Review

The cell biology of the insulin receptor*

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Summary. Following initial binding to specific cell surface receptors insulin is internalized in target cells. The fate of the internalized insulin-receptor complexes and how the processes involved are regulated is reviewed. The implications of these events in the effects of insulin on its target cells and in the

physiopathology of diabetes and insulin resistance states are also considered.

Key words: Insulin receptor, endocytosis, diabetes, internalization.

Introduction and historical perspective

The cells of a multicellular organism are bathed in an aqueous medium derived from the blood. This medium contains a very large number of substances present in exceedingly low concentration. Each cell needs definite quantities of these substances. Thus, each cell must be equipped with a recognition system allowing selection from the extracellular medium of the exact amount of the substances it needs.

The concept that cells possess receptive entities whose primary function is specific recognition and binding of other molecules arose more than a century ago when Langley wrote: "... *We may, without much rashness, assume that there is some substance or substances in the nerve endings or gland cells with which both atropin and pilocarpin are capable of forming compounds. On this assumption then the atropin or pilocarpin compounds are formed according to some law of which their relative mass and chemical affinity for the substances are factors.*" [1].

Twenty years later, Ehrlich extended this concept and postulated the existence at the cell surface of side chains designed to react with nutrients necessary to maintain life [2]. These side chains were named receptors and the substances that bind to them were called ligands (from Latin *ligare*=to bind).

In the case of insulin, evidence has been produced in the past 40 years that the first step in the action of this hormone is binding to specific receptor sites on the plasma membrane of the cell [3, 4]. However, this evidence was indirect and inferred from observations of subsequent biological responses to the hormone. The demonstration that the number of insulin receptor sites per cell was finite, that the receptors were on the outer surface of the cell, and that they were specific for insulin was made possible only in 1971, when Freychet et al. [5] and Cuatre-

casas [6] introduced insulin labelled with ^{125}I iodine (^{125}I), which allowed the precise characterization of the ligand-receptor interaction. At that time, studies were carried out at low temperature to minimize hormone degradation. Thus, attention was focused on the cell surface and the various effects of insulin were envisioned as the result of an equilibrium between the surface insulin receptor and insulin present in the external milieu.

The surface interaction of insulin with its receptor was visualized for the first time by Orci et al. [7] who detected insulin-dextran-ferritin complexes specifically bound to hepatocyte membranes prepared by the freeze-etching technique. Then, in 1976, we prepared to determine the fate of insulin following its binding to the insulin receptors. For this purpose, we selected ^{125}I -labelled insulin as the ligand since this molecule could be detected by both morphological (see below) and biochemical techniques in a combined structure-function analysis. ^{125}I -insulin was localized by quantitative high resolution autoradiography on electron micrographs. According to this technique, thin sections of cells containing the radioactive ligand are covered with a photographic emulsion. The silver bromide crystals hit by the radioactivity are then transformed by photographic processing into black silver grains. These grains thus localize, on the thin sections, the cellular site containing the radioactive ligand. Due to the dispersion of the radioactivity, a quantitative analysis of a large number of autoradiographic grains is required to accurately determine, in each condition, the exact location of the radioactive source.

Our initial results, obtained by incubating cultured human lymphocytes of the IM-9 line with ^{125}I -insulin, confirmed the binding of the hormone to the cell surface [8]. Shortly afterwards, however, we found that when ^{125}I -insulin was incubated with freshly isolated rat hepatocytes at 37°C , the autoradiographic reaction was not only present at the cell surface as expected, but was also detectable in the cell's interior [9]. The apparent

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Fig. 1a-e. Association of ^{125}I -insulin (detected by autoradiography) with cell surface differentiations. Autoradiographic grains are seen in relationship with microvilli (mv) (a and b); with coated pits (cp) (b and c); in d) ^{125}I -Epidermal growth factor (autoradiographic grains) and ferritin-LDL (arrows) are present in the same coated pit (cp); e) shows the freeze-fracture image of a coated pit (cp). It appears as a circular invagination of the membrane face containing large protein particles. a: $\times 35,000$; b: $\times 40,000$; c: $\times 40,000$; d: $\times 105,000$; e: $\times 95,000$

discrepancy between this and the IM-9 studies turned out later to be due to the incubation and temperature conditions since we found that IM-9 cells do internalize insulin [10], but at a slow rate and to a very small extent compared to hepatocytes [11].

