*Molecular and Cellular Endocrinology*, 32 (1983) 91–100
Elsevier Scientific Publishers Ireland, Ltd.



MCE 01043

# INSULIN RESISTANCE IN H-35 RAT HEPATOMA CELLS IS MEDIATED BY POST-RECEPTOR MECHANISMS

Nancy L. KRETT *, Joanne H. HEATON and Thomas D. GELEHRTER

*Departments of Human Genetics and Internal Medicine, University of Michigan, Ann Arbor, MI 48109 (U.S.A.)*

Received 31 March 1983; accepted 2 June 1983

Incubation of H-35 cells with 300 ng/ml (50 nM) of insulin causes a 3–4-fold induction of tyrosine aminotransferase at 4–6 h of incubation. At 24 h the activity of transaminase returns to basal levels despite the presence of sufficient insulin to stimulate a maximal response. Furthermore, addition of 300 ng/ml of fresh insulin fails to stimulate the induction of transaminase. In contrast, the addition of 0.1 μM dexamethasone to insulin-treated cells stimulates the induction of tyrosine aminotransferase, indicating that the loss of responsiveness is specific to insulin action. Incubation of H-35 cells with insulin also causes a 25–30% decrease in insulin binding. This modest decrease in receptor binding is not sufficient to explain the virtually complete loss of insulin responsiveness. Hence, in H-35 hepatoma cells insulin-induced desensitization to insulin action is mediated primarily by post-receptor events.

*Key words*: desensitization; tyrosine aminotransferase; insulin binding.

Insulin resistance can be defined as a clinical or experimental state in which normal concentrations of insulin produce a less than normal biological response (Kahn, 1978). Insulin resistance may result from a decrease in insulin sensitivity (or rightward shift in dose-response curve) which suggests alterations in insulin binding, or from a decrease in maximal response to insulin, which suggests alterations at post-binding steps, or from a combination of both these alterations (Kahn, 1978; Pollet and Levey, 1980). Insulin has been reported to regulate the concentration of its plasma membrane receptor in a number of experimental and clinical states (for reviews see Pollet and Levey, 1980; Kahn et al., 1981; Jacobs and Cuatrecasas, 1981). However, there are few

* To whom reprint requests should be addressed: Department of Human Genetics, Box 015, University of Michigan Medical School, An Arbor, MI 48109 (U.S.A.)

studies that attempt to correlate a decrease in insulin binding with alterations in insulin action (Marshall and Olefsky, 1980; Heaton and Gelehrter, 1981; Amatruda et al. 1982; Capeau et al. 1982; Pederson et al., 1982). In order to understand the regulation of insulin responsiveness, it is important to investigate simultaneously alterations in both insulin binding and insulin action.

One approach to investigating the regulation of insulin responsiveness is to study insulin resistance in an in vitro cell culture system. H-35 hepatoma cells are a minimally deviated rat hepatoma line that retains several liver-specific functions (Deschatrette and Weiss, 1974). These cells are highly sensitive to insulin (Wicks et al., 1980; Koontz and Iwahashi, 1981; Hoffman et al., 1980; Iwamoto et al., 1981), and have a number of insulin responses including the induction of tyrosine aminotransferase (TAT) (Deschatrette and Weiss, 1974; Pitot et al., 1964; Wicks et al., 1980; Iwamoto et al., 1981; Lee et al., 1970). Thus H-35 cells are a suitable system for this study. We report here that incubation of H-35 cells with insulin causes a virtually complete desensitization to insulin action. This loss of responsiveness is specific for insulin since the addition of dexamethasone to insulin-treated H-35 cells elicits a full induction of transaminase. Insulin also decreases insulin binding by 25–30%. This modest decrease in binding does not adequately explain the near-complete loss of responsiveness. Therefore, post-receptor steps are involved in mediating insulin-induced desensitization to insulin action.

