Rapid Effects of Insulin on the Cycling of the Insulin Receptor in a Human Monocyte Cell Line (U-937)

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SUMMARY

The insulin receptor and its regulation by insulin was studied in U-937 monocytes, a human cell line with properties similar to those of normal peripheral blood monocytes. Treatment of this cell with insulin for 8–16 h produced an overall loss in the insulin receptor, i.e., a loss of receptors from the cell surface and internal pools. In contrast, short-term insulin treatment (15–30 min) caused a reduction in cell surface receptors but an increase in the internal receptors, as judged by pronase treatment at 4°C to distinguish receptor location. After the removal of insulin and pronase, the internalized receptors were rapidly reinserted back into the cell surface after warming to 37°C.

Further studies showed an insulin-mediated increase in fluid-phase pinocytosis as measured by horseradish peroxidase (HRP) uptake. The amount of HRP accumulation and the time course for this stimulation were similar to those for receptor internalization. These features plus other results suggest that the insulin-stimulated internalization of insulin receptors may require an acceleration in the rate of pinocytic vesicle formation. DIABETES 1985; 34:403–408.

The peripheral monocyte is the most accessible cell in man that contains a significant number of insulin receptors. Unlike erythrocytes, which have few receptors,[1] or fibroblasts, which must be established in tissue culture,[2] monocytes are readily isolated in amounts that allow an accurate assessment of the number and binding affinity of the insulin receptor in normal and abnormal conditions.[3] Although a large number of studies have been done with this cell type, they have focused almost entirely on the hormone binding characteristics of the receptor. Consequently, little direct information is available regarding the fundamental processes that control the insulin receptor in the monocyte. The primary reason for this lack of information is the difficulty in isolating enough monocytes to conduct such investigations.

Because of this limitation, we undertook studies of a human monocyte-like cell line (U-937). This cell line has insulin receptors that demonstrate typical insulin binding characteristics[4] and tyrosine kinase activity[5] in addition to having other features of circulating monocytes.[6–11] In the present report, the effect of insulin on the movement of the insulin receptor into the cell interior and its reappearance on the cell surface was examined. We also examined fluid-phase pinocytosis and the effect of insulin on this process. These studies demonstrated a rapid cycling of the insulin receptor after insulin stimulation, which occurs in conjunction with an increase in fluid-phase pinocytosis.

MATERIALS AND METHODS

Materials. RPMI 1640 Medium, medium 199, penicillin, and streptomycin were purchased from Gibco Laboratories, Grand Island, New York. Sterile Systems Inc. (Logan, Utah) supplied fetal calf serum. Porcine insulin was a gift from Eli Lilly and Company, Indianapolis, Indiana. Na-^{125}I and Triton X-100 were obtained from New England Nuclear, Boston, Massachusetts. Fischer Scientific (Silver Springs, Maryland) supplied n-butylphalate oil. Horseradish peroxidase was supplied by Boehringer Mannheim, Indianapolis, Indiana.

U-937 cell line. The U-937 cell line was obtained originally from Dr. Peter Ralph, Sloan-Kettering Institute for Cancer Research, Rye, New York. The cells were maintained at a concentration of 0.5–1.2 × 10^6 cells/ml in a continuous spinner culture in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.[6] Studies were performed on cells taken from cultures late in the logarithmic phase of growth.

Insulin binding assays. Insulin binding studies to intact cells (e.g., cell surface binding) were conducted in an Earles balanced salt solution that contained 5 mM Tris-HCl, pH 7.4, and 0.1 g/dl bovine albumin. Approximately 2 × 10^6 cells

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TABLE 1
Insulin receptor of U-937 monocytes

Insulin binding characteristics
Curvilinear Scatchard plot:
High-affinity component: Kd of 0.5 nM, 3900 sites/cell
Low-affinity component: Kd of 50 nM, 26,000 sites/cell
Insulin competition studies: 50% displacement by 1 nM insulin
Presence of "negative cooperativity," i.e., accelerated dissociation of bound ^{125}I-insulin by native hormone
pH optimum of 8.0 for insulin binding
Structural characteristics
Stokes radius of 69 Å by Sepharose 6-B chromatography
Affinity-labeling techniques demonstrate:
Insulin binding subunit of M_r 125,000
Holo (nonreduced) receptors of M_r 280,000 and 300,000

were incubated in 350 μl with ^{125}I-insulin (5×10^{-11} M) in the absence or presence of the indicated concentration of native insulin and at the indicated temperature. The binding assay was terminated by layering the cells over 100 μl of n-butylphthalate oil and centrifugation in a Beckman microfuge for 3 min. The cell pellet was excised and the radioactivity determined by gamma counting.

