Insulin-induced Internalization of the Insulin Receptor in the Isolated Rat Adipose Cell

DETECTION OF THE INTERNALIZED 138-KILODALTON RECEPTOR SUBUNIT USING A PHOTOAFFINITY \(^{125}\)I-INSULIN*

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A photoactive insulin analogue (N-Bz-(2-nitro-4-azidophenylacetyl)insulin) which specifically and covalently labels the 138-kDa insulin receptor subunit, is used here to examine the effect of insulin on the subcellular distribution of insulin receptors in the isolated rat adipose cell. The photolabeled 138-kDa receptor subunit in the plasma and Golgi-enriched membrane fractions was quantitated by Na dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. When intact cells are photolabeled, subsequent incubation for 30 min at 37 °C with saturating native insulin induces a 30% loss of the labeled receptor subunit from the plasma membrane fraction. Greater than 50% of the lost receptor subunits can be specifically recovered in the Golgi-enriched membrane fraction. Qualitatively and quantitatively similar results are obtained when the 138-kDa receptor subunit is labeled in the membrane fractions following their preparation. However, the 138-kDa receptor subunit in the Golgi-enriched membrane fraction can only be labeled when the vesicles in this fraction are made permeable to the insulin analogue by the presence of 0.01% digitonin. The appearance of the 138-kDa receptor subunit in the Golgi-enriched membrane fraction is rapid, with a half-time of 2 min, and achieves a steady state within 10 min. This effect is also insulin concentration-dependent, with half-maximal and maximal effects at 6 and 30 nM, respectively, and is markedly, but not completely, inhibited at 16 °C. These results suggest that insulin induces a rapid and insulin concentration- and temperature-dependent translocation of its own receptor from the plasma membrane to an intracellular membrane fraction in the isolated rat adipose cell, and that this translocation represents internalization of the insulin receptor through an endocytic-like process.

The internalization and intracellular disposition of insulin initially bound to its specific plasma membrane receptor have been extensively characterized in a variety of cell types (1–3).

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In many cell types, insulin is rapidly taken up by receptor-mediated endocytosis following ultimately by lysosomal degradation. In the isolated rat adipose cell, plasma membrane-bound \(^{125}\)I-insulin rapidly appears in a subcellular fraction which sediments during linear sucrose density gradient ultracentrifugation between the endoplasmic reticulum- and Golgi-enriched membrane fractions (4). The appearance of ferritin-labeled insulin in small vesicular structures in the cytoplasm of this cell can readily be demonstrated using electron microscopic techniques (5). A significant association of insulin with lysosomes is observed only in the presence of lysosomotropic agents such as chloroquine (6).

Internalization of the insulin receptor itself has been directly demonstrated in freshly prepared rat hepatocytes using a photoaffinity \(^{125}\)I-insulin analogue and autoradiography (7). Plasma membrane receptors appear to be internalized primarily to lysosomal structures. In contrast, in vivo studies using rat liver suggest a rapid insulin-induced internalization of insulin receptors from the plasma membrane to the Golgi apparatus, as determined by measuring the distribution of Triton X-100-extractable insulin binding sites among subcellular membrane fractions (8). In the isolated rat adipose cell, internalization of the insulin receptor has been indirectly demonstrated by measuring the loss of trypsin-sensitive cell surface receptors from intact cells in response to insulin (9). Receptors lost from the cell's surface are partially recovered when the trypsin-treated cells are solubilized. However, these experiments were performed in the presence of Tris, a buffering agent known to promote an insulin-induced "down regulation" of the insulin receptor in rat adipose cells (9). Using similar experimental conditions and a photoaffinity \(^{125}\)I-insulin analogue to prelabel the cell surface receptors, an apparent processing of the trypsin-insensitive receptor is also observed (10).

Insulin has been shown to stimulate glucose transport in the isolated rat adipose cell primarily through a rapid and reversible translocation of glucose transport systems from a large intracellular pool, associated with a Golgi-enriched low density microsomal membrane fraction, to the plasma membrane (11, 12). These studies were undertaken in order to determine if insulin induces a similar, but directionally opposite, internalization of the insulin receptor. The distribution of the insulin receptor between the plasma membrane and Golgi-enriched membrane fractions has been examined by photoaffinity labeling the 138-kDa insulin receptor subunit with Bz-NAPA-insulin' (13).

