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# Insulin-Dependent Regulation of Insulin Receptor Concentrations: A Direct Demonstration in Cell Culture

**(lymphocytes/extracellular fluid/hormone)**

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**ABSTRACT** **Chronic (5–16 hr) exposure of cultured human lymphocytes to $10^{-8}$ M insulin at 37° *in vitro* produced a decrease in insulin receptor concentrations unaccounted for by simple occupancy of sites; acute exposure (0–2 hr) was without effect. These results reproduced observations *in vivo* where chronic hyperinsulinemia (e.g., $10^{-8}$ M insulin in the circulation of obese insulin-resistant hyperglycemic mice) is associated with a substantial reduction in the concentration of insulin receptors per cell, while acute hyperinsulinemia *in vivo* has no effect on receptor concentration. These data suggest a reciprocal relationship between insulin in the extracellular fluid and the concentration of insulin receptors per cell, which is mediated at the target cell itself by intracellular insulin-sensitive regulatory processes and directly affects target-cell sensitivity to hormone.**

Using new methods (1–9), we and others have studied directly the binding of many polypeptide hormones to specific receptors on cells (10). The specificity of binding correlates well with the biological activity of the hormone. Typically, binding is rapid, saturable, and reversible (e.g., Fig. 1) and represented as a simple biomolecular reaction, hormone + receptor $\rightleftarrows$ hormone–receptor complex.

Recently, Archer, Goldfine, Kahn, and others have found that the concentration of insulin receptors per cell fluctuates in response to altered conditions *in vivo* (11–23). Fasting or hypophysectomy is followed by an increase in the concentration of insulin receptors per cell (20, 22) associated with the hypoinsulinemia and the supernormal sensitivity to injected insulin that characterize these states; the increase in receptor concentration per cell correlates with increased sensitivity to insulin *in vitro* (20). Conversely, obesity or glucocorticoid excess, states that are characterized by chronic hyperinsulinemia and insulin resistance, are associated with large decrements in insulin receptors but not of other hormone receptors (11, 13, 14, 21). The loss of receptors is similar in liver, fat, and lymphocytes. Appropriate treatment of the obesity or of the glucocorticoid excess ameliorates the hyperinsulinemia and insulin resistance at the same time that the receptor concentration is restored (15, 16, 22). In contrast to chronic hyperinsulinemia, acute elevation (up to 1 hr) in plasma insulin in all cases had no effect on the concentration of insulin receptors (11, 13, 14).

In the present study we reproduce this phenomenon *in vitro*. Cultured lymphocytes that are exposed to chronic elevations in the ambient insulin concentration in the physiological range have a decrement in the concentration of insulin receptors per cell; acute elevations of the ambient insulin concentrations are without effect. Other hormone (e.g., growth hormone) receptor concentrations are unaltered. The time, concentration, and temperature dependence of the process, as well as its reversal, indicates a reciprocal regulatory relationship between the concentration of insulin in the extracellular fluid and the concentration of insulin receptors per cell, and this relationship *in vivo* affects the sensitivity of the target cell to the hormone.

## MATERIALS AND METHODS

*Insulin.* Solutions of porcine insulin (PJ5589, gift of Eli Lilly) were prepared in 25 mM Tris·HCl with 0.1 mg/ml of bovine-serum albumin, pH 7.6 ("Tris-albumin buffer"). Biologically active [$^{125}$I]insulin was prepared at specific activities of 100–200 $\mu$Ci/$\mu$g (9, 24).

*Growth of Cultured Lymphocytes.* Human lymphocytes (line no. IM-9) were maintained at 37° in Eagle's minimal essential medium supplemented with 10% fetal-calf serum (Grand Island Biological Supply) by feeding twice weekly (25). For detailed information on these cells, consult Fahey *et al.* (26). Cell viability, monitored by trypan blue dye exclusion (27), exceeded 90% in all experiments.

*Preincubation of Cells with Insulin.* Cultured lymphocytes, 48 hr after feeding, in midexponential phase of growth, were pooled in a sterile 500-ml glass bottle. From this homogeneous pool ($1.5 \times 10^6$ cells per ml), 50-ml aliquots were transferred to sterile Falcon culture flasks, to which insulin was added in a total volume of 30 $\mu$l of Tris-albumin buffer. Cells were then preincubated at 37° for 16 hr, unless otherwise indicated. All of the above operations were performed aseptically.