The ^{125}I-insulin (0.8 Ci/μmol) was prepared as previously described using the chloramine-T method.[12] Insulin degradation was monitored by trichloroacetic acid precipitation.[13]

Insulin binding by the Triton X-100 solubilized receptor was determined using the polyethylene glycol precipitation method previously described.[14]

Studies of insulin regulation of the insulin receptor. The cells were washed and resuspended under sterile conditions in medium 199 that contained 0.1 g/dl bovine albumin and 1 mg/ml bacitracin used to inhibit insulin degradation. The incubations with insulin were carried out at 37°C under 5% CO_2/95% air for the indicated period of time in the presence of 0.1 μM insulin. After the incubation, the insulin was removed using an acid buffer composed of 0.9% sodium chloride, 15 mM sodium phosphate, and 0.1% bovine albumin, pH 6.0,[15] and two washes at 37°C. After the second wash, the cells were incubated for 15 min at 37°C in the pH 6.0 buffer before centrifugation and resuspension in the buffer used for the insulin binding studies.

Details of the pronase treatment used to examine the movement of the receptor after insulin stimulation are given in the legends to the figures.

Uptake of horseradish peroxidase (HRP). These studies were carried out under the conditions used to investigate the effects of insulin on the movement of the insulin receptor. Briefly, 25×10^6 cells were suspended in 1 ml of ice-cold medium 199 that contained 0.75 mg of HRP and the indicated concentrations of insulin. Uptake of HRP was begun by rapidly warming the cells to 37°C; the incubation was then terminated by the addition of ice-cold medium 199. Extracellular HRP was removed by washing the cells four times at 4°C with medium 199. In studies of the insulin dose-response effect on HRP uptake, the cells were incubated with insulin for 12 h at 4°C before conducting the uptake studies.

After removal of extracellular HRP, the cells were lysed by the addition of 125 μl of 2% (vol/vol) Nonidet P-40 to the cell pellet. Particulate matter was removed by centrifugation and the HRP activity in the supernatant measured as described by Steinman and Cohen.[16]

RESULTS

Characteristics of the U-937 insulin receptor. The insulin binding characteristics and certain structural features of the insulin receptor of U-937 monocytes are summarized in Table 1. These findings agree with those recently reported by Robert et al.,[4] who examined the insulin binding properties of the U-937 cell. Other features of this insulin receptor, including its structural characteristics, are similar to the insulin receptors found in other cell types.[17-20]

Effects of insulin treatment on insulin binding activity and receptor location. A prominent feature of several insulin target cells is the loss of insulin receptors after treatment with a high concentration of insulin, i.e., insulin "downregulation."[21] Figure 1A demonstrates the effect of treating U-937 cells for 16 h with 0.1 μM insulin. This treatment reduces high-affinity insulin binding activity by approximately 60%, although there is little change in low-affinity binding. In Figure 1B, the effect of insulin treatment on Triton X-100 soluble insulin binding is shown. As illustrated, the amount of insulin binding activity extracted from the U-937 cell is markedly reduced by insulin. The reduction is approximately 50%,