The abbreviations used are: Bz-NAPA-insulin, N-Bz-(2-nitro-4-azidophenylacetyl)insulin; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.
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EXPERIMENTAL PROCEDURES

For each experiment, adipose cells were isolated from the whole epididymal fat pads of at least 48 adult libitum-fed (standard NIH rat chow), 150 to 200-g male rats (CD strain, Charles River Breeding Laboratories) by the method described by Rodbell (14) as modified by Cushman (15). All incubation media were Krebs-Ringer bicarbonate/HEPES buffer (standard Krebs-Ringer bicarbonate buffer reduced to 10 mM HCO3 and supplemented with 30 mM HEPES (Sigma), pH 7.4, containing 10 mg of untreated bovine serum albumin/ml (Bovine Serum Albumin Powder, Fraction V, Reheis Chemical Co.).

Following isolation, the pooled adipose cells were incubated according to either of two protocols. In order to examine the subcellular distribution of insulin receptors prelabeled in the plasma membrane of intact cells, the cells from 48 rats were distributed in 10-m1 volumes to four 950-ml polypropylene jars containing 20 ml of incubation medium and incubated for 15 min at 16 °C, 22 μCi of B23-NAPA-125I-insulin (116 μCi/ng) were added to all four suspensions and sufficient native insulin to suspensions 1 and 2 to achieve a final concentration of 700 nM; the suspensions were incubated for an additional 30 min at 16 °C in the dark; and all four suspensions were exposed for 80 s at 16 °C, with gentle swirling, to ultraviolet radiation from a super pressure mercury lamp (Model ALH 215, Osram HRO 100 w/2, Photocchemical Research Associates, Inc.) at a distance of 30 cm. Native insulin (final concentration of 700 nM) was then added to suspensions 2 and 4; suspensions 3 and 4 were warmed to 37 °C, while suspensions 1 and 2 were left at 16 °C; and all four suspensions were incubated for a final 30 min.

In order to examine the subcellular distribution of unlabeled insulin receptors, the isolated adipose cells were distributed in 10-m1 volumes (the cells from 12 rats) to 950-ml jars containing 20 ml of incubation medium and incubated for 15 min. For time course experiments, sufficient native insulin was then added to achieve final concentrations of 0 or 6 nM, and the suspensions were incubated for an additional 0, 2, or 30 min. For insulin concentration experiments, sufficient native insulin was then added to achieve final concentrations of 0, 1, 6, or 30 nM, and the suspensions were incubated for an additional 30 min. Samples incubated at 16 or 37 °C were maintained at these respective temperatures throughout the entire 45-min period.

Following incubation, both the prelabeled and unlabeled adipose cells were washed once and homogenized in a buffer containing 20 mM Tris-HCL, 1 mM EDTA, and 255 mM sucrose, pH 7.4, at approximately 20 °C. All further steps in the fractionation procedure were carried out in this same buffer at 4 °C. Plasma membrane and Golgi-enriched low density microsomal membrane fractions were then prepared by differential ultracentrifugation as previously described (11) and resuspended to final concentrations of 2 to 4 mg of membrane protein/ml and 1 to 2 mg of membrane protein/ml, respectively.

Insulin receptors in the membrane fractions from the unlabeled adipose cells were labeled with B23-NAPA-125I-insulin following the subcellular fractionation procedure as follows: 40 to 80-μg samples of membrane protein were incubated overnight at 4 °C in a total volume of 150 μl of homogenization buffer containing 25 ng of B23-NAPA-125I-insulin (3 to 4 μCi), 0.1% bacitracin, 0.01% digitonin, and 0 or 1 μM native insulin. Under the conditions employed here, the quantity of native insulin associated with the membranes in each subcellular fraction of intact cells to the intact plasma membrane was sufficiently small that the effects of its release from the membranes during the binding of the B23-NAPA-125I-insulin were insignificant (16). Each sample was then exposed for 45 s at 4 °C to ultraviolet radiation from a super pressure mercury lamp at a distance of 30 cm, and a membrane pellet was prepared by centrifugation at 220,000 × g, and aspiration of the supernatant.

Identification and quantitation of the 125I-labeled proteins in the membrane fractions prepared either from the prelabeled adipose cells or by labeling following the fractionation of the unlabeled cells were carried out using 7.5% Na dodecyl sulfate-polyacrylamide gels in the presence of 50 μM dithiothreitol and autoradiography as described by Laemmli (17). Protein was determined by the Coomassie brilliant blue method described by Bradford (18) (Bio-Rad protein assay, Bio-Rad) as modified by Simpson and Sonne (19), using crystalline bovine serum albumin (Sigma) as the standard. Native porcine insulin (crystalline Zn insulin, Eli Lilly and Co.) was kindly provided by Dr. Ronald E. Chance and B23-NAPA-insulin by Dr. Dietrich Brandenburg of the Deutsches Wollforschungsinstitut, Aachen, Germany. The latter was iodinated using chloramine-T as previously described (13).