*Washing Procedure Before [$^{125}$I]Insulin Binding.* After preincubation, the contents of each flask were transferred to 50-ml plastic conical centrifuge tubes, and spun at $600 \times g$ for 10 min (Beckman model PR-6). The cells were immediately resuspended in 40 ml of 25 mM Tris·HCl that contained 120 mM NaCl, 1 mg/ml of bovine-serum albumin, and 1 mM dextrose, pH 7.6 ("wash buffer"). The cells were sedimented again ($600 \times g$, 5 min) and resuspended in a fresh 40-ml aliquot of wash buffer; after 25 min at 23°, the sedimentation and resuspension were repeated. After an additional 25 min at 23°, the cells were again centrifuged for 5 min and re-

suspended in "assay buffer" [25 mM Tris·HCl, 120 mM NaCl, 1.2 mM magnesium sulfate, 1 mM disodium ethylenediaminetetraacetate (EDTA), 10 mM dextrose, 15 mM sodium acetate, and 1 mg/ml of bovine-serum albumin, pH 7.6]. Final cell concentrations at the end of the washings were 1.5 to 2.5 $\times$ $10^7$ cells per ml. Aliquots from this suspension of washed cells were used to study [$^{125}$I]insulin binding. All washings were conducted in the same vessel with less than 3% loss of cells and no loss of viability.

*[$^{125}$I]Insulin Binding to Washed Cells.* For binding studies, [$^{125}$I]insulin (0.005–0.05 nM) was incubated with washed cells (5 to 10 $\times$ $10^6$) in a total volume of 0.5 ml at 15° in the absence and presence of unlabeled insulin. After 75–90 min, the cells were sedimented and separated from the assay buffer, as described (9). Radioactivity that was bound to cells in the presence of 50 $\mu$g/ml of unlabeled insulin was considered "nonspecific" binding and was subtracted from the total binding (8).

## RESULTS

*Effect of Preincubation with Insulin on [$^{125}$I]Insulin Binding to Lymphocytes.* To mimic *in vivo* conditions, we preincubated lymphocytes at 37° in growth medium with insulin at $10^{-8}$ M, which approximated the hormone concentration in the plasma of obese mice who manifest insulin resistance and a diminution in insulin receptors per cell (11–13). Under these conditions, binding of insulin is rapid, reaching a steady state in less than 30 min, and receptor-bound [$^{125}$I]hormone is readily dissociated after addition of an excess of unlabeled hormone (Fig. 1).

After preincubation and washing, we determined the insulin receptor concentration per cell by incubating cells for 90 min at 15° with [$^{125}$I]insulin in the absence and presence of various concentrations of unlabeled insulin (Fig. 2). [$^{125}$I]Insulin binding to cells that were preincubated with $10^{-8}$ M insulin for 0–2 hr was identical to that of cells that had not been exposed to insulin (Fig. 2). When the preincubation was extended to 5 hr, [$^{125}$I]insulin binding was decreased by 30%; at 16 hr the decrement in binding was 55% (Figs. 2 and 3). Since all five curves in Fig. 2 are identically shaped, we concluded that the decrease in binding was accounted for by a decrease in the effective concentration of receptors and not by significant alteration in affinity.§ This conclusion was confirmed by more formal analysis of the data (28) (Fig. 2, *inset* and legend). A lower concentration of insulin ($10^{-9}$ M), although capable of filling a significant fraction (29) of insulin receptors in lymphocytes and producing biological effects *in vivo* and *in vitro* (30), had no effect during 16 hours of preincubation (Fig. 4). Concentrations of insulin greater than $10^{-8}$ M ($10^{-7}$ and $10^{-6}$ M) were more effective more quickly (Figs. 3 and 4).

These data parallel results *in vivo*. With insulin at $10^{-8}$ M,

§ From data in these and other studies, we and others (9) have assumed that under the steady-state conditions during which we measure [$^{125}$I]hormone binding, equilibrium conditions are well enough approximated to permit formal analysis of the binding data such as those described by Scatchard (28). Under conditions where we have detected decreases in [$^{125}$I]insulin binding, the analyses indicate a comparable decrease in receptor concentrations, but, because of the limitations in these types of analyses we have not entirely excluded a decrease in the affinity of the sites for hormone which could also account for the decreased binding.

