INSULIN-INDUCED RECEPTOR LOSS IN THE CULTURED HUMAN LYMPHOCYTE: QUANTITATIVE MORPHOLOGICAL PERTURBATIONS IN THE CELL AND PLASMA MEMBRANE

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SUMMARY

When cultured human lymphocytes (IM-9) are exposed to 10^{-9}M porcine insulin for 6 h, washed, and incubated with ^{125}I-insulin, the ability of the cell to bind the labelled hormone is reduced by a mean of 78%. Under these experimental conditions that induce insulin-receptor loss in this cell there is a mean 95% increase in microinvaginations in the plasma membrane revealed by electron microscopy on freeze-fractured replicas of the cell. At the same time, horseradish peroxidase uptake, a marker of endocytosis, is increased in the cells incubated with insulin.

Coupled with our recent EM autoradiographic evidence that labelled insulin is acutely internalized by this cell, these studies are consistent with the possibility that endocytosis represents a mechanism by which receptor is removed from the cell surface.

INTRODUCTION

Regulation of the concentration of polypeptide hormone receptors by the homologous hormone is an important mechanism controlling the cell’s sensitivity to the hormone. This phenomenon was first shown for the insulin receptor and has subsequently been shown for many other hormones (see references Lesniak & Roth, 1976; Carpenter & Cohen, 1976; and Hsueh, Dufau & Catt, 1977, for a partial list). Hyperinsulinaemic states are frequently associated with decreased insulin receptor concentration in many tissues of the rodent (Kahn et al. 1972; Freychet et al. 1972; Kahn, Neville & Roth, 1973 and Le Marchand, Jeanraud & Freychet, 1978) and in monocytes (Archer et al. 1973; Archer, Gorden & Roth, 1975; Bar et al. 1976; Olefsky, 1976) and adipocytes (Olefsky, 1976; Harrison, Martin & Melick, 1976) in man. Decrease in plasma insulin concentration is associated with an increased concentration of insulin receptors. Both in animals (Soll, Kahn, Neville & Roth, 1975) and in humans (Bar

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Gorden and others (1976) the insulin receptor concentration is inversely proportional to the chronic (but not acute) ambient plasma insulin concentration.

To test more directly the relationship between ambient insulin and receptor concentration it was demonstrated *in vitro* in the cultured human lymphocyte that the insulin-receptor concentration decreases with chronic exposure to insulin (Gavin et al. 1974). Though binding of the hormone to its receptor is required to initiate the process, physical occupancy of the receptor on the cell surface is not the mechanism responsible for reduced hormone binding.

Recently we have shown by quantitative EM autoradiography that when $^{125}$I-insulin is incubated with cultured human lymphocytes, the ligand is internalized in a temperature-dependent fashion (Carpentier et al. 1978). In addition, labelled insulin is internalized as a function of time and temperature in isolated rat hepatocytes and the internalized labelled material preferentially associates with lysosomes. The internalization process is evident by 2 min of incubation in both cultured human lymphocytes and isolated rat hepatocytes and continues through steady-state binding (i.e., for up to 1 h at 37 °C) (Gorden et al. 1978b, c). If the internalization process proceeds by way of endocytosis, the hormone-receptor complex is likely to be internalized as well; this would provide a mechanism by which the receptor is lost from the cell surface.

Since the cultured human lymphocyte requires large concentrations of insulin to induce receptor loss, it is not possible to use a labelled ligand to induce receptor loss. We have, therefore, taken an indirect approach to look for evidence consistent with endocytosis under the exact experimental conditions in which insulin induces loss of its receptor in the cultured human lymphocyte.

**MATERIALS AND METHODS**

**Incubations**

Cultured human lymphocytes of the IM-9 cell line were maintained at 37 °C in RPMI 1640 medium supplemented with 10% foetal calf serum and glutamine (0.29 mg/ml) and used in the late log or early stationary phase of growth as previously described (Gavin et al. 1974).

