BINDING OF ^{3}H-INSULIN TO CULTURED HUMAN LYMPHOCYTES - Further Evidence for Heterogeneity of Insulin Receptors or Negative Cooperativity

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SUMMARY

Insulin was tritiated by exposure to tritium gas activated by microwave radiation. ^{3}H-insulin competed with ^{125}I-insulin for binding to cultured human lymphocytes and to anti-insulin antibody to the same extent as did native insulin. The affinity constant for the binding of ^{3}H-insulin to specific receptors on cultured human lymphocytes was 0.48 X 10^{9} M^{-1} (SD=0.06). The affinity constant for the binding of ^{125}I-insulin was 0.57 X 10^{9} M^{-1} (SD=0.23). As was the case with ^{125}I-insulin, the Scatchard plot of the binding of ^{3}H-insulin to human lymphocytes was curvilinear, suggesting the presence of a heterogeneous population of receptors, or of a homogeneous population of receptors that exhibit negative cooperativity. The similarity observed between ^{3}H-insulin and ^{125}I-insulin helps refute the argument that distortion of the insulin molecule caused by introduction of an iodine atom may interfere with its binding to insulin receptors.

Research in the binding of insulin to insulin sensitive cells has relied almost exclusively on the use of radioiodinated insulin. While Freychet (1) and Cuatrecasas (2) have reported that their preparations of iodinated insulin were as biologically potent as the unmodified hormone, other workers have shown that even mild degrees of iodination may result in the loss of biological activity (3,4).

Scatchard analysis has shown that radioiodinated insulin binds to two or more specific receptors of different affinities (5,6) or that the affinity of the insulin receptor decreases with increasing degrees of receptor occupancy, i.e., the property of negative cooperativity (7). Taylor has suggested that this apparent complexity may be an artifact arising from differences in receptor affinity between native insulin and the radioiodinated tracer (8). The present

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study was undertaken to determine if a different tracer, ^{3}H-insulin, would behave differently from ^{125}I-insulin. Scatchard analysis of our results on the binding of ^{3}H-insulin to human lymphocytes is also consistant with heterogenity of insulin receptors or with negative cooperativity. This observation supports the view that the interaction between insulin and its receptor(s) is complex, and that this complexity does not arise from a false assumption in methodology.

METHODS AND MATERIALS

Beef insulin was tritiated by Biochemical and Nuclear Corporation, Burbank, California, according to a modification of the method of Hembree et al. (9). Briefly, insulin was placed into a 20 cc chamber which was evacuated with vacuum. The chamber was flushed with dry N_2 and again evacuated. Anhydrous tritium gas (58 Ci/m M) was admitted into the system at room temperature to a pressure of 10 mm Hg. Microwave discharge was initiated by a forward power of about 20 watts from a Raytheon Microwave generator (PGY-10X1, 2450 MHz). After 15 minutes, the tritium was removed by vacuum and the chamber flushed with N_2. Labile tritium was removed by exhaustive dialysis. The insulin was lyophilized and shipped to the University of Florida. The crude insulin was dissolved in 1 mM HCl and purified by gel filtration on a Sephadex G-50 column eluted with 1 M acetic acid. The purified ^{3}H-insulin (40 µCi/mg) was stored at -20°C for six months in aliquots containing 30 µg/ml of 1 M acetic acid. The material was indistinguishable from pure beef insulin by radioimmunoassay (10) and by bioassay on isolated fat cells (11)[a]. After six months of storage no loss of potency was noted. Ninety percent of the radioactivity remained insoluble in 5% TCA. As shown in Figure 1, about 90% of the radioactivity continued to elute with the insulin peak from a Sephadex G-50 column. The radioactivity also eluted as a single peak from a Dowex 50 column (12). Monoiodinated insulin was prepared fresh each month according to the method of Freychet (13). Its specific activity was 135-150 µCi/µg of which greater than 95% was soluble in 5% TCA.