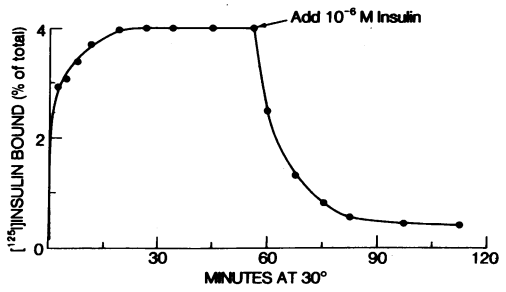


FIG. 1. Time course of [$^{125}$I]insulin binding to cultured human lymphocytes in complete growth medium. Cells (5 $\times$ $10^6$/ml) were incubated with [$^{125}$I]insulin (adjusted to $10^{-8}$ M concentration) at 30°. At 60 min, unlabeled insulin was added in a small volume (20 $\mu$l) to give a final concentration of $10^{-6}$ M. Each point has been corrected for "nonspecific" binding (8).

the magnitude of the decrement observed here *in vitro* is the same as that observed with hepatocytes and lymphocytes isolated from hyperinsulinemic insulin-resistant mice (13), and in both circumstances chronic but not acute elevations in insulin concentration affected the concentration of insulin receptor. *In vitro*, as *in vivo*, the decrease in [$^{125}$I]insulin binding appeared to be a regulatory event subsequent to binding and not due to simple occupancy or contamination. Exposure of cells to $10^{-8}$ M hormone for 2 hr at 37° produced no decrement in subsequent binding of [$^{125}$I]insulin, indicating that the decrement in [$^{125}$I]insulin binding after 5 hr of preincubation must have been an event subsequent to binding and could not be attributed to "contamination" from insulin in the preincubation. Contamination should be detected after 30 min or 2 hr of preincubation, as well as after 5 hr, and shoud not increase further, since binding of insulin to the cells under the conditions of preincubation is maximal within 30 min (Fig. 1). The competition curves in Fig. 2 offer further sup-

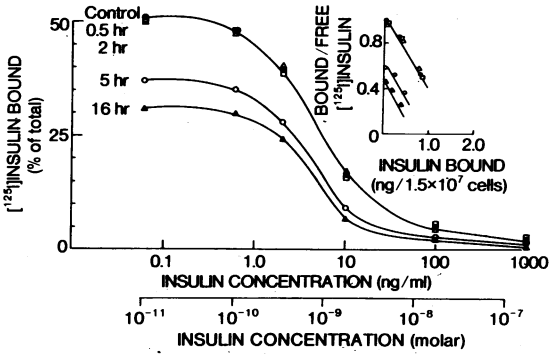


FIG. 2. [$^{125}$I]Insulin binding as a function of the insulin concentration with cells that had been preincubated with $10^{-8}$ M insulin. Cells (1.5 $\times$ $10^6$/ml) were preincubated for 16 hr at 37° in complete medium. Insulin ($10^{-8}$ M) was added to the medium 16 hr (▲), 5 hr (○), 2 hr (△), 0.5 hr (□), or not at all (control, ●) before the completion of the preincubation. After separation of cells from growth medium, washing, and resuspension in Tris-albumin assay buffer, binding of labeled insulin was studied. Cells (1.5 $\times$ $10^7$/ml) were incubated with 5 $\times$ $10^{-12}$ M [$^{125}$I]insulin in the presence of unlabeled insulin for 75 min at 15°. Each point represents the average of two experiments. *Inset:* Scatchard plot (28) of data with insulin concentrations in the range of 0.06–3.0 ng/ml, representing the region of the "high-affinity" binding sites (29). Extension of the analysis to the data at higher insulin concentrations revealed a second ("lower") order (29) of sites; results with both sets of sites were quite similar.

