Effects of Insulin Incubation on Insulin Binding, Glucose Transport, and Insulin Degradation by Isolated Rat Adipocytes

EVIDENCE FOR HORMONE-INDUCED DESENSITIZATION AT THE RECEPTOR AND POSTRECEPTOR LEVEL

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ABSTRACT We have examined the effect of in vitro hyperinsulinemia on insulin binding, glucose transport, and insulin degradation in isolated rat adipocytes. When cells were incubated with insulin for 2 or 4 h at 37°C, followed by washing in insulin-free buffer to remove extracellular and receptor-bound insulin, a time and dose-dependent decrease in insulin receptors was observed, which was accompanied by a reduced ability of cells to degrade insulin. Furthermore, the quantitatively predicted rightward shift in the insulin-glucose transport dose-response curve could be demonstrated. In addition to this reduction in insulin sensitivity, a striking decrease in maximal insulin-stimulated glucose transport was observed in the 4-h insulin-treated cells, indicating an abnormality distal to the insulin receptor. Thus, in vitro insulin-induced insulin resistance in adipocytes is caused by both receptor and postreceptor abnormalities. The postreceptor defect is most likely at the level of the glucose transport system per se because the insulinlike agents, spermine and antiinsulin receptor antibodies, also had a markedly reduced ability to stimulate glucose transport in 4-h insulin-treated cells. On the other hand, when cells were incubated with 100 ng/ml insulin for up to 4 h, after which time 2-deoxy glucose uptake was measured without removing buffer insulin or allowing receptor-bound insulin to dissociate, no decrease in maximal insulin-stimulated glucose transport was found.

In conclusion, (*a*) insulin leads to a dose-dependent loss of insulin receptors in freshly isolated adipocytes accompanied by the predicted functional consequence of decreased receptors, i.e., a rightward shift in the insulin-glucose transport dose-response curve, (*b*) prolonged incubation with insulin causes a marked postreceptor defect in the glucose transport system, (*c*) maintenance of the activated state of the glucose transport system prevents the expression of the postreceptor defect, (*d*) the location of the postreceptor abnormality is most likely in the glucose transport system per se, and (*e*) insulin-induced receptor loss is accompanied by a decrease in insulin degradation.

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INTRODUCTION

Numerous studies have indicated that insulin can inversely regulate the number of cellular insulin receptors. The first direct evidence for hormone-induced receptor loss was provided by the studies of Gavin et al. (1), who demonstrated that incubation of cultured human lymphocytes with insulin resulted in a time and dose-dependent loss of insulin receptors. Subsequent reports have shown that, in vitro, an insulin-induced loss of receptors can also be demonstrated in cultured hepatocytes (2), fibroblasts (3), and fat tissue explants (4, 5), thus confirming the original observations of Gavin et al. These in vitro results are also supported by a variety of in vivo observations. For example, human and animal obesity are associated with hyperinsulinemia (6, 7), and various tissues from obese animals and man demonstrate a decrease in the number of cellular insulin receptors (8–13). Furthermore, amelioration of the hyperinsulinemia leads to a reversal of the receptor loss (13). Many other hyperinsulinemic states have similarly

been associated with decreased insulin binding to insulin target tissues (14, 15).

Insulin resistance is another characteristic feature of most hyperinsulinemic states, and occurs concomitantly with reduced insulin receptors (8–13). However, the relationship between the loss of insulin receptors and overall insulin resistance has been difficult to quantitate in the in vivo situation as a result of a number of confounding factors, such as circulating insulin antagonists, postreceptor abnormalities in insulin action, and other in vivo factors. Consequently, although insulin-induced receptor loss or "down regulation" is a well-recognized in vitro and in vivo phenomenon, relatively little direct quantitative information concerning the relationship between receptor loss and changes in biologic function is available. In the current studies, we have developed an in vitro system for inducing a loss of insulin receptors by preincubating freshly isolated adipocytes with insulin, and correlating this receptor loss with insulin's biologic action in these insulin-treated cells. Using this system, we have quantitated the rapid effects of insulin to mediate the reduction in the number of cellular insulin receptors; we have also evaluated the relationship between this decrease in insulin binding, and insulin's subsequent biologic effects to promote glucose transport. The results demonstrate that insulin treatment leads to a state of in vitro cellular insulin resistance caused by a loss of insulin receptors as well as a marked postreceptor defect in insulin action.

METHODS

Materials. Porcine monocomponent insulin was generously supplied by Dr. Ronald Chance of the Eli Lilly & Co., (Indianapolis, Ind.), Na[^{125}I]and L-1-[^{3}H]glucose were purchased from the New England Nuclear (Boston, Mass.), 2-deoxy-D-[^{3}H]glucose from Amersham Corp. (Arlington Heights, Ill.), bovine serum albumin (fraction V) from Armour Pharmaceuticals (Chicago, Ill.), collagenase from Worthington Biochemical Corp., (Freehold, N. J.), and spermine tetrahydrochloride from ICN Nutritional Biochemicals Div. (Cleveland, Ohio).

Iodination of insulin. ^{125}I-insulin was prepared to be a specific activity of 100–150 μCi/mg according to the modification by Freychet et al. (16) of the method of Hunter and Greenwood (17) as previously described (18).

