

Insulin-induced Alterations in Insulin Binding and Insulin Action in Primary Cultures of Rat Hepatocytes

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SUMMARY

The exposure of primary cultures of hepatocytes to insulin, 10^{-8} M, for 16 h results in a decrease in high affinity insulin binding with no alterations in lower affinity binding. This is reflected in a decrease in the sensitivity, but normal responsiveness, of cultured hepatocytes to the acute effect of insulin on the uptake of aminoisobutyric acid. The shift in sensitivity, however, can only be partially explained by the decrease in insulin binding. With regard to lipid synthesis, hepatocytes cultured in the presence of insulin, 10^{-8} M, are normally sensitive and hyperresponsive to the acute effects of insulin. These data indicate that the insensitivity or resistance of a given tissue to insulin may be specific for the biologic response being evaluated, that postbinding events may be more important than alterations in insulin binding in determining both the sensitivity and responsiveness of a tissue to insulin, and that generalizations concerning the sensitivity or responsiveness of a tissue to insulin based on binding data alone may be unwarranted.
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Several studies have evaluated alterations in insulin binding in cells from animals and humans with altered metabolic states. While there are numerous exceptions,¹⁻¹² in some cases the level of insulin binding is inversely related to plasma insulin concentrations.¹²⁻¹⁶ Such downregulation of the insulin receptor by insulin has also been demonstrated in vitro in several cell systems including IM-9 lymphoblastoid cells,¹⁷ human fibroblasts,¹⁸ rat adipose tissue explants,¹⁹ and hepatocytes from regenerating²⁰ and normal rat liver.²¹ The biologic effectiveness of insulin in such downregulated cells, however,

has been evaluated in fat only, and no one to date has studied more than a single biologic response in cells down regulated in vitro. Furthermore, studies evaluating the biologic consequences of in vitro downregulation in the liver, a metabolically important target tissue for insulin, have not previously been reported.

Our laboratory has reported downregulation of the insulin receptor in primary cultures of hepatocytes after exposure to high concentrations of insulin, 10^{-7} M, for 16 h.²¹ Such incubations result in a decrease in insulin binding at all concentrations of insulin with no change in the apparent affinity of the insulin receptor for insulin. In the present studies, we have evaluated insulin binding and action in primary cultures of rat hepatocytes following a 16-h exposure to lower concentrations of insulin, 10^{-8} M. In addition, because of the possibility that different metabolic processes may respond differently to hyperinsulinemia, we have evaluated two biologic responses, amino acid uptake and lipid synthesis. The data indicate that the exposure of hepatocytes to 10^{-8} M insulin for 16-h results in profound alterations in insulin action, only some of which can be explained by changes in the insulin-receptor interaction. Furthermore, the data support the concept that postbinding events are of great importance in considerations of insulin responsiveness and that, just as different tissues may respond differently to the same metabolic perturbation, different biologic responses in the same tissue may also respond differently.

MATERIALS AND METHODS

Male Sprague-Dawley rats (150-250 g) fed ad libitum are used for liver-cell isolation as previously described.¹² Primary cultures of isolated rat hepatocytes are performed on collagen-coated plates by the method of Kletzien et al.²² in serum free medium.²¹ Rat tail collagen is prepared by extraction with acetic acid.²³

Insulin, 10^{-8} M, is added after the first medium change 4 h after plating, at which time 56% (2.1×10^6 cells/plate) of the cells had adhered to the plates as a monolayer. Insulin is also re-added every 6 h thereafter. Sixteen hours after the first medium change (4 h after the last insulin addition) the

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cells are washed twice at 37°C with culture medium in the absence of insulin with two 30-min periods at 37°C to allow the dissociation of any prebound insulin.²¹

Following the wash procedure, cultures are incubated in culture medium in the absence and presence of insulin (10^{-10} – 10^{-7} M) for 2 h at 37°C, after which fresh Hanks' Hepes buffer, pH 7.4, containing 8 mM glucose and 10 mg/ml albumin in the absence or presence of insulin is added and ¹⁴C-aminoisobutyric acid (AIB) (0.1 mM, 0.3 μ Ci/ml) uptake is measured over 10 min. Under these conditions AIB uptake is linear for 20 min. The reaction is terminated by washing the plates with ice-cold phosphate—saline, pH 7.4, following which the cells are scraped off the plates with a rubber policeman and centrifuged for 15 s in a Beckman microfuge, Beckman Instruments, (Fullerton, California). The bottom of the tube containing the cells is cut; the cells removed with a disposable pipet tip; and the cells, microfuge tube and pipet tip are added to 10.0 ml of Scintiverse and counted in a liquid scintillation counter.

