Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 427–431, January 1982
Cell Biology

Evidence for insulin-induced internalization and degradation of insulin receptors in rat adipocytes

(down-regulation/receptor degradation/chloroquine)

ALLAN GREEN AND JERROLD M. OLEFSKY

University of Colorado Health Sciences Center, 4200 East Ninth Avenue, B151, Denver, Colorado 80262

Communicated by David W. Talmage, September 8, 1981

ABSTRACT **We have investigated the theory that the insulin-induced loss of insulin binding from adipocytes is due to internalization of insulin receptors. Cell-surface receptors were assessed by the binding capacity of intact cells at 16°C. Total (i.e., cell-surface plus intracellular) receptors were assessed by solubilizing the cells in 1% Triton X-100 and then measuring binding by the solubilized extract. Intracellular receptors were measured by treating the cells with trypsin before solubilizing them. The trypsin treatment removed >90% of the cell-surface binding, so that any significant binding by soluble extracts of these cells must represent intracellular receptors. Adipocytes were incubated with insulin (100 ng/ml) with or without chloroquine (0.2 mM) for 4 hr. Insulin alone resulted in a 62% loss of cell-surface receptors, but only a 46% loss of total receptors, and a 170% increase in intracellular receptors, suggesting that the lost cell-surface receptors were internalized, where some were degraded. Insulin in the presence of chloroquine resulted in a 34% loss of cell-surface receptors, but no loss of total receptors, and a 300% increase in intracellular receptors. Thus, in the presence of chloroquine receptors were internalized but not degraded. The loss of cell-surface receptors and appearance of intracellular receptors were time and dose dependent and were linearly related. These results demonstrate that insulin causes translocation of insulin receptors from the cell surface to the cell interior, where they can be degraded (or inactivated) by a chloroquine-sensitive process.**

In many physiological and pathological conditions involving hyperinsulinemia, tissues possess a markedly reduced number of insulin receptors per cell (1–5). *In vitro* evidence supports the theory that it is the hyperinsulinemia that causes the loss of receptors, and this process has been termed "down-regulation" (6). Thus, insulin-induced receptor loss has been demonstrated in cultured human lymphocytes (6, 7), adipose tissue (8, 9), isolated adipocytes (10), cultured fibroblasts (11), and cultured hepatocytes (12). However, the fate of the lost receptors is unknown. After binding to its receptor the hormone is taken up into the cell and degraded (13–19). Some authors have suggested that the receptor is internalized along with its ligand (20–22) and that this is one mechanism underlying the eventual decrease in cellular insulin receptors. However, the data to support this hypothesis are circumstantial, and direct evidence to validate this idea is lacking. Therefore, to test this hypothesis, we have assessed the translocation of insulin receptors from the cell surface to the cell interior.

MATERIALS AND METHODS

Materials. Porcine monocomponent insulin was generously supplied by Ronald Chance of Eli Lilly; $Na^{125}I$ was purchased from New England Nuclear; bovine serum albumin (fraction V), from Armour Pharmaceuticals (Chicago, IL); collagenase, from Worthington; polyethylene glycol (approximate molecular weight 6000), bovine gamma globulin, bacitracin, Triton X-100, and trypsin, from Sigma; soybean trypsin inhibitor, from GIBCO; and talc tablets (50 mg), from Ormont (Englewood, NJ).

Methods. Iodination of insulin, preparation of rat adipocytes, and measurement of insulin binding to intact cells were carried out as described (10, 23).

Insulin Pretreatment and Dissociation Procedures. Adipocytes suspended in 10 ml of pH 7.6 buffer containing 35 mM Tris, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 2.5 mM KCl, 10 mM dextrose, 24 mM NaOAc, and 1% bovine serum albumin (Tris/albumin buffer) were incubated in the presence or absence of various concentrations of insulin with or without chloroquine (0.2 mM) in 25-ml polypropylene flasks. Cells were gently agitated in a shaking water bath for 0–6 hr as indicated in the text and figure legends, at 37°C. At the end of the incubation period, cells were washed three times in Tris/albumin buffer (pH 7.0), and receptor-bound insulin was allowed to dissociate at pH 7.0 for 1 hr at 37°C. It has been demonstrated previously that all receptor-bound insulin and any insulin internalized (including subsequently generated degradation products) is effectively dissociated or released by this procedure (10). After the 1 hr dissociation, cells were washed and resuspended in Tris/albumin buffer, pH 7.6. Control cells underwent similar incubation, dissociation, and washing procedures.

