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Cell Biology

Regulation of insulin receptors: Evidence for involvement of an endocytotic internalization pathway

(transglutaminase/human fibroblasts/receptor regulation)

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ABSTRACT **Cultured human fibroblasts degrade insulin by a receptor-mediated process. When intracellular hormone degradation is inhibited by chloroquine, ^{125}I-labeled insulin internalizes and accumulates intracellularly. In contrast, cultured IM-9 lymphocytes do not degrade receptor-bound insulin or accumulate ^{125}I-labeled insulin in the presence of chloroquine. Insulin-induced receptor loss occurs in both cell types, and chloroquine inhibits this process in fibroblasts but not in IM-9 lymphocytes. Transglutaminase is a membrane-associated enzyme thought to mediate the process of ligand-induced receptor aggregation and internalization; levels of this enzyme were high in fibroblasts but barely detectable in IM-9 lymphocytes. Furthermore, dansylcadaverine—a potent inhibitor of transglutaminase—blocked insulin-induced receptor loss in fibroblasts but was without effect in IM-9 lymphocytes. These results support the concept that insulin receptor regulation is mediated via an endocytotic internalization pathway in human fibroblasts and that the mechanisms of this process differ among cell types.**

Recent reports suggest that, after initial cell surface binding, insulin receptor complexes are internalized by absorptive endocytosis (1, 2). Following this, insulin is degraded at one or more intracellular sites, possibly involving lysosomes (3–7). The fate of the internalized receptor is unknown at present, although it is most likely degraded or recycled back to the plasma membrane or both. Gavin *et al.* (8) were the first to report that insulin could mediate the regulation of its own receptors; they found that, when IM-9 cultured lymphocytes were incubated with insulin, a marked reduction in insulin binding occurred. Since this initial report, it has been confirmed that insulin can lead to the loss of cell surface receptors in other cell types such as hepatocytes, adipocytes, and fibroblasts (9–11). Several workers have speculated that the internalization pathway for the insulin receptor may mediate the process of insulin-induced receptor loss. In order to examine the relationship between insulin internalization and receptor regulation, we have studied these processes in two cultured cell lines: normal human fibroblasts and the transformed cell line IM-9 lymphocytes.

MATERIALS AND METHODS

Materials. Minimal essential medium and trypsin were purchased from GIBCO. Fetal calf serum and bovine serum albumin were purchased from Reheis (Kankakee, IL). Hepes, *N*-tris[(hydroxymethyl)methylglycine] (Tricine), and chloroquine were purchased from Sigma. Na^{125}I (carrier free) was purchased from New England Nuclear. Dansylcadaverine was purchased from Fluka A. G. (Buchs, Switzerland). Single-component crystalline porcine insulin was the kind gift of Ronald Chance (Eli Lilly).

Methods. Normal human fibroblasts obtained from forearm punch biopsy or from foreskin resection were cultured at 37°C and 100% humidity under 5% CO_2/95% air. The growth medium was minimal essential medium at pH 7.4 supplemented with 10 mM Hepes, 26 mM $NaHCO_3$, biotin at 1 μg per liter, and 15% fetal calf serum. Cells were routinely subcultured (1:3 split) every 6 days in 25-ml flasks and reached confluence at 4 days. For experiments, confluent cells were subcultured (1:2 split) into 60 × 15 mm plastic dishes and were used on the sixth day after subculture. Cells were counted with a Coulter Counter (model ZB); counts ranged from 0.8 to 1.4 × 10^6 cells per dish. Cell viability was >95% as determined by trypan blue exclusion, even after incubations with the various drugs.