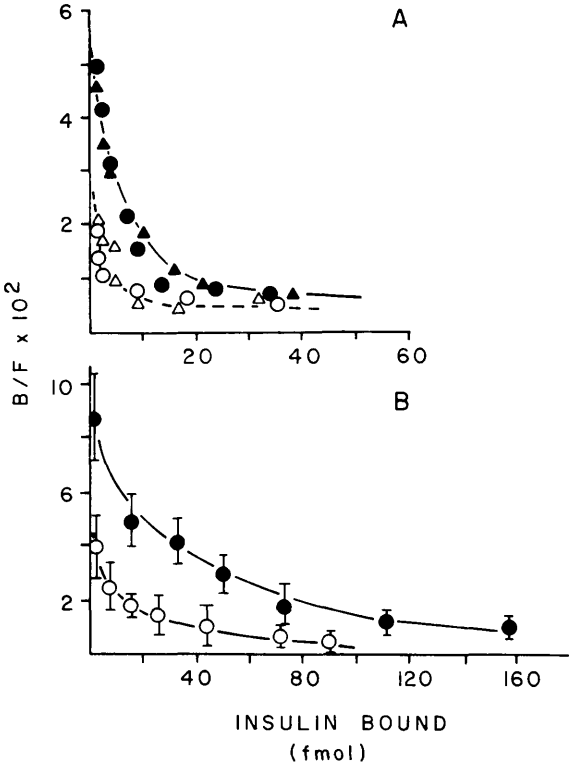

FIGURE 1. Effect of chronic insulin treatment on insulin binding activity of U-937 cells. (A) Cells were incubated for 16 h in the presence (open symbols) or absence (closed symbols) of 0.1 μM insulin. Insulin binding to the intact cells was measured as described in the text. The results are from two separate experiments. (B) Cells were incubated with (○) or without (●) 0.1 μM insulin as in panel A. The cells were then solubilized with Triton X-100 and insulin-binding activity in the soluble fractions was measured (see text). The results are the mean ± SEM of four separate experiments.

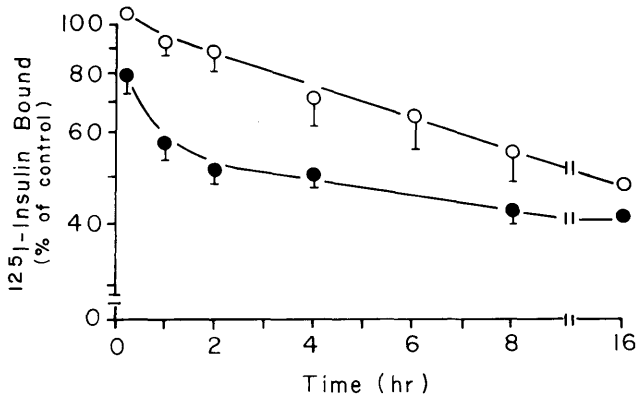

FIGURE 2. Time courses for the insulin-mediated loss of cell surface receptors and total receptor activity. Cells were treated with 0.1 μM insulin for the indicated times. The cells were then washed and insulin binding to the intact cells (●) was determined. Conversely, the cells were solubilized with Triton X-100 and insulin binding to the solubilized extract (i.e., total receptor activity) was measured (○) as described in the text. The results are expressed as percent of control (100) obtained from cells incubated for the appropriate times in the absence of insulin. The data are the mean ± SEM of three separate experiments.

which agrees with the loss of cell surface high-affinity insulin binding activity.

Additional studies were conducted to further characterize the effects of insulin on the receptor. Figure 2 illustrates the time course for the loss of cell surface binding activity after insulin treatment and contrasts these effects with the time course for the change in total receptor content as determined by Triton X-100 extraction. Loss of cell surface receptors is very rapid after insulin addition. The rapid loss is followed by a more gradual decline in surface binding, which reaches a "steady-state" level between 8 and 16 h. In contrast, total receptor content is not affected by insulin treatment during the first 30 min when cell surface receptors are rapidly lost. Total binding slowly decreases after 2 h of insulin treatment and approaches the relative amount lost from the cell surface at about 8 h.

To determine if the cell surface receptors were being driven into the cell interior during the early phase of insulin treatment, studies with pronase were conducted (Figure 3). In these experiments, the cells were treated with insulin for various periods of time, then they were washed and chilled to 4°C. These cells were then incubated with pronase at 4°C as described by Karin and Mintz,[22] which destroys 94% of the insulin binding activity (see legend to Figure 3). Figure 3 shows that the insulin treatment rapidly increases, in a time-dependent fashion, the amount of insulin binding activity that is protected from the protease enzymes. Within 15 min of insulin treatment, the internal insulin binding activity increased by 1.9-fold. Note that the cells normally have internal receptors in the absence of insulin treatment that account for approximately 10% of the total receptor population (see legend to Figure 3). Thus, insulin treatment causes a rapid redistribution of the receptors between the cell surface and the internal compartment.

