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Minkowski Award, 1987, Leipzig

Dr. Carpentier obtained his M.D. degree in 1972 from the University of Liège, Belgium. Interested in the morphological aspects of the mode of glucagon secretion, he moved to Geneva, Switzerland to work with Dr. Lelio Orci. A collaboration with Dr. Phillip Gorden changed the orientation of his scientific carrier sinc, he succeeded in localizing the insulin receptor at the electron microscope level by quantitative autoradiography and in showing that following binding to a surface receptor, insulin entered its target cells. Subsequent studies allowed Dr. Carpentier to dissect the pathway followed by insulin inside the cell, to track the fate of the insulin receptor in the absence or presence of insulin, to identify the physiological implications of the intracellular journey of insulin and its receptor, and to discover abnormalities of these processes in pathological conditions such as diabetes or insulin resistance. His more recent work has focused on dissecting the molecular and cellular mechanisms governing insulin receptor internalization in normal and pathological conditions. Dr. Carpentier is currently Associate Professor in the Department of Morphology of the University of Geneva.

Insulin receptor internalization: molecular mechanisms and physiopathological implications

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Summary The initial interaction between insulin and its receptor on target cell surface is followed by a series of surface and intracellular steps which participate in the control of insulin action. Abnormalities of any of these steps could result in mishandling of the receptor leading to defective modulation of receptor number on the cell surface and to inappropriate cell sensitivity to the hormone. Thus, the identification of each of these steps as well as understanding the mechanisms governing them is obligatory to unravel some aspects of the pathogenesis of insulin resistance states. This was the goal of the studies we have carried out during recent years using combined molecular and cellular biology as well as biochemical techniques. These studies allowed us to propose the following ordered sequence of events: 1) insulin binds to receptors preferentially associated with microvilli on the cell surface; 2) insulin triggers receptor kinase activation and autophosphorylation which not only results in initiation of the various biological signals leading to insulin action but also in redistribution of the hormone-receptor complex in the plane of the membrane; 3) on the non-villous domain of the cell surface, insulin receptors anchor to clathrin-coated pits through specific "internalization sequences" present in their cytoplasmic juxtamembrane domain; 4) insulin-receptor complexes are internalized together with other receptors present in the same clathrin-

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Abbreviations: NIDDM, Non-insulin-dependent diabetes mellitus; CHO, Chinese hamster ovary; EGF, epithelial growth factor.

coated pits through the formation of clathrin-coated vesicles; 5) the complexes are delivered to endosomes, the acidic pH of which induces the dissociation of insulin molecules from insulin receptors and their sorting in different directions; 6) insulin molecules are targetted to late endosomes and lysosomes where they are degraded; 7) receptors are recycled back to the cell surface in order to be reused. [Diabetologia (1994) 37 [Suppl 2]: S117–S124]

Key words Insulin receptor, endocytosis, diabetes mellitus, insulin resistance, internalization.

Following binding to a specific receptor on the cell surface, insulin enters the cell together with this receptor through a process called receptor-mediated endocytosis [1–4]. Internalization of the insulin-receptor complex is a multistep process involving surface and intracellular events. It results in the targeting of insulin to lysosomes where it is degraded and in the recycling of the receptor back to the cell surface where it will be reused [5, 6]. This intracellular journey of the insulin receptor may be implicated in the transmission of the biological signal of insulin by allowing the activated receptor to gain access to plasma membrane-inaccessible substrates, but at present, this possibility remains a subject of debate. By contrast, it is widely accepted that insulin receptor internalization participates in the control of insulin action by dissociating insulin from its receptor and inactivating insulin (and thus ending insulin action) and by modulating the number of surface insulin receptors (and thus controlling cell sensitivity to insulin) [5, 6].

Thus, a better understanding of insulin action requires the elucidation of the cellular and molecular mechanisms of insulin receptor internalization and recycling. In the present review (for a more extensive review see [6]), I will focus on recent findings which improve our knowledge of these mechanisms and will consider the physiopathological implications of these events in NIDDM and insulin resistance states.