Experimental Procedure. Binding experiments were performed with cultured human fibroblast monolayers in 60 × 15 mm plastic dishes as follows. After aspiration of growth media, monolayers were washed twice with 3 ml of cool (22°C) binding buffer (minimal essential medium, pH 7.4/25 mM Hepes/25 mM Tricine/1% bovine serum albumin). Then, ^{125}I-labeled insulin (^{125}I-insulin) at 0.3–0.4 mg/ml, insulin standards, other reagents, and buffer were added to a total volume of 2 ml and the dishes were incubated in a shaking water bath (50 oscillations per minute). After incubation, an aliquot of the buffer was withdrawn for estimation of total radioactivity, and the remaining buffer was aspirated and discarded. The monolayers were washed four times with 3 ml of ice-cold Hanks' buffer; then, 1.5 ml of 1 M NaOH was added to each dish. The dishes were rotated (100 oscillations per minute) for 15 min and the NaOH was replaced by another 1.5 ml for another 15 min. These two 1.5-ml portions of NaOH, containing all cell-associated radioactivity, were pooled and assayed in an automatic gamma counter with a 7.62-cm (3-inch) crystal. Thus, the amount bound contained all of the ^{125}I-insulin bound from the original entire 2 ml incubation volume. All data are normalized to a cell concentration of 10^6 cells per dish.

^{125}I-Insulin was prepared by a modification (12) of the method of Freychet *et al.* (13) as described (6).

Cultured IM-9 Lymphocytes. These cells were grown in suspension culture in modified minimal essential media as described (8). Cells were subcultured every 3–4 days, and all experimental procedures were carried out on cells at 3 days after subculture. Insulin binding measurements were performed as described by Gavin *et al.* (8).

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Assay of Cellular Transglutaminase. IM-9 lymphocytes were concentrated by centrifugation and washed twice in 20 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM EDTA (TBS/EDTA), resuspended, and lysed by brief sonication. Human fibroblasts were grown to confluency, the media were removed, and the monolayers were rinsed twice in Tris/EDTA prior to collection by scraping. Cells were lysed by brief sonication. Human peripheral blood lymphocytes were prepared from 100 ml of fresh defibrinated blood by sedimentation onto a Ficoll/Hypague gradient; cells were collected and washed twice with TBS/EDTA. Differential counts indicated >95% lymphocytes with occasional monocytes. Lymphocytes were also lysed by sonication.

Cell lysate (50 μl) containing 0.5–10 mg of protein per ml was added to 150 μl of 20 mM Tris, pH 7.5/5 mM 2-mercaptoethanol/0.8 μM [^{3}H]dansylcadaverine (1500 dpm/pmol) containing 2 mg of *N,N*-dimethylcasein per ml and either 5 mM Ca^{2+} or 5 mM EDTA. Incubation was at 30°C, and 25-μl aliquots were removed at intervals and spotted on filter papers. Protein-bound ^{3}H was determined as described (14). Under these conditions, all assay results were linear with time and with protein concentration. Background radioactivity determined in the absence of Ca^{2+} (5 mM EDTA) was subtracted from all samples. The K_m and V_{max} were determined from plots of reaction velocity versus substrate concentration when the dansylcadaverine concentration was increased by adding unlabeled dansylcadaverine.

RESULTS AND DISCUSSION

Chloroquine is a lysosomotropic agent which inhibits intralysosomal proteolysis and may also inhibit intracellular hormone degradation at other steps—i.e., by impairing fusion of endocytotic vesicles and lysosomes (15) or by preventing degradation within vesicles that have not yet matured into recognizable lysosomes (16). When ^{125}I-insulin is incubated with chloroquine-treated hepatocytes or adipocytes, there is a marked accumulation of intact intracellular insulin and a concomitant decrease in the rate of insulin degradation (3, 4, 6, 7, 15). In studies with chloroquine-treated human fibroblasts (Fig. 1*A*), there was a 2-fold increase in cell-associated ^{125}I-insulin after a 2-hr incubation at 30°C compared to control cells. Previous reports have demonstrated that in adipocytes all of the chloroquine-mediated increase in radioactivity is nondissociable, is inaccessible to trypsin, and represents intracellular insulin (6, 7), and we have found the same results with cultured fibroblasts (unpublished data).

