. A highly purified and well characterized fat cell membrane preparation has been shown to retain the insulin binding characteristics of intact fat cells. Two major binding sites were identified, one a high affinity, low capacity site (K_D = 5 x 10^{-10}M) and the other a lower affinity, high capacity site (K_D = 3 x 10^{-9}M). Insulin binding to the membranes was prevented by insulin analogues (desoctapeptide and desalanine insulin) and proinsulin in direct relationship to their biological activities.

Several laboratories have recently reported studies of the interaction of insulin with its receptor site on the fat cell membrane. Cuatrecasas has described a homogenous population of high affinity (K_D = 5 x 10^{-11}M) insulin receptors in his fat cell and fat cell membrane preparations and has found close parallelism between the saturation kinetics of his receptor system and biological responses to insulin (1,2). Kono and Barham (3) and Gliemann and Gammeltoft (4) in contrast have found the dominant insulin-receptor interaction to be characterized by a K_D of approximately 5 x 10^{-9}M, substantially higher than the half saturation point exhibited by many biological responses to insulin. Kono has nonetheless suggested a model whereby these lower affinity receptors might be relevant to the mechanism of insulin action (3). Our observations concerning the binding of insulin to isolated fat cells and to a highly purified and well characterized fat cell membrane preparation differ substantially from those previously reported but may suggest a means whereby these apparent discrepancies can be reconciled.

Materials and Methods. Insulin preparations - Porcine insulin (lot #5682) 23.1 U/mg was generously supplied by Dr. M. Root of Eli Lilly. Porcine

proinsulin, desoctapeptide insulin, and desalanine insulin were kindly provided by Dr. Ronald Chance also of Eli Lilly.

Preparation of ^{125}I-Insulin: Insulin was iodinated by a modification of the method of Freychet et al (5). The initial reaction mixture contained 4ug of insulin and 1.5 - 2.0 mCi of carrier-free ^{125}iodine (Industrial Nuclear) in 100 ul of 0.1N phosphate buffer, pH 7.4. The reaction was followed by TCA precipitation of aliquots of the reaction mixture. Successive aliquots (10ul) of dilute chloramine-T solution (28ug/ml of water) were added until the desired specific activity was attained. The reaction was then stopped by the addition of sodium metabisulfite. The ^{125}I-insulin was adsorbed to talc, washed, and eluted according to the method of Cuatrecasas (1). The ability of ^{125}I-insulin to suppress epinephrine stimulated lipolysis was used as a measure of biological activity.

Preparation of Fat Cells and Fat Cell Membranes: Isolated fat cells were prepared from the epididymal fat pads of male Wistar rats after the method of Rodbell (6). Fat cell membranes were prepared from isolated fat cells by the method of McKeel and Jarett (7). This technique yields membranes which are less than 10% contaminated by microsomes and other subcellular particles. Membrane preparations have been shown to be stable with regard to insulin binding for several weeks under storage conditions at -70° C.

Binding studies: Isolated fat cells or fat cell membranes were incubated for 30 minutes at 24° C with ^{125}I-insulin in the presence or absence of unlabeled insulin in a total volume of 0.5 ml of Krebs-Ringer bicarbonate buffer containing 1% BSA. Separation of bound hormone from free was achieved by the method of Cuatrecasas (1), using a Millipore filtration manifold and EGWP (Millipore) cellulose acetate filters. Further experimental details are given in the text or legends.

Results. The biological activity of ^{125}I-insulin preparations was indistinguishable from that of native insulin. Figure 1 demonstrates suppression

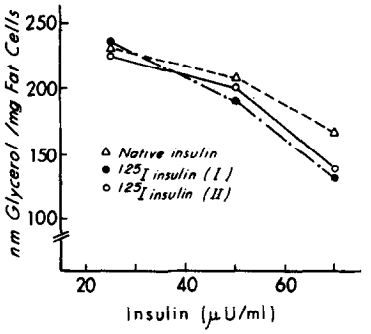

Fig. 1.