Triggering of insulin receptor internalization

Since 1979, a series of studies has pointed out that insulin-induced down-regulation of surface insulin receptors and insulin receptor internalization were related processes thus suggesting that insulin receptor internalization was insulin-induced [7–10]. On the other hand, others suggested that insulin simply shared, after binding to surface receptors, a continuously operating process [11]. These discrepancies could be linked to lack of straightforward relationship between down-regulation and internalization since receptor recycling also participates in the control of surface receptor number. In an attempt to eliminate this parameter, we blocked recycling by monensin and looked for a correlation between internalization and down-regulation. In the absence of insulin, monensin only slightly reduced the number of surface insulin receptors [12]. By contrast, when the experiments were repeated in the presence of

10^{-7} mol/l insulin, a significant reduction of the number of surface receptors was noted (Fig. 1) [12]. Taken together with the observations that the rate of insulin receptor internalization is accelerated following the addition of insulin to FAO cells [13], these results demonstrate that insulin receptor internalization is induced by insulin binding.

The discovery that insulin binding to the α-subunit of the receptor results in activation of a tyrosine kinase intrinsic to the β-subunit and to autophosphorylation of the receptor, led to the hypothesis that these enzymatic activations mediated insulin-induced internalization of its receptor. Since earlier data collected on this subject were controversial [14–19], we reconsidered the question by following directly, at the electron microscopic level, ^{125}I-insulin internalization in a battery of CHO cells expressing human insulin receptors which had been mutated such that kinase activation was affected in several different ways [20, 21]. In cell lines with a defect of insulin receptor kinase activation, ^{125}I-insulin internalization was inhibited compared to that observed in cells expressing normal human insulin receptors or insulin receptors with mutations that do not affect kinase activation (Fig. 2) [20, 21]. Further studies revealed that kinase activation was not sufficient to promote insulin receptor internalization in response to insulin

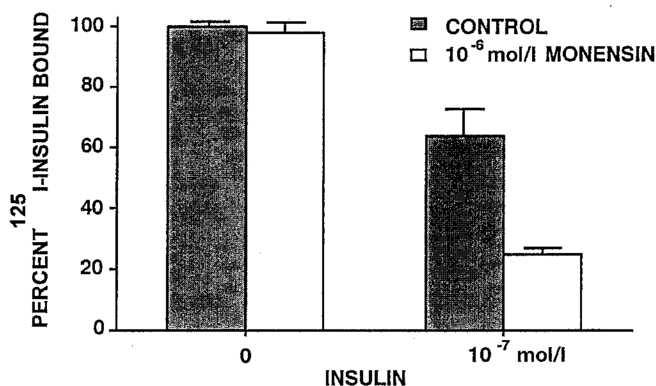

Fig. 1. Effect of monensin and insulin on the down-regulation of insulin receptors on U-937 monocytes. Adapted from Carpentier et al. [12]

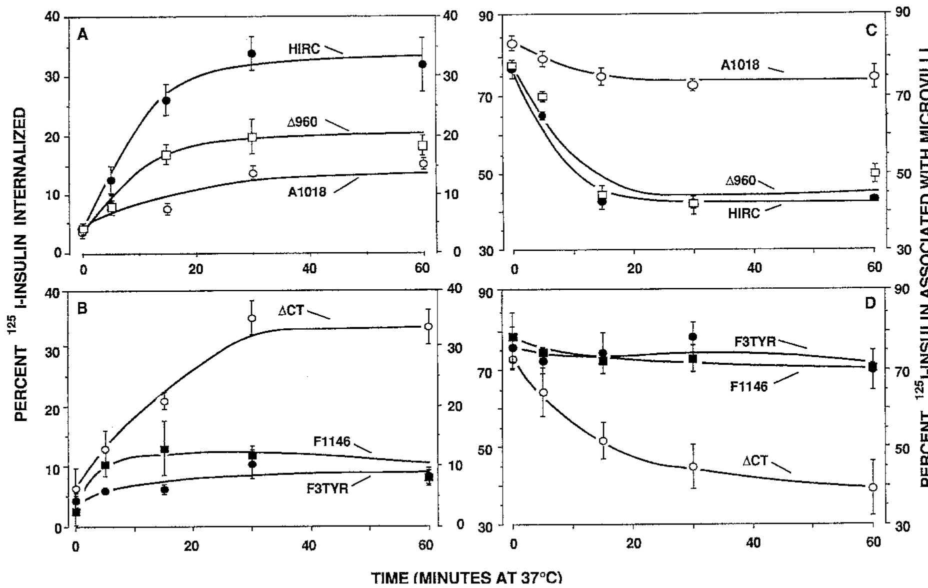

Fig. 2A–D. A and **B** ^{125}I-insulin internalization in CHO cells transfected with normal (HIRC) or mutated (so as to affect receptor kinase activation or receptor autophosphorylation) insulin receptors. **C** and **D**: Surface redistribution of ^{125}I-insulin on CHO cells transfected with normal (HIRC) or mutated (the others) insulin receptors. Adapted from Carpentier et al. [21]