Preparation of isolated adipocytes. Male Sprague-Dawley rats weighing 160–225 g were used for all experiments. Rats were stunned by a blow to the head, decapitated, and epididymal fat pads removed. Isolated fat cells were prepared by shaking at 37°C for 60 min in Krebs-Ringer bicarbonate buffer containing collagenase (3 mg/ml) and albumin (40 mg/ml) according to the method of Rodbell (19). Cells were filtered through nylon mesh (250 μM), centrifuged at 400 rpm for 2 min, and washed twice in Tris buffer. Adipocyte counts were performed according to a modification of method III of Hirsch and Gallian (20), in which the cells were fixed in 2% osmium tetroxide in 0.05 M collidine buffer (made isotonic with saline) for 24 h at 37°C and then taken up in a known volume of 0.154 M NaCl for counting. Counting was performed with a model ZB Coulter Counter (Coulter Electronics Inc., Hileah, Fla.) with a 400-μM aperture.

Insulin pretreatment and dissociation procedures. 10 ml of adipocytes (4–6 × 10^5 cells/ml) suspended in pH 7.6 buffer containing 35 mM Tris, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.0 mM CaCl, 2.5 mM KCl, 10 mM dextrose, 24 mM NaAc, and 1% bovine serum albumin (Tris-BSA buffer)[1] were incubated with various concentrations of insulin in 25-ml polypropylene flasks. Cells were then gently agitated in a shaking water bath for 2 or 4 h at 37°C. At the end of the incubation period, cells were transferred to 16 × 125-mm polystyrene tubes, centrifuged at 200 rpm for 2 min, and the insulin-containing buffer removed and replaced with insulin-free Tris-BSA buffer, pH 7.0. Adipocytes were again centrifuged, resuspended in Tris-BSA buffer, transferred to 25-ml flasks, and receptor-bound insulin allowed to dissociate at pH 7.0 for 1 h at 37°C. Measurements at the end of this dissociation period (at the lowered pH, 7.0) demonstrated that there was complete loss of cell-associated radioactivity (data not shown). Thus, all receptor bound insulin and any insulin internalized (including subsequently generated degradation products) were effectively dissociated or released by this procedure. After the 1 h dissociation, cells were washed and resuspended in 10 ml Tris-BSA buffer, pH 7.6, for determination of ^{125}I-insulin binding. When 2-deoxy glucose uptake was to be measured, cells were resuspended in Krebs-Ringer bicarbonate, pH 7.4, containing BSA (10 mg/ml). Control cells underwent similar washing, dissociation, and centrifugation procedures as insulin-treated cells, and glucose uptake measurements were performed concomitantly for both groups of cells.

Binding studies. Isolated adipocytes (2–3 × 10^5 cells) were incubated in a total volume of 1 ml (Tris-BSA buffer, pH 7.6) with ^{125}I-insulin in the absence and presence of 50 μg of unlabeled insulin. Incubations were performed in polypropylene tubes (17 × 100) in a shaking water bath at the indicated temperatures and times. The binding reactions were terminated, and free ^{125}I-insulin separated from cell-bound radioactivity by removing aliquots (300 μl) from the cell suspension and rapidly centrifuging the cells in plastic microtubes, into which 100 μl of silicone oil had been added (21). Silicone oil has a specific gravity intermediate between buffer and cells; therefore, after centrifugation, three layers result: cells on top, oil in the middle, and buffer on the bottom. The cells were then removed and the radioactivity was determined. Adipocyte-associated radioactivity was determined in triplicate from each incubation tube, and, unless otherwise indicated, each experiment is a representative example of at least three similar experiments. Insulin degradation was monitored by assessing the ability of ^{125}I-insulin remaining in the incubation media to precipitate with 10% trichloroacetic acid. With this method (22), TCA precipitable radioactivity is considered to be intact insulin, and TCA soluble material is taken as degraded insulin.

Nonspecific binding. In these experiments, nonspecific binding is defined as the amount of ^{125}I-insulin remaining in the cell layer in the presence of a large excess (50 μg/ml) of unlabeled insulin. When cells equilibrate with a tracer concentration of ^{125}I-insulin (0.1–0.6 ng/ml), only 3 of 7% of the bound insulin represents nonspecific binding. For all samples, total and nonspecific binding was determined and the total binding corrected to reflect specific binding.

Glucose transport studies. Control and insulin-treated

[1] *Abbreviation used in this paper*: BSA, bovine serum albumin.

adipocytes (2–3 × 10^5 cells/ml) were incubated in Krebs-Ringer bicarbonate, 1% BSA, pH 7.4, in the absence and presence of various concentrations of insulin, as previously described in detail (23). Incubations were performed in polypropylene tubes (total vol 1 ml) in a shaking water bath at 37°C for 1 h, unless otherwise indicated. Glucose uptake was then measured by adding 10 μl of 2-deoxy glucose (12.5 mM) containing 0.2 μCi 2-deoxy-[1-^{3}H]glucose. The reaction was terminated at the end of 3 min by transferring 300-μl aliquots from the incubation mixture to plastic microfuge tubes containing 100 μl silicone oil. The tubes were centrifuged for 30 s in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, Calif.) and the assay was considered terminated when centrifugation began. This assay measures the total uptake of radiolabeled 2-deoxy glucose and is based on the principle that, although 2-deoxy glucose is transported and phosphorylated by the same process as D-glucose, it cannot be further metabolized. For all experiments, glucose uptake for each point represents the mean of triplicate samples, and each experiment is a representative example of at least three similar experiments unless otherwise indicated. The distribution space of radiolabeled L-glucose was determined in all experiments, and used to correct the uptake data for nonspecific carry-over of radioactivity with the cells, and the uptake of hexose by simple diffusion.