## MATERIALS AND METHODS

### *Cells*

H-35 cells were provided by Dr. W.D. Wicks, University of Tennessee. The cells were grown in monolayer culture in antibiotic-free α-MEM (Gibco) supplemented with 5% fetal bovine serum, 5% calf serum, 2 mM glutamine and 30 mM sodium bicarbonate. The cells were maintained in a 37°C humidified incubator under an atmosphere of 5% $CO_2$ and 95% air. 18 h prior to the start of an experiment the growth medium was removed from confluent monolayers and replaced with serum-free MEM containing 0.1% bovine serum albumin, 2 mM $CaCl_2$, 50 μg/ml neomycin and 2 mM glutamine.

### *Reagents*

Crystalline porcine insulin (25.4 units/mg) was a gift from Dr. R.E. Chance, Lilly Research Laboratories; dexamethasone was a gift from Dr.

Walter Gall, Merck and Co.; $^{125}$I-labeled insulin (80–100 mCi/mg) was purchased from New England Nuclear; bovine serum albumin and Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) were purchased from Sigma Chemical Co.; all other chemicals used were of reagent grade.

*Tyrosine aminotransferase assay*

Tyrosine aminotransferase (EC 2.6.1.5) was assayed as described by Spencer and Gelehrter (1974). Transaminase activity is reported as milliunits/mg of soluble protein; one milliunit catalyzes the formation of one nmole of *p*-hydroxyphenylpyruvate per minute at 37°C. Protein was determined by the method of Lowry et al. (1951).

*Insulin binding assay*

Insulin binding to cells in monolayer culture was measured in 0.1 M Hepes, pH 8.0, 0.12 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 10 mM glucose, 1 mM EDTA and 1.0% bovine serum albumin, as described by Heaton and Gelehrter (1981). $^{125}$I-labeled insulin was used at a concentration of 0.04 μCi/ml (0.4–0.6 ng/ml). Nonspecific binding was determined in the presence of 10 μg/ml unlabeled insulin. After 3 h of incubation at 15°C, the cells were washed 3 times with phosphate-buffered saline, pH 7.4, at 4°C. The cells were dissolved in 1.0 N NaOH and the radioactivity was determined in a gamma spectrometer.

## RESULTS

*Effect of insulin on the insulin induction of tyrosine aminotransferase*

Insulin (300 ng/ml) stimulates a 3–4-fold induction of TAT activity which is maximal by 5–6 h of incubation (Fig. 1A). This is similar to previously published data (Iwamoto et al., 1981; Lee et al., 1970). By 24 h of incubation, however, transaminase activity has returned to basal levels despite the continued presence of sufficient insulin to stimulate a maximal response. The concentration of insulin (measured by radioimmunoassay) remaining in the medium after 24 h of incubation with H-35 cells is 150 ng/ml, which is more than the maximally effective concentration of 20 ng/ml (see Fig. 2, upper curve).

At 24 h (Fig. 1B) the medium was removed from the cell monolayers and replaced with fresh serum-free medium containing either no insulin or 300 ng/ml insulin. Additional insulin stimulated little or no induction of transaminase activity in insulin-treated cells; hence these cells are desensitized to insulin action. When insulin was added to control cells

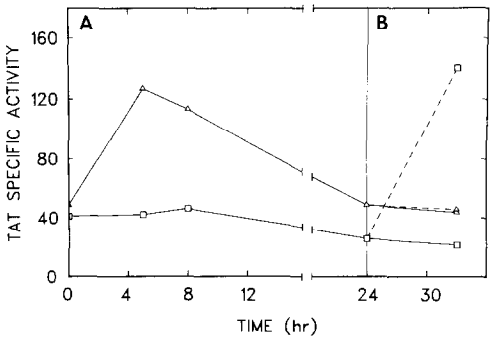


Fig. 1. Insulin-induced desensitization to insulin. Monolayer cultures were incubated in serum-free medium for 18 h. The medium was then replaced with fresh serum-free medium with or without 300 ng/ml insulin and the incubation continued for 24 h. Tyrosine aminotransferase specific activity was determined at the times indicated (A). At 24 h the medium was replaced with either fresh serum-free medium or medium containing 300 ng/ml insulin. Transaminase activity was determined after 6 h of incubation (B). Each point represents the average of duplicate assays of duplicate cultures: □———□, control cultures; △———△, insulin-treated cultures; □— — —□ insulin added to control cultures; △— — —△, insulin added to insulin-treated cultures.