Figure 4 demonstrates that the internalized receptors can be reinserted on the cell surface. In these studies, the cells were treated with insulin for 30 min to move receptors into the cell interior. They were then treated with pronase at 4°C, washed, and rapidly warmed to 37°C for the indicated period of time in the presence of cycloheximide. The cells were then chilled to 4°C to inhibit further movement of receptors and insulin binding was evaluated at this temperature. The results indicate that, within 15 min at 37°C, the internalized receptors moved from a pronase-protected environment to the cell surface. The effect peaked at 30 min and then decreased at 60 min. At 30 min, the cells previously treated with insulin had almost a sixfold greater amount of cell surface receptors than untreated cells.

It is possible to argue that cell surface receptor binding of insulin confers protection from pronase action. However, in studies of this possibility, we found that pronase treatment of the cells in the presence of 0.1 μM insulin rapidly destroyed cell surface binding activity to essentially the same extent as nontreated cells (data not shown). Moreover, in an experiment similar to the one shown in Figure 3, insulin-bearing cells that were not warmed and consequently had little internalized receptor gave the same findings as the control (non-insulin-treated) cells. Finally, the time dependency of the increase in cell surface binding with 37°C incubation argues that the effect depends on a process in-

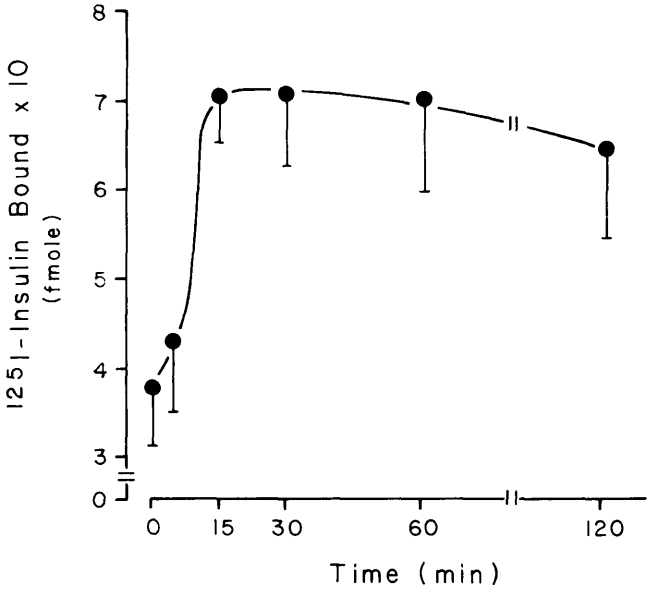

FIGURE 3. Effects of insulin treatment on internal insulin-binding activity. U-937 cells were treated with 0.1 μM insulin at 37°C for the indicated period of time. The cells were then washed to remove insulin and chilled to 4°C. Pronase (100 μg/ml) was added and the incubation continued at 4°C for 2 h. (This treatment reduced cell surface binding from 2.0 ± 0.05 fmol insulin bound per 4 × 10^6 cells to 0.13 ± 0.01 fmol using a ^{125}I-insulin concentration of 75 pM.) Pronase was removed by three buffer washes at 4°C and the cells were solubilized with Triton X-100. Insulin binding was measured in the detergent extract by incubating the soluble receptor for 16 h at 4°C with 75 pM of ^{125}I-insulin. In the absence of the pronase treatment, total insulin binding in the Triton X-100 extract of control cells (4 × 10^6) was 2.1 ± 0.2 fmol. Pronase treatment of control cells reduced the binding activity to 0.22 ± 0.1 fmol, indicating that the internal pool of insulin receptors is approximately 10% of the total cellular receptor content. The presence of insulin (0.1 μM) during the 4°C treatment of the cells with pronase did not affect the results, i.e., insulin bound to the receptor does not protect the receptor from the effects of pronase. The results are expressed as the mean ± SEM of eight separate determinations.