RESULTS AND DISCUSSION

B23-NAPA-125I-insulin has previously been demonstrated to specifically label the 138-kDa subunit of the insulin receptor in the intact isolated rat adipose cell (13). The efficiency of covalent labeling of the receptor is approximately 20%. The subcellular distribution of this photolabeled receptor subunit is illustrated in Fig. 1. Prior to fractionation, prelabeled intact cells were incubated for 30 min at 16 or 37 °C in the presence of a saturating concentration (700 nM) of native insulin. Because of the low number of 138-kDa receptor subunits in the Golgi-enriched membrane fraction, approximately 2-fold more Golgi-enriched membrane protein was applied to the gel than plasma membrane protein; the quantitative interpretation of the labeling patterns observed in Fig. 1 must take these differences in membrane protein into account.

When prelabeled adipose cells are incubated at 16 °C, the 138-kDa insulin receptor subunit in the plasma membrane fraction is prominently and specifically labeled together with a faintly labeled band of Mr ~ 45,000. This same 138-kDa subunit is only faintly labeled in the Golgi-enriched membrane fraction and probably reflects the roughly 5% contamination of this latter fraction with plasma membranes (20). Following incubation of the intact cells for 30 min at 37 °C, however, the labeling of the 138-kDa subunit in the plasma membrane fraction decreases while that in the Golgi-enriched membrane fraction simultaneously increases. Quantitation of these changes by densitometric scanning of the autoradiographs or by slicing the gels and counting indicates an approximately 30% loss of the 138-kDa receptor subunit from the plasma membranes.
membrane fraction after 30 min at 37 °C, of which approximately 50 to 60% is recovered in the Golgi-enriched membrane fraction. Virtually identical labeling patterns are obtained when intact isolated rat adipose cells are incubated for 30 min at 16 or 37 °C in the absence or presence of a saturating concentration of native insulin, and the two subcellular membrane fractions are prepared and labeled directly with B₂-NAPA-¹²⁵I-insulin in the presence of digitonin (not illustrated).

Thus, insulin appears to induce a translocation of at least the 138-kDa subunit of the insulin receptor from the plasma membrane to a Golgi-enriched low density microsomal membrane fraction in the isolated rat adipose cell. Qualitatively and quantitatively similar results have more recently been obtained by iodinating the proteins in these membrane fractions using the lactoperoxidase/Na¹²⁵I technique and immunoprecipitating the insulin receptor using a specific antiserum (21). The latter provide evidence that both the 138 and 95-kDa insulin receptor subunits are translocated together.

Figs. 2 and 3 demonstrate the time course and insulin concentration dependency, respectively, for the insulin-induced appearance of the 138-kDa insulin receptor subunit in the Golgi-enriched membrane fraction. The appearance of the 138-kDa receptor subunit in the Golgi-enriched membrane fraction at 37 °C is rapid, with a half-time of approximately 2 min, and appears to reach a steady state level within approximately 10 min (Fig. 2). The appearance of the 138-kDa band
is also directly dependent upon the insulin concentration to which the intact cells are exposed, with half-maximal and maximal effects observed at approximately 6 and 30 nM, respectively (Fig. 3). Incubation of the intact cells at 16 °C inhibits the appearance of the 138-kDa receptor subunit in the Golgi-enriched membrane fraction in the presence of both 6 nM (Fig. 2) and 30 nM (Fig. 3) insulin. While the time course of these processes is somewhat slower than that of the binding of 6 nM insulin to its specific receptor in the plasma membrane of the intact cell at 37 °C, the insulin concentration dependency of these processes parallels receptor occupancy (22). A similar time course and insulin concentration and temperature dependency for the appearance of receptors in the Golgi-enriched membrane fraction and concomitant disappearance of receptors from the plasma membrane fraction have previously been observed in this laboratory by directly measuring 125I-insulin binding to these subcellular fractions (16).