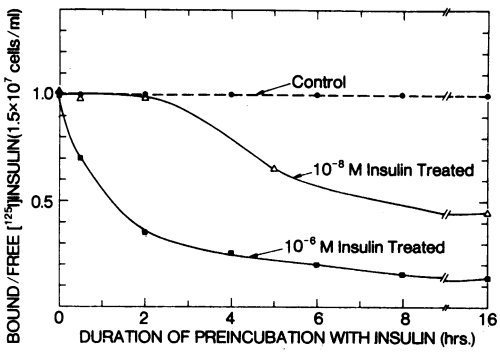


FIG. 3. Effect of duration of exposure to insulin on the binding of [$^{125}$I]insulin. Cells were preincubated for 16 hr at 37°; at intervals, a small volume of Tris-albumin buffer, with and without insulin, was added. At the completion of the preincubation, the cells were separated, washed, resuspended ($1.5 \times 10^7$ cells per ml), and exposed to [$^{125}$I]insulin ($10^{-12}$ M) for 90 min at 15°. The bound/free ratio of the [$^{125}$I]insulin is plotted as a function of duration of the preincubation period after addition of Tris-albumin with or without insulin.

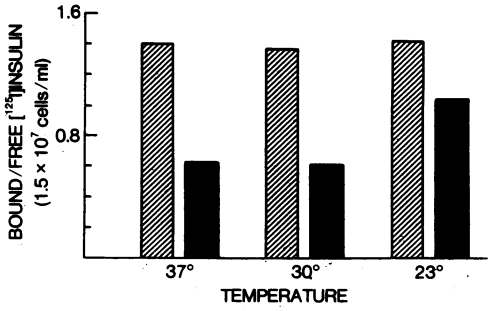


FIG. 5. [$^{125}$I]Insulin binding to lymphocytes after 16 hr preincubation with $10^{-8}$ M insulin at various temperatures. Cells in complete media with and without $10^{-8}$ M insulin were preincubated for 16 hr in water baths maintained at 37°, 30°, or 23°. The cells were then separated from media, washed, resuspended in assay buffer, and incubated with [$^{125}$I]insulin for 90 min at 15°. The bound/free ratio of [$^{125}$I]insulin at 15° is plotted as a function of the temperature of the preincubation, in the absence (*hatched bar*) and presence (*solid bar*) of $10^{-8}$ M insulin. Each *bar* represents the average of two experiments.

port. In each of the five curves, the binding of [$^{125}$I]insulin was inhibited significantly by as little as 0.6 ng/ml of unlabeled insulin ($10^{-10}$M) and half-maximal inhibition of binding occurred with about 6 ng/ml ($10^{-9}$ M) of unlabeled hormone (Fig. 2). Contamination would be expected to manifest itself in these curves as a reduction in binding at low hormone concentrations, while at moderately high insulin concentrations all five curves would overlap, but this was not observed.

To show that the decreased binding of labeled insulin cannot be ascribed to unlabeled insulin released from the cells into the medium during the binding assay, cells were preincubated with $10^{-8}$ M insulin at 37° for 16 hr, washed, and incubated in assay buffer for 90 min at 15° (without [$^{125}$I] insulin). The supernatant from that experiment, when incubated with [$^{125}$I]insulin and fresh cells for 90 min at 15°, had no effect on the binding of [$^{125}$I]insulin.

*Effect of Temperature During Preincubation.* Cells were preincubated at 37, 30, or 23° for 16 hr in the absence and presence of insulin ($10^{-8}$ M), after which they were washed at 23° and incubated with [$^{125}$I]insulin for 90 min at 15°. Preincubation at 30° produced the same reduction in receptor concentrations as preincubation at 37°, whereas preincubation at 23° produced a much smaller decrement in receptor concentrations (Fig. 5). Since at 23°, under the conditions of the preincubation, insulin bound to receptor was actually greater than at 30° or 37° (29), it would appear that the decrement in insulin receptor concentrations is a temperature-dependent cellular event subsequent to and distinct from physicochemical binding of hormone to receptor.

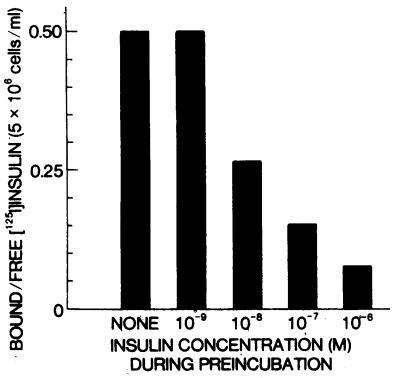


FIG. 4. Effect of insulin concentration during preincubation on the binding of [$^{125}$I]insulin. Cells were preincubated with insulin (0–$10^{-6}$ M) for 16 hr, washed, resuspended ($5 \times 10^6$/ml), and incubated with [$^{125}$I]insulin ($10^{-11}$ M) for 75 min at 15°. "Nonspecific binding" was practically identical for each set, and results have been corrected for this contribution. Each *bar* represents the average of two experiments.

