Insulin Stimulates the Assembly of Cytosolic Clathrin onto Adipocyte Plasma Membranes*

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The effects of insulin on the subcellular distribution of the heavy chain of clathrin and on the insulin-like growth factor II (IGF-II) mannose 6-phosphate receptor have been investigated in isolated rat adipocytes. Plasma membranes, intracellular membranes, and cytosol were separated by differential centrifugation, and the concentration of clathrin and receptor in each fraction was quantified by sequential immunoblotting with monoclonal and polyclonal antibodies against these proteins. A 3-fold increase in the amount of clathrin heavy chain associated with isolated plasma membranes was found after treatment of cells with low concentrations of insulin. This effect was complete within 2 min of stimulation at 37 °C and was abolished at 5–10 °C. The insulin-mediated increase in the cell surface concentration of receptors for IGF-II/mannose 6-phosphate displayed a similar time course and temperature dependence. A concomitant decrease in the concentration of IGF-II/mannose 6-phosphate receptors in intracellular membranes was observed. In contrast, no significant changes in the concentration of clathrin in this fraction could be detected. Instead, a marked decrease in the level of unassembled cytosolic clathrin was observed in insulin-treated cells compared with controls. These results suggest that insulin induces an increase in the assembly of cytosolic clathrin onto the plasma membrane in conjunction with its ability to increase the concentration of receptors on the cell surface.

The receptor-mediated internalization of numerous macromolecules, growth factors, and nutrients occurs through coated pits at the plasma membrane (1). These specialized structures are composed predominantly of clathrin and of a group of polypeptides that appear to interact with the cytoplasmic domains of various receptors (2). The formation of coated pits is thought to involve the continuous assembly of these components onto the plasma membrane followed by their disassembly from internalized coated vesicles. Several hormones and growth stimuli appear to regulate the formation of clathrin-coated membranes in their target cells. For example, an increase in the number of coated pits in the membrane of PC-12 pheochromocytoma cells occurs within minutes after stimulation by nerve growth factor (3). In addition, a marked increase in the levels of assembled clathrin is observed early after mitogenic stimulation of mouse lymphocytes (4).

The mechanisms that trigger the formation of clathrin-coated membranes under basal conditions and the mechanisms whereby growth factors or mitogens stimulate this process are not known. However, it has been recently shown that hyperexpression of transferrin receptors in mouse L cells is accompanied by an increase in the number of coated pits (5), suggesting that the cytoplasmic tail of the receptor may trigger or facilitate coated pit formation.

Recent work has shown that stimulation of adipocytes or 3T3-L1 cells by insulin causes a 3–5-fold increase in the cell surface concentration of receptors for transferrin, insulin-like growth factor II/mannose 6-phosphate (IGF-II/Mann-6-P), and α2-macroglobulin (6–10), and a significant stimulation in the uptake of IGF-II, and α2-macroglobulin by the cell. These observations suggested the hypothesis that the insulin-mediated increase of the concentration of receptors at the cell surface would be accompanied by an increase in clathrin-coated membrane formation. Isolated rat adipocytes are well suited as a model system in which to examine this possibility, because these cells are extremely sensitive and responsive to insulin. In addition, methods for rapid subcellular fractionation of these cells have been developed (11–14). In this study, an effect of insulin to increase the concentration of clathrin assembled on the plasma membrane of rat adipocytes is demonstrated.

EXPERIMENTAL PROCEDURES

Cell Isolation and Fractionation—Adipocytes were isolated by collagenase digestion (15) from the epididymal fat pads of 100–125-g male Sprague-Dawley rats (Taconic Farms, Inc.). Cells (approximately 3 x 10⁶ cells/condition) were resuspended in 5 ml of Krebs-Ringer Hepes, pH 7.4, supplemented with 2 mM pyruvate and 20 mg/ml bovine serum albumin (Intragen), and placed in 15-ml conical culture tubes. Cells were then incubated for 20 min in a 37 °C water bath, and insulin (crystalline porcine, Lilly) was added at the concentrations and for the times indicated in each figure. The cell suspensions were then centrifuged for 10 x g at 500 x g, the buffer aspirated, and the cells homogenized in a Teflon/glass homogenizer using 6 volumes of an ice-cold buffer composed of 250 mM sucrose, 10 mM Tris, 5 mM EDTA, 50 mM sodium fluoride, 100 mM sodium vanadate, 50 mM sodium pyrophosphate, 1 mM 1,10-phenanthroline, 10 μg/ml leupeptin, and 1 mM benzamidine, adjusted to pH 7.5. Plasmamembranes, high density microsomes, low density microsomes, and cytosol were prepared from the homogenates by a previously characterized procedure of differential centrifugation (11–14). This method provides a plasma membrane fraction highly enriched in 5-nucleotidase, an activity associated specifically with the adipocyte plasma membrane (8, 11, 12). The low density microsomal fraction and the high density microsomal fraction obtained by this procedure are highly enriched (6–8-fold) in UDP-galactose:N-acetylgalactosamine galactosyltransferase and NADPH-dependent cytochrome c reductase activities, which are markers of the Golgi apparatus and of the endoplasmic reticulum, respectively (8). Both the low and high density microsomal fractions obtained by this method are devoid of 5-nucleotidase activity (8, 12), indicating that they are free of plasma membrane contamination. The plasma membrane fraction shows no enrichment in Golgi or endoplasmic reticulum markers (8, 12) and

