Insulin Down-regulates Insulin Receptor Number and Up-regulates Insulin Receptor Affinity in Cells Expressing a Tyrosine Kinase-defective Insulin Receptor*

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We examined the effect of insulin treatment on HTC cells transfected with large numbers of either normal insulin receptors (HTC-IR) or insulin receptors defective in tyrosine kinase (HTC-IR/M-1030). In both HTC-IR and HTC-IR/M-1030 cells, 20 h of insulin treatment (1 μM) at 37 °C resulted in a 65% decrease in the number of binding sites with a reciprocal 6-fold increase in affinity. In contrast, treatment with 10 nM insulin (20 h, 37 °C) also increased receptor affinity but had a smaller effect on the number of binding sites. 125I-Insulin binding to soluble receptors from HTC-IR and HTC-IR/M-1030 cells pretreated with insulin showed results similar to those obtained in intact cells. In both HTC-III and HTC-IR/M-1030 cells, insulin enhanced insulin receptor degradation. In HTC-IR/M-1030 cells a 1-h incubation with insulin did not change receptor number and had only a small effect on receptor affinity; also there was no effect of insulin after a 20 h incubation at 15 °C. Inhibiting protein synthesis by pretreatment with cycloheximide (100 μM) did not block either the decrease in receptor number or the increase in receptor affinity. Both HTC-IR and HTC-IR/M-1030 cells exhibited a very slow rate of insulin and insulin receptor internalization and no differences were seen in this parameter when HTC-IR cells were compared to HTC-IR/M-1030 cells. These studies indicate, therefore, that in cells expressing kinase-defective insulin receptors, insulin down-regulates insulin receptor number via enhanced receptor degradation, and up-regulates receptor affinity. These effects were time- and temperature-dependent, but not dependent on new protein synthesis, and suggest that activation of tyrosine kinase may not be a prerequisite for certain mechanisms whereby insulin regulates its receptor.

The initial interaction of insulin with target cells is via a glycoprotein receptor located in the plasma membrane (1–4). This receptor is the αβ2 disulfide-linked tetrameric glycoprotein, composed of two α-subunits (130 kDa) which are extracellular and contain the insulin binding sites, and two β-subunits (95 kDa) which are transmembrane and contain tyrosine kinase activity on their intracellular domains (1–4).

One α- and one β-subunit are derived from a common precursor protein that has a minimum molecular mass of 153 kDa and is subsequently cleaved (5, 6). Some tissues produce a larger variant of the insulin receptor by alternative exon splicing (6); this variant has 12 additional amino acids at the COOH-terminus of the receptor α-subunit. When insulin binds to the α-subunit of either form of the insulin receptor, tyrosine kinase is activated and insulin action ensues (1–4).

In most cells insulin also down-regulates its receptor via two mechanisms (7, 8). First, after insulin binds to its receptor, the insulin-receptor complex is rapidly internalized thus removing the receptor from the cell surface. Second, in the cell interior, the receptor is slowly degraded (7–10) thus reducing the cellular insulin receptor content. In addition, in certain cell types incubation of insulin receptors with insulin leads to an increase in insulin receptor affinity (9, 11). Prior studies with insulin receptor tyrosine kinase mutants have suggested that tyrosine kinase activity is necessary for insulin to induce insulin receptor internalization (12–14). The relationships, however, between insulin activation of receptor tyrosine kinase, insulin-induced receptor degradation, and insulin-induced regulation of receptor affinity have not been defined. The tyrosine kinase activity of the insulin receptor β-subunit is dependent upon a key lysine residue1 at position 1030 in the ATP binding site (1). This amino acid, conserved among the protein kinase family members, is thought to provide a salt bridge to βγ-phosphates of ATP. In the present study we have transfected normal and mutated human insulin receptors into rat HTC cells via an expression vector. The mutated receptor contains a methionine in place of lysine at position 1030. This substitution inactivates tyrosine kinase activity, but since lysine and methionine have similar bond lengths and angles the overall structure of the insulin receptor β-subunit should be preserved (15). Herein we demonstrate that insulin can increase insulin receptor degradation and increase receptor affinity in HTC cells transfected with both normal and mutant insulin receptors.