binding and that autophosphorylation of three tyrosine residues of the regulatory or kinase domain of the molecule was required [20, 21]. In contrast, the two tyrosines present in the C-terminal tail and which are autophosphorylated in response to insulin binding, are not required for internalization since a receptor with a deletion of the last 43 amino acids of the β-subunit (ΔCT) was internalized normally (Fig. 2) [20, 21].

Stimulation by phorbol myristate acetate or diacylglycerol of protein kinase C, also increased insulin receptor internalization [22, 23]. This effect applies to many other receptors including those which do not require ligand binding to be internalized but, in the case of the insulin receptor, the stimulation was only observed when the receptor was occupied by insulin [22]. This suggests that activation of protein kinase C acts at a relatively non-specific step of the internalization process [22]. Together with the observation that binding of the chemoattractant formyl methionyl leucyl phenylalanine (fMLP) to its receptor induced an increased internalization of CR1 (C3b receptor) in human neutrophils via production of diacylglycerol and stimulation of protein kinase C, these data suggest the existence of cross-talks between receptors of different origins allowing interreceptor control of receptor-mediated endocytosis via protein kinase C activation [24].

Cell surface events controlling insulin receptor internalization

The demonstration that kinase activation and receptor autophosphorylation were required for insulin to trigger internalization of its receptor could appear intriguing inasmuch as many receptors which do not contain a tyrosine kinase and which are not autophosphorylated are nevertheless efficiently internalized via clathrin-coated pits. But, as proposed by Goldstein et al. [4] and Brown et al. [25], surface receptors can be subdivided in two classes. Class I receptors, including transport protein receptors i. e. transferrin or LDL receptors, are spontaneously present in clathrin-coated pits and cycle continuously between the surface and the interior of the cell. By contrast, class II receptors which include many polypeptide hormone receptors, i. e. insulin or EGF receptors, are outside clathrin-coated pits in their unoccupied form, and require binding of their specific ligands to trigger their internalization [6, 25]. These two classes of receptors differ also by their kinase activity and their capacity to be autophosphorylated [6]. Receptor kinase activation could thus be the motor allowing surface redistribution of class II receptors which would represent the ligand-specific step of the internalization process. This has been confirmed since ^{125}I-insulin redistributed from microvilli to the non-villous domain of CHO cell expressing normal human insulin receptors at 37 °C, whereas this shift was not observed in cells expressing insulin receptors with an inactive kinase (A1018) or following substitution of the tyrosines of the regulatory or kinase domain of the receptor (F1146, F3TYR) (Fig. 2) [20, 21]. Thus, surface redistribution of the insulin receptor from microvilli to the non-villous surface is ligand-dependent and mediated by receptor kinase activation and autophosphorylation of specific tyrosine residues of the regulatory domain of the β-subunit.

Cytoskeleton elements which are particularly abundant in microvilli could be involved in insulin receptor surface redistribution. Their role could be to maintain the unoccupied receptors on microvilli. The role of insulin would either be to release this