Thus, in 1977, it became clear that following initial binding to specific receptors at the cell surface, insulin was internalized in the target cells in a way similar to that recently discovered for low density lipoproteins [12]. It was also soon recognized that many polypeptide hormones and growth factors, as well as other unrelated ligands such as $\alpha 2$ -macroglobulin, asialoglycoprotein or transferrin, followed essentially the same pattern of internalization after initial binding to their respective cell surface receptors [13–18].

The finding that insulin was internalized upon binding to its receptor opened a whole new research field that I will review under four main chapters:

1. Fate of insulin and of the insulin receptor following their interaction at the cell surface
2. Implication of insulin internalization in the effects of the hormone on target cells

3. Triggering and regulation of insulin receptor internalization

4. Implication of insulin internalization in the pathophysiology of diabetes and insulin resistance states

Fate of insulin and the insulin receptor following their interaction at the cell surface

The detailed examination of the location of the autoradiographic grains at the cell surface coupled to a statistical evaluation of this distribution with respect to the geometry of the cell periphery revealed that initial binding did not occur randomly. Autoradiographic grains specifically associate at the earliest steps of the incubation with thin digitations of the cell periphery extending in the extracellular medium, the microvilli (Fig. 1) [19–21]. From this initial location, insulin-receptor complexes appear to move in the plane of the plasma membrane to reach membrane invaginations which are frequently detected at the base of microvilli. These invaginations, or pits (Fig. 1), are characterized by the presence of a protein coat associated with the cytoplasmic leaflet of the membrane, hence their name coated invaginations or coated pits. The coat is made of the protein clathrin [22, 23]. Easily detectable on thin sections for electron microscopy because of their membrane coat, coated pits can also be recognized on freeze-fracture replicas due to their high concentration of intramembrane protein particles (Fig. 1) [24]. The detection of coated pits on freeze-fracture replicas is important because it allows the characterization of the plasma membrane distribution of the receptor-ligand complex by the fracture label technique [25].

Fig. 2a-e. Association of ^{125}I -insulin and its receptor with intracellular compartments during internalization. Autoradiographic grains seem in relationship with **a**) a coated vesicle (cv); **b**) with endosomes (e); **c**) with a multivesicular body (mvb) and **d**) with a lysosome (Ly); **e**) illustrates a small uncoated, clear vesicle implicated in the recycling of ^{125}I -photoreactive insulin-receptor to the cell surface. **a:** $\times 58,000$; **b:** $\times 45,000$; **c:** $\times 46,000$; **d:** $\times 45,000$; **e:** $\times 56,000$

Following the concentration of ^{125}I -insulin in coated pit, the bound ligand is internalized through endocytosis, a process resulting in the formation of a coated vesicle free in the subplasmalemmal cytoplasm (Fig. 2) [26–28]. The life time of a coated vesicle is short (1–2 min or less) since, by yet unknown mechanisms, the coat is shed from the membrane delimiting the vesicle.

The next station in the internalization pathway is represented by a heterogeneous, complex set of tubules and vacuoles called endosomes [27]. These recently characterized prelysosomal organelles are located in the peripheral cytoplasm. Endosomes are endowed with proton pumps capable of acidifying the internal milieu of the organelle [29]. This acid pH is crucial for the dissociation of the ligand from the receptor (see below). The next intracellular structure containing labelled internalized insulin is the multivesicular body (Fig. 2), also called late endosome [30]. This organelle is larger than the initial endosome and is constituted by small vesicles enclosed within

a membrane vacuole bearing a limited coated segment. Unlike the initial or early endosome, the multivesicular body may contain lysosomal enzymes and mannose-6 phosphate receptors [30, 31]. How insulin gains access to multivesicular bodies is presently unknown. The terminal station of the internalization pathway of insulin is represented by digestive vacuoles, or secondary lysosomes where insulin will ultimately be degraded [32, 33]. This sequence of events is summarized in Fig. 3.

