Antibody against the insulin receptor causes disappearance of insulin receptors in 3T3-L1 cells: A possible explanation of antibody-induced insulin resistance
(hormone desensitization/type B syndrome of insulin resistance and acanthosis nigricans/cultured fat cells/deoxyglucose uptake)

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**ABSTRACT** The effect of a rabbit antibody against the rat insulin receptor (RAR) was tested using cultured 3T3-L1 fat cells. As previously seen with antibodies against the insulin receptor from patients with the type B syndrome of insulin resistance and acanthosis nigricans, RAR acutely mimicked the action of insulin by stimulating deoxyglucose uptake. After prolonged exposure of 3T3-L1 cells to RAR, insulinomimetic activity was lost and the cells became resistant to the action of insulin. This state of insulin resistance is similar to that seen with the human autoantibodies. However, unlike antibody from the patients, RAR did not acutely inhibit the binding of insulin to its receptor; rather, RAR increased the binding of insulin to its receptor by a mechanism consistent with an increase in the affinity of the receptor for insulin. With prolonged exposure to RAR there was a dramatic decrease in insulin-receptor binding on the treated 3T3-L1 fat cells. These results suggest that antibody against the insulin receptor induces insulin resistance by a mechanism that involves loss of cell-surface insulin receptors.

Antibodies directed against the insulin receptor are found in the antiserum of patients with the type B syndrome of insulin resistance and acanthosis nigricans (HAR) (1, 2). Such patients show a marked decrease in the binding of insulin to circulating monocytes (2). When studied acutely in vitro, HAR was shown to inhibit the binding of insulin to its receptor but also to mimic all of the known actions of insulin (1, 3).

A model for the induction of insulin resistance by these anti-receptor autoantibodies has been developed using 3T3-L1 fat cells maintained in tissue culture (4, 5). As in other tissues, HAR acutely mimics the action of insulin in 3T3-L1 cells but, with prolonged exposure to HAR in culture, the insulinomimetic activity disappears and the cells are rendered resistant to the actions of insulin (4, 5). This does not represent simple "down-regulation," as 3T3-L1 cells studied under these conditions do not down-regulate in response to insulin (6). Because HAR also acutely inhibits the binding of insulin to its receptor, it has not been possible to follow the fate of the insulin receptor during antibody-induced insulin resistance. When cells are treated with saturating amounts of HAR, no difference is seen between the acute inhibition of binding and the residual binding after the induction of insulin resistance (4). Hence, for lack of a clear mechanism, this process was termed "antibody-induced desensitization of the insulin receptor." The process was shown to be time and temperature dependent but was not inhibited by a variety of inhibitors of specific cell functions (5).

This paper examines the effect of an antibody raised in rabbits against the rat hepatic insulin receptor (RAR) that does not acutely inhibit the binding of insulin to its receptor (7, 8). The use of this antibody makes it possible for the first time to dissociate the short-term effects of the anti-receptor antibody on insulin binding from the longer term induction of insulin resistance by anti-receptor antibody in 3T3-L1 cells. The data indicate that antibody against the insulin receptor causes the loss of cell-surface insulin receptors during antibody-induced desensitization.

**METHODS**

**Materials.** Dexamethasone was purchased from Sigma. 1-Methyl-3-isobutylxanthine was purchased from Aldrich. Bovine serum albumin was purchased from Armour Pharmaceutical (Phoenix, AZ). Pork insulin was obtained from Elanco (Indianapolis, IN). 2-Deoxy[3H]glucose (30 Ci/mmole) and carrier-free Na[3H]I were from New England Nuclear; Petri plates and fetal calf serum were obtained from Flow Laboratories (Rockville, MD).

Rabbit antiserum produced against RAR was provided by Steven Jacobs (Burroughs—Wellcome) (7, 8). RAR and control serum were used after heat inactivation. HAR was obtained from two patients with the type B syndrome of insulin resistance and acanthosis nigricans (National Institutes of Health patients B-2 and B-8) and was partially purified as described (4).

3T3-L1 cells were provided by Howard Green (Massachusetts Institute of Technology). The cells were grown in Dulbecco—Vogt modified Eagle’s medium containing 10% fetal calf serum (DV medium) as described (6). Differentiation was induced by treating confluent cells for 2 days with DV medium, supplemented with 0.5 mM 1-methyl-3-isobutylxanthine/0.25 μM dexamethasone/insulin (1 μg per ml) as described (9).