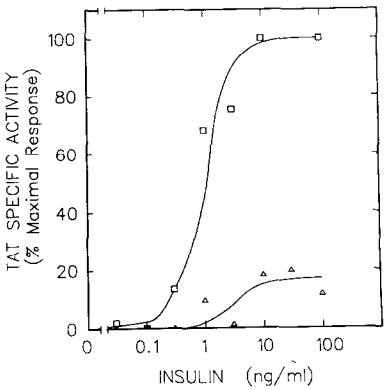


Fig. 2. Effect of insulin on the concentration dependence of insulin induction of tyrosine aminotransferase. Monolayer cultures were incubated in serum-free medium for 18 h. The medium was replaced with either serum-free medium or serum-free medium containing 300 ng/ml insulin and the incubation continued for 24 h. At this time the medium in both control and insulin-treated cultures was replaced with fresh serum-free medium or serum-free medium containing insulin ranging in concentration from 0.03 to 100 ng/ml. Transaminase-specific activity was determined after 6 h and the data expressed as the percentage of activity in control cells treated with 100 ng/ml insulin. Each point represents the average of duplicate assays of duplicate cultures: □, control; △, insulin-treated.

there was a maximal stimulation of tyrosine aminotransferase activity, indicating that cells are still capable of responding to insulin after 24 h of incubation under these experimental conditions.

The concentration dependence of insulin induction of tyrosine amintransferase is shown in Fig. 2. The concentration of insulin which causes half-maximal induction of transaminase is 1 ng/ml (167 pM). This concentration of insulin is comparable to the portal venous concentration of insulin in normal fasting humans (Blackard and Nelson, 1971). Cells previously incubated with insulin show little induction of transaminase even at high concentrations of insulin.

*Specificity of desensitization*

One possible explanation for the lack of responsiveness to insulin in desensitized cells is that the insulin treatment has impaired the cellular mechanisms involved in the regulation and expression of tyrosine aminotransferase. In order to test this possibility, we examined the dexamethose induction of this enzyme in insulin-desensitized cells. De-

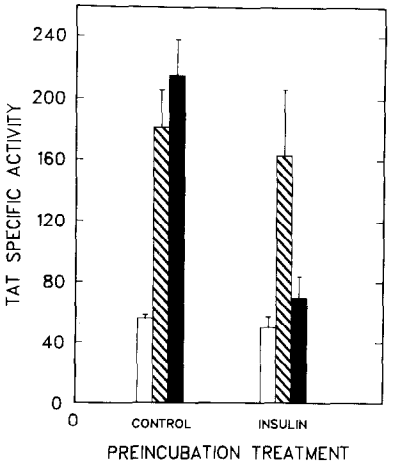


Fig. 3. Specificity of desensitization. Monolayer cultures were incubated in serum-free medium for 16–18 h. The medium was then replaced with either fresh serum-free medium or fresh serum-free medium containing 300 ng/ml insulin. The incubation was continued for 24 h and this was designated the preincubation treatment. The medium from each preincubation was then replaced with either serum-free medium, serum-free medium containing 300 ng/ml insulin, or serum-free medium containing 0.1 $\mu$M dexamethasone. Tyrosine aminotransferase was assayed after a 6 h incubation. The data represent the mean $\pm$ SEM of 6 determinations from 3 separate experiments. Open bars, control; hatched bars, dexamethasone; shaded bars, insulin.

xamethasone interacts with different receptors than insulin and induces transaminase activity in hepatoma cells by a different mechanism than does insulin (Lee et al., 1970; Gelehrter, 1979). Monolayer cultures were incubated in serum-free medium for 24 h in the absence or presence of 300 ng/ml insulin. Medium was then replaced with etiher serum-free medium alone, serum-free medium containing insulin (300 ng/ml), or serum-free medium containing 0.1 $\mu$M dexamethasone, and aminotransferase activity assayed after 6 h of incubation. As shown in Fig. 3, cells which are insensitive to insulin remain sensitive to the induction of transaminase by dexamethasone. This implies that desensitization is not the result of a defect in tyrosine aminotransferase induction per se, but reflects a failure at some step in insulin action.