Fig. 4 illustrates that the appearance of the 138-kDa insulin receptor subunit in the Golgi-enriched membrane fraction is detectable by directly labeling this membrane fraction following its preparation only in the presence of low concentrations of digitonin. Digitonin is known to specifically interact with the cholesterol in the membrane in a way which increases the membrane’s permeability (23). Digitonin is not required, on the other hand, for the direct labeling of the 138-kDa receptor subunit in the plasma membrane fraction (Fig. 4) nor for visualizing the 138-kDa band in the Golgi enriched membrane fraction of the prelabeled cells (Fig. 1). Concentrations of digitonin higher than those employed here solubilize both membrane fractions and prevent their recovery by centrifugation. This requirement for digitonin in labeling the 138-kDa receptor subunit in the Golgi-enriched membrane fraction, but not in the plasma membrane fraction, suggests that the orientation of the receptor is reversed in the former relative to the latter and that the insulin-induced translocation of insulin receptors represents internalization through an endocytic like process.

Considerable evidence supports the concept that insulin and a variety of other ligands are at least in part degraded through a pathway of which the initial steps are represented by receptor-mediated endocytosis (1-6). This concept presumes that ligand and receptor are internalized together as a complex. In two instances, however, detailed studies of ligand binding and degradation have been interpreted to suggest that the receptors mediating ligand uptake and degradation continuously recycle between the plasma membrane and intracellular compartment (24, 25) and, in the case of insulin and its receptor, that ligand and receptor may actually be degraded by separate mechanisms (24). During the degradation of both insulin by chick liver cells (24) and asialo-orosomucoid by rat hepatocytes (25), 20 to 30 molecules of ligand are calculated to be internalized and degraded per receptor/h in the presence of saturating concentrations of ligand. The present results in the isolated rat adipocyte cell are qualitatively and quantitatively consistent with these previous observations and provide direct evidence for the concept that insulin and its receptor are internalized together. In addition, while the re-translocation of internalized receptors back to the plasma membrane in this cell type remains to be established, the achievement of a steady state level of internalization is suggestive of continuous receptor recycling.

The identity of the intracellular membrane species containing the internalized insulin receptor and the precise quantitative relationship between the appearance of insulin receptors in the Golgi-enriched membrane fraction and their disappearance from the plasma membrane fraction remain to be clarified. Kono et al. (4) and Suzuki and Kono (6) identified an intracellular membrane fraction containing internalized insulin between the endoplasmic reticulum- and Golgi-enriched membrane fractions on a linear sucrose gradient. Since this subcellular fraction was not enriched in marker enzyme activities characteristic of either of the former two membrane species or of the plasma membrane, these workers suggested that this subcellular fraction represents specialized membrane vesicles such as those of endocytic origin. The failure of these investigators to detect an insulin-induced internalization of 125I-labeled cell surface proteins further suggests that any endocytosis occurring specifically in response to insulin either comprises an extremely small proportion of the cell’s total plasma membrane and/or is superimposed on a relatively large constitutive endocytic activity. Preliminary results in this laboratory favor the latter interpretation. The results of the present studies, in which the internalized insulin receptor has been demonstrated in a Golgi-enriched membrane fraction prepared by differential ultracentrifugation, are fully compatible with those of Kono and his collaborators but do not further identify the internalized membrane species nor localize its origin in the intact cell. The membrane vesicles may, however, correspond to the ferritin-labeled insulin-containing endocytic vesicles demonstrated in the rat adipocyte cell by Hammons and Jarrett (5) using electron microscopic techniques and/or the “receptorsome” tentatively identified in the fibroblast by Willingham and Pastan (26).

At steady state in the presence of a saturating concentration of insulin, approximately 30% of the insulin receptors in the plasma membrane fraction are lost per mg of membrane protein. Of these, greater than 50% are recovered in the Golgi-enriched membrane fraction/mg of membrane protein. These values roughly agree with those obtained by Suzuki and Kono

Fig. 4. Effects of digitonin on photoaffinity labeling of 138-kDa insulin receptor subunits in subcellular membrane fractions of the adipose cell. Isolated rat adipose cells were incubated at 37 °C for 30 min in the presence of 30 nM native insulin. Plasma and Golgi-enriched membrane fractions were then prepared, photoaffinity labeled at 4 °C with Bmax-NAPA-125I-insulin in the presence of 0 to 0.03% digitonin and in the absence or presence of 1 μM native insulin, and solubilized, and the labeled proteins were separated by Na dodecyl sulfate-polyacrylamide gel electrophoresis and identified by autoradiography as described under “Experimental Procedures.” 40 μg of plasma membrane protein and 80 μg of Golgi-enriched membrane protein were applied to the indicated lanes. Con, control; 138K, 138K, 138K.