Cells were incubated with $1 \times 10^{-8}$ M porcine insulin for 6 h at 37 °C in the growth medium. Other variations included an incubation at 15 °C for 6 h; an incubation with $1 \times 10^{-8}$ M porcine proinsulin for 6 h at 37 °C; an incubation with $1 \times 10^{-8}$ M guinea-pig insulin for 6 h at 37 °C, and an incubation with $1 \times 10^{-8}$ M porcine insulin at 37 °C in the presence of 0.5 mg/ml of horseradish peroxidase (HRP) (Worthington Biochemicals). For each individual experiment control cells were incubated under identical conditions except that insulin or proinsulin was excluded from the incubation medium.

At the end of the incubation the cells were centrifuged at 500 g for 5 min and suspended in phosphate-buffered saline. An aliquot of cells was removed for electron-microscopic studies and the remaining cells were thoroughly washed and assayed for residual insulin binding as previously described (Gavin et al. 1974).

**Electron-microscopic studies**

Following the incubation, cells were washed twice by gentle suspension in buffer. Buffer was aspirated from the cell pellet, replaced by 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and allowed to stand for 4 h at room temperature. The glutaraldehyde was then
removed and replaced by 0.1 M phosphate buffer, pH 7.4, and stored at 4 °C until further processed.

The fixed cells were treated as follows:

(a) Processed for transmission electron microscopy as previously described (Carpentier et al. 1978).

(b) Cells incubated with HRP were first reacted with diaminobenzidine and hydrogen peroxide (Graham & Karnovsky, 1966) before being processed as in (a).

(c) Fixed cells were immersed in 30 % glycerol buffered with 0.1 M phosphate buffer, pH 7.4, for 30 min, rapidly frozen in Freon 22, cooled in liquid nitrogen, then fractured and shadowed in a Balzers BAF 301 apparatus (Balzers, Liechtenstein) (Moor & Mühe, 1963). The freeze-fracture replicas were then cleaned in a sodium hypochlorite solution for 2 h, rinsed in distilled water, and mounted on copper grids.

(d) For autoradiography cells were incubated with $5 \times 10^{-10}$ M $^{125}$I-insulin in growth media (see above) for 2 min through 3 h and processed exactly as previously described (Carpentier et al. 1978).

All samples were subsequently examined and photographed on a Philips EM 300 electron microscope (Philips, Eindhoven, The Netherlands). For the methods used to quantitate microinvaginations from freeze-fractured replicas, see legend to Fig. 3, and for the method used to estimate horseradish peroxidase (HRP) uptake, see legend to Fig. 4.

RESULTS

When cultured human lymphocytes of the IM-9 cell line are incubated at 37 °C in the presence of $10^{-8}$ M porcine insulin for 6 h, extensively washed, and resuspended in assay buffer, the binding of tracer concentrations of $^{125}$I-insulin is reduced by a mean of 78 ± 2 % as compared to control cells where porcine insulin was excluded from the incubation (Fig. 1). This is similar to previously reported data for this cell line under the same conditions (Gavin et al. 1974).

When cells, at the end of the incubation, are fixed, processed, sectioned and examined by transmission electron microscopy, small flask-shaped microinvaginations can be seen in the plasma membrane of cells from both the insulin-treated and control incubations (Fig. 2). Because of the small amount of membrane exposed on thin section, it is not possible to quantitate these structures. Using freeze-fracture techniques, however, large areas of the membrane-fractured surface are exposed and surface events can be quantitated. Microinvaginations as shown in Fig. 2 appear as small pits on the protoplasmic or P-face of the fractured membrane and as small mounds on the extracellular or E-face of the membrane (Fig. 3). A given freeze-fracture replica may show very few membrane invaginations or many structures may be evident (Fig. 3). Since all the control replicas contain varying numbers of microinvaginations (i.e., incubations in the absence of insulin) replicas from the experimental or insulin-containing incubations are compared only against their specific control (Fig. 1).

Under these circumstances membrane microinvaginations are increased by a mean of 95 % over control under conditions of insulin-induced receptor loss (Fig. 1) (mean invaginations per $100 \mu m^2$: control = 90 ± 12 vs 139 ± 6 for insulin exposed, $P < 0.005$).