EXPERIMENTAL PROCEDURES

Materials—The following chemicals were purchased: bacitracin, HEPES,2 cycloheximide, phenylmethylsulfonyl fluoride (PMSF), glucose oxidase type X, trypsin type XII, and soybean trypsin inhibitor type I-S from Sigma; lactoperoxidase from Boehringer Mannheim; bovine plasma albumin (fraction V) from Reheis, Chicago, IL; '*'I-Insulin (1 uCi/ml) from Boehringer Mannheim; bovine carbonic anhydrase type X, trypsin type XII, and soybean trypsin inhibitor (to P. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 According to the numbering system of Ebina et al. (6).
2 The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; DME, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol.
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**Introduction**

In order to measure exogenous substrate phosphorylation, 15 μl of \( ^{32}P \)-labeled insulin were added to each of the labeled and lysed cell samples. The reaction was allowed to proceed at 22°C for 30 min. The reaction was then terminated by adding 3 ml of ice-cold PBS, and the cell lysates were subjected to analysis by SDS-polyacrylamide gel electrophoresis followed by autoradiography (31, 22).

**Tyrosine Kinase Activity**

The tyrosine kinase activity of the receptor in solubilized cell lysates was assessed by measuring the autophosphorylation of the insulin receptor β-subunit using a phospho-specific antibody (25) and autoradiography. The radioactive bands corresponding to the insulin receptor β-subunit were excised from the gel and counted to determine the total radioactivity associated with the receptor. Nonspecific binding, determined in the presence of 1 μM unlabeled insulin, was subtracted to give the specific binding.

**Degradation**

The metabolic fate of the insulin receptor was assessed by measuring the rate of insulin receptor degradation in cells treated with cycloheximide. The rate of receptor degradation was determined by measuring the decrease in insulin receptor protein levels over time using Western blot analysis. The results were expressed as the percentage of the initial receptor protein level remaining at each time point.

**Insulin Receptor Internalization**

The method of Hori and Roth (13) was used. Confluent HTC-IR and HTC-IR/M-1030 cells in 150-mm dishes were washed three times with PBS, 5 ml of PBS was added to the flasks followed by 200 μl of D-glucose (1 M), 50 μl of lactoperoxidase (5 mg/ml), 10 μl of glucose oxidase (8 mg/ml), and 1 mCi of \(^{125}I\) insulin. The reaction time was 30 min at 22°C. Cells were washed twice with PBS, 5 ml of 10 mM sodium acetate, 150 mM NaCl (pH 4.5) for a total of 6 min in order to remove surface-bound \(^{125}I\) insulin. After the incubation, cells were quickly put at 4°C and washed three times. In order to remove the insulin receptor from the cell surface, the cells were trypsinized (0.5 mg/ml) for 15 min at 15°C. Soybean trypsin inhibitor (10 mg/ml) was added, and the cells were washed three times with PBS. The pellets thus derived were resuspended in 0.8 ml of PBS containing 1% Triton X-100, 1 mg/ml bacitracin, and 1 mM PMSE. After solubilization for 60 min at 4°C, the suspensions were centrifuged at 100,000 × g for 15 min at 4°C. The pellets were washed twice with 5 ml of 1% PMSE and counted in a γ-counter to determine the “total cell-associated” \(^{125}I\) insulin, or washed twice with 0.5 M sodium acetate, 150 mM NaCl (pH 4.5) in order to remove “surface-bound” \(^{125}I\) insulin. After the acid wash, the cells were washed three times with PBS and lysed with 0.03% SDS. In this latter case the radioactivity of the lysates reflected “internalized \(^{125}I\) insulin.” Non-specific \(^{125}I\) insulin binding was determined in the presence of 1 μM unlabeled insulin. Binding was normalized per 1 mg of cell protein. Degradation of the extracellular insulin was assessed by 10% trichloroacetic acid precipitation as described previously (13).