These results are consistent with the interpretation that cell surface-bound insulin becomes internalized via endocytosis and is degraded within cells, and the degraded products are rapidly released. Because chloroquine inhibits degradation of internalized insulin within lysosomes, and possibly at other sites, it leads to an intracellular accumulation of ^{125}I-insulin. However, chloroquine had no effect on cell-associated ^{125}I-insulin in IM-9 lymphocytes (Fig. 1*B*), indicating that the endocytotic internalization pathway is not fully intact in these cells.

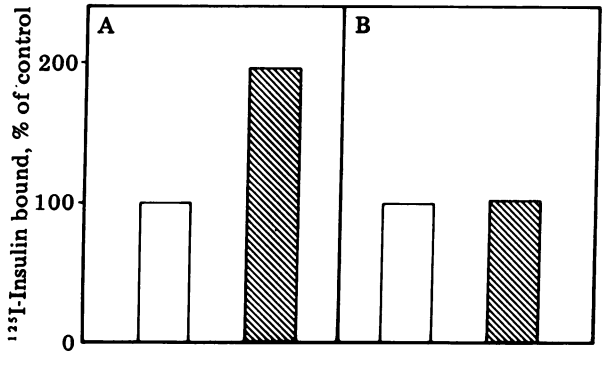

FIG. 1. Effect of chloroquine on ^{125}I-insulin binding. (*A*) ^{125}I-Insulin (0.2 ng/ml) was incubated with cultured fibroblasts (10^6 cells per dish) in monolayer for 120 min at 30°C in the absence (□) or presence (▨) of 0.2 mM chloroquine. (*B*) IM-9 lymphocytes were incubated with ^{125}I-insulin in the absence (□) or presence (▨) of chloroquine for 240 min at 37°C.

After endocytosis and internalization, insulin is processed within cells, and this pathway is at least one mechanism by which cells can degrade insulin. Results of studies of hormone degradation in these two cell lines are consistent with this formulation. Cells were incubated with ^{125}I-insulin until steady-state binding conditions were reached. Then the cells were washed to remove extracellular insulin, and bound insulin was allowed to dissociate into insulin-free buffer. After 60 min at 30°C, 41% of the previously bound ^{125}I-insulin was released from fibroblasts as degradation products (Fig. 2*A*), whereas only 4% of the insulin released from IM-9 lymphocytes was degraded (Fig. 2*B*). Under these experimental conditions, receptor-bound insulin is the only possible substrate for the degradative mechanisms. Therefore, one can conclude that receptor-mediated insulin degradation is a major pathway in fibroblasts but is absent or minimal in IM-9 lymphocytes.

Morphologic evidence suggests that IM-9 lymphocytes may internalize receptor-bound insulin to some degree (17, 18). However, the fact that these cells neither accumulate nor degrade appreciable amounts of ^{125}I-insulin suggests that intracellular insulin is released intact at a rate equal to its rate of uptake or the amount of internalized insulin processed through the intracellular degradative pathway is too small to be detected. Thus, in fibroblasts and other nontransformed cells, receptor-bound insulin is internalized and degraded intracellularly whereas, in IM-9 lymphocytes, internalized receptor-bound insulin must be rapidly released without being appreciably degraded.

Studies of insulin-mediated receptor loss in human fibroblasts and IM-9 lymphocytes revealed further differences between these cells. The rates of receptor loss were comparable between the two cell types, and incubation with insulin (100 ng/ml) led to an approximately 50% loss of insulin receptors by 6 hr. To evaluate the relationship of the endocytotic internalization pathway to the process of insulin-induced receptor loss, cells were incubated with insulin (100 ng/ml) with or without chloroquine (0.2 mM) for 6 hr. When the subsequent ability of