What about the insulin receptor? There are both biochemical and morphological [34–37] data indicating that the receptor is internalized following ligand binding. However, unlike insulin which is degraded following internalization, the receptor has an alternative pathway which is direct recycling to the cell surface. The recycling of the receptor implies that the latter is uncoupled from its specific ligand previous to reaching the lysosomal degradative compartment. This step is assumed to occur in the acidic environment of the endosome. Separation of ligand and receptor seems not, however, to be an obligatory prerequisite for receptor recycling. When insulin is irreversibly bound to its receptor by covalent linkage, one detects, following internalization of the ligand-receptor complex, the reexpression of the labelled ligand at the cell surface over prolonged incubation time [38, 39]. This process occurs at a much slower rate than the rapid, presumably physiologic, recycling of the receptor occurring

Fig. 3. Schematic representation of the intracellular pathway of insulin following binding to its plasma membrane receptor on microvilli. Also represented is the recycling route of the receptor from the endosomal/lysosomal compartment and the possible insertion of newly-synthesized receptors. mvb = multivesicular body

Fig. 4. Schematic view of the insulin receptor with its two external binding domains (α -subunits) linked by disulfide bonds to two transmembrane domains (β -subunits) extending into the cytoplasm. On the later portion of the β -subunits tyrosine residues become phosphorylated (P-Tyr) upon insulin binding to the α -subunits

when dissociation from the ligand is not prevented experimentally. The compartment in which covalently-bound insulin can be visualized, by autoradiography, on its way to be recycled to the plasma membrane appears

composed of small vesicles with an electrolucent content and an uncoated limiting membrane [39] (Fig. 2).

Implication of insulin internalization on the effects of the hormone on target cells

The formation of the insulin-receptor complex triggers diverse responses in the target cell: a) induction of a biological signal; b) degradation or inactivation of the hormone to terminate the signal; c) regulation of insulin binding which implies a regulation of the sensitivity and number of surface insulin receptors.

The induction of a biological signal in the target cell upon ligand binding to its specific receptor requires transduction mechanism between the cell surface event and the cell cytoplasm. Recently, clues to the nature of this mechanism have been obtained by the finding that the insulin receptor is a tyrosine specific protein kinase [40–42]. This property is shared by plasma membrane receptors for polypeptide ligands as diverse as epidermal growth factor (EGF) [43], platelet derived growth factor (PDGF) [44, 45], insulin-like growth factor I (IGF-I) [46, 47] and colony stimulating factor I (CSF-I) [46]. The insulin receptor is composed of two external binding domains (α -subunits) and two transmembrane domains (β -subunits) (Fig. 4). All these subunits are linked by disulfide bonds. A current hypothesis of transduction is that insulin binding to the α -subunit stimulates autophosphorylation of tyrosine residues on the intracellular domain of the β -subunit which, in turn, triggers the activation of phosphotransferases [49–51] and catalyses tyrosine phosphorylation of specific substrates. This hypothesis has gained support recently by the discovery of various substrates which undergo tyrosine phosphorylation during insulin binding [52–56]. Moreover, inhibition or deletion of the insulin receptor tyrosine kinase blocks insulin action [57–59]. Thus, it appears plausible that insulin-induced receptor autophosphorylation is the starting point of a cascade of phosphorylation leading to the biological actions of the hormone. Such a cascade could be initiated either at the cell surface or inside of the cell. The latter possibility would require previous internalization of the activated receptor for the phosphorylation of substrates inaccessible from the plasma membrane. At present, data concerning the phosphorylation state of the insulin receptor during its internalization are still fragmentary [60]. However, we succeeded in the visualization, by immunofluorescence and protein A-gold immunocytochemistry, of the state of phosphorylation of the EGF receptor in A-431 cells. Using an antiphosphotyrosine antibody, we localized phosphotyrosine antigenic sites on the inner surface of the plasma membrane and, following internalization, on the outer leaflet of the cytoplasmic vacuoles (Fig. 5). This suggests that the EGF receptor is internalized in its phosphorylated or activated form and remains phosphorylated at least up to the multivesicular bodies step [61]. Assuming a similar process following insulin binding, one can speculate that the