*Regeneration of Insulin Binding Capacity.* Cells were preincubated with $10^{-8}$ M insulin for 16 hr, separated from the insulin-containing growth medium, and washed as described. One set of insulin-treated cells was resuspended in assay buffer and shown to have decreased binding capacity (see Fig. 4). When a companion set of cells was resuspended in fresh insulin-free growth medium and incubated for an additional 16 hr at 37°, the cells recovered their full insulin-binding capacity. When the second preincubation was performed in fresh medium but with $10^{-9}$ M insulin added, binding capacity increased but was not restored to normal. Thus, $10^{-9}$ M insulin, which was insufficient to depress the receptor concentration (Fig. 4), was sufficient to prevent full restoration of the receptor binding capacity. This is consistent with observations *in vivo* where a given level of plasma insulin that was insufficient to depress the concentration of receptors was sufficient to inhibit full repair (16).

## DISCUSSION

In some conditions *in vivo*, chronic (but not acute) elevations of circulating insulin are associated with a reduction in the number of receptor sites that are available for insulin binding. The present experiments reproduced this phenomenon directly *in vitro*. The insulin concentration used to produce the effect and the receptor deficiency observed are quite comparable to those observed *in vivo* in severe insulin-resistant states, e.g., obese mice.

Insulin appears to have two distinct effects on receptors. The first step is a physicochemical interaction in which in-

sulin binds rapidly to a fixed set of specific sites on the cell surface. Subsequently the insulin-receptor complex at physiological temperatures in intact cells, through a series of unknown steps, generates the biological responses of the cell to insulin. The present data suggest that one of the effects of insulin on cells is the transmission of a regulatory signal to decrease the concentration of receptors available for binding, and the magnitude of this effect is related to the level of hormone and duration of exposure. The features that distinguish the physicochemical binding process from the hormone receptor-regulatory process are outlined in Table 1.

The decrease in receptors cannot be ascribed to simple contamination of receptors by insulin added during the preincubation. If it had occurred, there should have been no delay in its onset upon treatment of the cells with $10^{-8}$ M insulin and no progression with time, and it should have been more marked with preincubation at 23° than at 37° (29). Further, a reduction in binding due to contamination might be expected to alter the shapes of the competition curves, but this did not occur.

The intracellular events initiated by insulin that lead to reduction in the number of sites are unknown, as is the fate of the lost receptors. Possible mechanisms include decreased synthesis, accelerated degradation, release into the medium, translocation to an intracellular site, inaccessibility due to insulin-induced aggregation of the receptor structures, and a metabolic event that includes irreversible binding of insulin to the receptor or intracellular accumulation of insulin. It should be pointed out that "movement" of plasma-membrane surface proteins of lymphocytes can occur upon interaction of these moieties with specific binding proteins added to the incubation medium (31–33). This observation has also been extended to specific surface proteins in fibroblasts (34). Insulin at high concentrations may induce similar perturbations in the morphology of lymphocyte membranes, leading to loss of available receptors. It is also possible that the insulin bound to the receptors may be transported to intracellular compartments by mechanisms similar to those reported for surface immunoglobulins (31).

The physiological system may be pictured as literally self-modulating. Protracted elevations of hormone levels cause a decrease in the number of receptors available for insulin binding. Decreases in the number of receptors, and therefore of insulin-mediated messages, may then reduce the insulin effects, including a reduction in the drive to decrease receptors. Thus, each steady-state insulin concentration will produce, after some hours, a steady-state concentration of receptors. We expect that this modulation will be observed at both increased and subnormal insulin concentrations. Even in systems where only a minority of receptor sites need to be filled to achieve the maximal biological response (i.e., where there are "reserve receptors"), the sensitivity of the system to hormone will be altered by an alteration in the receptor concentration. In addition to its modulating effect, this mechanism may be viewed as a safety valve, protecting against persistently high hormone concentrations.