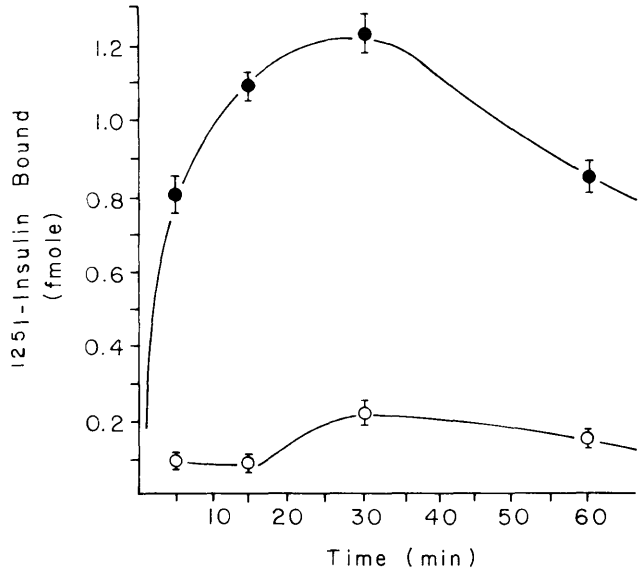

FIGURE 4. Reinsertion of insulin receptors on the surface of U-937 cells. The cells were incubated with (●) or without (○) insulin (0.1 μM) for 30 min at 37°C. They were then chilled to 4°C and treated for 2 h with 100 μg/ml pronase. The cells were washed and resuspended in medium 199 that contained 0.1 mM cycloheximide and then warmed to 37°C for the indicated period of time. They were then rapidly chilled to 4°C to inhibit further movement of receptors, and insulin-binding studies were conducted with the intact cells at this temperature. In other control studies, cells treated with insulin (0.1 μM) at 4°C rather than at 37°C and then subjected to the same protocol as above had similar cell surface binding activity as the control cells (○). The results are the mean ± SEM of three separate determinations.

trinsic to the U-937 monocyte. For these reasons, it is not possible to explain the findings by invoking insulin protection of the cell surface receptor from pronase digestion.

Effect of insulin on fluid-phase pinocytosis. Since cell surface proteins are generally felt to enter the cell via the formation of endocytotic vesicles,[23] we undertook studies to examine the rate of fluid-phase pinocytosis and to determine whether this process is influenced by insulin treatment. Horseradish peroxidase (HRP) was chosen as the extracellular marker to monitor fluid-phase pinocytosis.[24] Preliminary studies showed that the amount of HRP taken up by the cells during a 30-min period was directly proportional to the concentration of enzyme in the medium, using concentrations that ranged from 75 μg/ml to 1.5 mg/ml (data not shown). Furthermore, HRP did not absorb onto the cells; after a 30-min incubation at 4°C followed by the normal wash procedure, no enzyme activity was detected associated with the cells. Thus, these findings indicate that the uptake of HRP is by fluid-phase pinocytosis and not by adsorptive pinocytosis.

Figure 5 illustrates the effect of insulin on HRP uptake. With warming of the cells to 37°C, there is an initial burst of uptake, an observation also made in other cell systems.[23] In the absence of insulin, the short period of acclerated uptake was followed by a period of slow accumulation of HRP. Insulin treatment, however, elicited a continued accelerated uptake of HRP over a 20-min period. After 30 min, the rate of uptake slowed to the rate found with the control cells. It is interesting to note that the amount of internalized HRP after 15 min of incubation is approximately twofold greater in insulin-treated cells than in controls, which agrees with the twofold increase in the internal receptor pool found at this time point.

The dose-response relationship for the insulin effect is shown as an insert in Figure 5. Half-maximal stimulation is obtained with an insulin concentration of approximately 1 nM, the Kd value for insulin binding as measured by insulin competition experiments. These results suggest that accelerated pinocytosis is initiated by the binding of insulin to the insulin receptor.

The U-937 monocyte also has an Fc receptor,[7,8] which, like that of normal circulating monocytes, fails to internalize monomeric IgG.[25] We therefore examined the effect of IgG (1 mg/ml) on HRP uptake. In the presence of IgG, HRP uptake was modestly inhibited, i.e., 0.27 ± 0.002 ng/15 min/10^6 cells versus 0.34 ± 0.003 for IgG-treated and control cells, respectively, mean ± SEM of three experiments. Therefore, at least in the two instances we have examined, a positive correlation exists between receptor-ligand internalization and the stimulation of fluid-phase pinocytosis.