RESULTS

Insulin binding to receptors. To determine whether insulin can acutely regulate its own receptors in isolated adipocytes in vitro, cells (3 × 10^5) were preincubated with varying concentrations of insulin for 2 h at 37°C, washed to remove buffer insulin, and receptor-bound insulin dissociated as described in the Methods section. Fig. 1 presents the subsequent time courses of ^{125}I-insulin binding at 16°C (A) or 37°C (B) after preincubation with the indicated insulin concentrations. When binding was measured at 16°C, preincubation with 1, 5, 25, and 100 ng/ml insulin reduced ^{125}I-insulin binding by 9, 19, 38, and 47%, respectively; when binding was measured at 37°C, the corresponding reductions were 12, 25, 41, and 49%. Thus, insulin preincubation led to a marked dose-dependent decrease in the ability of adipocytes to bind insulin. Additionally, because the results at 16°C were comparable to those at 37°C, further changes in insulin receptors, such as regeneration or ongoing loss, did not occur during the binding phase of the experiment because these processes are absent at 16°C. When adipocytes were preincubated with 25 ng/ml insulin at 16°C for 2 h, and subsequent binding of ^{125}I-insulin measured at 16°C, no reduction in insulin binding was observed, indicating that the loss of insulin binding is a temperature-dependent process (data not shown). Additionally, because binding was the same with or without the 16°C insulin preincubation, this experiment also shows that no free or bound insulin was carried through from the preincubation to the binding phase of the experiment with the washing and dissociation conditions employed.

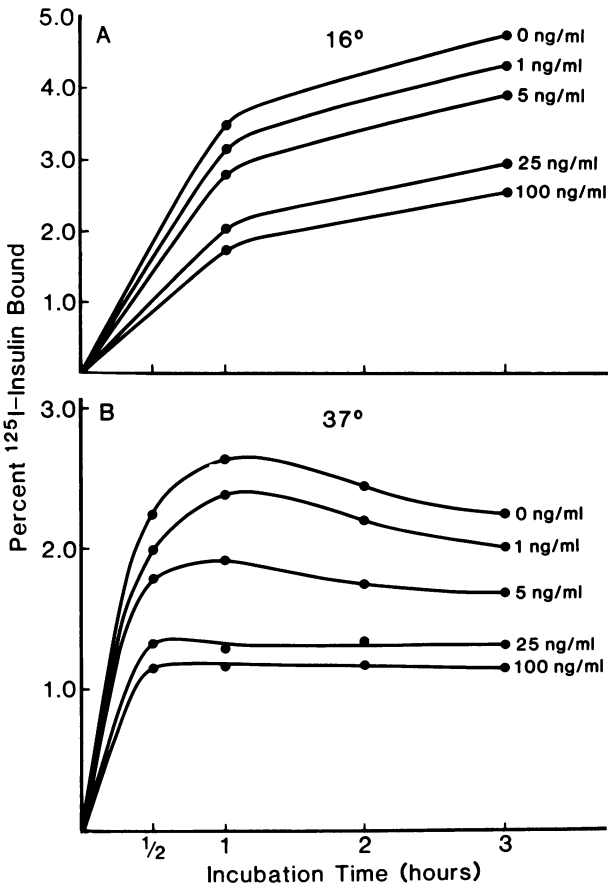

FIGURE 1 Effect of insulin preincubation on the time-course of specific ^{125}I-insulin binding. Adipocytes (3 × 10^5 cells/ml) were preincubated for 2 h at 37°C with 0, 1, 5, 25, and 100 ng/ml of insulin, and washed twice to remove buffer insulin; receptor-bound insulin was then dissociated at pH 7.0 for 1 h at 37°C. Cells were then incubated at either 16°C (A) or 37°C (B) in the presence of 0.3 ng/ml ^{125}I-insulin. At the indicated times, triplicate 300-μl aliquots were removed, and the cells separated from the buffer by centrifugation through 100 μl of silicone oil. The concentration of insulin in the preincubation buffer is noted in nanograms per milliliter to the right of each curve. Nonspecific binding was determined for each point from a duplicate set of tubes into which ^{125}I-insulin and 50 μg of unlabeled insulin had been added.

To determine whether the insulin-induced decrease in insulin binding was due to a reduction in receptor number, affinity, or both, competition binding curves over a wide range of insulin concentrations were carried out using control cells and adipocytes, which had been preincubated with 100 ng/ml insulin for 2 or 4 h at 37°C. As can be seen (Fig. 2A), the ability of insulin-treated cells to subsequently bind insulin is decreased at all points on the curve; the reduction is greatest in cells preincubated with insulin for 4 h. Scatchard plots of these insulin binding data are seen in Fig. 2B, and although precise interpretation of these curvilinear plots is difficult, the generally comparable