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1 The abbreviations used are: IGF, insulin-like growth factor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TAPS, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; Cmcl, clathrin heavy chain.
by electron microscopy appears free of intracellular membranes (11). In addition, insulin has been shown to have no effect on the distribution of membrane marker enzymes nor on the total protein recovered in any of the membrane fractions (8, 12, 14). The association of surface IGF-II/Man-6-P receptors with the plasma membrane fraction obtained by this procedure has been demonstrated by cell surface labeling of isolated adipocytes, followed by subcellular fractionation and immunoprecipitation with an anti-IGF-II receptor polyclonal antisera. These experiments have demonstrated that all of the receptor present on the cell surface copurifies with the plasma membrane fraction (13). Similar results have been obtained after affinity cross-linking of $^{125}$I-IGF-II to the cell surface of control or insulin-treated adipocytes followed by homogenization and subcellular fractionation. In the experiments described here, the cytosolic fraction was centrifuged one additional time at 350,000 x $g$ for 30 min to ensure the complete sedimentation of any particulate material. All of the unassembled clathrin was detected in the supernatants from the centrifugation. The membranes were resuspended in 20 mM TAPS, pH 9.0, supplemented with the protease inhibitors described above and assayed for protein by the method of Bradford (16).

Electrophoresis and Immunoblotting—Aliquots (100 µg of protein) of each fraction obtained from control or insulin-treated cells were boiled for 2 min in electrophoresis sample buffer at a final concentration of 3% sodium dodecyl sulfate, separated on 6% polyacrylamide gels, and electrophoretically transferred onto nitrocellulose paper. After blocking in 5% bovine serum albumin, the blots were incubated overnight with anti-clathrin heavy chain monoclonal antibody Chc 5.9 (Boehringer Mannheim) at a final concentration of 2.5 µg/ml. The blots were then washed and developed with a polyclonal goat anti-mouse immunoglobulin coupled to alkaline phosphatase (Promega). Color development was performed according to the manufacturer’s instructions. The intensity of the colored bands was quantified using a LKB Ultrascan XL laser densitometer. Standard curves constructed with purified rat liver-coated vesicles indicated that this procedure was sensitive to as little as 0.1 µg and linear to up to 10 µg of coated vesicle protein (see Ref. 4). Standard curves of purified clathrin were run periodically to ensure that the signal obtained from each subcellular fraction was within a linear range in each experiment. To estimate the concentration of IGF-II/Man-6-P receptors in the cytosolic fraction, the concentration of clathrin heavy chain was first estimated by densitometry. The intensity of the bands was measured using an LKB laser densitometer. To estimate the total amount of clathrin in each fraction, the values obtained by densitometry were multiplied by the total amount of protein recovered in each fraction, which in this experiment was 264 and 256 µg of control or insulin-treated plasma membranes, 2.0 and 2.1 mg of control or insulin-treated cytosol, 198 and 200 µg of control or insulin-treated high density microsomes, 176 and 228 µg of control or insulin-treated low density microsomes, respectively. The results of these calculations were used to estimate the relative proportion of total cellular clathrin present in each fraction of control (C) or insulin-treated (I) cells (bar graph).

RESULTS AND DISCUSSION

The subcellular distribution of clathrin was analyzed in isolated rat adipocytes. Fig. 1 shows the results of an experiment in which these cells were incubated in the absence or presence of insulin for 10 min at 37 °C and then fractionated into plasma membranes, high and low density intracellular membranes, and cytosol. Aliquots from each fraction (100 µg of protein) were separated by polyacrylamide gel electrophoresis and analyzed by quantitative immunoblotting with a monoclonal antibody to the clathrin heavy chain (Chc 5.9), followed by a second antibody coupled to alkaline phosphatase. A single polypeptide of approximately 180 kDa, corresponding to the clathrin heavy chain (Chc 5.9), was detected in each fraction (Fig. 1, top insets). The intensity of the colored bands was quantified by laser densitometry, and the values obtained were multiplied by the total amount of protein recovered in each fraction (legend to Fig. 1). The sum of the normalized values was taken to represent 100% of the total cellular clathrin. The relative proportion of clathrin present in each fraction in intact cells is represented by the bars (Fig. 1, bar graph). Insulin caused a 2-3-fold increase in the concentration of clathrin heavy chain associated with the plasma membrane (Fig. 1). The concentration in the cytosolic fraction, which in control cells amounted to approximately 20% of the total, decreased to virtually undetectable levels in response to insulin. Approximately 60–70% of the total cellular clathrin was found associated to intracellular membranes of high and low density, which by marker enzyme analysis appear to correspond to endoplasmic reticulum and Golgi membranes, respectively (8, 12). Insulin did not detectably alter the abundance of clathrin associated with these membrane fractions.