Lipid synthesis is assessed under identical conditions as AIB uptake except that the final preincubation in the absence and presence of insulin is for 30 min after which sodium acetate (5 mM, 2.2 μ Ci/ml) is added in the absence and presence of insulin (10^{-10} – 10^{-7} M). Following a 30-min incubation, the plates are washed with cold phosphate-saline, pH 7.4, and the cellular lipids extracted by the method of Bligh and Dyer²⁴ as previously reported.²¹ In some experiments, the incorporation of (1-¹⁴C)-acetic acid into fatty acids was measured by saponification with extraction into petroleum ether as described by Tarlow et al.²⁵

Insulin binding is measured after washing the cultures twice at 37°C as described above, except that Hanks' Hepes buffer containing glucose (8 mM) and albumin (10 mg/ml), pH 7.4, is used instead of culture medium. This procedure has been previously shown to remove prebound insulin.²¹ Insulin binding is measured after a 45-min incubation at 25°C with ¹²⁵I-labeled insulin, 10^{-10} M, in the presence of increasing concentrations of native insulin in the Hanks' Hepes buffer containing glucose and albumin. Binding is expressed as specific binding, i.e., the ¹²⁵I-labeled insulin bound after subtraction of the insulin which remains bound in the presence of a large excess (10^{-6} M) of unlabeled hormone. Insulin is iodinated with chloramine T according to the method of Cuatrecasas.²⁶

Carrier-free Na¹²⁵I, α (1-¹⁴C) aminoisobutyric acid (51.6 mCi/mmol), and (1-¹⁴C) acetic acid (2.9 mCi/mmol) were obtained from New England Nuclear (Boston, Massachusetts), crude collagenase (CLS II, 48H141, 152 U/mg) from Worthington Biochemical Co. (Kankakee, Illinois), α -aminoisobutyric acid from Calbiochem (San Diego, California), and all natural amino acids from Sigma Chemical Co. (St. Louis, Missouri). Crystalline porcine insulin was kindly provided by Dr. Ronald Chance of Eli Lilly and Co. (Indianapolis, Indiana).

RESULTS

Insulin binding was assessed in primary cultures of hepatocytes incubated in the absence and presence of insulin, 10^{-9} M, for 16 h. Scatchard analyses of these data are illustrated in Figure 1 and demonstrate that the binding of insulin to hepatocytes which have been exposed to insulin is significantly decreased at tracer concentrations of hor-

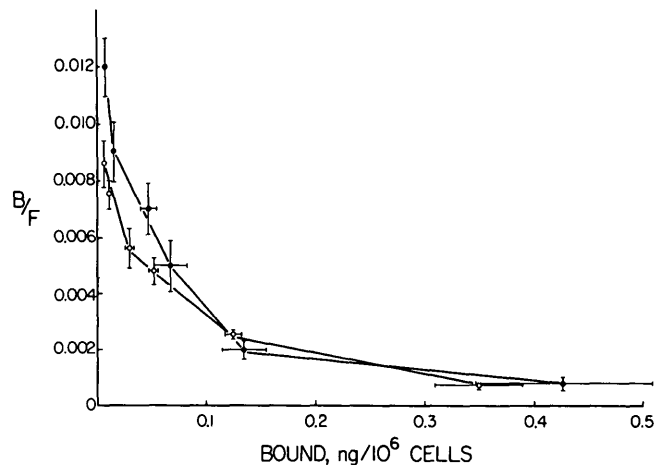


FIGURE 1. Scatchard analyses of insulin binding to primary cultures of hepatocytes incubated for 16 h in the absence (●) and presence (○) of insulin, 10^{-9} M. The cultures are washed prior to assessing insulin binding as described in METHODS. The data represent the mean \pm SEM of four separate experiments.

none, 10^{-10} M ($P < 0.005$). There is no significant difference, however, in insulin binding to treated and untreated cells at higher concentrations of insulin. This indicates that the 16-h exposure to insulin has either decreased the affinity of the insulin receptor for insulin or decreased the number of high affinity binding sites. As previously reported, this decrease in binding is not due to an increase in insulin degradation, since this parameter is unaltered in insulin-treated cells.²¹

To determine the effect of the exposure of hepatocytes to insulin on basal and insulin-stimulated metabolism, we evaluated two metabolic parameters, amino acid uptake and lipid synthesis. Two responses were chosen because of the possibility that different metabolic pathways in the same tissue may respond differently to the same chronic hormonal stimulus.