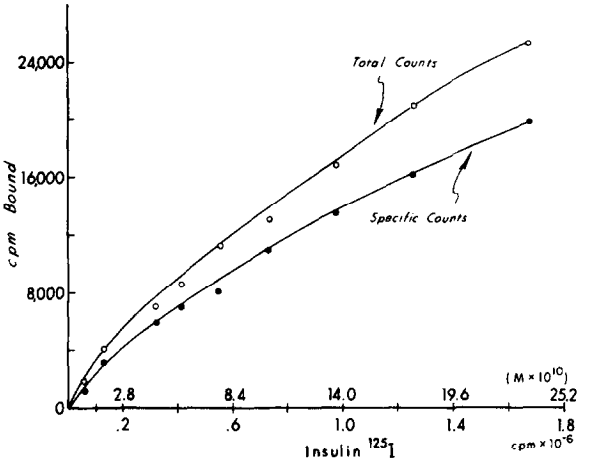

Fig. 2.

Figure 1 - Biological activity of ^{125}I-insulin. Adipocytes were incubated in the presence of 0.125 ug epinephrine/ml and various concentrations of native insulin or of two representative ^{125}I-insulin preparations. Each point represents the mean of triplicate determinations. The rate of glycerol release was used as a measure of lipolysis as previously described (15). The amount of ^{125}I-insulin added was based on specific activity and assumed full biological activity.

Figure 2 - Binding of ^{125}I-insulin by intact fat cells. Increasing amounts of radio-iodinated insulin were incubated with 3 x 10^5 fat cells for 30 min. at 24° C. Total counts bound represent the mean of triplicate determinations. Counts bound in the presence of 25ug unlabeled insulin (not shown) were also determined in triplicate and considered "non-specific." Specific counts were then computed by subtraction of non-specific counts from total as in the studies of Cuatrecasas (1).

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of epinephrine stimulated glycerol release by two representative preparations.

The insulin-fat cell interaction was initially studied by the method of Cuatrecasas (1), utilizing increasing amounts of radioiodinated insulin (Fig. 2). A reproducible inflection point at approximately 10 $^{-10}$M was observed, but saturation was not apparent at 2 x 10 $^{-9}$M, the maximum concentration easily attained using ^{125}I-insulin. For this reason the remainder of the studies were conducted using more traditional competitive binding techniques, with a fixed concentration of ^{125}I-insulin and increasing concentrations of unlabeled hormone. As illustrated in Figure 3, competitive binding curves for isolated fat cells and purified fat cells membranes were

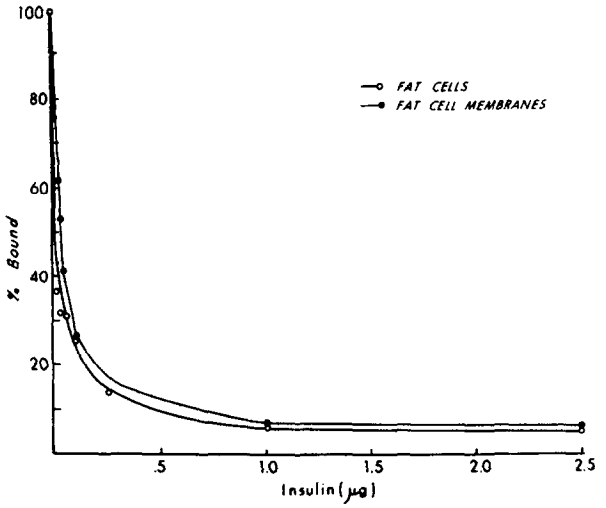

Figure 3 - Effect of unlabeled insulin on binding of ^{125}I-insulin by fat cells and fat cell membranes. ^{125}I-insulin (10^{-10}M) and increasing concentrations of unlabeled insulin were added simultaneously to 6 x 10^5 fat cells or to fat cell membranes (150 ug membrane protein) and incubated at 24° C for 30 min. Binding is expressed as percent maximum (no cold insulin). Residual binding with 25 ug cold insulin (1.2% of total) was considered non-specific and subtracted from each point.

indistinguishable. The one-half maximal saturation found in four studies with both adipocytes and membranes was 3 x 10^{-9}M. Scatchard analysis confirmed this value for the predominant dissociation constant and also revealed binding sites of still lower affinity (K_D = 3 x 10^{-7}M) which are presumably non-specific. From these analyses the number of binding sites (K_D = 3 x 10^{-9}M) was calculated to be approximately 30,000 per cell. The specificity of these binding sites for the biologically active form of the insulin molecule was tested in the series of experiments illustrated in Figure 4. Desoctapeptide insulin was a hundred fold less potent than native insulin in displacing iodinated insulin from its receptor, consistent with its lack of biological activity (8) and minimal contamination with more active derivatives. Proinsulin is twenty-fold less potent and desalanine insulin is equipotent when compared to native insulin, consistent with the known biological potency of these two species (9) (10).