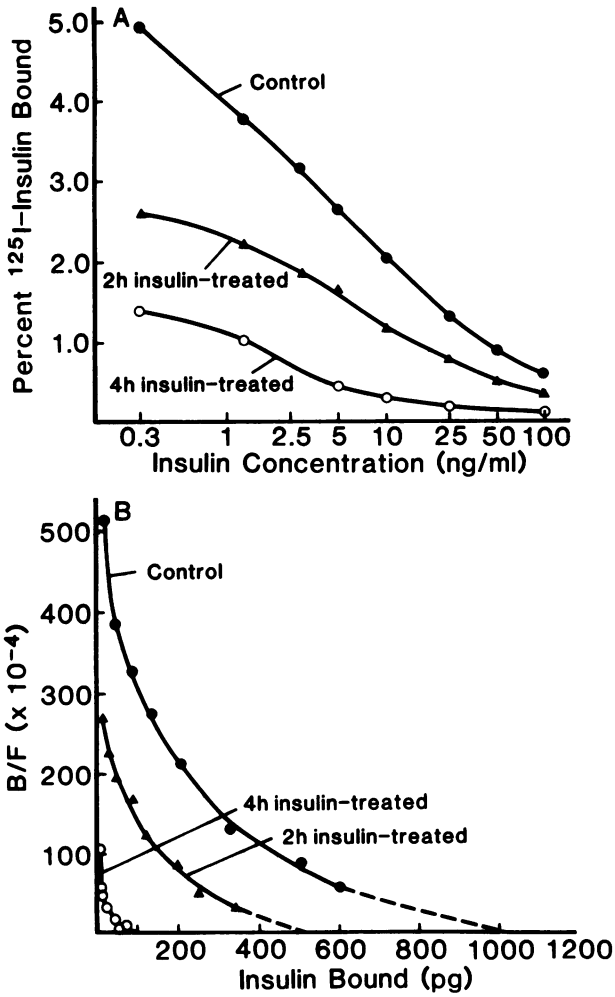

FIGURE 2 Ability of 2- and 4-h insulin-treated adipocytes to specifically bind ^{125}I-insulin at various insulin concentrations. A Adipocytes (2.5×10^5) were preincubated either in the absence of insulin (●) or in the presence of 100 ng/ml insulin for either 2 h (▲) or 4 h (○) at 37°C, and prebound insulin dissociated as previously described. Cells were then incubated for 3 h at 16°C with 0.3 ng/ml ^{125}I-insulin, plus unlabeled insulin, to give the indicated total insulin concentrations. All data are corrected for nonspecific binding. B Scatchard plots of these insulin binding data for control (●), 2 h (▲), or 4 h (○) insulin-treated cells. The ratio of bound to free insulin (B/F) is plotted on the ordinate, and bound insulin on the abscissa.

shapes of the curves indicate that the major cause of the decrease in insulin binding is a reduction in insulin receptor number.

Glucose transport studies. The predicted functional consequence of a decrease in insulin receptors is a rightward shift in the biologic function dose-response curve, with no change in the maximal effect of insulin. Therefore, to assess the effect of the insulin-induced receptor loss on insulin sensitivity, and to examine the relationship between insulin binding and action, the ability of insulin to stimulate 2-deoxy-glucose uptake in insulin-treated cells was studied. Adipocytes were preincubated with 100 ng/ml insulin for 2 or 4 h at 37°C; after this, buffer insulin was removed and receptor-bound insulin was allowed to dissociate, as described earlier. Cells were then incubated at 37°C for 1 h with various concentrations of insulin, after which time 2-deoxy glucose uptake was determined (Fig. 3A). The ability of 2 h insulin-treated adipocytes to take up 2-deoxy glucose was decreased at all insulin concentrations. However, because the basal rate of 2-deoxy glucose uptake was also reduced, the percent increase in uptake above basal was similar at the higher insulin concentrations for control and 2-h insulin-treated adipocytes. Thus, as a percent of the basal value, insulin-stimulated 2-deoxy glucose uptake was decreased at submaximal insulin concentrations in treated cells, whereas maximal stimulation was 810 and 830% above basal in control and insulin-treated cells, respectively. When adipocytes were incubated with insulin for a longer period of time (4 h), the effects on glucose transport were more pronounced. Thus, 2-deoxy glucose uptake was depressed at all insulin concentrations (Fig. 3A), including maximally effective insulin levels, and this decrease in maximal responsiveness to insulin is apparent whether the data are presented in absolute terms or as percent increase above basal (not shown). This pronounced reduction in maximal insulin effectiveness cannot be explained by a loss of insulin receptors, and indicates a marked postreceptor defect.

To better analyze the functional form of these curves, the data were plotted as a percent of the maximal insulin effect (Fig. 3B). With this analysis, it can be seen that the dose-response curve was shifted to the right in the insulin-treated cells with a 1/2 maximal insulin effect at 1.3 and 2.0 ng/ml after 2 and 4 h, respectively, as compared with 0.5 ng/ml in control cells. Thus, the expected functional effect of a decrease in insulin receptors was observed, i.e., a progressive rightward shift of the insulin glucose transport dose-response curve (24, 25). To further examine the functional relationship between insulin receptor occupancy and activation of glucose transport, the amount of insulin bound was plotted against the percent insulin effect (Fig. 3C) for all groups of cells. The resulting curves are quite comparable, indicating that coupling between insulin receptors and glucose transport units is normal in the insulin-treated cells, and that the decrease in insulin binding entirely accounts for the rightward shift of the dose-response curve.

The results in Fig. 3 demonstrate that when cells are preincubated with insulin for a longer interval, the appropriate functional abnormality is expressed (i.e., a further rightward shift in the dose-response curve) and coupling between receptors and transport