Since receptor loss is a function of occupancy of the receptor and of temperature of incubation, the effects of these 2 parameters were studied. Proinsulin and guinea-pig
Fig. 1. Relationship of microinvaginations to receptor loss. Each symbol represents a separate incubation. Microinvaginations in the control and insulin-exposed incubations as well as the percentage receptor loss for each incubation is shown by the same symbol. For each control and insulin-exposed condition (1 x 10^{-6} M insulin for 6 h at 37 °C), 50 photographs of membrane of 50 different lymphocytes were taken in at least 2 separate replicas. The number of microinvaginations was counted and expressed per 100 μm² of membrane surface. Thus, for the 9 experiments shown 450 membranes were photographed in the control and 450 in the insulin-exposed. The microinvaginations tabulated here have a mean diameter of 46 nm.

Fig. 2. IM-9 cultured human lymphocyte. Typical flask-shaped invagination occurring at the cell surface (arrow) with an average diameter of 46 nm. × 56000.
insulin in equimolar concentration to insulin occupied fewer receptors and produced less receptor loss than insulin (Kosmakos & Roth, unpublished observations). There is a small increase in microinvaginations seen with proinsulin but no increase with guinea pig or the 15 °C incubation (Fig. 4). The number of microinvaginations per 100 μm² reaches about the same maximum in all insulin incubations and the differ-
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2-fold preferential association of grains with vesicles of the Golgi area (Fig. 6) (Table 2). Note the similarity in distribution of developed grains with the distribution of HRP.

In an additional experiment carried out under conditions of insulin-induced

Table 1. Horseradish peroxidase uptake (HRP)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Insulin</th>
<th>Increase over control, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells labelled with HRP</td>
<td>P &lt; 0.001*</td>
<td>53/356</td>
<td>91/334</td>
</tr>
<tr>
<td></td>
<td>15%</td>
<td>27%</td>
<td></td>
</tr>
<tr>
<td>Volume density HRP</td>
<td>P &lt; 0.001*</td>
<td>1.8</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* The statistical significance between the 2 conditions was assessed by the $\chi^2$ test.

Fig. 5. IM-9 cultured human lymphocytes after 6 h of exposure to horseradish peroxidase (HRP). HRP reaction product typically locates (p) in vesicles in the Golgi region. To obtain the data shown in Table 1, HRP-exposed cells were scanned at 7500 x. All cells scanned and all cells containing HRP were tabulated as shown in Table 1. In addition, the first 30 cells found to contain HRP were photographed from control and experimental samples respectively. The negatives were projected on a table projector unit at a final magnification of 40,000 x. The volume density of HRP-containing vacuoles was determined by a point-counting method (Weibel, 1969) and expressed per unit volume of cytoplasm. × 6400 approx.
Fig. 6. Selected images of developed autoradiographic grains associated with vesicles. In A and B, grains are situated over membrane-bounded vesicles containing amorphous and membranous material. These vesicles, associated mainly with the Golgi region, are considered as lysosomes. Of the 3.7% grains related to vesicles (Table 2) 28% were related to such structures. In C an autoradiographic grain is associated with an 'empty' vesicle. Of the 3.7% grains related to vesicles (Table 2) only 0.9% were related to such structures. While the incubation conditions employed in these experiments are essentially identical to those of a recently reported study (Goldfine et al. 1978) the results are different for unexplained reasons. ×36000.
Table 2. Relationship of autoradiographic grains to intracellular organelles

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Volume density organelle</th>
<th>% grains related to organelle</th>
<th>Ratio</th>
</tr>
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<tbody>
<tr>
<td>Mitochondria</td>
<td>6.4</td>
<td>6.2</td>
<td>0.97</td>
</tr>
<tr>
<td>Vesicles*</td>
<td>1.7</td>
<td>3.7</td>
<td>2.18</td>
</tr>
<tr>
<td>RER</td>
<td>1.6</td>
<td>1.6</td>
<td>1.00</td>
</tr>
<tr>
<td>Nucleus</td>
<td>32.9</td>
<td>31.0</td>
<td>0.94</td>
</tr>
<tr>
<td>Residual cytoplasm</td>
<td>57.4</td>
<td>57.5</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Under the term 'vesicles' are included the 2 types of structures illustrated in Fig. 6.

receptor loss (i.e., exposure to 10⁻⁶ M insulin at 37 °C) for 2 h, there was an approximate 60% receptor loss and a 60% increase in microinvaginations (data not shown).