**Insulin Action and Insulin Binding.** Insulin stimulation of 2-deoxyglucose uptake and the binding of 125I-insulin were studied under identical conditions on 3T3-L1 cells adherent to tissue culture plates by previously described methods (4, 9). All assays were performed in Krebs—Ringer phosphate buffer containing bovine serum albumin (20 mg/ml)/1.3 mM CaCl2, pH 7.4 (1x KR buffer). Both insulin binding and insulin stimulation of deoxyglucose uptake were measured at 22°C. For deoxyglucose uptake, cells were incubated for 20 min in 1x KR buffer or in 1x KR buffer supplemented with insulin (1 μg/ml) or antibody to the indicated dilutions. Up—

Abbreviations: HAR, antibody against the insulin receptor found in patients with the type B syndrome of insulin resistance and acanthosis nigricans; RAR, antibody against the insulin receptor induced in rabbits by immunization with purified rat liver insulin receptor; DV medium, Dulbecco—Vogt modified Eagle’s medium containing 10% fetal calf serum; 1x KR buffer, Krebs—Ringer phosphate buffer containing bovine serum albumin (20 mg/ml) and 1.3 mM CaCl2.
take was then measured by a 20-min pulse of 0.2 mM deoxy[3H]glucose (0.4 Ci/ml). The reaction was terminated by washing with Dulbecco's phosphate-buffered saline at 4°C, and the cells were prepared for liquid scintillation counting as described (4, 9).

125I-labeled insulin was prepared by modification of the chloramine-T method to a specific activity of 150-200 μCi/mg or ≈0.5 iodine per insulin (10). Cells were incubated with 125I-labeled insulin (50 pM) under conditions identical to those of the deoxyglucose-uptake assay, except that the incubation was carried out for 10 min in 1× KR buffer. The cells were then washed with Dulbecco's phosphate-buffered saline and prepared for gamma counting as described (4, 9).

Densensitization Protocol. For desensitization, cells were exposed to antibody against the insulin receptor or control serum in DV medium for 7 hr at 37°C in a 5% CO2/95% air incubator. Cells were then rinsed and insulin binding or insulin action was studied in KR buffer as described above. This protocol is identical to that used previously when studying HAR from patients with the type B syndrome of insulin resistance and acanthosis nigricans (4, 5).

RESULTS

The Acute Effects of Rabbit Anti-receptor Antibody on Insulin Action. As previously seen with other cell types, RAR acutely mimicked the action of insulin in 3T3-L1 cells. After a 20-min incubation at 22°C, both RAR and HAR from patient B-8 were able to stimulate deoxyglucose uptake to the same extent as insulin (Fig. 1). Control serum had no effect on deoxyglucose uptake at these dilutions (data not shown).

The Effect of Prolonged Exposure of 3T3-L1 Cells to RAR on Insulin Action. 3T3-L1 cells were exposed to either RAR or HAR from patients B-2 or B-8 for 7 hr at 37°C. When basal deoxyglucose uptake was then measured, the insulinomimetic activity had disappeared (Fig. 2, open bars). In addition, after this prolonged exposure to either RAR or HAR, insulin was no longer able to stimulate deoxyglucose uptake in the treated 3T3-L1 cells (hatched bars). Thus, RAR caused a state of insulin resistance in 3T3-L1 cells similar to that seen when the cells are exposed to HAR from patients with the type B syndrome. Exposure to control serum had no significant effect on basal or insulin-stimulated deoxyglucose uptake compared with cells not exposed to serum (data not shown).

The Acute Effect of RAR on Insulin Binding. RAR did not inhibit the binding of insulin to its receptor during acute exposure. In fact, when 3T3-L1 cells were acutely exposed to RAR, tracer insulin binding increased (Fig. 3, Inset). The maximal increase in insulin binding was seen at an antibody titer of 1:100, similar to that required for the maximal stimulation of deoxyglucose uptake by RAR (data not shown). In several experiments, the increase in insulin tracer binding varied from 220% to 300% of control. The effect of RAR on insulin binding was then analyzed by means of a Scatchard plot (Fig. 3). The binding of insulin to its receptor on RAR-treated cells was increased at low concentrations of insulin but approached that of control cells at higher concentrations. This is consistent with RAR acutely increasing insulin binding by increasing the affinity of the receptor for insulin.

The Effect of Prolonged Exposure to RAR on Insulin Binding. To determine the fate of the insulin receptor during the induction of insulin resistance by RAR, cells were exposed in culture to RAR for 7 hr in DV medium at 37°C, a time sufficient to induce insulin resistance. Prolonged exposure to increasing amounts of RAR led to a decrease in tracer insulin binding (Fig. 4, Inset). When the decrease in binding was analyzed by a Scatchard plot (Fig. 4), the data were consistent with a decrease in insulin receptor number.

DISCUSSION

In this paper, the effects of acute and prolonged exposure of 3T3-L1 cells to rabbit antibody against the insulin receptor were studied. During acute treatment, RAR mimicked the action of insulin, having the same maximal effect as insulin or HAR on stimulating deoxyglucose uptake. The concentration of RAR required for stimulation of deoxyglucose uptake
in 3T3-L1 cells was slightly higher than that required for stimulation of glucose oxidation in rat adipocytes or lipid synthesis in rat hepatocytes (7, 11). This is consistent with either the decreased sensitivity of 3T3-L1 cells compared to rat adipocytes or a difference in species specificity (3T3-L1 cells are a mouse line and may not crossreact as well with RAR, an antibody raised against purified rat hepatic insulin receptors).