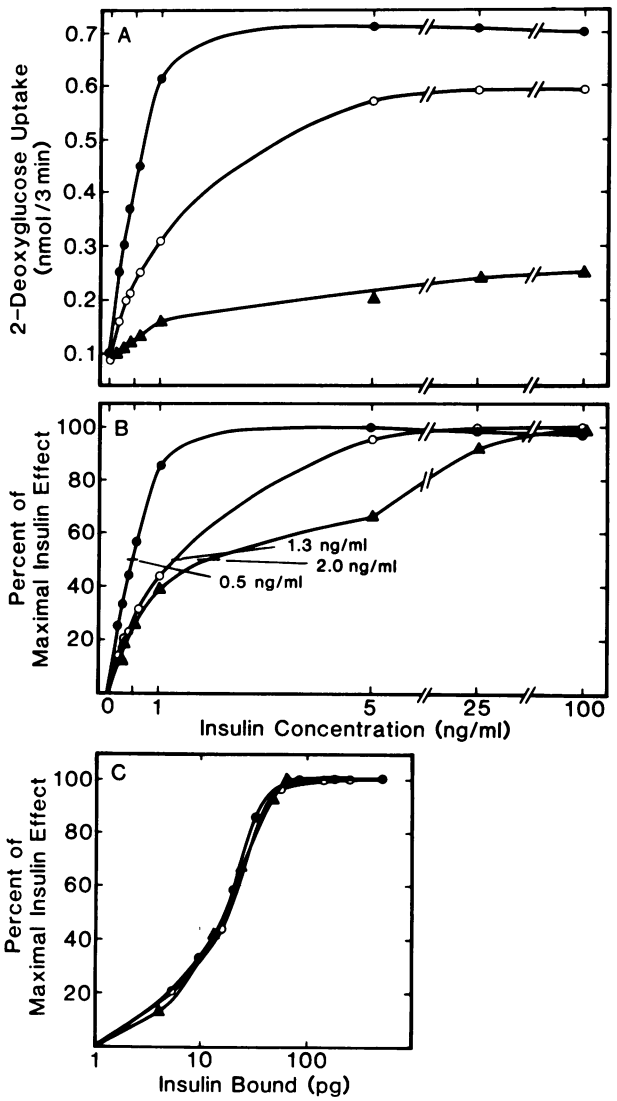

FIGURE 3 Insulin-stimulated glucose transport of control, 2-h and 4-h insulin-treated adipocytes. A Cells were preincubated in the absence (●) and presence of 100 ng/ml insulin for 2 h (○) or 4 h (▲) at 37°C, and prebound insulin dissociated as described in Fig. 1. Control and insulin-treated cells were then incubated at 37°C for 1 h, with or without insulin (at the indicated concentrations), and 2-deoxy glucose uptake determined after a 3-min incubation with 0.125 mM 2-deoxy glucose. B The 2-deoxy glucose uptake data for control (●) and 2-h (○) and 4-h (▲) insulin-treated cells is plotted as a percentage of the maximal insulin effect. Calculations were made by dividing the increment in 2-deoxy glucose uptake (absolute minus basal value), at the indicated insulin concentration, by the maximal increment in uptake (at 100 ng/ml insulin). C Relationship between amount of insulin bound (picogram) and percentage insulin effect on 2-deoxy glucose uptake by control (●) and 2-h (○) and 4-h (▲) insulin-treated adipocytes.

is normal. The most striking finding, however, is the marked decrease in maximal insulin responsiveness, indicating a postreceptor defect in insulin action. To evaluate whether this postreceptor defect observed in 4-h insulin-treated cells is localized to the glucose transport effector system, and whether this defect is specific for the insulin action mechanism, experiments with different insulin-like agents were conducted. Spermine is an insulinlike agent that stimulates glucose transport via a mechanism independent of the insulin receptor (26). On the other hand, antiinsulin receptor antibodies derived from patients with severe insulin resistance (27, 28) mimic insulin action via the receptor in a manner analogous to the native hormone. As Table I illustrates, at maximally effective concentrations, insulin, spermine, and the antiinsulin receptor antibody have a reduced ability to stimulate 2-deoxy glucose uptake in 4-h insulin-treated cells, as compared with controls. This indicates that the postreceptor defect is not unique to the insulin action mechanism and likely resides in the actual glucose transport system.

Previous reports have shown that stimulation of adipocyte glucose oxidation by insulin is linear for several hours at 37°C (29). At first approximation, this appears incongruous with our observations of a time-dependent postreceptor defect induced by preincubation with insulin. Therefore, to evaluate the possibility that the continued presence of insulin (with no washing, insulin dissociation, or deactivation of glucose transport before measurement of uptake) could prevent the expression of the postreceptor abnormality, the experiments presented in Fig. 4 were conducted. Cells were incubated in the presence or absence of 100 ng/ml insulin for 1, 2, and 4 h at 37°C. At these times, 2-deoxy glucose uptake was measured without washing the cells free of buffer insulin, and without allowing receptor-bound insulin to dissociate. With this experimental design, the glucose transport system was maximally activated in the insulin-treated cells, and was not allowed to deactivate before the measurement of glucose transport. Cells preincubated with insulin for 2 and 4 h had a reduced number of insulin receptors, as previously demonstrated (Figs. 1 and 2), but there was no decrease in the maximal insulin effect to simulate 2-deoxy glucose uptake at any of the indicated times (Fig. 4). Therefore, once glucose transport is stimulated by insulin, it remains maximally active regardless of the length of the incubation (up to 4 h) provided the insulin stimulus is continuous. However, once receptor-bound insulin dissociates and rates of transport return to basal levels, a postreceptor defect in insulin action is expressed upon restimulation with the hormone (Fig. 3A).