**Insulin Receptor Degradation**

HTC-IR/M-1030 and HTC-IR cells were plated in 75-cm² flasks in DME H-16 supplemented with 10% fetal bovine serum. The flasks were washed twice in PBS, 5 ml of methionine-free medium was added, and the cells were pulsed by the addition of 2 μCi of \(^{35}S\) methionine. After 4 h at 37°C, the monolayers were washed twice in PBS and then chased in methionine-free medium with 25 μM insulin for 24 h at 37°C. The cells were then rapidly cooled to 4°C and washed twice with 10 ml of 0.5 M sodium acetate, 150 mM NaCl (pH 4.5) for a total of 6 min in order to remove surface-bound insulin. After washing three times with PBS, the cells were then scraped off and solubilized for 60 min with Triton X-100 plus protease inhibitors (PMSF and bacitracin). After solubilization the suspensions were centrifuged at 100,000 × g for 60 min to remove insoluble components. Labeled receptors were then immunoprecipitated using a monoclonal antibody (25) that binds to the C-terminal tail of the insulin receptor β-subunit.
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under reducing conditions, and subjected to SDS-PAGE and autoradiography.

RESULTS

Receptor Kinase Assays—The ability of insulin to activate receptor kinase activity, as measured by either β-subunit autophosphorylation or by phosphorylation of the exogenous substrate poly(Glu-Tyr), was studied in equal numbers of nontransfected wild type HTC cells (HTC-WT), and transfected HTC-IR and HTC-IR/M-1030 cells. In all cell lines insulin increased β-subunit autophosphorylation (Fig. 1A). Although HTC-IR/M-1030 cells had 50 times more insulin receptors than nontransfected HTC cells, receptor autophosphorylation was not increased. In contrast receptor autophosphorylation in HTC-IR cells was greater than 20-fold that of either HTC-WT or HTC-IR/M-1030 cells (Fig. 1A). Insulin-stimulated receptor kinase activity toward poly(Glu-Tyr) was enhanced in HTC-IR cells but reduced by more than 50% in HTC-IR/M-1030 cells when compared to HTC-WT cells (Fig. 1B).

Down-regulation of Insulin Receptors in Transfected Cell Lines—To measure insulin receptor down-regulation, HTC-IR and HTC-IR/M-1030 cells were incubated in the presence and absence of 1 μM insulin for 20 h at 37 °C. Next, the cells were washed at pH 4.5 to remove unlabeled hormone, and the specific binding of 125I-insulin to its receptors measured at 4 °C in order to inhibit insulin receptor processing and internalization. Scatchard analysis of binding data (Fig. 2A) revealed that insulin decreased the number of receptors by 64.7 ± 7 and 66.1 ± 5% (mean ± S.E., n = 4) in HTC-IR/M-1030 and HTC-IR cells, respectively.

We previously reported that insulin binding to wild type HTC cells is linear function reflecting the presence of little or any site-site interactions (16). In cells transfected with both HTC-IR and HTC-IR/M-1030 cells, Scatchard plots of untreated cells were also linear (Fig. 2A). However, after insulin treatment, Scatchard plots became curvilinear; the affinity of the high affinity receptors (when compared to low affinity receptors) increased from 2.34 ± 0.32 nM (mean ± S.E., n = 4) to 0.36 ± 0.09 nM (mean ± S.E., n = 4) in HTC-IR/M-1030 cells and from 1.82 ± 0.26 nM (mean ± S.E., n = 4) to 0.29 ± 0.06 nM in HTC-IR cells.

To determine whether these changes in receptor number and affinity were present at lower insulin concentrations, the same experiments were carried out at 10 nM insulin. In both HTC-IR/M-1030 and HTC-IR cells insulin induced major changes in the shapes of the Scatchard plots and in receptor affinity (Fig. 2B). However, only a small effect was seen on receptor capacity (Fig. 2B).