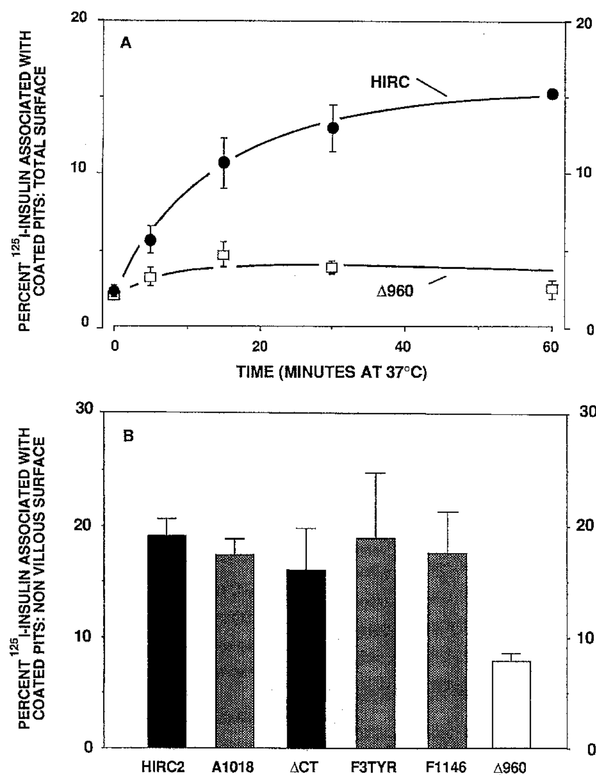

Fig. 3 A, B. Clathrin-coated pits association of ^{125}I-insulin on the surface of CHO cells expressing normal human insulin receptors (HIRC) or mutated insulin receptors (the others). Results are expressed either in terms of total autoradiographic grains associated with the cell surface (**A**) or in terms of the fraction of these receptors which are present on the non-villous domain of the plasma membrane (**B**). Adapted from Carpentier et al. [21]

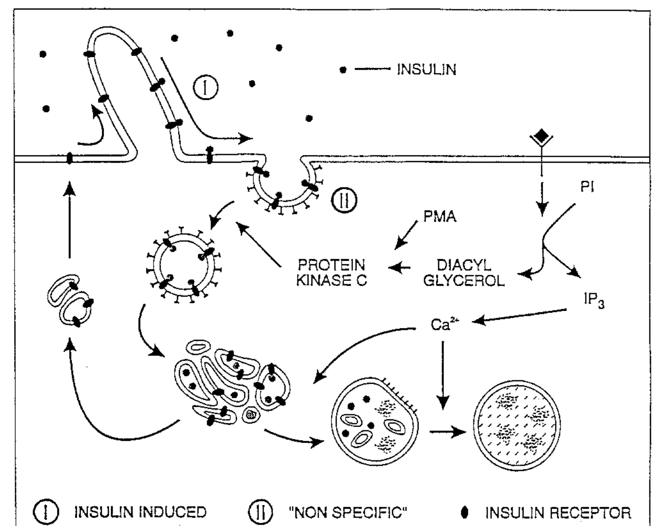

Fig. 4. Schematic representation of the intracellular pathway of insulin and its receptor following their interaction on the cell surface. The various sites of regulation of the internalization process described in the text are illustrated. PMA, Phorbol myristate acetate

brake and free the insulin receptor in the plane of the membrane or be more active and participate in driving the receptor from the microvilli down to the non-villous domain of the cell surface.

Following their shift to the non-villous domain of the cell surface, insulin receptors are sequestered into clathrin-coated pits which represent the internalization gates. The major constituents of the clathrin coat are clathrin triskelions which are three-legged structures consisting of three heavy chains (≈ 190 kDa) and three tightly associated light chains of two classes (≈ 23 kDa and ≈ 27 kDa) [26]. Clathrin-coated pits do not discriminate receptors since representatives of the two receptors classes concentrate in the same clathrin-coated pits before being internalized [27]. Thus, all these receptors must contain some common recognition signal(s) which allows anchoring to these specialized surface domains. Recent studies have identified tetra- or hexapeptidic internalization motifs included in the cytoplasmic tail of a series of receptors. They have in common one or two aromatic amino acids (preferentially tyrosines) presented in the context of a β-turn [28–34]. Recent evidence suggest that these amino acid sequences interact with adaptor proteins which represent the second major class of clathrin-coat proteins [35–37]. Two such internalization motifs are present in the juxtamembrane cytoplasmic domain of the insulin receptor [38–41]. To get further insight on the exact role of one of them (NPEY) [38–42], we analysed the consequence of a deletion of 12 amino acids of the juxtamembrane cytoplasmic domain including the NPEY sequence (CHO.Δ960) on the surface localization of this receptor [21]. This mutated receptor has an active kinase and, consequently, was normally redistributed from microvilli to the non-villous domain of the cell in response to insulin binding (Fig. 2). Whereas normal insulin receptors progressively concentrated in clathrin-coated pits as a function of incubation time at 37 °C, this type of concentration was not observed in the case of insulin receptors expressed in Δ960 cells which results in an impaired internalization (Fig. 3) [21]. The deficient association with clathrin-coated pits was particularly evident when the morphological quantification was restricted to autoradiographic grains present on the non-villous domain of the cell (Fig. 3B).