Fig. 5. **a** Antiphosphotyrosine antibody detected by immunofluorescence stains the peripheral cytoplasm as well as intracellular structures of semi-thin sections of A-431 cell in culture exposed for 60 min at 37°C to epidermal growth factor (100 nmol/l). **b** Antiphosphotyrosine antibody revealed at the electron microscope level by the protein A-gold technique can be detected on the outer aspect of the membrane delimiting multivesicular bodies (mvb). Arrows point to individual gold particles. Conditions of incubation of cells as in **a**. **a**: $\times 600$; **b**: $\times 60,000$

internalization of the phosphorylated insulin receptor is required for the transmission of at least some biological signals inside the target cell.

The postulate that the binding of insulin to its specific receptor triggers hormone action implies an associated mechanism to terminate this action. Insulin degradation might represent such a mechanism. We have noted, previously, the association of insulin-derived radioactivity (detected by autoradiography) with the degradative organelles of the cell, the lysosomes [32, 33]. The internalization step following insulin binding is most likely to be the way by which insulin is delivered to this degradative compartment. The insulin degradative role of lysosomes could be evidenced by the observation that agents such as chloroquine or ammonium chloride which raise intralysosomal pH and perturb digestion by acidic hydrolases, inhibited insulin degradation [62]. The implication of neutral (presumably cytosolic) proteases in this process [63] seems difficult to conceive since these enzymes should not normally have access to the receptor-bound hormone, that, as far as we know, is entirely segregated in the endosomal-lysosomal compartment.

Beside probably mediating signal transduction and ligand degradation, the sequence of insulin binding to its cell surface receptor followed by the internalization of the insulin-receptor complex could provide a way by which insulin can regulate the number of its own receptors at the cell surface. Indeed, since the internalization of insulin is concomitant with the internalization of the receptor, each time one insulin molecule is removed from the cell surface, one receptor molecule follows the same way. However, we have seen that unlike insulin, the re-

ceptor can avoid degradation and be recycled to the cell surface. Thus, one has to assume that the number of surface receptors is ultimately modulated by both the rates of internalization and recycling. To assess the respective contribution of either process we have blocked endocytosis by a hypertonic medium [64], and recycling by monensin [65]. In each condition, we have measured the insulin-induced loss of surface insulin receptors (down regulation). In the condition of reduced ^{125}I -insulin internalization (0.3 mol/l NaCl incubation of HL60 myelomonocytoid cells), the down regulation of insulin receptors was significantly decreased (Fig. 6). Conversely, in the presence of monensin which inhibits recycling, down regulation was markedly increased (Fig. 6). Thus, on the basis of these inhibition studies, it is evident that at any given time the turnover of insulin receptors between the inside and the surface of the cell determines their number available at the plasma membrane for binding extracellular insulin. In addition, one has to take into account the de novo synthesis of receptors which has been shown to participate in this modulation [66].

Triggering and regulation of insulin receptor internalization

Monensin, which as mentioned above, inhibits receptor recycling, alters the number of surface insulin receptors over short periods of time only if insulin is present in the incubation medium [65]. This indicates that the insulin receptor is not (or is slowly) turning over in the absence of the ligand. Similar conclusions were recently reached by Backer et al. [60] who found an acceleration in the rate of insulin receptor internalization following the addition of insulin. Thus, endocytosis of the insulin receptor is induced by insulin binding.