Trypsinization. After the procedures outlined above, adipocytes were treated with trypsin (200 μg/ml) for 10 min at 37°C. Soybean trypsin inhibitor (400 μg/ml) was added, and the cells were washed three times in Tris/albumin buffer, pH 7.6.

Solubilizing Procedure. After samples of the cell suspension had been taken for measurement of intact cell binding, the adipocytes were centrifuged and the buffer was removed. A volume (0.5–2 ml as indicated) of Tris/albumin buffer (pH 7.0) containing Triton X-100 (1%) and bacitracin (2 mg/ml, to inhibit proteolysis) was added and the cells were agitated at 37°C for 1 hr. The low pH and warm temperature were used to ensure that any remaining intracellular insulin was dissociated from intracellular insulin–receptor complexes. At the end of the 1-hr solubilizing step, the suspensions were transferred to 1.5-ml centrifuge tubes and spun in a Beckman Microfuge B for 2 min. The aqueous layer was removed and transferred to a second centrifuge tube. Talc was added (50 mg for each 1 ml of extract) and the extract was mixed vigorously. The suspension was then centrifuged (10 min, 3000 rpm) and the supernatant was used for measurement of insulin binding (see below). The talc precipitation was used to remove any traces of insulin.

Solubilized Cell Binding Studies. Samples (50 μl) of the soluble extracts were added to Tris/albumin buffer (pH 7.6) (final volume 0.5 ml) with ^{125}I-labeled insulin (^{125}I-insulin) (0.3 ng/ml) in the presence or absence of unlabeled insulin (50 μg/ml,

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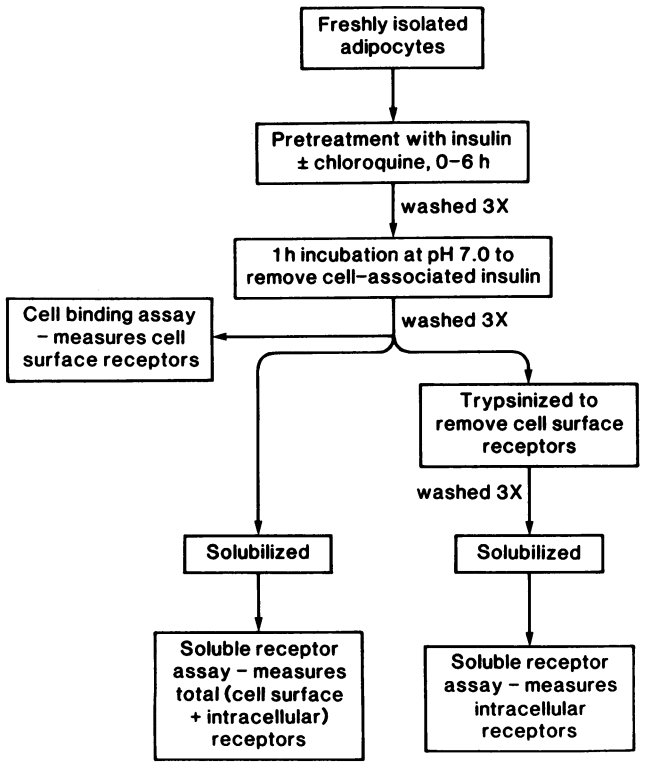

FIG. 1. Flow diagram describing the methods used for measuring cell-surface and intracellular receptors.

for determination of nonspecific binding). The tubes were incubated at 4°C for 16 hr, and bound ^{125}I-insulin was precipitated by adding 0.5 ml of Tris buffer (pH 7.6) containing 0.2% gamma globulin followed by 1 ml of 20% (wt/vol) polyethylene glycol (24). All assays were performed in triplicate, and the results have been corrected for nonspecific binding.

Summary of Methods. A flow diagram describing the way in which the methods have been used is presented in Fig. 1.