Insulin degradation. Adipocytes can degrade insulin by a receptor-mediated mechanism (22) and, therefore, hormone degradation can be considered another function of the insulin receptor. Thus, the ability of control and insulin-treated adipocytes to degrade ^{125}I-insulin was assessed. When cells were

TABLE I

Effects of Insulin and Insulin Mimickers on 2-Deoxy Glucose Uptake

Additions	2-Deoxy glucose uptake		Percent of insulin-stimulated uptake	
	Control cells	4-h insulin-treated cells	Control	4-h insulin-treated cells
	pmol/3 min		%	%
Basal	105±5	93±7	—	—
Insulin (100 ng/ml)	540±30	280±10	100	100
Spermine (25 μM)	320±20	180±40	49	46
Spermine (100 μM)	455±25	230±30	80	73
Spermine (200 μM)	450±20	240±26	79	78
Antireceptor antibody (1:50)	522±37	287±18	96	103

Isolated adipocytes were preincubated in the presence or absence of 100 ng/ml insulin for 4 h at 37°C. Receptor-bound and buffer insulin were removed from treated cells as described in Methods, and insulin-stimulated glucose transport allowed to deactivate to basal levels. Control and insulin-treated cells were then incubated for 1 h at 37°C in the presence or absence of insulin (100 ng/ml), spermine tetrahydrochloride (25, 100 or 200 μM), and serum containing antiinsulin receptor antibodies (1:50 dilution). Glucose uptake was determined after a 3-min incubation as previously described, and the indicated values represent the mean±SEM of two separate experiments. Uptake data for the insulin mimickers is also expressed as a percentage of the maximal insulin-stimulated glucose uptake.

preincubated with 5 or 25 ng/ml insulin for 2 h, washed, and incubated at 37°C with 0.3 ng/ml ^{125}I-insulin, the rate of degradation of the labeled insulin was reduced in a dose-dependent manner (Fig. 5A). Thus, at 2 h, the percentage of degraded insulin in the buffer was reduced by 14 and 22% in cells that were preincubated with 5 and 25 ng/ml, respectively. Increasing the length of the preincubation with 25 ng/ml insulin to 3 h led to a further reduction in the ability of insulin-treated cells to degrade insulin (Fig. 5B).

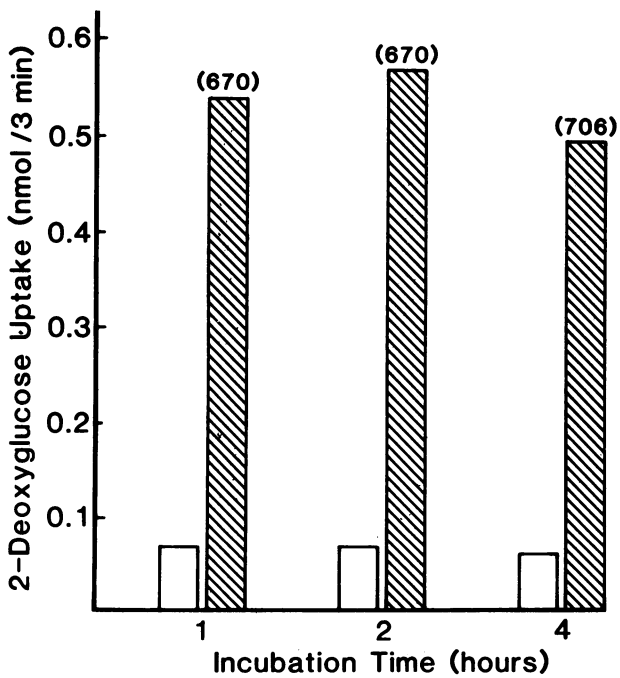

FIGURE 4 Basal and insulin-stimulated 2-deoxy glucose uptake at various incubation times. Adipocytes were incubated at 37°C for 1, 2, or 4 h in the absence (white bars) or presence (hatched bars) of 100 ng/ml insulin, after which time 2-deoxy glucose uptake was determined. Values above hatched bars indicate the percent increase in glucose uptake above basal value.

DISCUSSION

The present studies clearly demonstrate that in vitro incubation of isolated rat adipocytes with insulin can induce a rapid loss of insulin receptors. Furthermore, the functional counterpart of this reduction in insulin receptors was demonstrated when insulin's ability to stimulate glucose transport was measured. Thus, a rightward shift in the insulin dose-response curve was observed, and this is the predicted consequence of a decrease in the number of insulin receptors. More prolonged incubations of cells with insulin led to a marked abnormality in the ability of adipocytes to maximally transport glucose, consistent with a post-receptor defect. Therefore, in vitro insulin treatment results in a hormone-induced insulin-resistant state in isolated adipocytes at both the receptor and post-receptor levels.

Decreased insulin responsiveness is defined as a decrease in the maximal biologic response under study, and results from a postreceptor defect in insulin action. Decreased insulin sensitivity is defined as a decreased biological response to submaximal concentrations of insulin, with a normal response to maximal concentrations, and is a result of decreased insulin receptors (30). Thus, the predicted functional consequence of decreased insulin receptors is a rightward shift in the insulin-biologic function dose-