Measurement of specific insulin binding to IM-9 cultured human lymphocytes was performed according to a modification of the method described by deMeyts (14). Experiments designed to compare the ability of ^{3}H-insulin and unlabeled insulin to compete with ^{125}I-insulin for binding to insulin receptors were performed as follows: 0.1 ng of ^{125}I-insulin and approximately 3 X 10^{7} cells were incubated in 1.0 ml of Hepes buffer, pH 8.1. ^{3}H-insulin or unlabeled insulin were added in concentrations ranging from 5-200 ng/ml. After 90 minutes of incubation at 4°C, 200 µl aliquots were obtained in triplicate and layered on 150 µl of fresh medium in 400 µl polypropylene microfuge tubes (Beckman). The washed cellular pellet was collected by centrifugation and counted in a Searle gamma counter. Radioactivity bound to the cellular pellet was expressed as a percent of the total radioactivity in the incubation mixture. Non-specific binding was defined as the radioactivity bound in the presence of 50 µg/ml of unlabeled insulin. Data were expressed as specific binding (total binding minus non-specific binding) per 3 X 10^{7} cells. In experiments designed to assess the binding of ^{3}H-insulin to insulin receptors, cells were incubated in 1.5 ml polyethylene microfuge tubes in 1.0 ml Hepes buffer containing from 5.0 to 250 ng of ^{3}H-insulin. At the end of the incubation period, the cells were collected by centrifugation, washed with 0.5 ml cold Hepes buffer, and suspended

[a] bioassay kindly performed by Dr. Stacey Psychoyos, Ciba Geigy, Ardsley, N.Y.

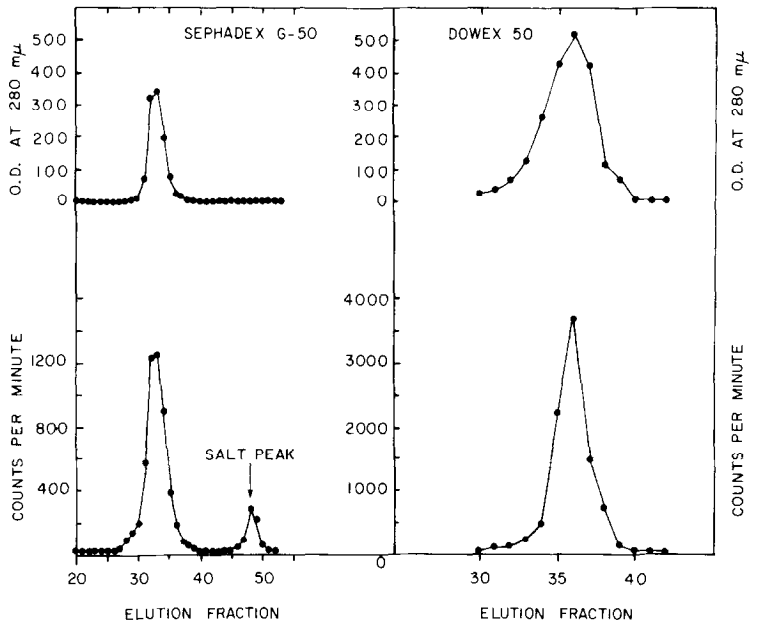

FIGURE 1. RADIOACTIVE PURITY OF ^{3}H-INSULIN AFTER SIX MONTHS OF STORAGE. (left) 2.5 μg of ^{3}H-insulin and 2 mg of crystalline insulin were placed on a 1.1 X 54 cm Sephadex G-50 column in 1.0 ml of 1 mM HCl and eluted with 1 M acetic acid in 1.0 ml fractions at a flow of 3 ml/hr. The salt peak was identified by the appearance of a precipitate with 50 μl of saturated $AgNO_3$. (right) 5 μg of ^{3}H-insulin and 3 mg of crystalline insulin were placed on a 0.8 X 35 cm Dowex 50 W column in 20 ml of 1 M HCl. The column was washed with 50 ml of 1 M NH_4Cl (pH 5). Insulin was eluted with 1 M NH_4OH in 1.5 ml fractions at a flow rate of 3 ml/min.

in 1.0 ml of 1 mM HCl. The material was transferred to scintillation vials containing 10 ml 3a70b RPI scintillation cocktail, and counted in a Packard liquid scintillation counter. As described above, non-specific binding was determined in the presence of 50 μg/ml of unlabeled insulin. The quantity of ^{3}H-insulin bound to the cells was calculated from the radioactivity of the ^{3}H-insulin added to the incubation medium. Initially, this was determined by adding 10 ng of ^{3}H-insulin to a pellet of 3 X 10^{7} cells, suspending the material in 1.0 ml of 1 mM HCl, and counting as usual with 10 ml of scintillation fluid. It was later found that the counting efficiency (about 20%) was not altered by the presence of the cells.