RESULTS

Effects of Insulin and Chloroquine on the Distribution of Insulin Receptors Between the Cell Surface and the Cell Interior. The data shown in Fig. 2*A* demonstrate that cells treated with insulin (100 ng/ml) at 37°C for 4 hr showed a 62% loss of insulin binding capacity compared to controls. As in our previous reports (10), Scatchard analysis demonstrated that this loss of binding capacity was due to a decreased receptor number (data not shown). When cells were treated with insulin in the presence of chloroquine (0.2 mM), the effect of insulin to cause a loss of receptors was inhibited, such that only a 35% decrease in binding capacity was observed. Incubation of cells with chloroquine alone had no effect on insulin binding (data not shown). It is important to note that the measurements of ^{125}I-insulin binding were performed at 16°C; because internalization is blocked at this low temperature (18) the results reflect binding to cell-surface receptors. The cells were then solubilized and the insulin binding capacity of the solubilized extracts was measured (Fig. 2*B*). This approach provides a measure of the cell-surface plus intracellular insulin receptors—i.e., the total insulin binding capacity of the cells. When studied in this way, the insulin treatment of intact cells resulted in only a 46% loss of total binding capacity, which is significantly less ($P < 0.01$) than the 62% loss of cell-surface receptors (Fig. 2*A*). The cells treated with insulin in the presence of chloroquine showed no significant loss of total binding capacity. When the results from the intact and solubilized cells are considered together, it is evident that if total binding capacity is measured (solubilized cells) the insulin-induced decrease in insulin binding is smaller than when only cell surface binding (intact cells) is measured. This indicates that insulin treatment induces a loss of receptors from the cell surface and that some of these receptors can be recovered in an intracellular location. These effects are even more pronounced when chloroquine is used. Thus there is es-

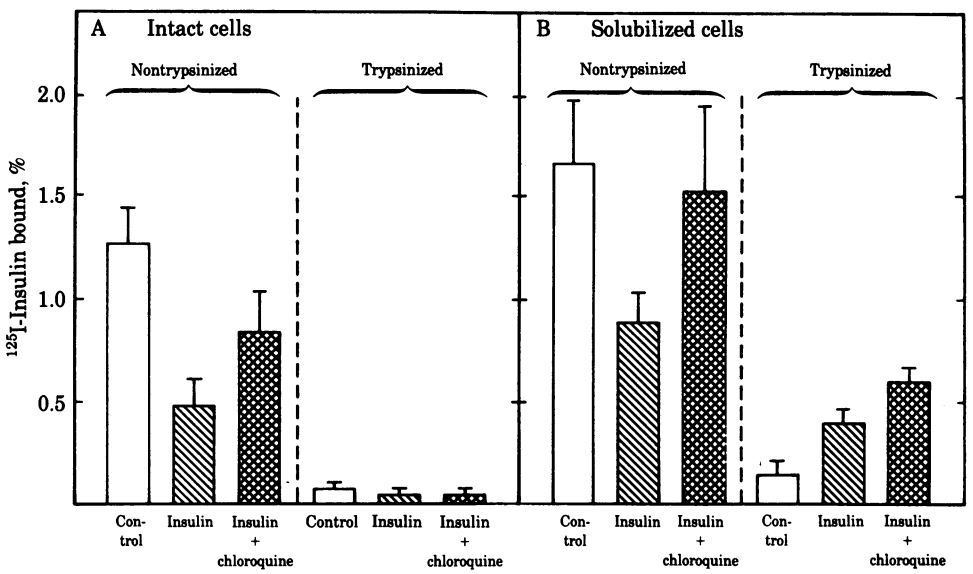

FIG. 2. Effect of 4-hr incubation of adipocytes with insulin or insulin plus chloroquine on distribution of insulin receptors between the cell surface and the cell interior. Adipocytes (approximately 8×10^5 per ml) were incubated with no additions, insulin (100 ng/ml), or insulin (100 ng/ml) plus chloroquine (0.2 mM) for 4 hr. Cells were then washed, and bound insulin was allowed to dissociate. After further washes, each group of cells was divided into two, and one half was trypsinized to remove cell-surface receptors. Samples (approximately 2×10^5 cells) were taken for measurement of intact cell binding. The remaining cells were then solubilized and insulin binding to the soluble extract was measured, using a volume of extract equivalent to the number of cells (i.e., approximately 2×10^5) used in the intact cell binding assay. Results are the mean ± SEM of three separate experiments performed on different days.

sentially no loss of total insulin binding capacity when cells are exposed to insulin in the presence of chloroquine. This suggests that when cells are incubated with insulin plus chloroquine a loss of receptors from the cell surface occurs, and these receptors are essentially quantitatively recovered within the cells. It should be noted that it is difficult to quantitatively compare the intact cell binding data with results on solubilized cells, because the assays differ in several respects. Therefore, the experiments have been performed such that quantitative comparisons are made only within groups of intact cells or solubilized cell assays.