Insulin Binding to Control and Down-regulated Solubilized Transfected Cell Lines—To determine whether the decrease in surface receptors after insulin treatment (as measured by insulin binding in intact cells), was associated with a concomitant reduction in total cellular insulin receptors, 125I-insulin binding to solubilized receptors was carried out in both cell lines. After incubation in the presence or absence of 1 μM insulin for 20 h at 37 °C, followed by

Fig. 1. Insulin receptor kinase activity in untransfected wild type HTC cells (HTC-WT) and in HTC cells transfected with normal (HTC-IR) and mutant (HTC-IR/M-1030) cells. In this study partially purified insulin receptors were prepared from equal numbers of all three types of HTC cells. After preincubation with 1 μM insulin, [32P]ATP was added and receptor β-subunit phosphorylation (A) and the phosphorylation of poly(Glu-Tyr) (B) analyzed.

Fig. 2. Insulin binding to control and down-regulated transfected cell lines. HTC-IR/M-1030 and HTC-IR cells were preincubated in the absence and presence of either 1 μM insulin (A) or 10 nM insulin (B) for 20 h at 37 °C. After acid washing to remove bound insulin (see "Experimental Procedures") the cells were incubated with 6 pM 125I-insulin and varying concentrations of unlabelled insulin. Scatchard plots (24) of binding data are presented. Results are the means of duplicates and a representative of four experiments is shown.
by acid washing, cells were solubilized and the receptors precipitated with human γ-globulin and polyethylene glycol. Next the receptors were resuspended and specific ¹²⁵I-insulin binding measured. Scatchard plots revealed a 38% decrease of the HTC-IR/M-1030 receptors and a 62% decrease of HTC-IR receptors after insulin treatment (Fig. 3). In both cell lines insulin treatment changed the shape of the Scatchard plots and increased receptor affinity: HTC-IR/M-1030 (Kd = 0.61 ± 0.013 and 0.25 ± 0.018 nM, control versus insulin treatment, mean ± S.E., N = 3); HTC-IR (Kd = 0.97 ± 0.02 h and 0.60 ± 0.016 nM).

Effect of Temperature, Time, and Cycloheximide Treatment on Down-regulation of Insulin Receptors in HTC-IR/M-1030 Cells—To further investigate the characteristics of the insulin-induced down-regulation in HTC-IR/M-1030 cells, the effects of incubation temperature and time of incubation were examined. Cells were incubated with 1 μM insulin for either 20 h at 15 °C, or 1 h at 37 °C (Fig. 4). Next specific ¹²⁵I-insulin binding was carried out. Compared to studies for 20 h at 37 °C, Scatchard analysis of binding data indicated that there were relatively small differences between control and insulin-treated cells when the incubation was carried out at either 15 °C for 20 h or after 1 h at 37 °C.

Next the effect of cycloheximide, an inhibitor of protein synthesis, was studied. Pretreatment of HTC-IR/M-1030 cells with 100 μM cycloheximide (at which concentration >95% of protein synthesis was inhibited) followed by treatment with 1 μM insulin for 20 h at 37 °C did not change the effect of insulin on either receptor affinity or receptor capacity (Fig. 5).

Insulin Receptor Degradation Studies—In order to determine whether insulin accelerated receptor degradation, HTC-IR and HTC-IR/M-1030 cells were placed into methionine-free medium, pulsed with [³⁵S]methionine for 4 h, and then chased with media containing unlabeled methionine for 24 h in the absence or presence of 1 μM insulin (Fig. 6). In both cell types after 24 h of chase, labeled insulin receptors fell to levels of approximately 30% of that at time 0. Addition of unlabeled insulin during the chase period accelerated the loss of insulin receptors in both cell types (Fig. 6).

Insulin Internalization and Degradation—To study insulin internalization, radiolabeled insulin was incubated with HTC-WT, HTC-IR/M-1030, and HTC-IR cells for 60 min at 37 °C. Both total cell-associated radioactivity and intracellular radioactivity (acid-resistant) were measured and the percentage of ¹²⁵I-insulin internalized calculated (Fig. 7A). In all cell lines

Fig. 3. Insulin binding to solubilized receptors from control and down-regulated HTC-IR/M-1030 and HTC-IR cells. Cells were incubated in the absence or presence of 1 μM insulin for 20 h at 37 °C. After acid washing the cells were solubilized and the receptors precipitated with human γ-globulin and polyethylene glycol (see "Experimental Procedures"). Soluble receptors were then incubated with 30 pm ¹²⁵I-insulin and varying concentrations of unlabeled insulin. Scatchard plots of binding data are presented. Results are the means of duplicates and a representative of three experiments is shown.