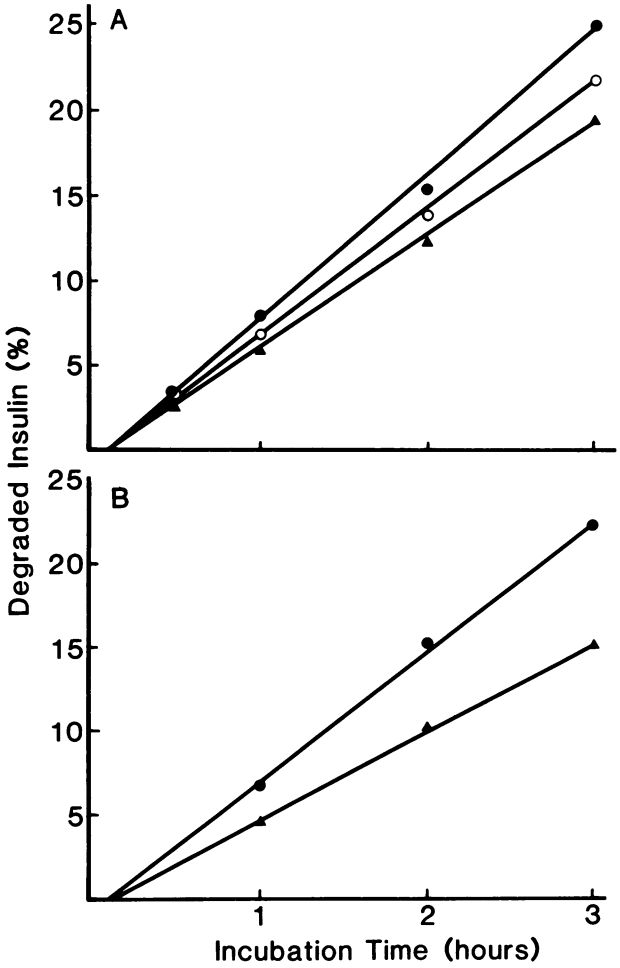

FIGURE 5 Effect of insulin-induced loss of insulin receptors on the subsequent ability of adipocytes to degrade ^{125}I-insulin. Adipocytes were preincubated in the absence of insulin (●) or in the presence of 5 (○) or 25 ng/ml (▲) insulin for 2 h at 37°C, and prebound insulin dissociated as described in Fig. 1. Cells were then incubated with 0.3 ng/ml ^{125}I-insulin at 37°C and insulin degradation determined at the indicated times. Degradation of insulin was quantitated by the percentage of buffer radioactivity precipitable in 10% trichloroacetic acid. B Cells were preincubated in the absence (●) and presence (▲) of 25 ng/ml insulin for 3 h at 37°C, receptor bound insulin dissociated, and ^{125}I-insulin degradation subsequently measured at the indicated times.

response curve, and this was observed (Fig. 3B) when 2-deoxy glucose uptake was assessed in adipocytes that had lost 50 and 75% of their receptors after preincubation with insulin for 2 and 4 h, respectively. Half-maximal stimulation of 2-deoxy glucose uptake occurred at 0.5 ng/ml in untreated cells as compared with 1.3 and 2.0 ng/ml after 2 and 4 h insulin pretreatment. The loss of receptors was quantitatively correlated to the magnitude of the rightward shift in the dose-response curve, demonstrating that the binding sites that were lost were biologically significant insulin receptors.

In addition to decreased insulin sensitivity, a striking decrease in maximal insulin-stimulated glucose transport was observed in 4-h insulin-treated adipocytes, indicating a marked decrease in insulin responsiveness. Because maximum 2-deoxy glucose uptake occurs when only ~10% (25) of the normal number of insulin receptors are occupied, and because 25% of the control number of receptor was still present in 4-h insulin-treated cells, an adequate number of receptors was still present to elicit the full biologic response. Consequently, the decreased rate of 2-deoxy glucose uptake at maximally effective insulin concentrations indicates an abnormality distal to insulin binding, i.e., a post-receptor defect in insulin action. Thus, the insulin-induced insulin resistance of isolated adipocytes is caused by changes in both insulin sensitivity and responsiveness, secondary to receptor and post-receptor abnormalities.

The postreceptor defect in insulin-treated adipocytes may reside in either the "coupling step" between insulin-receptor complexes and glucose transport units, or in the glucose transport system itself. The efficiency of coupling was examined by plotting the percent of the maximal insulin effect as a function of the amount of insulin bound (Fig. 3C), and the resulting curves were found to be comparable in control and insulin-treated cells. These results suggest that the interaction between occupied receptors and glucose transport is normal in insulin-treated cells, and that the postreceptor defect represents a defect in the glucose transport system per se.

To further localize this postreceptor defect, and to determine whether this abnormality was specific for the insulin action mechanism, the ability of anti-insulin receptor antibodies and spermine to stimulate glucose transport in insulin-treated cells was assessed. Because both of these agents had a markedly reduced ability to stimulate glucose uptake in 4-h insulin-treated adipocytes (Table I), this defect is not unique to the insulin action mechanism and most likely resides in the glucose transport system itself.

Insulin-stimulated glucose oxidation has been shown to be linear for several hours at 37°C (29), and this appears inconsistent with the development of an insulin-induced time-dependent postreceptor defect. Therefore, we examined the possibility that continuous insulin stimulation could prevent the expression of this postreceptor abnormality. Cells were incubated with 100 ng/ml insulin for up to 4 h, after which time 2-deoxy glucose uptake was measured without removing buffer insulin or allowing receptor-bound insulin to dissociate. Under these conditions there was no decrease in maximal insulin-stimulated glucose transport, indicating that once glucose transport is activated it remains active regardless of the length of the incubation, provided the insulin stimulus is continuous. In contrast, once insulin is allowed to dissociate from its receptors, and the rate of transport

deactivates to basal levels, a postreceptor defect in the glucose transport system can then be observed upon restimulation with either insulin, spermine, or antireceptor antibodies.

These results allow some tentative inferences about the relationship between the insulin receptor and the basic mechanisms of glucose transport activation. First, once glucose transport is maximally stimulated by 100 ng/ml insulin, transport remains maximal despite the fact that 50–75% of the insulin receptors are lost, indicating that the insulin receptor and the glucose transport protein are independent structures in the membrane, as previously suggested (31). Furthermore, the finding that a reduction in the glucose transport system can be prevented by maintaining the system in an active state indicates that the activated glucose transport units are regulated in a different manner than the unstimulated system. This conclusion is analogous to previous reports demonstrating that insulin's ability to activate glucose transport is ATP dependent, but once activated, the transport system can remain in the activated state despite depletion of cellular ATP or cell disruption (32–34).

