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Computer Assisted Analysis of Ferritin-Insulin Receptor Sites on Adipocytes and the Effects of Cytochalasin B on Groups of Insulin Receptor Sites

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Summary. A computerized quantitative technique was used to analyze the distribution of ferritininsulin receptor sites on rat adipocytes and the effects of cytochalasin B on groups of receptor sites. Computer analysis of separation distances between receptor sites established that insulin receptor sites on adipocytes did not have a random distribution but have a distinct tendency to exist in groups with a maximum separation distance between particles of 400 Å. A peak in the distribution of separation distances occurred at 100–200 Å. Cytochalasin B, but not cytochalasin D, treatment of adipocytes resulted in a decrease in the number of large groups of receptor sites and a corresponding increase in single and paired receptor sites without affecting the separation distance between the remaining grouped receptors. This suggested that when cytochalasin B disrupted the bond holding receptor sites together, it caused complete disruption. These observations provided additional information on the ultrastructural characteristics of the insulin receptor. Further application of these techniques to the analysis of insulin receptors may provide the necessary structural correlates to the biochemically observed differences in insulin action in other tissues and diseased states.

The development of monomeric ferritin-insulin as an electron microscopic marker for the insulin receptor has proven useful in morphologically characterizing the insulin receptor on rat adipocytes [4–8] and human placenta [10]. In both tissues insulin receptor sites appeared to be in naturally occurring groups prior to and independent of occupancy be ferritin-insulin. The insulin receptor groups on the adipocyte were distributed over the whole cell surface [5] while those on the placenta were restricted to the distal portion of the microvilli of the syncytial trophoblast [10]. It was shown that cytochalasin B partially disrupted the groups of insulin receptor sites on rat adipocytes [8]. Since cytochalasin D did not cause this effect, it has been suggested that a structural or chemical linkage other than microfilaments were involved in holding these groups together.

Gershon et al. [2] have developed a computerized quantitative technique to analyze the distribution of proteins or particles on membranes. The method was designed to determine if a given pattern of particle aggregation was statistically significant or simply due to random occurrence and to provide information on the various types of aggregates present and the importance of the distances separating the particles within the aggregates. This analytical technique was used to study the distribution of insulin receptors on rat adipocytes and the effect of cytochalasin B on these groups. As a result, this communication, using a quantitative rather than qualitative approach, confirms several observations reported previously and provides additional information about the ultrastructural characteristics of the insulin receptor on this insulin-sensitive tissue.

Materials and Methods

Morphological Procedures

The morphological procedures have been described in detail in previous publications [5, 8]. Monomeric ferritin-insulin (Fm-I) was prepared by covalently linking insulin to ferritin with glutaraldehyde and was purified by column chromatography on BioGel A 1.5 M [7]. Adipocytes, prepared with modifications [8] of the Rod-

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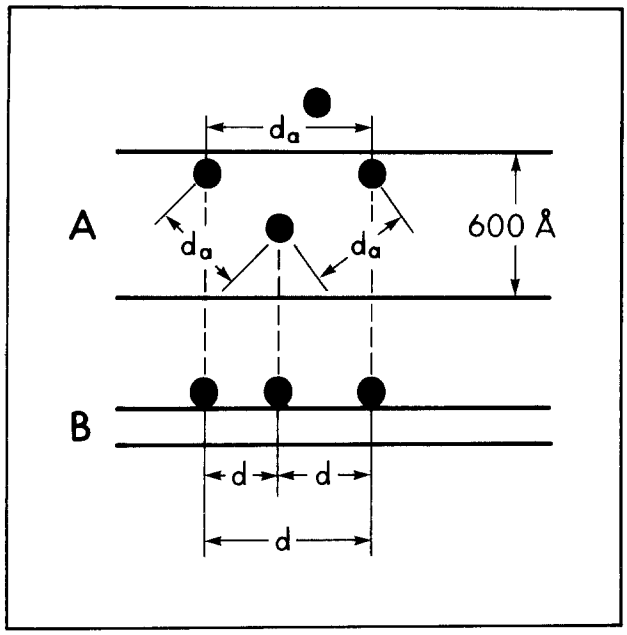