In an attempt to delineate receptors with affinity closer to insulin

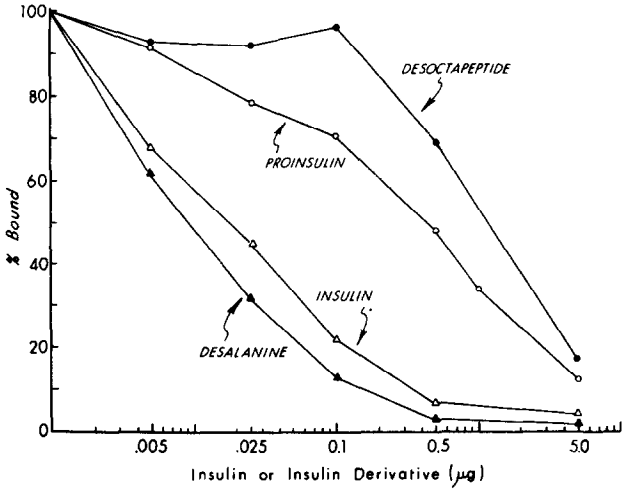

Figure 4 - Effect of insulin derivatives on binding of ^{125}I-insulin by fat cell membranes. ^{125}I-insulin (10^{-10}M) was incubated with fat cell membranes (150 ug membrane protein) and unlabeled insulin or insulin derivatives as indicated for 30 min. at 24° C. Binding is expressed as in Figure 3. Maximum binding was 13% of total ^{125}I-insulin added. Non-specific binding was 1.5% of total.

concentrations attained in vivo, the response of this system was examined with very small doses of insulin (Fig. 5). These studies have revealed a population of receptors with a K_D of 5 x 10^{-10}M. From Scatchard analysis the number of these receptors was calculated to be less than one-tenth that of the lower affinity (K_D 3 x 10^{-9}M) receptors.

Discussion. In studying hormone-membrane interaction the use of a highly purified and well characterized membrane preparation has obvious advantages. Carter et al (11) using preparations of fat cell membranes identical to that used in the present study were unable to demonstrate insulin facilitation of glucose transport and questioned the integrity of the insulin receptor in these preparations. The demonstration of insulin binding to the membranes in the present study would suggest that the defect was in the effector rather than the hormone receptor mechanism. The characteristics of this system with regard to affinity and specificity make it likely that the biologically relevant receptor was being studied.

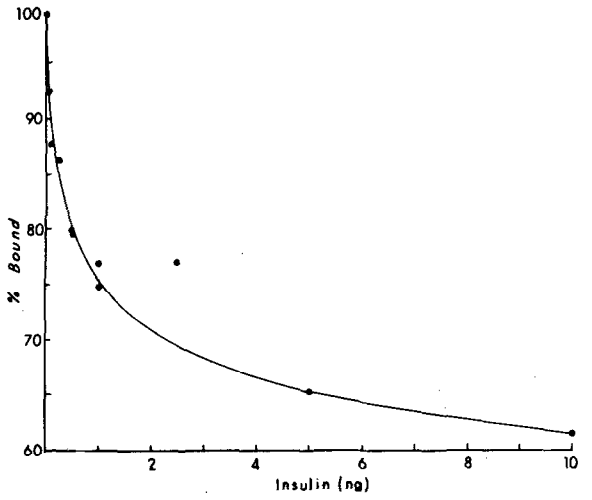

Figure 5 - Effect of small doses of native insulin on binding of ^{125}I-insulin by fat cell membranes. ^{125}I-insulin (5×10^{-11}M) and increasing concentrations of unlabeled insulin were added simultaneously to fat cell membranes (150 ug membrane protein) and incubated at 24° C for 30 min. Binding is expressed as in Figure 3. Maximum binding was 14.2% of total counts added; non-specific binding was 1.1%.

The heterogeneity of binding sites encountered here is somewhat at variance with the observations of some investigators (1,2) (3) (4), but is in excellent agreement with the studies of Freychet et al regarding the nature of insulin binding to liver membranes (12). A duality of receptor molecules has been demonstrated by Lefkowitz et al for the ACTH receptor of adrenal (13) and by Shlatz and Marinetti for several hormones in liver (14). The significance of the lower affinity receptors is unclear at present. However, their wide-spread presence in hormone responsive tissues and their demonstrated specificity for the biologically active hormone structure may indicate a significant role in the mechanism of hormone action.

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