To evaluate amino acid uptake, we used a nonmetabolizable amino acid analogue, α -aminoisobutyric acid (AIB), which is transported through the insulin responsive "A" system.²⁷ The insulin dose-response relationships for the uptake of AIB are illustrated in Figure 2. Hepatocytes that have been exposed to insulin are significantly less responsive to concentrations of hormone that elicit a submaximal effect, 10^{-10} and 10^{-9} M, ($P < 0.01$ to $P < 0.02$) but equally responsive to a maximal stimulating concentration of insulin, 10^{-7} M. This decreased responsiveness at submaximal insulin concentrations leads to a shift to the right in the dose-response curve that is characteristic of decreased sensitivity to insulin. The similarity of the maximal effect of insulin in untreated and treated cells indicates that insulin responsiveness is unaltered.

Contrary to our observations concerning the uptake of aminoisobutyrate, the incorporation of (¹⁴C)-acetate into lipids is markedly enhanced in cells treated with insulin at all the concentrations of hormone tested (Figure 3). This increase in responsiveness ranges from 2.3 to 3-fold at insulin concentrations from 10^{-10} to 10^{-7} M ($P < 0.05$ to $P < 0.02$). There is no significant difference, however, in the sensitivity of lipid synthesis to insulin in untreated and treated cells. Studies in our laboratory indicate that in freshly isolated hepatocytes from normal rats and in primary cultures of hep-

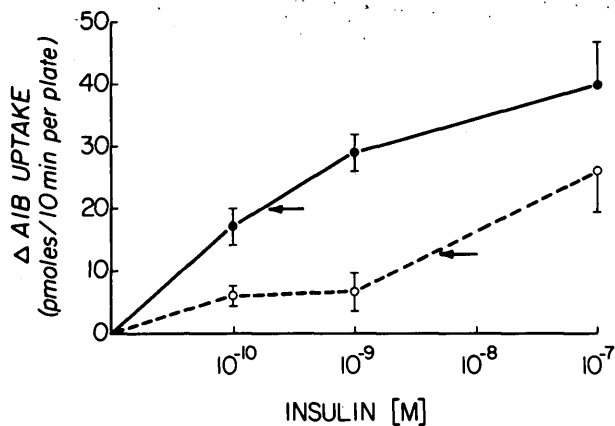
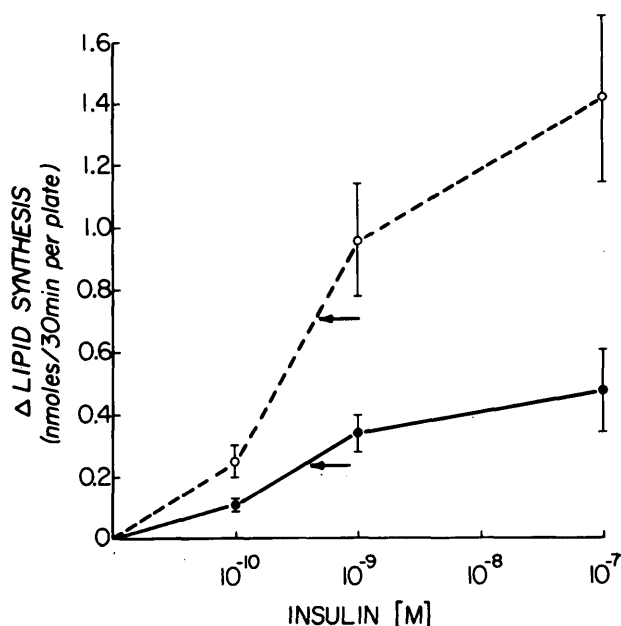


FIGURE 2. Dose-response curves for insulin-stimulated uptake of aminoisobutyrate in primary cultures of hepatocytes incubated for 16 h in the absence (●) and presence (○) of insulin, 10^{-8} M. The data represent the mean \pm SEM of three separate experiments. The basal rate of AIB uptake is 38 ± 2 pmol/10 min/plate for control cells and 65 ± 12 pmol/10 min/plate in cells treated overnight with insulin ($P < 0.2$).

atocytes from normal and 72-h fasted rats incubated for 20-h with insulin, lipid synthesis from (14 C)-acetate and $^3\text{H}_2\text{O}$ are directly related (Amatruda, J. M., unpublished observation). In the present studies, the response to insulin, 10^{-7} M, is increased 3- and 2.5-fold in cultures incubated for 16 h with 10^{-8} M insulin when measured from 14 C acetate and $^3\text{H}_2\text{O}$, respectively (data not shown). These observations substantiate the usefulness of (14 C)-acetate in these studies and indicate that potential alterations in substrate pool sizes do not significantly alter the data. Also, despite a 16-h incubation with insulin and greatly enhanced insulin responsiveness, basal lipid synthesis is not altered (mean \pm SEM = 0.94 ± 0.16 vs. 1.19 ± 0.19 nmol acetate