Fig. 1. Schematic representation of a section cut from an adipocyte showing ferritin-insulin (Fm-I) binding to the cell membrane viewed (A) perpendicular to the membrane surface showing actual particle separations (d_a) and (*B*) parallel to the membrane as seen on electron micrographs. It is noted that the perceived distance, *d*, between particles which lie outside the plane of the section will not be observed; therefore, the reported number of the insulin receptor molecules per group will be less than or equal to the actual number of receptor sites

bell technique [11], were incubated for 15 min at 37 °C in the presence or absence of 5 μM cytochalasin B or D prior to the addition of 500 μU Fm-I per ml. The cells were then incubated at 37 °C for 30 min, washed to remove free Fm-I, and prepared for electron microscopic examination as previously described [5, 8]. Sections of cells containing Fm-I particles were photographed, and all negatives were printed to a final magnification of 89,000× or 135,000×.

Analytical Procedures

Electron micrographs of intact adipocytes were analyzed and the *x* and *y* coordinates were determined for each Fm-I particle using a Tektronics 4953 digitizer. The distances between particles were calculated from their *x* and *y* coordinates and the known magnification of the micrographs. The number of interparticle distances within different intervals (e.g., between 150 and 200 Å, between 250 and 300 Å, etc.) were counted, and the results of many different micrographs were divided by the total number of particles. This yielded the average distribution of the number of neighbors *vs.* the distance from a particle. For group analysis we used the following procedure. On each micrograph the particles were separated into groups by the following criterion: A maximal distance, *d*, between two consecutive particles in a group was chosen. If two consecutive particles were separated by a distance shorter than *d* then they belonged to the same group, and vice versa. The number of groups of each size were calculated over all the given micrographs, for $d=200$, 300, 400, 500, and 600 Å. At least 600 Fm-I particles were examined for each experimental condition. The data was subjected to several types of analysis.

Results

Analysis of data generated by determining the distances between Fm-I particles on electron micrographs necessarily is limited by certain theoretical considerations. Micrographs are two-dimensional representations of three-dimensional structures since sections cut from the cells have a finite depth. This distortion potentially results in several types of errors. It is therefore prudent to recognize and deal with these problems.

Evidence to date showed that the groups of insulin receptors were located on the entire adipocyte surface [5]. For the sake of simplicity it can be assumed that a group of ferritin-insulin particles could be randomly located at any depth within a section with each particle having an actual distance from another particle of d_a as represented in Fig. 1*A*. The measurements in this study were made from a position perpendicular to the plane of the section (Fig. 1*B*), therefore the perceived distance, *d*, as represented on the two-dimensional projection of the three dimensional membrane, would always have been less than or equal to the actual distance. It was also possible that the plane of sectioning would pass through a group in such a way as to miss one or more ferritin-insulin particles associated with the group. Thus this type of artifact would cause the reported size of the groups to be less than or equal to the actual size. In this context it should be noted that ferritin-insulin will only identify occupied insulin receptor sites. Since saturating concentrations of ferritin-insulin have not been used in these studies, all insulin receptors have not been occupied or identified.

The selection of sections for this study was random and the assumption made that the distribution of the groups of insulin receptor sites was also random. Therefore, the possible errors due to the plane of sectioning and the plane of observation would have no effect on comparative studies, as are being reported here, as long as section thickness, plane of observation, and method of measurement remained constant and sufficient data were analyzed to prevent significant bias in the result from these random errors. The analyses performed in this study have taken these points into consideration. All subsequent data have been reported in terms of *d*, or perceived distances, and as the number of receptors per group. The reader should be aware that these are estimates for both cases and should only be compared to data where comparable levels of receptor occupancy have been achieved.