DISCUSSION

The U-937 monocyte cell line established by Sundstrom and Nilsson[26] has many properties of circulating human monocytes. These properties include the production of lysozyme, strong esterase activity, *p*-glucuronidase activity, weak

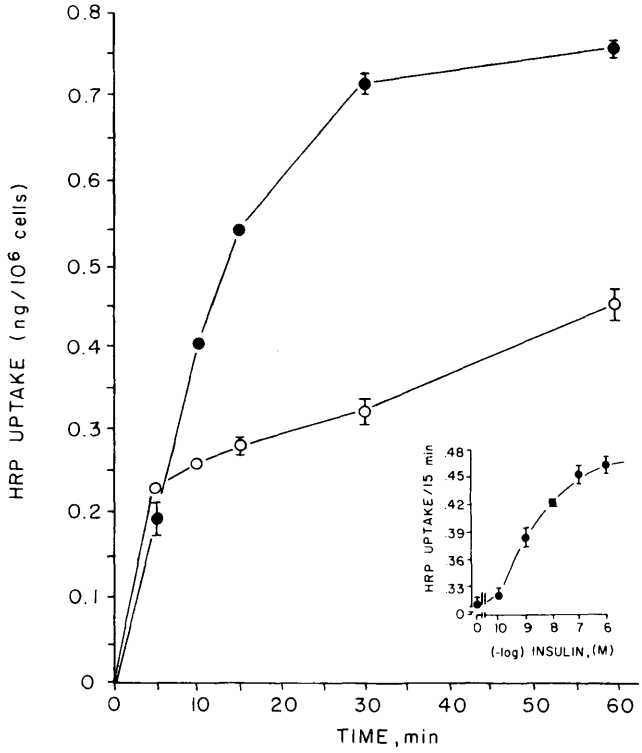

FIGURE 5. Effect of insulin on HRP uptake. U-937 monocytes were incubated at 37°C with HRP in the presence (●) or absence (○) of 0.1 μM insulin as indicated in the text. At the indicated times, the cells were rapidly chilled to 4°C and washed to remove extracellular HRP (see text). The cells were lysed and HRP activity was determined. Insert: The concentration dependency for insulin stimulation of HRP uptake. Insulin was incubated with the cells in the absence of HRP for 12 h at 4°C to insure the attainment of steady-state occupancy of the insulin receptor. HRP was then added and the cells were rapidly warmed to 37°C.

phagocytic function, and the presence of receptors for complement, IgG and IgE.[7] Furthermore, many characteristics of the U-937 Fc receptor for IgG are concordant with the receptor of normal monocytes.[6,8] The exposure to supernatants from mixed lymphocyte cultures[9–11] or to 12-tetradecanoylphorbol-13-acetate[9] results in apparent morphologic and functional differentiation of these cells to a more mature form. Such cells have macrophage-like qualities, including adherence, vigorous phagocytosis, and an increased capacity for antibody-dependent cytotoxicity.

These similarities to normal monocytes make the U-937 cell line a potentially valuable system to investigate the insulin receptor. Their value is enhanced by the extensive use of monocytes to study the human receptor, particularly since little direct information is available regarding the regulation of the monocyte receptor. As described in Table 1 and by other recent studies,[4,5] the binding properties of the insulin receptor in U-937 cells correspond closely to the known properties of the human monocyte receptor.[27,28] These findings, along with the other features of the U-937 cells, argue that it is an appropriate model in which to conduct studies of the insulin receptor of human monocytes.

The first studies were designed to examine the effect of "chronic" insulin treatment (e.g., 16 h) on the level of the insulin receptor. As reported for a number of cell systems[29] including the U-937 monocyte,[4] insulin treatment reduces cell surface insulin binding activity. Furthermore, we find that the reduction in binding was present in Triton X-100 extracts of treated U-937 cells. Therefore, insulin treatment for 16 h produced an overall loss in insulin binding activity, i.e., the effect was not caused simply by a change in the distribution of the receptors between the cell surface and an internal receptor pool.

This feature of downregulation in monocytes differs from the insulin-mediated changes reported in myocytes[30] and hepatocytes,[29] in which lysis of the cells exposes all of the insulin binding activity lost from the cell surface. In myocytes, this binding activity can be recovered even after a 20-h treatment with insulin. Thus, differences exist in the way that various cell types process the insulin receptor.