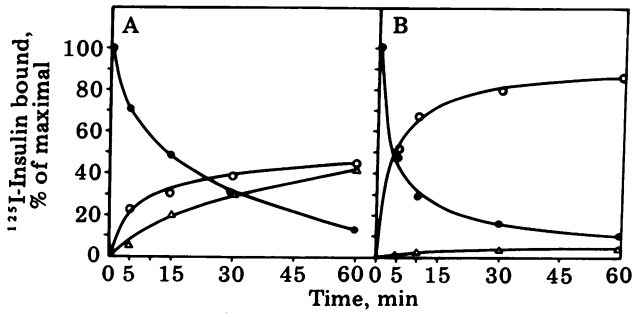

FIG. 2. Receptor-mediated ^{125}I-insulin degradation. Human fibroblasts in monolayer (*A*) or IM-9 lymphocytes in suspension (*B*) were incubated at 30°C with ^{125}I-insulin (0.4 ng/ml) to reach steady-state binding conditions (90 min). Cells were then washed and incubated with fresh insulin-free medium at 37°C. The cell-associated radioactivity (●) was allowed to dissociate, and the radioactive material that appeared in the medium during the following 60 min was measured. Radioactivity soluble in 15% trichloroacetic acid was considered to be ^{125}I-insulin degradation products (△) and acid-precipitable material was considered to be intact insulin (○).

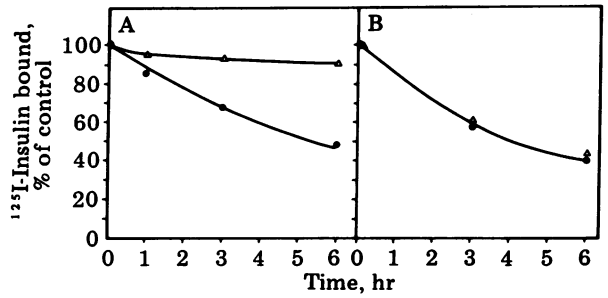

FIG. 3. Time course of insulin-induced receptor loss. Cultured human fibroblasts in monolayer (*A*) or IM-9 lymphocytes (*B*) were incubated with native insulin (100 ng/ml) in the presence (△) or absence (●) of 0.2-mM chloroquine for 6 hr at 37°C. At the indicated times, cells were washed and ^{125}I-insulin binding was measured over a 3-hr period at 15°C. ^{125}I-Insulin binding to control cells incubated for 6 hr at 37°C in medium alone or chloroquine alone was also measured and found to be unchanged.

these cells to bind insulin at 15°C was measured, chloroquine inhibited 80% of the insulin-induced receptor loss in fibroblasts, whereas it had no effect on this process in IM-9 lymphocytes (Fig. 3). These findings clearly demonstrate that the fate of receptor-bound insulin is different in these two cell types and that the mechanisms underlying receptor regulation involve processes that differ quantitatively and possibly qualitatively.

Recently, Goldstein *et al.* (19), Davies *et al.* (14), and others (20) presented evidence that the mechanism for receptor loss in cultured fibroblasts involves ligand-induced internalization of receptors through specialized areas of the plasma membrane termed "coated pits." Furthermore, these workers (14, 20) suggested that this process is mediated by transglutaminase. The proposed mechanism is that transglutaminase is a plasma membrane enzyme that may crosslink receptors in the area of coated pits, thereby facilitating aggregation and internalization. In view of this hypothesis, and the reported differences between transglutaminase activity of normal and transformed cells (21, 22), we measured the enzyme activity in IM-9 lymphocyte homogenates and compared it with the activity of normal human lymphocytes and fibroblasts. Although the apparent K_m for the substrate dansylcadaverine (2–6 μM) was comparable between the cell types tested, the values for V_{max} were substantially different (Table 1). V_{max} in IM-9 lymphocytes was less than $\frac{1}{10}$th that in normal lymphocytes and $\frac{1}{40}$th that in normal human fibroblasts. The V_{max} in normal human fibroblasts was similar to the V_{max} in other fibroblastic cell lines such as BALB, 3T3, or normal rat kidney fibroblasts (14). This is consistent with previous reports demonstrating low levels of transglutaminase in transformed cell lines (21, 22) and represents an important further difference between fibroblasts and IM-9 lymphocytes, supporting the formulation that internalization and intracellular processing of hormone and receptor

Table 1. Cellular levels of transglutaminase

Cell type	Enzyme activity	
	K_m, μM	V_{max}*
Normal human fibroblasts	2	11.9
Human peripheral blood lymphocytes	3	3.3
IM-9 lymphocytes	6	0.29

* Data are expressed as pmol of dansylcadaverine incorporated per min per mg of lysate protein. K_m and V_{max} were determined from plots of reaction velocity versus substrate (dansylcadaverine concentration).