Beef insulin was the gift of Dr. Ronald E. Chance of Lilly Research Laboratories Crystalline bovine insulin (Lot 615-D63-5, 25.4 Units/mg) was used for calibration in column chromatography, and for determination of non-specific binding. Highly purified bovine insulin (Lot 615-R110-238-2, containing less than 0.001% proinsulin) was used otherwise.

RESULTS

Figure 2 shows the specific binding of ^{125}I-insulin to IM-9 cultured human lymphocytes in the presence of increasing concentrations of ^{3}H-insulin or unlabeled insulin. As was the case with the radioimmunoassay and isolated fat

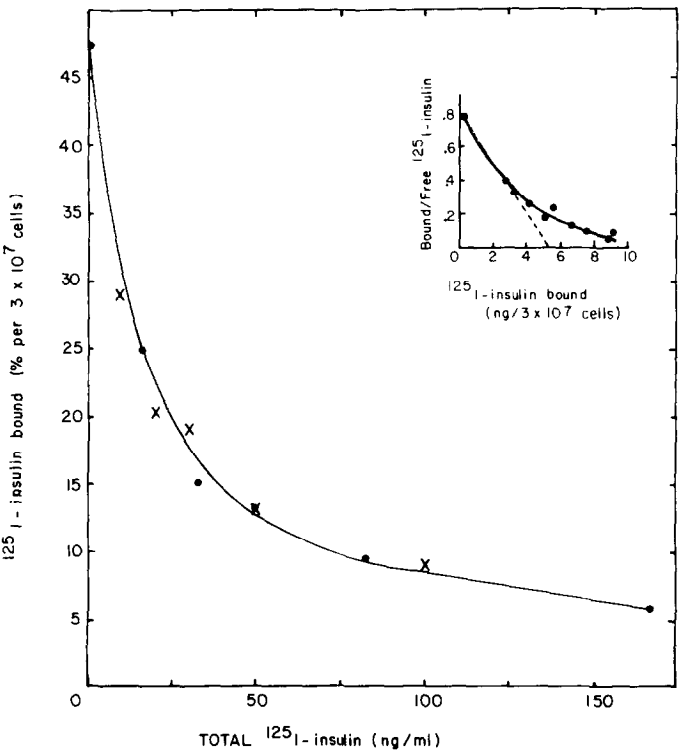

FIGURE 2. SPECIFIC BINDING OF ^{125}I-INSULIN IN THE PRESENCE OF ^{3}H-INSULIN (✕—✕) OR UNLABELED INSULIN (●—●). Cells were incubated with 0.1 ng ^{125}I-insulin as described under Methods. Data represent specific binding (total binding minus non-specific binding) of ^{125}I-insulin at different concentrations of ^{3}H-insulin or unlabeled insulin expressed as a percent of total radioactivity in the incubation medium. The insert is a Scatchard plot of the same data. The affinity constant is 0.83 X 10^9 M^{-1}.

cell bioassay, the radio-receptor assay did not distinguish this preparation of ^{3}H-insulin from unlabeled insulin. The Scatchard plot of the data shown in the insert is linear at low concentrations of insulin, but is curvilinear at high concentrations. The shape of this curve was initially interpreted by Kahn et al. (5) and Gavin et al. (6) as suggesting the presence of "high affinity" and "low affinity" insulin receptors. More recently, deMeyts et al. have suggested the curve demonstrates the property of negative cooperativity, whereby the affinity of the receptor for insulin decreases with increasing receptor occupancy (7). From the slope of the linear portion of the curve, the initial affinity constant was calculated as described by Kahn et al. (5) [See Legend].