The U-937 monocyte rapidly internalizes the insulin receptor after insulin treatment. This effect begins within minutes and reaches a maximum by 15 min. Again, the monocyte differs from myocytes in that the maximum increase is a doubling of the internal receptor pool, whereas the increase in myocytes is 4–6-fold.[30]

During the first 15-min period of insulin treatment, very little loss of total receptor content in U-937 monocytes can be detected. However, by 1 h a decrease is found and this effect proceeds as an apparent first-order process until 8 h, after which further changes are minimal (see Figure 2). These results do not allow a description of the process responsible for receptor loss. However, since internalization of the receptor precedes receptor loss, the process may require the receptor to move into the cell interior to promote receptor degradation and/or the inhibition of receptor synthesis.

Although the fate of the internalized receptor probably includes its degradation, this structure has the ability to move back to the cell surface once insulin is removed from the incubation medium. Reinsertion of these receptors into the cell surface was rapid at 37°C and the expansion of the internal pool by insulin treatment allowed the cells to insert a sixfold greater number of receptors than untreated cells. It was difficult to compare the quantity of internalized receptors with the amount reinserted on the cell surface, since two different types of insulin binding assays are required and one, which uses intact cells, is more sensitive than the other. However, given this limitation, the results suggest that the majority of internalized receptors can be recycled to the cell surface.

Although other studies have presented evidence that the receptor is internalized and can recycle after binding the hormone, this concept is based in part on experiments that follow the movement of ^{125}I-insulin into the cell (reviewed in ref. 29). Some studies have used affinity labeling techniques to covalently attach ^{125}I-insulin to the receptor and have shown receptor internalization[31–33] and cycling.[34,35] These results directly show movement of the insulin receptor, although permanent attachment of the ligand to the receptor may alter some aspect of this process.

Knutson et al.[36] recently used trypsin treatment at 25°C for 5 min to examine the effect of insulin treatment on the amount of receptor internalized in 3T3-C2 fibroblasts. These findings agree with those of the present study in that insulin treatment rapidly moved insulin receptors into a trypsin-protected environment. They also deduced that the internalized receptors were reinserted on the cell surface using an indirect assessment based on the loss of internal receptors after insulin removal. Our study is the first, however, to directly quantitate the rates of internalization and reinsertion of the unmodified receptor into the cell surface by following a functional property of this structure (e.g., insulin binding).

Although the present results point out several interesting features of the insulin receptors of U-937 monocytes and their use as a model system for examining receptor recycling, they do not provide any information regarding the basic processes that act to internalize the receptor. For this reason, we conducted studies of HRP uptake, which is a measure of fluid-phase pinocytosis.[24]

The studies of the HRP uptake demonstrate a stimulatory effect of insulin that is hormone concentration dependent and quite similar to the binding kinetics of insulin to the insulin receptor. Studies with monomeric IgG showed that its binding to the Fc receptor of U-937 cells did not stimulate HRP uptake. Treatment of these cells with IgG also does not produce insulin receptor internalization (data not shown) nor is the IgG-Fc receptor complex internalized.[25] Therefore, with these two receptor systems there is a correlation between the ligand (e.g., insulin) that produces receptor internalization and accelerated pinocytosis and the ligand (IgG) that fails to stimulate either event.

Insulin-induced stimulation of HRP uptake has also been described by Gorden et al.[37] in IM-9 lymphocytes. In this study, HRP was identified by electron microscopy after an incubation period of 6 h with 1 μM insulin. The enhanced uptake occurred in conjunction with an increase in microinvagination in the cell surface and loss of insulin receptors.

In the U-937 monocyte, insulin-stimulated pinocytosis is maintained for about 15–20 min after which the rate of HRP uptake decreases to that of unstimulated cells. At this time, the amount of intracellular HRP is twofold greater in the

insulin-treated cells than in the controls. These results correlate well with the insulin-mediated increase in intracellular receptors, since the internal receptor pool rapidly doubles in size. Although these results do not prove that the insulin receptors enter by insulin-stimulated pinocytosis, because receptors were not directly demonstrated in the pinocytic vessels, they strongly support this concept.

In summary, U-937 monocytes offer an excellent model in which to investigate the movement of insulin receptors after insulin stimulation. The present studies directly show that the level of the cell surface receptor is controlled by the rates for receptor internalization and its reinsertion into the cell surface, and by a process that alters the balance between receptor synthesis and degradation. Finally, insulin treatment provokes an increase in the rate of pinocytosis that correlates with receptor internalization. Although not directly proven, an early event in receptor internalization may involve an insulin-mediated acceleration in the rate of pinocytosis.

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