The mechanism by which insulin triggers endocytosis of its receptors, however, is unknown. It was initially suggested, on the basis of inhibition studies, that transglutaminases were involved [67]. This view was based on the observation that several chemical agents inhibited both transglutaminase activity and endocytosis. It is now clear

Fig. 6. Opposite effect of monensin (10^{-6} mol/l) (black columns) or hypertonic medium (0.3 mol/l NaCl) (stippled column) on the insulin-induced (10^{-7} mol/l) down regulation of surface insulin receptors (= Loss of insulin receptors present at the cell surface after a 60 min of incubation in the presence of insulin). Control conditions include incubations in the absence of insulin and the drug (white columns), and incubations in the presence of 10^{-7} mol/l insulin for 60 min (stripped columns). Experiments designed to test the role of the hypertonic medium were performed in myelo-monocytic HL-60 cells and those testing the role of monensin were performed in U-937 monocyte-like cells

Fig. 7. Effect of intracellular calcium concentration (low, resting, high) and phorbol myristate acetate (PMA) (50 nmol/l) (black columns) on 125 I-insulin internalization in HL-60 cells. Internalization appears independent of intracellular calcium concentration (white columns) while PMA significantly increases 125 I-insulin internalization at all levels of intracellular Ca^{2+} concentration

Fig. 8. Comparison of the internalization of 125 I-insulin and 125 I-glucagon in hepatocytes freshly isolated from normal (white columns), diabetic (streptozotocin-treated) (striped columns), and diabetic rats after insulin treatment (black columns). Note that streptozotocin diabetes (low insulin, high glucagon in plasma) decreases the internalization of insulin receptors, but increases internalization of glucagon receptors. Treatment of diabetes with insulin corrects both changes

that these processes are not related [68]. More recently there was a surge of interest in the role of the autophosphorylation of the receptors. Various polypeptide hormone and growth factor receptors which undergo a specific ligand-induced autophosphorylation, are internalized at the same time by target cells. Furthermore, endocytosis of polypeptide ligands is an energy consuming process, as is phosphorylation, and phorbol esters which induce phosphorylation of serine or threonine amino acids also promote receptor internalization [69–71]. CHO cells expressing mutated insulin receptor lacking kinase activity were shown to poorly internalize insulin [58, 72]. Despite the suggestive evidence pointing out autophosphorylation of the receptor as the triggering mechanism for ligand-receptor complex internalization, the situation cannot be considered as definitively settled in the case of the insulin receptor. Indeed, when we compared the internalization of 125 I-insulin with that of a 125 I-labelled anti-insulin receptor antibody (the latter does not induce autophosphorylation of the receptor), we found the two

internalization curves fully superimposable [73]. Similarly, recent studies confirmed that autophosphorylation of the insulin receptor is not required for its internalization [74–76]. Thus, autophosphorylation of the insulin receptor does not seem to be the general mechanism for triggering insulin receptor internalization.

To investigate the possible mechanism(s) regulating insulin receptor internalization, we have studied the effect of changing the concentration of the cytoplasmic free calcium and the level of protein kinase C. For this purpose, intracellular concentration of calcium was modulated in HL60 cells by quin2 in the presence or absence of extracellular calcium. As in previous experiments, the rate of insulin internalization was evaluated by quantitative high resolution autoradiography. Our findings were that irrespective of the level of intracellular free calcium, insulin internalization proceeded at the same rate (Fig. 7) thereby ruling out a direct effect of calcium on the regulation of internalization [70]. In contrast, the stimulation of protein kinase C by phorbol myristate acetate (PMA) significantly increased 125 I-insulin internalization at all levels of intracellular calcium concentration (Fig. 7). To test the general relevance of these findings on the mechanism of regulation of receptor internalization, we repeated the experiments with another receptor, the transferrin receptor [70]. These experiments indicated a higher degree of regulation complexity among different receptors than previously thought. Indeed, while the behaviour of the transferrin receptors was fully comparable to that of the insulin receptor in the presence of their respective ligands, the behaviour differed entirely in the absence of the ligands. We tested the capacity of HL60 cells to bind the ligand at low temperature (= no internalization) after a period of pretreatment during which the receptor internalization was enhanced by PMA at 37°C in the absence of the ligand. PMA-pretreatment had no effect on subsequent 125 I-insulin binding at low temperature, while it caused a 50% decrease of 125 I-transferrin binding. In other words the number of insulin receptors present at the cell surface was unchanged while that of transferrin receptors was reduced by 50%. Stated differently, insulin receptor internalization was not stimulated by PMA in