**DISCUSSION**

Insulin-induced receptor loss involves a new steady-state concentration of receptors on the cell surface at approximately 25% of their original concentration. This new steady state is achieved at about 6 h with 10⁻⁶ M insulin and over a longer period with lower concentrations. It is apparent that a full understanding of this process must include events involved in receptor synthesis, possible storage, and insertion in the membrane; presently available data, however, suggest that the primary initial process is receptor loss.

To our knowledge, there have been no previous studies investigating morphological perturbations of the cell under conditions in which a hormone such as insulin induces loss of its receptors. Microinvaginations in the plasma membrane of cells and HRP uptake by cells are non-specific processes. Both of these, however, are increased during the process of insulin-induced receptor loss.

Invaginations in the plasma membrane represent the earliest morphologically detectable phase of an endocytotic event, and HRP uptake has been used as an indicator of endocytosis (Silverstein et al. 1977). In acute experiments using physiological concentrations of ¹²⁵I-insulin, we have shown that the ligand is internalized in both cultured human lymphocytes and isolated rat hepatocytes at 60 min of incubation at 37 °C. In rat hepatocytes, the labelled insulin preferentially associates with lysosomes in the Golgi region. In the present experiments using a longer period of incubation with a higher concentration of labelled insulin, we find also a preferential localization of grains to vesicles of the Golgi area.

While no receptor loss is demonstrable in the cultured lymphocyte with the insulin concentration used for our autoradiographic studies, at 10⁻⁶ M insulin, 30-60% receptor loss occurs by 1 to 2 h. At these same times there is an increase in the concentration of microinvaginations in the plasma membrane.

Epidermal growth factor (EGF) has been shown to induce the loss of its specific receptor in human fibroblasts (Carpenter & Cohen, 1976). We have shown by
quantitative EM autoradiography that EGF localized to the plasma membrane of human fibroblasts at low temperatures but at 37 °C is internalized in a time- and temperature-dependent fashion and preferentially associates with lysosomal structures (Gorden et al. 1978a-c). Further, Schlessinger, Shechter & Willingham (1978), using an elegant new fluorescent-microscopic technique, have demonstrated that both rhodamine-labelled insulin and EGF undergo patching prior to internalization and Haigler, Ash, Singer & Cohen (1978) have demonstrated the internalization of a fluorescent derivative of EGF in human carcinoma cells A-431.

Thus in acute studies there is direct evidence from a variety of visual probes that both insulin and EGF are internalized. EGF induces receptor loss in human fibroblasts at concentrations 2 to 3 orders of magnitude lower than does insulin in cultured lymphocytes. This is of interest since at steady state only about 20–25% of labelled insulin is internalized by the cultured lymphocytes (Carpentier et al. 1978), while under similar circumstances over 65% of labelled EGF is internalized by the fibroblast (Gorden et al. 1978a).

Any mechanism proposed for insulin-induced receptor loss must be compatible with the extraordinary specificity of this process. In the cultured human lymphocyte insulin induces loss of the insulin receptor but has no effect on the growth hormone receptor of this cell (Gavin et al. 1974). Growth hormone in turn induces loss of its unique receptor and has no effect on the insulin receptor (Lesniak & Roth, 1976). Both microheterogeneity and flow of membrane constituents are important properties of a fluid mosaic membrane structure (Singer & Nicolson, 1972; Singer, 1975). All studies carried out thus far employing ferritin-insulin conjugates suggest that insulin receptors appear singly or in groups (Jarett & Smith, 1977; Orci et al. 1975). The groups could be ligand-induced and involve movement in the plane of the membrane; they could exist naturally in the absence of the ligand, or both processes could be involved. In any of these cases, the specificity of the system would lie in the binding of insulin to its unique receptor. The binding of a single insulin molecule to a single receptor followed by endocytosis could induce the loss of one receptor or several receptors, depending on the distribution of receptor on the membrane segment.

We have presented data that directly demonstrate that insulin binding to its receptor is associated with intracellular translocation and lysosomal association of the labelled hormone, and in the present study we present further indirect data suggesting that adsorptive endocytosis may be an important mechanism involved in insulin-induced receptor loss.

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