To more specifically evaluate the intracellular receptor pool, cell-surface receptors were removed with trypsin. As shown in Fig. 2A, trypsin destroyed greater than 90% of the insulin-binding capacity of control, insulin-treated, or insulin plus chloroquine-treated intact cells. However, when insulin binding was measured with solubilized extracts of the trypsinized cells (Fig. 2B), the insulin-treated cells had a greater binding capacity than control cells, and cells treated with insulin plus chloroquine showed an even higher binding capacity. Again, chloroquine was without effect when added alone (data not shown). These results show that, in the control state, almost all of the cells' total binding capacity represents cell-surface receptors, with a relatively small intracellular pool. Insulin treatment leads to a decrease in cell-surface receptors and an increase in the number of receptors in the intracellular pool. Chloroquine partially inhibits the insulin-induced loss of surface receptors, but these receptors are now quantitatively recovered intracellularly and no net loss of total binding capacity is observed. This suggests that chloroquine inhibits the intracellular processing of internalized insulin receptors. The experiments described above were also conducted with another lysosomal inhibitor, NH_4Cl (20 mM). This agent gave results identical to those with chloroquine.

Specificity of the Intracellular Insulin Receptor. In these studies the binding isotherms and specificity of intracellular and cell-surface receptors were compared. The results shown in Fig. 2 demonstrate that approximately 90% of the insulin receptors on control cells are located on the cell surface. Therefore solubilized extracts of control adipocytes were used to assess insulin binding properties of cell-surface receptors. In order to prepare a solubilized extract containing large numbers of intracellular receptors and negligible cell-surface receptors, adipocytes were incubated with insulin plus chloroquine to increase the intracellular pool. These cells were then trypsinized to remove surface receptors and soluble extracts were prepared. Samples of these two solubilized preparations were then incubated with ^{125}I-insulin plus various concentrations of unlabeled insulin, insulin analogs, or other peptide hormones, and the results are shown in Fig. 3. Native porcine insulin inhibited ^{125}I-insulin binding to both preparations in an identical manner, and Scatchard analysis (*Insets*, Fig. 3) yielded comparably shaped curvilinear plots. Desoctapeptide insulin and two recently characterized low-affinity insulins (25), [Leu^{B24}]insulin and [Leu^{B25}]insulin, inhibited ^{125}I-insulin binding to the two solubilized receptor preparations in a comparable manner. The binding curves obtained with these low-affinity insulins are consistent with their previously reported binding potency (25). Growth hormone, glucagon, and prolactin, at concentrations of 10 μg/ml, did not inhibit ^{125}I-insulin binding to either preparation, further demonstrating their specificity for insulin (results not shown).

Time Course of Insulin Receptor Internalization. The time course of insulin-induced adipocyte cell-surface receptor loss is seen in Fig. 4A. Insulin induced a rapid loss of receptors that was half-maximal by about 1 hr and essentially complete by 4 hr. Chloroquine inhibited this loss of cell-surface receptors by about 30% at all time points. Together with the loss of cell-surface receptors was a time-dependent accumulation of intracellular receptors (Fig. 4B), and chloroquine markedly potentiated the generation of this intracellular receptor pool. To illustrate the quantitative and temporal correlation between the loss of cell-surface receptors and the increase of intracellular receptors, the increase in intracellular receptors was plotted as a function of loss of cell-surface binding (Fig. 4C). A highly significant correlation exists between these variables in both the absence and the presence of chloroquine.