Thus, the entry of insulin receptor inside target cells is preceded and governed by a series of surface events including: a) preferential association of the unoccupied receptor with microvilli; b) surface redistribution from microvilli to the non-villous domain of the cell surface; and c) anchoring to clathrin-coated pits where insulin receptors are sequestered with other receptors which are to be internalized. Two of these key steps (surface redistribution and association with clathrin-coated pits) depend on different domains of the receptor β-subunit (respectively the kinase and the juxtamembrane domain) and have dif-

ferent levels of specificity [21] (Fig. 4). The surface redistribution is ligand-specific and depends on insulin-induced receptor kinase activation and autophosphorylation of tyrosines 1146, 1150 and 1151, whereas the association with clathrin-coated pits is ligand-independent and is common to many receptors which in their cytoplasmic domain possess appropriate internalization motifs.

In the absence of kinase activation i. e. in cells expressing kinase-deficient insulin receptors, a small proportion of ^{125}I-insulin is internalized at a slower rate than in cells expressing kinase-sufficient insulin receptor (Fig. 2). This endocytotic process was followed by tagging the insulin receptor with a ^{125}I-labelled monoclonal antibody directed against the α-subunit which did not activate insulin receptor kinase. Morphological analysis carried out with this tool confirmed that in the absence of kinase activation, the vast majority of the receptors remained associated with microvilli and showed that the few receptors seen on the non-villous domain of the cell exhibited the same propensity as insulin-activated receptors to associate with clathrin-coated pits [43]. These results indicate that kinase activation is not required for insulin receptor to anchor to clathrin-coated pits and that constitutive internalization of this receptor occurs through the same internalization pathway (clathrin-coated pits) as that followed by insulin-triggered receptor.

In addition to the two above-mentioned domains of the insulin receptor, other receptor domains or factors extrinsic to the receptor could also participate in the control of insulin receptors entry into the clathrin-coated pit/clathrin-coated vesicles/endosome continuum. These include: receptor mobility on the cell surface, interaction of the unoccupied receptor with cytoskeleton elements of microvilli, aggregation of insulin receptors, interaction of insulin receptors with other surface receptors or exposed molecules, structure of the transmembrane domain of the receptor, glycosylated side chains, etc. Studies are in progress to determine the possible implication of these additional parameters in the control of insulin receptor internalization.

Regulation of the intracellular steps of insulin receptor internalization

Subsequent to its entry inside the cell through pinching off of clathrin-coated pits which results in the formation of clathrin-coated vesicles, the insulin-receptor complex is delivered to a network of tubules and vesicles termed endosomes [5, 6]. The gradual acidification of the endosomal lumen allows the dissociation of insulin from its receptor and the targetting of the components of the complex in different directions: insulin is routed to late endosomes and lysosomes where it is degraded while the insulin receptor is recycled back to the cell surface from where it can start a new journey inside the cell and back [5, 6] (Fig. 4). A number of intracellular steps occurring in the course of receptor-mediated endocytosis (such as the assembly of the clathrin-coat, the formation of clathrin-coated vesicles, the fusion of endosomal vesicles, etc.) were recently reconstituted in vitro using cell-free or broken-cell systems [44]. However, even if progress has been made, many of the mechanisms controlling these intracellular events remain poorly defined. In the case of polypeptide hormone and growth factor receptors the amount of information on this topic is particularly limited [45].

Varying the cytoplasmic free calcium concentration does not affect insulin receptor internalization [22]. In contrast, when $[Ca^{2+}]_i$ is lowered 10 times below normal resting levels both insulin degradation and insulin receptor recycling are inhibited [46]. As determined morphologically, these functional changes are accompanied by a reduced association of internalized ^{125}I-insulin with lysosomes and an increased association with endosomes which suggests that, as shown in other situations, $[Ca^{2+}]_i$ controls the fusion of early and late endosomes. These observations confirm that sorting of insulin and its receptor occurs at the early endosomal stage and that insulin has to be transferred to a subsequent compartment (late endosome, lysosome) in order to be fully degraded (Fig. 4).

Concerning the sorting mechanisms which allow signalling receptors to escape degradation in lysosomes and instead be rapidly recycled, kinase seems to play a role. Indeed, kinase activation is required for the sorting of EGF receptors towards lysosomes whereas kinase-inactive EGF receptors are rapidly recycled [47] and recent studies have proposed that the phosphorylation of annexin I could be involved in this process [48]. Indirect evidence also suggests that the lack of dephosphorylation of insulin receptors results in their targetting to lysosomes. Indeed under photoreactive insulin was covalently bound to its receptor by exposure to ultraviolet light, the complex was routed to lysosomes from where recycling was slow and inefficient [49, 50]. This mistargetting could be due to the fact that, under these conditions, the undissociable insulin maintains the receptor kinase in an active state which would prevent the receptor from being appropriately sorted and recycled. Experiments showing that a specific point mutation of the insulin receptor leads not only to a defect of insulin dissociation at acidic pH but also to an increased degradation of the receptor could be similarly interpreted. Altogether, these observations suggest that insulin receptor dephosphorylation is required for recycling.