FIGURE 3. Dose-response curves for insulin-stimulated lipid synthesis in primary cultures of hepatocytes incubated for 16 h in the absence (●) and presence (○) of insulin, 10^{-8} M. The data represents the mean \pm SEM of six separate experiments. Basal rates of lipid synthesis are given in RESULTS.



incorporated into lipids per plate for control and insulin treated cultures). These basal rates of lipid synthesis are similar to those previously reported from our laboratory^{21,28} and, as discussed, are substantially less than those of freshly isolated hepatocytes.²⁸ While not proven, this may be due to a decreased quantity and activity of lipogenic enzymes.²⁸ Under all the conditions tested, i.e., control and insulin-treated cultures with and without the acute addition of 10^{-7} M insulin, in three separate experiments, the percent contribution of fatty acids to total lipid synthesis ranged from 58% to 83% with no significant differences between treatment groups.

DISCUSSION

The present studies illustrate several concepts concerning downregulation, the importance of binding and postbinding events in the altered biologic effects of insulin, and the significance of evaluating multiple biologic responses in the tissue of question whether speaking of insulin sensitivity i.e., the response to insulin at submaximal concentrations of hormone, or insulin responsiveness, i.e., the response to insulin at maximal concentrations of hormone.

First, while most studies in vitro have demonstrated that downregulation represents a change in insulin binding at all concentrations of hormone,^{17,18,20,21} the changes in some altered metabolic states^{12,29-35} as well as in cells exposed to insulin in vitro¹⁹ can also be manifest as an alteration in binding only at low concentrations of hormone. Such changes have been shown to occur in association with fasting,^{12,15} exercise,²⁹ insulin receptor antibodies,³⁰ carbohydrate ingestion,³¹ acromegaly,³² insulin,³³ growth hormone,³⁴ and glucocorticoid³⁴ administration, and the time of day.³⁵ Our studies demonstrate that alterations in the binding of insulin at low concentrations of hormone are also present when primary cultures of hepatocytes are exposed to 10^{-8} M insulin for 16 h. Whether this decrease in high-affinity binding represents a loss of high affinity binding sites or a true change in affinity is presently unknown. Previous studies from our laboratory utilizing incubations with higher concentrations of insulin (10^{-7} M) have demonstrated a decrease in both high and low affinity insulin binding in primary cultures of rat hepatocytes.²¹ The reason for these differences is unknown but could represent primary and secondary events in the process of downregulation or a concentration effect. Since acute alterations in insulin binding in vivo are usually attributable to changes in high affinity binding,^{31,35} both the concentration of insulin and the time of exposure to insulin may be important in determining whether changes occur primarily high or both high and low affinity binding. It is possible that more prolonged incubations with 10^{-8} M insulin may lead to alterations in lower affinity insulin binding as well.

Primary cultures of hepatocytes provide the opportunity to evaluate the biologic consequences of downregulation of the insulin receptor in a metabolically important target tissue for insulin. In the present studies, we demonstrate the expected alteration in insulin sensitivity with regard to AIB uptake. A decrease in binding at 10^{-10} M insulin is associated with decreased biologic response at this concentration of insulin. The shift in sensitivity in insulin-treated cells, however, is also evident at higher concentrations of insulin. This suggests that postbinding events may also contribute

to the decrease in sensitivity. The importance of postbinding events is more dramatically illustrated by the lipid synthesis data. In this instance, we observed a marked increase in lipid synthesis in cells exposed to insulin overnight. Since both basal lipid synthesis and maximal insulin binding are unaltered, such changes result solely from alterations in the cells' responsiveness to insulin independent of any changes in insulin binding. Interestingly, our laboratory has demonstrated that hepatocytes from fasted rats, which bind slightly more insulin than hepatocytes from fed animals at low concentrations of hormone, are resistant to the ability of insulin to stimulate AIB uptake.¹² These changes, in conjunction with the present findings, emphasize the significance of postbinding events in the liver.

Finally, the present studies underscore the problems in drawing general conclusions from binding data alone and from data evaluating binding and a single biologic response. From the AIB uptake data, one could draw the conclusion that the loss of high-affinity sites leads to insulin insensitivity through receptor and postreceptor mechanisms. However, the lipid synthesis data do not support this conclusion and indicate that for lipid synthesis downregulated hepatocytes are equally sensitive and more responsive to insulin. As a result, we conclude that the insensitivity or resistance of a given tissue to insulin may be specific for the biologic response being evaluated. Generalizations from one tissue to another may also be unwarranted.

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