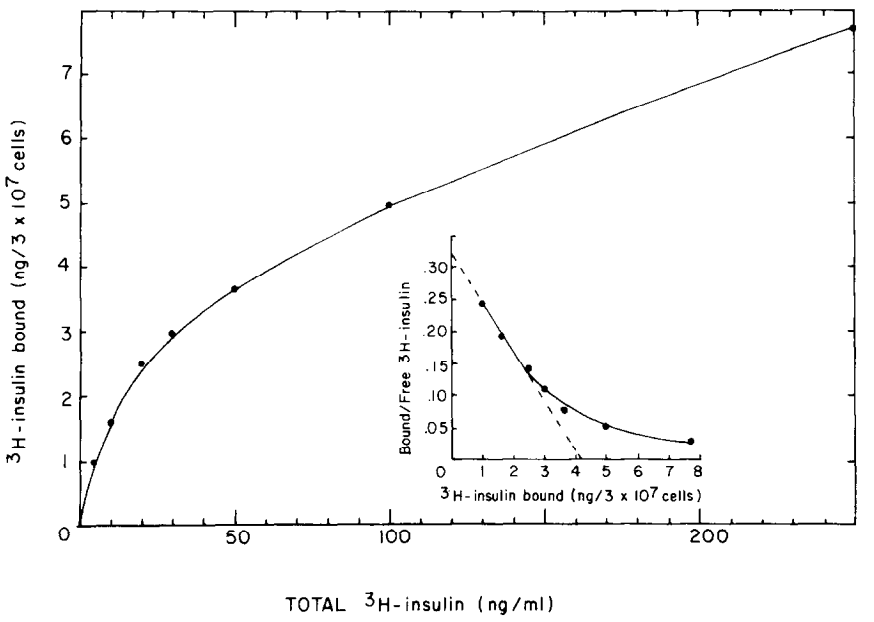

FIGURE 3. SPECIFIC BINDING OF ^{3}H-INSULIN. Cells were incubated with different concentrations of ^{3}H-insulin. Data represent the amount of ^{3}H-insulin specifically bound at each concentration. The insert is a Scatchard plot of the same data. The affinity constant of the insulin receptor complex is 0.48 X 10^9 M^{-1}.

The specific binding of ^{3}H-insulin to lymphocyte receptors is shown in Figure 3. A Scatchard plot of the data is shown in the insert. As was the case with ^{125}I-insulin, the plot is nearly linear at low concentrations of insulin, but became curvilinear at higher concentrations. The mean affinity constant calculated from the initial linear portion of the Scatchard plot is 0.48 X 10^9 M^{-1} (SD = 0.06) for ^{3}H-insulin, compared to 0.57 X 10^9 M^{-1} (SD = 0.23) for ^{125}I-insulin.

DISCUSSION

Analysis of data of the binding of a hormone to its receptor depends on the assumption that the affinity of the receptor is the same for the tracer as for the unmodified hormone (5). This assumption cannot be tested directly because the affinity of a hormone, or hormone analog, is itself determined by the degree to which it competes with the tracer hormone. Indirect support for this

assumption was provided by Freychet et al. (1) and Cuatrecasas et al. (2) who demonstrated that monoiodinated insulin stimulated glucose uptake by isolated fat cells to the same extent as did native insulin. However, Arquilla et al. (3) and Brunfeldt et al. (4) reported results to the contrary. Their data suggested that introduction of even a single iodine atom distorts the insulin molecule to such an extent that biological activity is altered. Taylor has shown that a non-linear Scatchard plot of the binding of radioiodinated insulin to insulin receptors would arise if the affinity of the radioiodinated insulin tracer for the receptor were less than that of native insulin. Were this the case, the interpretation that a curvilinear Scatchard plot represents heterogenity of receptors or negative cooperativity would be spurious. The importance of the present study is the observation that ^{3}H-insulin behaves similarly to ^{125}I-insulin. A curvilinear Scatchard plot was observed with both tracers and therefore cannot be attributed to distortion of the insulin molecule or insulin-receptor complex by the introduction of a large iodine atom.

The use of ^{3}H-insulin to study insulin binding poses formidable problems. The low specific activity of ^{3}H-insulin compared to ^{125}I-insulin requires that a much larger amount of tracer be used. The inconvenience and expense of liquid scintillation counting are also disadvantages. ^{3}H-insulin does have the advantage that the label is more evenly distributed. With the radioiodinated insulin currently used for binding studies, virtually all the radioactivity is distributed between the two tyrosine residues on the A chain (15). ^{3}H-insulin may therefore be useful in the study of insulin metabolism (16) and particularly in the identification of intracellular insulin fragments (17).

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