Insulin increases the concentration of transferrin and IGF-II/Man-6-P receptors on the plasma membrane at the expense of an intracellular pool which sediments with the low density microsomal fraction (6–9, 14). Fig. 2 shows an immunoblot of plasma membranes and low density microsomes from control or insulin-treated cells probed with a polyclonal antibody against the IGF-II/Man-6-P receptor (14, 17) followed by a second antibody coupled to alkaline phosphatase. The blot was then reprobed with anti-clathrin heavy chain monoclonal antibody Chc 5.9 as described above. An increase in both IGF-II/Man-6-P receptors and clathrin heavy chain could be detected in plasma membranes from insulin-treated cells (Fig. 2). A concomitant decrease in the abundance of IGF-II/Man-6-P receptors in the low density microsomes from insulin-treated cells could be observed. However, the concentration of clathrin in this fraction was not detectably decreased. These results suggest that the insulin-induced change in the
steady state distribution of IGF-II/Man-6-P receptors between the plasma membrane and intracellular membrane pools is not accompanied by a change in the steady state level of clathrin in intracellular membrane compartments. These results are consistent with the experiment shown in Fig. 1, which indicate that the increase in plasma membrane clathrin can be quantitatively accounted for by the decrease in the unassembled cytoplasmic pool.

The insulin-stimulated increase in clathrin assembly at the plasma membrane paralleled the increase in the cell surface concentration of receptors under various experimental conditions studied. Fig. 3 (left panel) shows that both effects occurred at a low concentration of insulin, with half-maximal increases observed at 0.1–1.0 nM insulin. Both effects were complete after 2 min of stimulation at 37 °C, and with the methodology employed, it was not possible to detect any consistent differences in the early kinetics of these two effects (Fig. 3, right panel).

To analyze whether insulin could elicit an increase in the assembly of clathrin at the plasma membrane in the absence of an increase in the cell surface concentration of receptors, experiments were performed with cells incubated at 5–10 °C. At this temperature insulin can bind and stimulate the tyrosine kinase activity and autophosphorylation of its receptor (18), and clathrin is competent to assemble in vitro into coated pits (19). Incubation of the cells at 5 °C blunted the ability of insulin to increase the cell surface concentration of IGF-II/Man-6-P receptors (Fig. 4). Under these conditions, no increase in the plasma membrane concentration of clathrin due to insulin could be detected. Thus, the insulin-induced increase in clathrin assembly could not be dissociated from the increase in cell surface concentration of receptors under these conditions. Nevertheless, the concentration of clathrin in control cells incubated at 5 °C was increased compared with those incubated at 37 °C (Fig. 4), suggesting that in these cells, as in fibroblasts (19), clathrin assembly proceeds at low temperature.

The results presented here demonstrate that in conjunction with increasing the cell surface concentration of receptors, insulin causes an increase in the assembly of clathrin onto the plasma membrane at the expense of a cytoplasmic unassembled pool. The results raise several possibilities concerning the mechanism of increased clathrin assembly and of its potential importance in mediating the effects of insulin on receptor distribution and macromolecule uptake. The increase in the assembly of clathrin onto the plasma membrane may be secondary to the increase in the concentration of cell surface receptors that occurs in response to insulin. In this case the increased number of receptors on the plasma membrane may nucleate the formation of coated pits by attracting...
specific adaptor molecules (2) and clathrin from an unassembled cytosolic pool. A second possibility is that insulin directly enhances the ability of clathrin or of the assembly polypeptides to polymerize onto plasma membranes. In this case, covalent modifications, such as phosphorylation, of one or several of these proteins could be involved. It is interesting that one of the major protein kinase activities found in coated vesicles is casein kinase II (20), which has been shown to be regulated by insulin (21). Other growth factors have been shown to transiently increase coated pit formation at the plasma membrane (3). Similarly to insulin, these hormones may induce a redistribution of receptors to the cell surface and a polymerization of cytosolic clathrin.

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S Corvera

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