As seen with rat adipocytes and hepatocytes, RAR did not acutely inhibit the binding of insulin to its receptor in 3T3-L1 cells. Rather, RAR increased the binding of insulin through its receptor by a mechanism that appears, by conventional Scatchard analysis, to involve an increase in the affinity of insulin for its receptor. It is possible that RAR acutely increases insulin binding by interfering with the interaction of the insulin receptor with the recently proposed affinity modulator (12, 13). However, Klotz and others have called attention to the difficulty in precisely quantifying receptor number or affinity (14, 15). Since saturation was not reached on

"Klotz" plots (data not shown), a change in receptor number cannot be ruled out. Alternatively, this increase in binding may be a reflection of differences in internalization (16). The acute increase in insulin binding induced by RAR is not a result of the higher concentrations of RAR needed for maximal effect in 3T3-L1 cells, as at least a 2-fold increase in insulin binding to 3T3-L1 cells was seen when using the same concentration as that used in rat hepatocytes (11).

With prolonged exposure of 3T3-L1 cells to RAR, the insulinomimetic activity of the antibody was lost and the cells were rendered severely resistant to the action of insulin. Since RAR did not acutely inhibit the binding of insulin to its receptor, it was therefore possible to follow the fate of the cell-surface insulin receptors during the induction of insulin resistance by RAR: cell-surface insulin binding decreased dramatically by a mechanism that probably involves a decrease in insulin-receptor number.

It has not been possible to determine the fate of cell-surface insulin receptor during induction of insulin resistance by HAR, as the antibody from patients with the type B syndrome acutely inhibits insulin binding (4, 5). Little difference was seen between the acute inhibition of insulin binding by saturating concentrations of HAR and the amount of insulin binding found after induction of insulin resistance by HAR in 3T3-L1 cells (4). It has previously been postulated that a normal insulin receptor might remain on the surface of cells of type B patients in vivo (17), as an acid wash capable of dissociating antibody-antigen complexes increased insulin binding on the surface of cells of monocytes from these patients. However, the acid wash was able to restore insulin binding to nearly normal only when cells were acutely treated with HAR for 1 hr; when circulating monocytes from type B patients that are chronically exposed to HAR in vivo were given the same acid wash, insulin binding was only slightly increased (17). Similar acid washes could not restore binding to normal in 3T3-L1 cells that had been exposed to HAR for prolonged periods (ref. 5; unpublished data). More recently, studies from this laboratory indicate that HAR from type B patients may also induce a progressive loss of cell-surface insulin receptors (unpublished data).

The mechanism by which antibodies against the insulin receptor induce the loss of cell-surface insulin binding is further defined. It is unlikely that this process is identical to the down-regulation seen with insulin for several reasons. First, insulin does not down-regulate its receptor in 3T3-L1 cells under the conditions (6, 18) that have been used for these studies. However, Ronnett et al. (19) reported that insulin can cause receptor down-regulation when 3T3-L1 cells are treated with insulin within 24 hr of removal of the insulin-containing medium used for differentiation; this corresponds to the period when insulin receptor number is "up-regulating" during differentiation to the adipocyte phenotype. Second, the insulin resistance seen in type B patients far exceeds that which can be attributed to down-regulation in hyperinsulinemic states (2). Finally, antibody-induced desensitization does not resemble insulin-induced down-regulation, as it occurs faster and is not inhibited by cycloheximide (4). However, it is not yet clear whether the loss of insulin receptors causes the desensitization to insulin or whether the insulin receptor is first inactivated and then lost.

Antibodies against the cholinergic receptor in myasthenia gravis decrease the number of cholinergic receptors by increasing the cellular degradation rate of the receptor (20). Antibodies against the insulin receptor could work by a similar mechanism; alternatively, they might induce internalization without degradation or cause shedding of the receptor into the medium. Although we cannot distinguish between these possibilities at this time, it is of note that HAR requires bivalence in order to induce insulin resistance (5); antibodies against the cholinergic receptor increase receptor degrada-
tion by a cross-linking process that also requires bivalence (20).

In conclusion, RAR was used in cultured fat cells to induce a state of insulin resistance similar to that induced by HAR from patients with the type B syndrome of insulin resistance and acanthosis nigricans. These studies indicate that the induction of insulin resistance by anti-receptor antibody is not dependent on the acute inhibition of insulin binding that is usually seen with anti-receptor antibodies from humans. Moreover, the induction of insulin resistance by RAR was accompanied by the loss of cell-surface insulin receptor binding. A similar mechanism may be responsible for the insulin resistance seen in the type B syndrome of insulin resistance and acanthosis nigricans.

**Note Added in Proof.** Since this manuscript was submitted, Roth et al. (21) and Taylor and Marcus-Samuels (22) have shown that antibodies against the insulin receptor cause down regulation of the insulin receptor in 1M-9 cells.

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