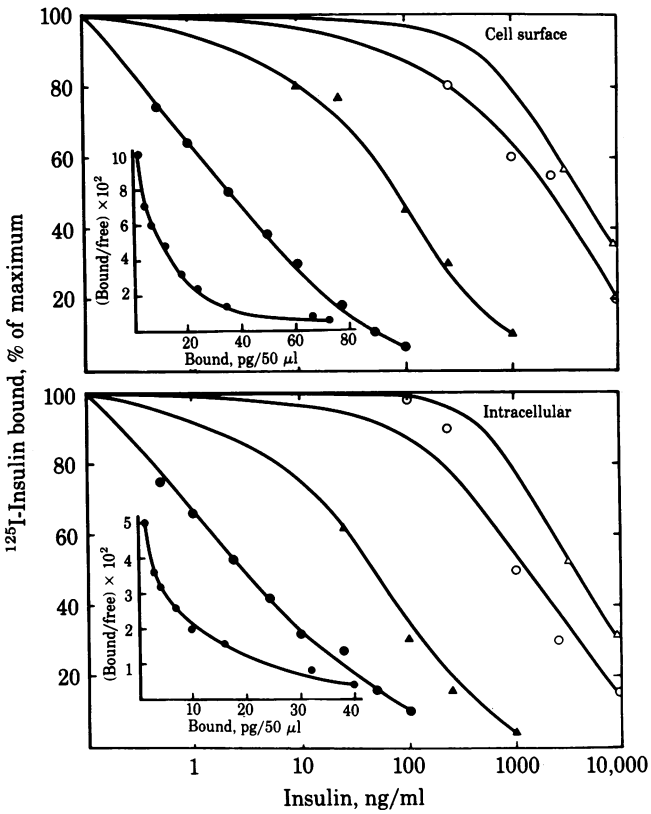

FIG. 3. Binding characteristics of cell-surface and intracellular insulin receptors. The extracts containing receptors were incubated with ^{125}I-insulin plus various concentrations of unlabeled insulin (●), [Leu^{B24}]insulin (▲), [Leu^{B25}]insulin (○), or desoctapeptide insulin (△), and binding was determined. Scatchard plots of the insulin binding data are shown in the *Insets*.

Dose–Response Relationship for Insulin Receptor Internalization. Fig. 5A shows that the insulin-induced loss of cell-surface receptors was concentration dependent, with maximal effects seen at an insulin concentration of 25 ng/ml, and a half-maximal effect at about 2.5 ng/ml. Chloroquine inhibited the insulin-induced receptor loss at all hormone concentrations, but the dose–response relationship was essentially the same. The generation of the intracellular insulin receptor pool was also related to the insulin concentration used and, as before, chloroquine potentiated the size of this intracellular pool. The relationship between loss of cell-surface receptors and appearance of intracellular receptors was highly significant (Fig. 5C).

DISCUSSION

It is now well recognized that incubation of various cell types with insulin leads to a decrease in the number of cell-surface insulin receptors, a process termed down-regulation (6–12). It is also well established that, after binding to cell-surface recep-