The data gathered from measuring interparticle distances were analyzed. Figure 2 shows the measured distances between Fm-I particles on control cells, up

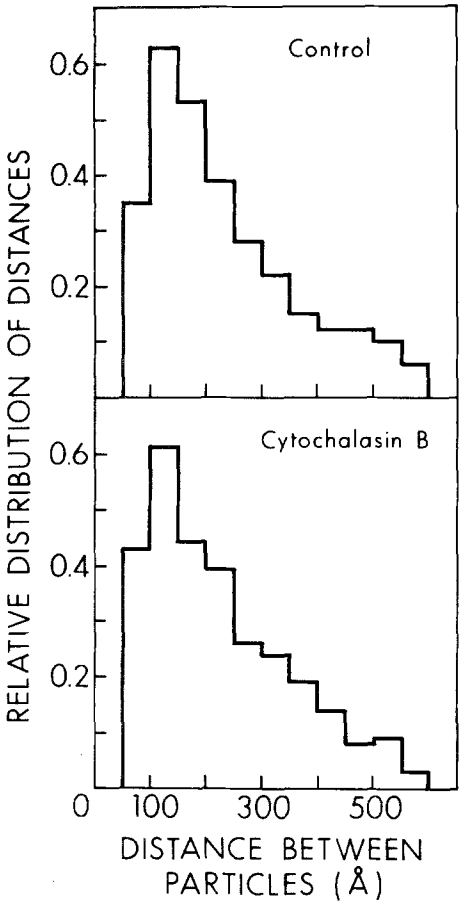

Fig. 2. Effect of cytochalasin B on the distribution of the distances among all Fm-I particles with separation distances of 50 to 600 Å. This analysis was performed by measuring the distance separating any two receptors on electron micrographs. Any given particle could be used for more than one measurement; therefore the total scale heights add to more than one. The distances between Fm-I particles showed a peak at 100–200 Å on control cells which was not effected by treatment of the adipocytes with 5 μM cytochalasin B prior to insulin bindung

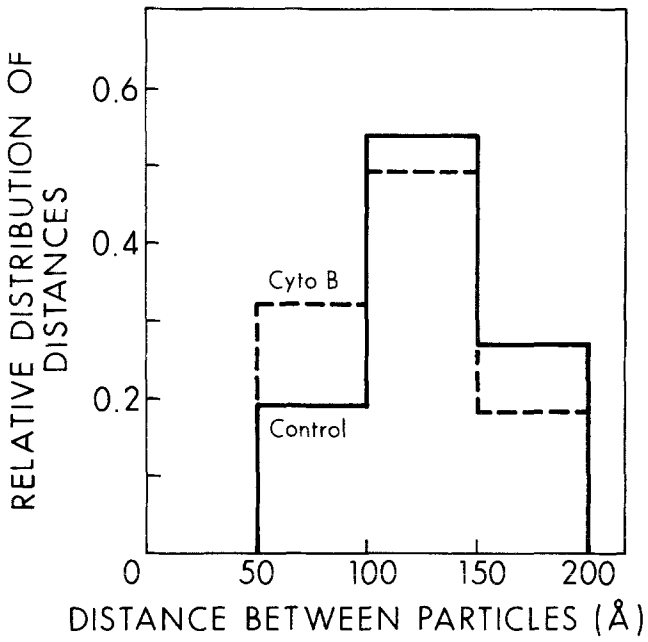

Fig. 3. Effect of cytochalasin B on the distribution of distances between members of pairs of receptor sites at distances up to 200 Å. This analysis was performed first by selecting all pairs and then measuring the distance separating members of pairs, up to a maximum of 200 Å. A given particle was used in only one measurement; therefore the sum of the peak height equals one. It was found that cytochalasin B had a negligible effect on the distance separating pairs of receptors

to a maximum separation distance of 600 Å. There was a definite peak in the distribution at a distance of 100 to 200 Å. The measurements from cytochalasin B-treated cells illustrate that incubating adipocytes with 5 M cytochalasin B prior to the addition of Fm-I had no appreciable effect on the measured distribution pattern as compared to controls (Fig. 2, lower panel).

Further analysis was made of a subgroup of the population consisting of the particles contained in pairs whose members were not separated by more than 200 Å. The distribution of the distances between members of pairs of this subpopulation is given in Fig. 3. This analysis also failed to reveal a significant effect of cytochalasin B on the distribution of distances between individual members of pairs of occupied receptor sites.