The present study illustrating insulin-induced desensitization at the receptor and postreceptor level fits well with the in vivo data demonstrating the development of receptor and postreceptor defects in insulin-resistant hyperinsulinemic states. Numerous investigators have reported an inverse relationship between elevated plasma insulin levels and decreased insulin receptors in a common insulin-resistant state such as obesity (8–13). However, it appears that the relationship between insulin binding and insulin resistance is more complex than can be accounted for by a reduction in insulin receptor number alone. For example, Kolterman et al. (35) have recently reported that the insulin resistance of human obesity is heterogeneous. Thus, mildly hyperinsulinemic subjects are insulin resistant solely as a result of a decrease in insulin receptors, whereas severely hyperinsulinemic subjects are insulin resistant as a result of both a receptor and a postreceptor defect. Lastly, Ciaraldi and Olefsky (36) have found that the postreceptor defect in severely hyperinsulinemic obese patients is associated with decreased intrinsic activity of the glucose transport system. This sequence of events is also consistent with certain animal studies, because when glucose metabolism was studied using soleus muscles obtained from genetically obese, hyperinsulinemic animals, or adipocytes from spontaneously obese old rats, it was found that insulin binding, hormone sensitivity, and hormone responsiveness were all reduced (24, 37), indicating receptor and postreceptor defects.

Our in vitro findings that the insulin-induced postreceptor defect in insulin action is expressed only when the glucose transport system is allowed to deactivate may be relevant to the therapy of diabetic ketoacidosis. Until recently, standard therapy for diabetic ketoacidosis consisted of intermittent "high dose" insulin injections, and it was felt that these patients were insulin resistant because very high doses of insulin were necessary to control ketoacidosis. Current treatment now employs "low dose" therapy in which a fixed amount of insulin is infused in a continuous manner. It is possible that the efficacy of the "low dose" therapy may also be related to the fact that the insulin simulus is constant, preventing the expression of the postreceptor defect, whereas intermittent insulin therapy may allow the glucose transport system to deactivate and become relatively unresponsive to subsequent injections of insulin.

We have recently reported that chronic hyperinsulinemia in the rat augments insulin-stimulated glucose uptake in adipocytes by increasing the V_{max} of glucose transport (38). In contrast, the present in vitro studies show that 4 h insulin treatment led to a decrease in maximal insulin-stimulated glucose transport. These differences in the ability of insulin to regulate glucose transport function indicate the existence of a separate acute and chronic mechanism for modulating the glucose transport system. For example, the chronic effect of insulin may be mediated through increased synthesis of the glucose transport carrier protein or increased insertion into the plasma membrane, whereas the acute effects of insulin may be to rapidly modulate insulin-stimulated activation of the carrier protein. These possibilities are under investigation.

The current data showing hormone-induced desensitization at the receptor and postreceptor levels find some support in other systems. For example, Tsuruhara et al. (39) have demonstrated hormone-induced desensitization of the testosterone response to human chorionic gonadotropin (hCG) in Leydig cells prepared from testicular tissue of rats treated with hCG. Overall, desensitization was mediated by both luteinizing hormone (LH) receptor loss, and a defect in the steroidogenic pathway distal to LH binding. Chang and Polakis (40) have reported that insulin-treated 3T3-C2 cells have fewer insulin receptors and decreased insulin-stimulated aminoisobutyric acid uptake, whereas Martin and Pohl (41) have shown that insulin-treated fibroblasts display only a rightward shift in the dose-response curve for α-aminoisobutyric acid uptake, with no change in maximal responsiveness. Livingston et al. (4, 5) incubated fat pad explants with low concentrations of insulin for 17 h and found a decrease in subsequent insulin binding with a rightward shift in the dose-response curve for glucose oxidation. It should be noted that in the latter study, insulin responsiveness was not depressed by insulin treatment, whereas in our study a decrease

in maximal insulin action was observed concomitant with reduced insulin binding. Combining the in vitro and in vivo results, one can speculate that insulin-induced insulin resistance in man represents a continuum of effects, with mild hyperinsulinemia causing loss of insulin receptors and decreased insulin sensitivity, whereas greater hyperinsulinemia results in a further loss of receptors and insulin sensitivity, plus a postreceptor defect in the glucose transport system.

Evidence has been provided that insulin is internalized and degraded intracellularly by a receptor-mediated mechanism (42). In the present studies, we have found that insulin can induce a rapid loss of adipocyte insulin receptors, and that this receptor loss is accompanied by a reduction in the ability of adipocytes to degrade insulin. Thus, the reduced ability of insulin-treated adipocytes to degrade the hormone reflects the fact that the absolute number of insulin receptors available for endocytosis is reduced and, consequently, less insulin is degraded by the receptor-mediated mechanism. However, it should be noted that the reduction in overall insulin degradation was only about half as great as the decrease in insulin receptors. This is consistent with the formulation that isolated adipocytes can degrade insulin by both receptor-mediated and nonreceptor-mediated mechanisms. Thus, the lack of quantitative correlation between the loss of insulin receptors and the decrease in insulin degradation could reflect the fact that only the receptor-mediated degradation component was affected by insulin pretreatment. A less likely possibility (but one that we cannot rule out with the present data) is that hyperinsulinemia enhanced the ability of cells to degrade insulin by a mechanism independent of the insulin receptor, which would partially counterbalance the reduction in receptor-mediated degradation mediated by the insulin-induced receptor loss.

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