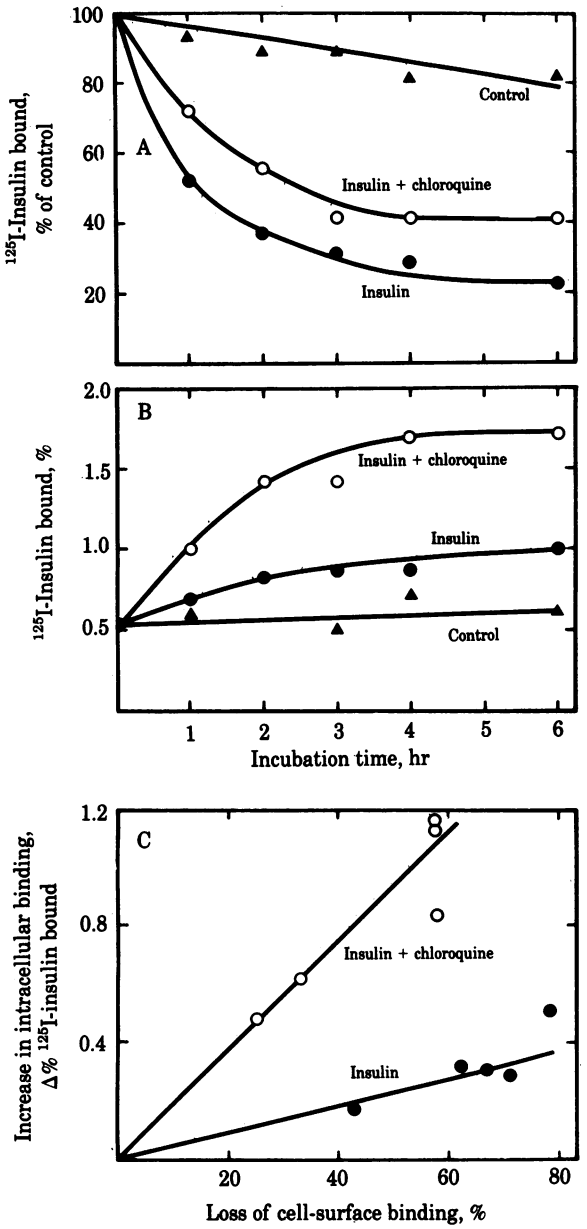

FIG. 4. Time course of insulin-induced internalization of insulin receptors. (*A*) Time course of cell-surface receptor loss; (*B*) time course of appearance of intracellular receptors. Adipocytes (approximately 6×10^5 per ml) were incubated with no additions (▲); with insulin at 100 ng/ml (●), or with insulin at 100 ng/ml + chloroquine at 0.2 mM (○). At the indicated times, cell-surface and intracellular insulin binding were measured. (*C*) Loss of cell-surface receptors is plotted as a function of the increase in intracellular binding. The correlation coefficient for insulin + chloroquine is $r = 0.96$ ($P < 0.01$). For insulin alone, $r = 0.90$ ($P < 0.02$).

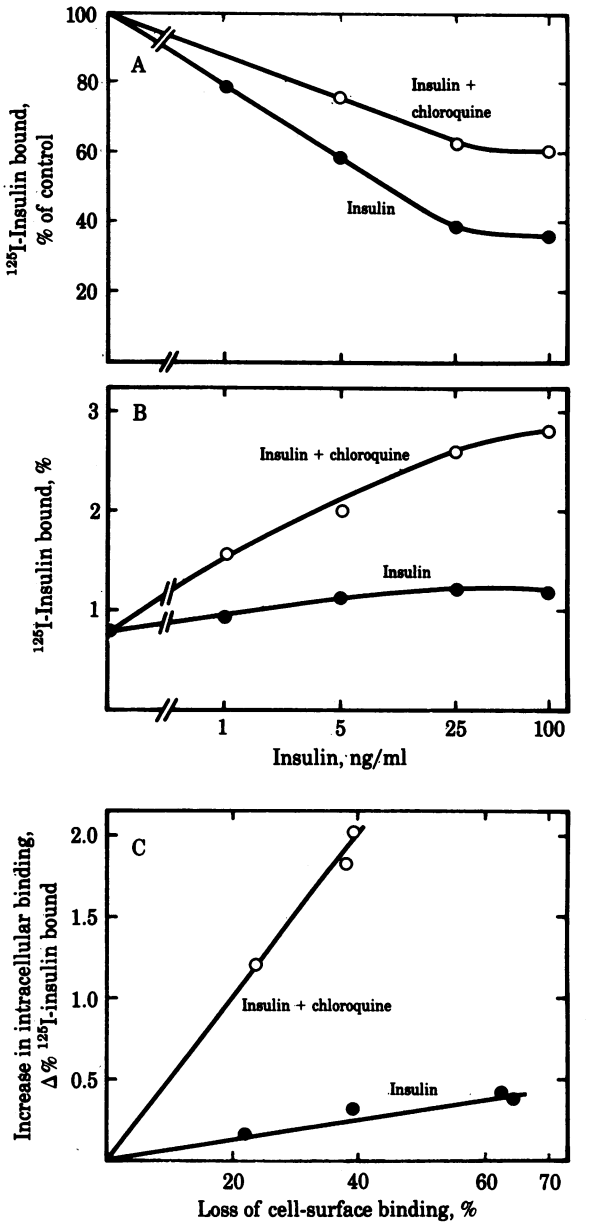

FIG. 5. Effect of various concentrations of insulin, in the presence and absence of chloroquine, on cell-surface receptors (*A*) and intracellular receptors (*B*). Adipocytes (approximately 6×10^5 per ml) were incubated with the indicated concentrations of insulin, with (○) or without (●) chloroquine (0.2 mM) for 3 hr, and cell-surface and intracellular insulin binding was measured. (*C*) Cell-surface receptor loss is plotted as a function of the increase in intracellular binding. For insulin + chloroquine, $r = 0.99$ ($P < 0.01$); for insulin alone, $r = 0.91$ ($P < 0.05$).

tors, the insulin molecule is internalized and is processed and degraded intracellularly (13–19). We (21, 22) and others (20) have suggested that receptor loss and hormone internalization are related and that insulin is internalized along with its receptor, thus initiating receptor loss or down-regulation. Although indirect evidence exists to support this notion, direct evidence demonstrating insulin-induced internalization of the insulin receptor has been lacking.