In insulin-resistant states, there is a close correlation between insulin resistance and the basal circulating insulin concentration (35, 36). It has been generally thought that the resistance is due to circulating peripheral antagonists or to insulin resistance at the tissue level, or both, and that the resistance or antagonism produces hyperinsulinemia by feedback stimulation of insulin secretion (35–37). Our finding that hyperinsulinemia itself is capable of decreasing insulin-receptor concentration, and thereby reducing tissue sensitivity to insulin, leads us to suspect that in some insulin-resistant states overproduction of insulin might be the primary defect and that tissue resistance follows. Further, in conditions where the primary defect is in the target cell, the hyperinsulinemia that results might act, in some of these states, to increase further the insensitivity of the tissue to insulin. Moreover, insulin itself may be one of the circulating insulin-antagonists sought after for the last decade or more (38, 39). Insulin-resistant states in which the hyperinsulinemia may, at least in part, be an early contributory condition include obesity, where overeating produces protracted insulin peaks and, probably more important, high valleys between peaks (40–42); patients treated with long-acting insulins; insulin-treated patients in whom circulating antibodies act as chronic reservoirs of insulin; patients with insulin resistance after insulin overdosage (Somogyi effect); and patients with insulin-secreting tumors.

TABLE 1. *Characteristics of physicochemical binding of insulin to lymphocytes compared to regulatory effects of insulin on receptor concentrations*

| Parameter | Physicochemical binding | "Regulatory" effect |
|---|---|---|
| Lowest insulin concentration at which observed | $10^{-12}$ M | $10^{-8}$ M |
| Onset of observed effect with $10^{-8}$ M insulin | $\leq$ 3 min | >2 hr |
| Progression with time | Steady state reached in 20 min | Continuous increase at 5–16 hr |
| Effect of lowering temperature | Significantly increased | Significantly decreased |

Recent studies have extended this phenomenon of receptor modulation to human growth hormone and its specific receptors (43). While growth hormone affects its own receptors, it produces no effects on the insulin receptors in these cells, and vice versa. Interestingly with growth hormone, a 50% decrease in receptor concentrations was detected[¶] after exposure of the cells to concentrations of hormone ($10^{-10}$ M) that produce occupancy of only 10% of the receptor sites (43). This may be a general mechanism of modulation for each of the polypeptide hormones. The following observations are cited as a few possible examples of this mechanism in action. In patients with choriocarcinoma or with medullary carcinoma of the thyroid, who have astronomical concentrations of biologically active chorionic gonadotropin or thyrocalcitonin in their circulation, manifestations of hormone excess are usually trivial or absent (44, 45), whereas, a tiny fraction of this hormone concentration in normal subjects produces significant physiologic effects. In mice that are treated with chorionic gonadotropin for 3 days the uterine weight increases in proportion to the dose of hormone up to a point. Doses in excess of that produce a fall off in the effect,

[¶] Lesniak, M. A., Gavin, J. R., III & Roth, J., manuscript in preparation.

so that at very high doses effects are markedly reduced.[‖] Similarly, ovaries acutely, *in vitro*, respond equally to luteinizing hormone and to prostaglandin $E_1$, measured as activation of adenylate cyclase or as steroidogenesis. Exposure of ovaries to luteinizing hormone for 16 hr *in vitro* abolished the hormone's responsiveness without any alteration in sensitivity to prostaglandin $E_1$ (46). Conversely, increased sensitivity to hormone after its removal, e.g., enhanced sensitivity of adrenals to adrenocorticotrophic hormone after hypophysectomy, may also be mediated by this system of direct modulation. Finally, although not every form of hyperinsulinemia produces the same fall in insulin-receptor concentrations (e.g., acromegaly) and not every example of target-cell resistance is due to changes in receptor concentrations (e.g., postnatal development), receptor modulation appears to be a widespread and important mechanism *in vivo*.

**Note Added in Proof.** The demonstration by De Meyts *et al.* (1973) [*Biochem. Biophys. Res. Commun.* **55**, 154–161] with these cells, as well as with purified plasma membranes from rat liver, that receptor sites for insulin interact with one another reinforces this caveat.

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