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2 M. J. Zarnowski, I. A. Simpson, and S. W. Cushman, unpublished observation.
(6) who measured the subcellular distribution of noncovalently bound $^{125}$I-insulin and simply calculated the ratio of counts in the two membrane fractions. Nevertheless, an accounting of the disposition of all of the cell’s insulin receptors and a determination of the true stoichiometry of the internalization process cannot be undertaken here in the absence of any means for calculating the recovery of receptors in the Golgi-enriched membrane fraction. These results do suggest, however, that few, if any, intracellular receptors exist in the rat adipose cell in the absence of insulin and that receptor internalization is not a constitutive process but requires the formation of a ligand-receptor complex.

The rapidity of the internalization process reported here and the achievement of a steady state distribution of insulin receptors between the plasma and Golgi-enriched membrane fractions are strikingly similar in every respect to the results obtained by Desbuquois et al. (8) in rat liver following injection of the intact animal with a bolus of insulin. However, these results somewhat contrast with those obtained by Fehlmann et al. (7, 27) in freshly prepared hepatocytes and markedly contrast with those obtained by Green and Olefsky (9) and Berhanu et al. (10) in the isolated rat adipose cell. In the hepatocyte, roughly 85% of the cell surface receptors labeled with a photoaffinity $^{125}$I-insulin analogue are rapidly lost from the cell in 30 to 60 min. Autoradiography indicates that of the remaining photolabeled receptors, approximately 70% are preferentially associated with lysosomal structures, although they slowly return to the plasma membrane over a subsequent 4-h incubation period.

Relative to the present results, Green and Olefsky (9) report a slower and progressive insulin-induced internalization of insulin receptors in the isolated rat adipose cell which is accompanied by a marked loss of the cell’s total number of receptors. Using a photoaffinity $^{125}$I-insulin analogue to prelabel the cell surface receptors, Berhanu et al. (10) further report an apparent alteration in the structure of the internalized receptor during this process. However, the loss of receptors appears to represent an insulin-induced down regulation which occurs over 2 to 4 h in the presence of Tris (28, 29), but only over 15 to 24 h in the absence of Tris (30). Thus, the internalization of the receptor described by Green and Olefsky (9) and the modification of receptor structure described by Berhanu et al. (10) may reflect a process specific to the Tris-treated adipose cell or a similar, but much slower, process occurring naturally in the adipose cell, in the absence of Tris. In addition, since internalized receptors are measured by solubilizing the entire intact cell, modified receptors may be localized specifically to the lysosomal compartment and reflect an early step in the degradation of receptors occurring only during the down regulation process.

The internalization process reported here comprises a rapid subcellular redistribution of insulin receptors which occurs during, and as a part of, the approach to steady state binding. It would not be observed by measurements of insulin binding to the intact cell since receptor-bound ligand is internalized together with the receptor (16), nor in studies in which the initial 10-min incubation period is not examined. Furthermore, this process does not appear to be accompanied by alterations in receptor structure, as assessed in this study by labeling the receptor subunit with a photoaffinity $^{125}$I-insulin analogue or, more recently, by labeling both the α and β receptor subunits using the lactoperoxidase/Na$^{125}$I technique and immunoprecipitating the internalized receptor subunits with a specific antiserum (21). However, lysosomal fractions were not prepared in either of these studies. Nevertheless, while this rapid internalization is not accompanied by any perceptible reduction in the cell’s total number of receptors, it may represent an early event in the down regulation process. The long term down regulation of insulin receptors probably represents an adaptive mechanism protecting the cell from an excess of metabolic stimuli in its environment.

The rapidity of the insulin-induced internalization of the insulin receptor observed here and the achievement of a steady state subcellular distribution of insulin receptors within 10 min at 37 °C are comparable, although directionally opposite, to the insulin-induced translocation of glucose transport systems from a membrane-associated intracellular pool to the plasma membrane (11). Insulin receptor internalization may, therefore, represent a functional link between insulin binding and the glucose transport response. However, the insulin concentration dependency of receptor internalization directly correlates with receptor occupancy and thus is considerably less sensitive to insulin than is the stimulation of glucose transport. The relevance of the insulin-induced internalization of its own receptor to insulin’s regulatory action on adipose cellular function will only be explained when the enigma of degenerate receptors is resolved.

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