Receptors were classified into groups of from one up to five or more receptor sites according to the distances between nearest neighbors by defining a separation distance of 200, 300, 400, 500 or 600 Å as the maximum distance to permit two nearest neighbors to be grouped together. Separation distances less than 200 Å were not used because of the diameter of ferritin particles themselves being approximately 150 Å. Thus if any groups exist with distances between receptor sites less than the 150 Å, they were not detected. Figure 4 represents the result of the analysis with only single receptor sites and groups of five or more illustrated since these showed the most dramatic changes. As might be expected, shorter distances resulted in the observation of more single receptor sites and fewer groups of five or more than did greater distances. It was interesting to observe that the changes in receptor distribution occurred only up to a separation distance of 400 Å, after which the distribution of receptor sites remained relatively constant. Similar results were observed for cytochalasin B-treated cells (data not shown).

In order to determine the effect of cytochalasin B on the number of insulin receptor sites per group, the number of receptor sites per group was determined as on the control cells at separation distances of 200 to 600 Å. Figure 5 presents the data from the

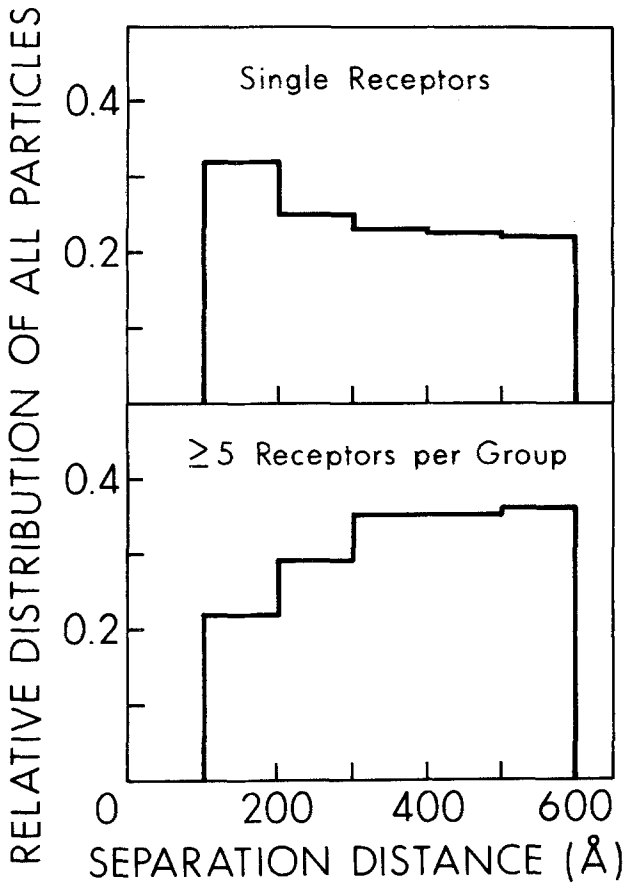

Fig. 4. Effect of the arbitrarily chosen maximum separation distance on the relative distribution of Fm-1 receptor sites. Fm-I molecules were classified as single molecules or in groups from two to five or more receptor sites per group based on arbitrarily selected separation distances of 200 to 600 Å. The top panel shows that more receptors were classified as single receptor sites when the separation distance was short. Similarly, in the lower panel fewer groups of five or more receptor sites were observed at short separation distances. Changes in the distribution of particles in groups were eliminated at separation distances of 400 Å or more. This may suggest that when distances shorter than 400 Å were chosen the receptor distribution was biased toward smaller groups of single-receptor sites and that 400 Å is the maximal distance separating Fm-I receptors on the cell surface