the absence of the ligand, while the transferrin receptor was stimulated. This major difference may relate to the different topographical distribution of these two classes of receptors: unoccupied insulin receptor is predominantly associated with microvilli [19, 20, 26] while free transferrin receptor is associated with coated pits [77]. Being present in coated pits, transferrin receptors would be continuously internalized and recycled irrespective of whether the ligand binding step has occurred. On the contrary, the insulin receptor does not move to coated pits unless it has bound insulin on microvilli. Little internalization of this receptor therefore should occur previous to ligand binding. However, once either type of ligand is concentrated in coated pits, the internalization route is similar since both insulin (or EGF) and transferrin (or LDL) receptors are recovered in the same coated pits (Fig. 1) and subsequently in the same endosomal-lysosomal intracellular compartment [78]. For insulin, ligand-specific regulation of receptor internalization should therefore occur previous to coated pit migration.

Implication of insulin internalization in the physiopathology of diabetes and insulin resistance

The data reviewed so far suggest that the process of internalization of the ligand-receptor complex is probably a key step in the overall response of the target cell to changes in the level of extracellular ligand. The study, by quantitative autoradiography of insulin internalization in pathological situations characterized by perturbed extracellular insulin levels, thus represents a good test case for the hypothesis. We studied insulin internalization in two conditions of decreased extracellular insulin level: streptozotocin diabetes in rats and Type 1 (insulin-dependent) diabetes mellitus in man. In both conditions, we observed a significantly reduced internalization rate of insulin in hepatocytes (streptozotocin diabetes) or blood monocytes (Type 1 diabetes). This reduction was corrected by restoration of the extracellular insulin level through infusion of insulin (Fig. 8) [79]. The fact that these changes were ligand-specific was evident from the observation that in streptozotocin-diabetic animals, which present an accompanying hyperglucagonaemia, ^{125}I -glucagon internalization by isolated hepatocytes was increased, a change that could also be reversed by insulin treatment which restores normal glucagonaemia (Fig. 8). The regulation of internalization can thus be viewed as a mechanism by which the cell is able to adjust the number of surface receptors to keep up with the level of extracellular ligand: augmentation of surface receptors in the case of a decreased signal (hypoinsulinaemia), and, conversely, reduction of the number of receptors when the hormone is present in excess (hyperglucagonaemia).

This apparently coherent pattern of receptor metabolism in hypoinsulinaemic, hyperglucagonaemic states would make one expect an increased internalization rate of insulin receptors in hyperinsulinaemic states. When

we tested this hypothesis in monocytes of obese, non-obese, lipoatrophic, and Type 2 (non-insulin-dependent) diabetic patients, we found however that ^{125}I -insulin internalization was decreased after 30 min of incubation at 37°C , although the decrease was no longer perceptible as compared with control subjects at later incubation times. In obese, non-diabetic control subjects, internalization at 30 min was slightly less than internalization in normal control subjects, but larger than that of the diabetic group. Fasting was associated with normalization of internalization in the obese group [80].

Thus, it seems that the situation of hyperinsulinaemia is complex and that in addition to a response of the receptor internalization to altered insulin levels, a relative defect in the mechanism of internalization is also present. In this respect, it is worth noting that recent data have indicated a defect in tyrosine kinase activity in Type 2 diabetes [81]. We have mentioned above the probable involvement of tyrosine phosphorylation for the endocytotic step following insulin binding. Thus, in obese and Type 2 diabetic patients, the delay in insulin internalization could be concomitant with the defect in tyrosine kinase activity.

Conclusions

The data we have reviewed indicate that insulin binds to a cell surface receptor. The complex then becomes involved in a series of steps that regulates both the ligand concentration and the receptor concentration on the cell surface and could participate in the transmission of the biological signal. This sequence of events is very similar for the interaction of polypeptide growth factors with their target cells, and what is most surprising they also closely resemble what is observed in the case of many other unrelated ligands such as low density lipoproteins, toxins, lectins and viruses. An understanding of the cell biology of the low density lipoprotein receptor has gone a long way to explain the regulation of blood cholesterol. Therefore, it seems reasonable to expect that a better understanding of the cell biology of insulin action and of insulin receptor regulation will contribute to a better understanding of disease states such as diabetes.

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