In these studies we have used intact cell binding at 16°C as a measure of cell-surface receptors, binding by solubilized cells as a measure of total (cell-surface plus intracellular) receptors, and binding by trypsinized and then solubilized cells as a measure of intracellular receptors. The results (Fig. 2) demonstrate that insulin leads to a greater loss of cell-surface than of total receptors, with an accumulation of intracellular receptors. However, there is some loss in total binding capacity, suggesting that, subsequent to internalization, some of the receptors are degraded or processed to a form that does not bind insulin. On the other hand, in the presence of chloroquine or NH_4Cl, there is no loss in total binding capacity, and the accumulation of intracellular receptors is potentiated. This suggests that these agents, which are known to inhibit intracellular insulin degradation, also inhibit processing of the insulin receptor.

Chloroquine and NH_4Cl clearly inhibit the intracellular processing of insulin receptors, and because these agents interfere

with intralysosomal proteolysis one can speculate that the lysosome is involved in the inactivation and degradation of the insulin receptor. However, these agents can also affect other intracellular processes, such as fusion of various vesicles with lysosomes (26), and it is also possible that the effect of these agents on intracellular receptors is due to a nonlysosomal intracellular action. In any event, the current data show that internalized insulin receptors are processed by a chloroquine- and NH_4Cl-sensitive step.

It seems likely that the small intracellular pool of insulin receptors in control cells is derived from both endogenous receptor synthesis and a slow turnover of cell-surface receptors in the basal state. However, after insulin treatment, with or without chloroquine, the increase in the intracellular receptor pool appears to be closely related to internalization of cell-surface receptors. This quantitative relationship is made clearer by the close correlation between the loss of cell-surface insulin receptors and the corresponding increase in intracellular receptors as a function of time, or insulin concentration, in the presence or absence of chloroquine (Figs. 4 and 5). Insulin is also taken up by adipocytes and is degraded intracellularly by a chloroquine-sensitive process (18). A close correlation between the loss of insulin receptors from the cell surface and internalization of insulin has been found (22), suggesting that the hormone and the receptor are internalized together and then processed intracellularly; the current data provide direct evidence for this pathway. However, because the number of insulin molecules degraded greatly exceeds the number of cell-surface receptors lost (15) and because not all internalized receptors are degraded, it is likely that after internalization the pathways for intracellular processing of insulin and the receptor diverge.

In order to compare the binding properties of the cell-surface receptor with those of the internalized receptor, solubilized cell extracts were prepared containing primarily cell-surface or primarily intracellular receptors as described in *Results*. The affinities of both receptor preparations for native insulin and for a number of insulin analogs were identical, suggesting that the receptor does not undergo any major alterations that affect its ability to bind insulin as it is internalized. On the other hand, binding of insulin is only one of the functional properties of the insulin receptor, and further detailed studies will be necessary to characterize all of its functional, chemical, and physical properties.

Both chloroquine and NH_4Cl inhibited the insulin-induced loss of cell-surface receptors from adipocytes. Although the reason for this inhibition is not clear from the current studies, the same phenomenon has been observed in cultured human fibroblasts (21). The quantitative accumulation of intracellular receptors in the presence of chloroquine makes it unlikely that this agent simply inhibits receptor internalization. Therefore, it seems possible that accumulation of intracellular insulin receptor complexes, or products thereof, inhibits internalization of further insulin receptors by a feedback mechanism. With this formulation the chloroquine-mediated accumulation of intracellular insulin and receptors could accentuate this feedback inhibition. Clearly, this hypothesis will require experimental validation, because it is possible that chloroquine and NH_4Cl inhibit receptor loss by a mechanism independent of their effects on insulin or receptor degradation.

This work was supported by Grants AM 25241 and AM 25242 from the National Institute of Arthritis, Metabolism and Digestive Disease.

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