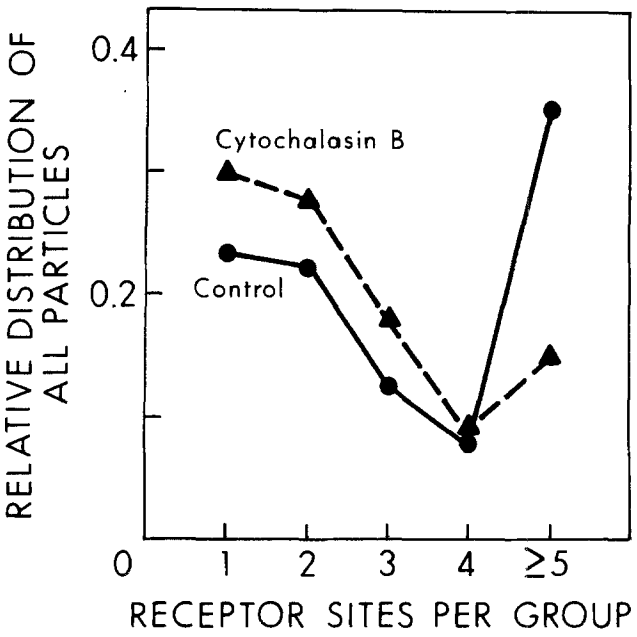

Fig. 5. Effect of cytochalasin B on the distribution of Fm-I receptor sites into groups of various sizes at a separation distance of 400 Å. Cytochalasin B (▲) was found to increase the number of receptor sites in small groups while causing a marked decrease in the number of groups of five or more receptor sites

Table 1. Effect of cytochalasin B on the distribution of ferritin-insulin receptor sites in groups at various interparticle separation distances

Separation distance (Å)	% Change in ferritin-insulin receptor sites/group		
	1	2	≦5
200	+26	+25	−55
300	+36	+17	−54
400	+30	+27	−57
500	+27	+18	−53
600	+18	+18	−49

Receptor sites were classified into groups of from one to five or more receptor sites according to the distances between nearest neighbors by defining a separation distance of 200, 300, 400, 500 or 600 Å as the maximum distance to permit two particles to be grouped together. The effect of cytochalasin B on the distribution of insulin receptors in groups was determined by comparing the percentage of the total receptors as single or paired particles or groups having five or more particles with similar groups on control adipocytes. Cytochalasin B increased the number of receptors in small groups and decreased the number of large groups, irrespective of the interparticle separation distance chosen for the analysis

analysis performed at a 400-Å maximum separation distance. Cytochalasin B caused about a 25% increase in the number of single and paired receptors. At the same time there was about 57% fewer receptors in groups of five or more receptor sites. The minimal effect seen in groups of four receptors was probably attributable to the balance between the decrease caused by their disruption and the increase as a result of the dispersion of larger groups. Increases in single and paired receptor sites and the decrease in large groups were observed at all separation distances up to 600 Å (Table 1).

Cytochalasin D did not have the same effect as cytochalasin B on the distribution of distances between Fm-I receptor sites or on the distribution of receptor sites into groups. This confirmed the earlier observation [8].

Discussion

The density of total specific insulin receptor sites on the adipocyte cell surface has been estimated at 20

to 100 per μm^2 [6]. If the receptors were randomly distributed, the average distance between two receptor sites on any random section would be greater than 1500 Å[1]. At the ferritin-insulin concentration used in these studies no more than 10–15% of the total receptor sites would be occupied. Therefore the distances observed between occupied receptor sites should be even greater than 1500 Å if they were truly randomly distributed. The distribution of distances between ferritin-insulin particles (Fig. 2) clearly demonstrated that this was not the case, leading to the conclusion that the observations made are indeed the result of a systematic aggregation of insulin receptor sites into groups. As reported previously, this aggregation occurred prior to occupancy and was not the result of ligand-induced aggregation [7]. An attempt was made to determine the actual separation distance between pairs of particles. It was observed that receptor distribution, in terms of the percent of total receptor sites in groups of various sizes, was affected, probably aberrantly, by the arbitrarily chosen separation distance when that distance was less than 400 Å (Fig. 4). It would appear therefore that naturally grouped insulin receptors are separated from each other by a distance of no more than approximately 400 Å.