Fig. 4. Effect of time and temperature on insulin-induced down-regulation of insulin receptors in HTC-IR/M-1030 cells. Cells were preincubated in the absence and presence of 1 μM insulin for either 20 h at 15 °C or 1 h at 37 °C. After acid washing, specific insulin binding was measured and Scatchard plots are shown. Results are the means of duplicate determinations.

Fig. 5. Effect of cycloheximide treatment on insulin-induced down-regulation of insulin receptors in HTC-IR/M-1030 cells. Cycloheximide (100 μM) was added to cell cultures; 30 min later the cells were preincubated in the absence or presence of 1 μM insulin for 20 h at 37 °C. After acid washing to remove bound insulin, ¹²⁵I-insulin binding was measured and Scatchard plots are shown. Results are the means of duplicate determinations and are representative of three experiments.

Fig. 6. Enhanced degradation of insulin receptors by insulin in HTC-IR and HTC-IR/M-1030 cells. Cells in methionine-free medium were pulsed with [³⁵S]methionine for 4 h and then chased 24 h with unlabeled methionine in the absence or presence of 1 μM unlabeled insulin. Cells were solubilized, and receptors were immunoprecipitated and subjected to SDS-PAGE under reducing and denaturing conditions. α, the receptors after the 4-h pulse. 24 h, the receptor after the 24-h chase. The location of the insulin receptor α-subunit (130 kDa) and the β-subunit (90 kDa) are shown.
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The percent of the bound hormone that was internalized was small, being approximately 3% of total in three separate experiments. Moreover, the percentage of labeled hormone that was internalized was the same for all three lines.

Previously we demonstrated that HTC cells have a very low rate of insulin degradation (16). To investigate whether 125I-insulin degradation was also low in HTC-IR and HTC-IR/M-1030 cells, we studied this parameter. The percentage of labeled hormone that was internalized was the same for all three lines.

Insulin Receptor Internalization—In addition we measured the rate of insulin receptor internalization and the influence of insulin upon this parameter. The surface proteins of HTC-IR and HTC-IR/M-1030 cells were labeled by 125I and then treated for 1 h with 1 μM insulin. Next, in one-half of the cells the labeled insulin receptors were solubilized, immunoprecipitated, and analyzed. To the other half of the cells trypsin was added to destroy the cell surface insulin receptors and the cells were then solubilized, immunoprecipitated, and analyzed. By this procedure with both HTC-IR/M-1030 (Fig. 8) and HTC-IR cells (not shown) less than 5% of insulin receptor was internalized.

**DISCUSSION**

In the present study we investigated the regulation of the insulin receptor in HTC cells expressing both normal human insulin receptors (HTC-IR) and insulin receptors mutated at lysine 1030 in the ATP binding site (HTC-IR/M-1030). For several reasons HTC cells are important for studying the effect of insulin on receptor degradation and affinity. First there is very little ligand degradation, so that the analyses of receptor binding and internalization are not complicated. Second, with HTC cells, in contrast to most cell types, there is little or no hormone induced receptor internalization. Thus, in these cells it is possible to separate the function of insulin-induced internalization from the function of insulin-induced receptor degradation. Finally, in these cells there are very few endogenous rat receptors and no related IGF-I receptors. However, it should be pointed out that since HTC cells are unique in several aspects, the data derived from them may not be applicable to all insulin-sensitive cells.

In the present study, by changing lysine 1030 in the ATP binding site to methionine, the tyrosine kinase activity of the transfected receptors was inactivated. In cell types expressing both this mutant human receptor and a normal human insulin receptor, a 20-h incubation with insulin at 37 °C both decreased receptor number and increased receptor affinity. Two lines of evidence indicated that insulin caused a decrease in total cellular insulin receptor content. First, studies with solubilized receptors gave results similar to that seen in intact cells. Second, metabolic labeling studies indicated that insulin accelerated insulin receptor degradation.