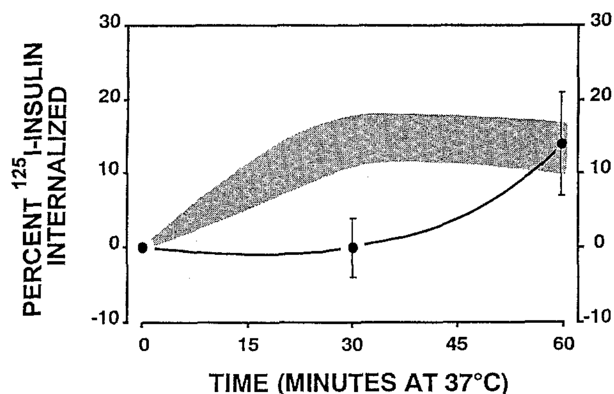

Fig. 5. Comparison of ^{125}I-insulin internalization in monocytes freshly isolated from NIDDM patients (•–•) and from normal volunteers (hatched area). Adapted from Grunberger et al. [52]

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

Abnormal functioning of the insulin receptor is involved in both extreme and moderate insulin resistances. Among the two major forms of extreme insulin resistance (immunologic and genetic) those of genetic origin (accompanied with mutations of the insulin receptor) are a good example of a direct implication of the insulin receptor in the pathological process. According to Taylor [51], mutations can induce insulin resistance by interfering either with: a) synthesis of the receptor (class 1), b) transport of the receptor to the cell surface (class 2), c) ability of the receptor to bind insulin (class 3), d) capacity of the receptor to transduce insulin signal (deficit in tyrosine kinase activity) (class 4), e) rate of internalization and recycling of the receptor (class 5).

We studied ^{125}I-insulin internalization in peripheral monocytes freshly isolated from patients with extreme insulin resistance due to a defective tyrosine kinase activity (class 4) and found a significantly reduced internalization of the ligand [52]. Preliminary observations of ^{125}I-insulin internalization in cultured cells transfected with insulin receptors with mutations reproduced from those observed in extreme insulin resistance states confirm a defect of ^{125}I-insulin internalization in situations with a defect of kinase activation. The inability of weakly activated receptors to gain access to potential intracellular substrates could contribute to the pathogenesis of insulin resistance.

An example of class 5 mutations has recently been reported by Kadowaki et al. [53]. It consists in a mutation of Glu^{460} detected in insulin receptors from a patient with leprechaunism. This mutation impairs insulin dissociation from its receptor at the acidic pH of endosomes. This lack of dissociation perturbs receptor recycling and favours its targetting to lysosomes where it is degraded. This increased degradation of the receptor leads to a decreased number of surface receptors and hence to reduced cell sensitivity to insulin and insulin resistance [54].

The most widely spread form of moderate insulin resistance is NIDDM. This pathological condition is characterized not only by insulin resistance but also by insulin deficiency [55–57]. Defects in insulin action in NIDDM may result from both decreased receptor tyrosine kinase activity and defects in postreceptor steps [58–60]. In monocytes freshly isolated from patients with NIDDM, ^{125}I-insulin internalization was impaired [52] (Fig. 5). Such inhibition could be linked to the defect in receptor autophosphorylation but, as mentioned, the decreased ability for the receptor to gain access to intracellular substrates could also be, at least in part, responsible for the pathological process.

In the case of insulin-dependent diabetes mellitus, insulin receptor internalization is clearly perturbed [61] but recent observations indicate that this abnormality is part of a general perturbation of all endocytotic processes [62]. This impairment of endocytosis found in hypoinsulinaemic diabetes leading to a decrease of the uptake of LDL by liver cells could be responsible, at least in part, for the increase in blood cholesterol observed in some diabetic patients. Similarly, the dramatic inhibition of fluid phase endocytosis could contribute to extracellular matrix accumulation of macromolecules normally taken up by this pathway.

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