Table 2. Effect of dansylcadaverine on insulin-induced receptor loss in normal human fibroblasts and IM-9 lymphocytes

Condition*	% insulin binding	
	Fibroblasts	IM-9 lymphocytes
Control	100	100
Insulin (100 ng/ml)	46	43
Insulin (100 ng/ml) plus dansylcadaverine (100 μM)	98	45

* Cells were incubated in the presence or absence of insulin (100 ng/ml) for 6 hr at 37°C. No change in insulin binding was observed in the control cells incubated in the absence of insulin; presence of insulin led to a 54–57% loss of receptors. When dansylcadaverine was added 30 min prior to insulin, receptor loss was blocked in fibroblasts but not in IM-9 lymphocytes.

do not proceed normally in IM-9 lymphocytes and that this may be a consequence of the low activity of transglutaminase in this transformed cell line.

Because dansylcadaverine is a potent inhibitor of transglutaminase (14, 20) and has been shown to inhibit the process of ligand-induced receptor aggregation and internalization for other hormone systems (23), we studied the effects of this agent on insulin-induced receptor loss in fibroblasts and IM-9 lymphocytes. This agent was completely effective in preventing receptor loss in human fibroblasts but had no effect on this process in IM-9 lymphocytes (Table 2).

These data provide strong evidence that, after binding to the fibroblast insulin receptor, insulin is internalized and degraded intracellularly and the products are released. It is likely that the insulin receptor is internalized along with the hormone and, because the process of receptor loss is sensitive to dansylcadaverine, we suggest that transglutaminase plays a role in insulin receptor internalization and subsequent receptor loss in normal human fibroblasts. In contrast, hormone internalization and receptor-mediated degradation are minimal or absent in IM-9 lymphocytes. Furthermore, these transformed cells contain very low levels of transglutaminase, and the process of insulin-induced receptor loss is not affected by dansylcadaverine. This may represent a qualitative difference between the cell types or a marked quantitative difference in the rates of the internalization-degradative process. This indicates that the mechanisms by which IM-9 lymphocytes regulate their surface receptors may be different than those in nontransformed cells and that internalization and intracellular processing may not play important roles. In this regard, Gavin *et al.* (24) showed that, when IM-9 lymphocytes were incubated in the presence of insulin, receptors were lost from the cell surface and could be recovered in the extracellular buffer.

The finding that chloroquine can inhibit hormone-induced receptor loss in fibroblasts demonstrates that internalization and intracellular processing of insulin and its receptor mediate the process of receptor regulation. Although the mechanism by which chloroquine inhibits receptor regulation is not known, certain inferences are possible. When a tracer concentration (picomolar) of insulin is incubated with chloroquine-treated fibroblasts, internalization (as evidenced by intracellular ^{125}I-insulin accumulation) is unhindered. On the other hand, chloroquine rapidly inhibits receptor loss induced by nanomolar concentrations of insulin. Because chloroquine acts to inhibit intracellular degradation and to increase intracellular, and possibly intralysosomal, insulin concentrations manyfold, we postulate that this large accumulation of intact intracellular insulin, together with its receptor, may mediate a feedback signal(s) that inhibits insulin-induced receptor loss. Thus, only a small amount of receptor loss occurs in the presence of chlo-

roquine before inhibition of these events takes place. With this formulation the intracellular level of insulin or its receptor and components may modulate receptor regulation and, thus, cellular insulin sensitivity.

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