Cytochalasin B caused a different distribution pattern of Fm-I than found with controls. This was interpreted to indicate that cytochalasin B disrupted some of the groups of receptor sites. In so doing, cytochalasin B could have completely disrupted a bond allowing redistribution but had no partial effect on a bond. This would explain the observation that the distribution of distances between receptor sites of remaining groups did not change but that the distribution of receptor sites in groups was altered (Fig. 5 and Table 1). An alternative but less likely explanation for these observations could be that cytochalasin B did not disrupt the receptor sites groups but only caused the Fm-I to distribute differently.

The physiological function of the insulin receptor groups remains an unanswered question. We currently believe that they are a structural feature of the adipocyte which may increase the probability of a cross-linking reaction that may be necessary for or intensifies insulin action. Kahn et al. [9] have shown that bivalent fragments of insulin receptor antibodies bind to the insulin receptor and mimic insulin action in rat adipocytes. Monovalent Fab fragments bound but did not initiate insulin-like effects unless anti-immunoglobulin was added to cross-link the Fab fragments. These data suggested that the insulin like activity of the anti-receptor antibody required cross-linking of insulin receptor sites. In the same study anti-insulin antibody, at specific concentrations, caused a slight increase in the biological response of adipocytes to insulin. Shechter et al. [12] have found that anti-insulin immunoglobulin over a specific range markedly increased ^{125}I-insulin binding to fibroblasts and liver plasma membranes but had no effect on ^{125}I-insulin binding to adipocytes. Similarly, the anti-insulin immunoglobulin increased the biological response of the fibroblast to insulin but had no effect on the response of the adipocyte. They concluded that the adipocyte already had insulin receptors in naturally occurring aggregates allowing maximum sensitivity, while receptors had to be aggregated on the fibroblast and liver membrane leading to enhanced biological response in fibroblasts. These findings are consistent with observations which demonstrate that the ferritin-insulin receptors on adipocytes are grouped while those on liver occur primarily as single molecules [13]. This latter observation would suggest that insulin itself does not cause aggregation of the insulin receptor sites, otherwise aggregation or groups of receptor sites would have been observed on liver plasma membranes. The data of Schechter et al. [12] support this concept since the anti-insulin immunoglobulin altered binding of ^{125}I-insulin on liver plasma membranes but not on adipocytes where receptor sites are naturally aggregated.

Cytochalasin B treatment of adipocytes, even though it disrupted groups of receptor sites, which would presumably interfere with subsequent cross-linking of the receptor, failed to inhibit the action of insulin on glucose transport, lipolysis, or protein synthesis [8]. It must be recognized, however, that a large number of groups remained intact (Fig. 5 and [8]), and these may have been sufficient to permit cross-linking of the receptors and therefore the insulin actions to occur. The mechanism by which cytochalasin B disrupts the groups is unclear, but it may involve breaking or interfering with the formation of disulfide bonds. The biochemical effect of various sulfhydryl reagents on the adipocyte glucose transport system and the insulin effector system have been well documented (for review *see* [1]).

Combined morphological and biochemical studies are in progress that may describe the role of disulfide bonds in the groups of insulin receptors on adipocytes and help determine the function of the grouped receptors in insulin action. Biochemical studies have shown that disruption of disulfide bonds by 1 mM dithiothreitol triples ^{125}I-insulin binding to adipocyte plasma membranes and converts the normally curvilinear or biphasic Scatchard plot of ^{125}I-insulin binding to a linear plot [13]. This same concentration of dithioth-

[1] This calculation was performed assuming 100 receptors per μm^2, which equals one receptor per 10^6 Å^2. Since the section thickness was 600 Å, it would be expected, on the average, to have, approximately one receptor site each 1666 Å along the section if the receptors were randomly distributed.

reitol had little effect on ^{125}I-insulin binding to liver plasma membranes and did not change the curvilinear shape of the Scatchard plot. These data certainly implicate disulfide and sulfhydryl groups in the insulin interaction with its receptor on adipocytes. These results coupled with other biochemical [12] and morphological data [4–8] as well as the present morphological data would suggest that the structural organization of the insulin receptor sites differs markedly between adipocytes and liver cells. This may help explain the different sensitivity of the two cell types to insulin.

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