The present studies with transfected HTC cells resemble prior studies of receptor number and affinity in untransfected Fao Reuber H-35 rat hepatoma cells (11). In that study, like the present study, insulin caused a time- and dose-dependent decrease in receptor number, and an increase in insulin receptor affinity. Moreover, as in the present study, the reduction of the incubation temperature to 15 °C blocked the effect of insulin on its receptor. In that study total cellular insulin receptors content and receptor turnover were not measured. However, when plasma membranes were isolated, insulin decreased receptor number in that compartment.

Previously, employing monoclonal antibodies to the α-subunit of the human insulin receptor, we investigated the relationship between enhanced insulin receptor internalization and insulin receptor degradation (10). In that study we found that the process of receptor internalization did not necessarily lead to enhanced receptor degradation. Rather one or more unknown cellular processes were needed to direct the endocytosed receptor into the degradation pathway. In the present study we find that the rate of 125I-insulin internalization (reflecting the endocytosis of the insulin-receptor complex) was relatively low and was similar in HTC cells expressing either normal insulin receptors or mutated receptors altered...
at the ATP binding site. These studies suggested therefore that activation of insulin receptor tyrosine kinase by insulin did not enhance insulin receptor endocytosis, and that endocytosis of insulin receptors in both cell types was by a slow constitutive pathway. However, in both cell types insulin reduced receptor number and increased receptor affinity. This observation indicated that: 1) insulin-induced insulin receptor degradation is a process that is independent of insulin-induced receptor internalization; and 2) the enhanced degradation of the insulin receptor does not require the activation of tyrosine kinase.

The possibility was considered that, in the HT1-IR/M1030 cells, binding of insulin to the native rat insulin receptor generated metabolic signals that in turn regulated the translocated mutant human insulin receptor. We believe, however, that the mechanism was not operative. It has been previously demonstrated that transfection of animal cell lines with human insulin receptors mutated at the ATP binding site impairs the ability of the native rat insulin receptor to regulate a variety of cellular function (12, 14). In the present study we found that transfection of HTC cells with a similar mutant receptor markedly inhibited the ability of the native rat receptor to phosphorylate an endogenous substrate. Phosphorylation of substrate by the insulin receptors is believed to be a key intermediate step in the regulation of cellular function by the insulin receptor (2, 3). Thus we believe that it is highly unlikely that the native rat receptor is regulating the translocated mutant receptor, when concomitantly, the latter receptor is markedly attenuating the rat receptor's ability to signal.

Several prior studies have shown that in Chinese hamster ovary cells (12, 13) and rat 1 fibroblasts (14) transfected with human insulin receptor cDNA and expressing normal human insulin receptors, insulin accelerates insulin receptor internalization. In two (12, 14) of these studies, the data indicated that insulin accelerated insulin receptor degradation. However, when these same cells expressed human insulin receptors mutated at the lysine in the ATP binding site, insulin failed to accelerate both insulin receptor internalization and appeared not to accelerate insulin receptor degradation.

There are several reasons why these two other groups did not observe degradation of the mutant insulin receptor by insulin. First, they substituted an alanine for lysine, whereas in the present study we used methionine which should theoretically maintain a more normal protein structure (15). Second, we analyzed receptor number by Scatchard analysis, whereas the other groups did not. If, in these other studies with mutant receptors, there was a reciprocal change in receptor affinity, down-regulation may not have been detected. In one previous study with mutant receptors, receptor labeling was carried out followed by analysis of its degradation rate (12). In that study insulin appeared to accelerate the degradation of its receptor.

Several other studies, concerning other receptors that have intrinsic tyrosine kinase activity, support the concept that activation of receptor tyrosine kinase is not necessary for enhanced receptor degradation. In the case of the platelet-derived growth factor receptor, mutation and inactivation of the tyrosine kinase domain does not block ligand-induced receptor down-regulation (26). Similarly, in the case of epidermal growth factor receptor, mutation and inactivation of the tyrosine kinase domain does not block ligand-induced receptor down-regulation (27). These studies, with both insulin receptors and other receptors containing tyrosine kinase, indicate therefore that activation of tyrosine kinase may not be prerequisite for ligand-induced down-regulation of receptors.

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