*Concentration dependence of desensitization*

The concentration dependence of desensitization was determined in order to further characterize insulin resistance in H-35 cells (Fig. 4).

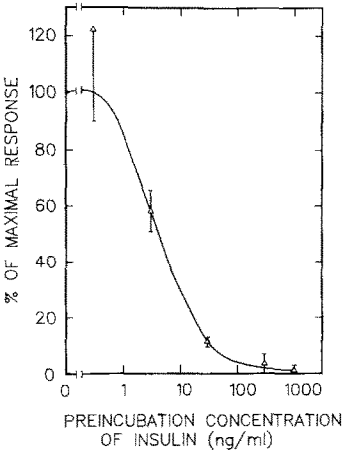


Fig. 4. Concentration dependence of desensitization. Monolayer cultures were incubated for 16–18 h in serum-free medium. The medium was removed and replaced with fresh serum-free medium or serum-free medium containing insulin ranging in concentration from 0.3 to 1000 ng/ml. The incubation was continued for 24 h and this is designated the preincubation treatment. At this time the medium was replaced in each preincubation group with either serum-free medium or serum-free medium containing 300 ng/ml insulin. After 6 h of incubation, tyrosine aminotransferase specific activity was determined by duplicate assays of duplicate cultures. Enzyme induction in control cultures (preincubation without insulin) is defined as 100% response and the induction for each preincubation insulin concentration is expressed as a percentage of that in control cultures. Each point represents the mean $\pm$SEM for 3 separate experiments.

Monolayer cultures were preincubated for 24 h with a concentration of insulin as specified in Fig. 4. The time-course of tyrosine aminotransferase induction at each insulin concentration was similar to the time-course in Fig. 1A (data not shown). At 24 h the monolayer cultures at each insulin concentration were divided into two groups. After removing the medium from all cultures, one group received fresh serum-free medium containing 300 ng/ml insulin and the other group received fresh serum-free medium. Transaminase induction in control cells (preincubation without insulin) is expressed as 100% response, and induction in cultures preincubated at each insulin concentration is expressed as a percentage of that in control cells. The concentration of insulin which caused half-maximal desensitization was approximately 4 ng/ml insulin (670 pM), which is comparable to the concentration which causes half-maximal induction of tyrosine aminotransferase.

*Effect of insulin on insulin binding*

Desensitization in insulin-treated cells might result from a change in insulin binding either at the level of receptor affinity or at the level of receptor concentration in the plasma membrane (Kahn, 1978; Pollet and Levey, 1980). However, desensitization might also result from alterations in post-receptor events (Kahn, 1978; Pollet and Levey, 1980). In order to distinguish among alternatives we investigated the effects of insulin on insulin binding.

Steady-state binding of [$^{125}$I]insulin in H-35 cells is achieved by 3 h of incubation at 15°C (data not shown). Under these conditions, 90–95% of the [$^{125}$I]insulin in the medium is precipitable by trichloroacetic acid, indicating that there is very little degradation of the [$^{125}$I]insulin. There is also very little internalization of insulin at 15°C, since 88% of the cell-associated radioactivity is removed by a 5 min incubation with 20 mM sodium barbital, pH 3.0 (Olefsky and Kao, 1982). Nonspecific binding is measured in the presence of 10 $\mu$g/ml unlabeled insulin and is less than 5% of total binding.

Unlabeled insulin competes with a tracer concentration of [$^{125}$I]insulin (85 pM) for binding to the insulin receptor in a concentration-dependent fashion (Fig. 5). Half-maximal competition occurs at 35 ng/ml unlabeled insulin in both control cells and cells that have been treated with insulin (300 ng/ml) for 24 h. Scatchard analysis (not shown) of these binding data yields curvilinear plots for both insulin-treated and control cells, as reported by others (Hoffman et al., 1980; Massague et al., 1982). Assuming the presence of two classes of binding sites (Pollet et al., 1977), the $K_d$ of binding for the high affinity site was $0.80 \pm 0.10$ nM ($n = 4$, mean $\pm$ SEM) for both insulin-treated and control cells, indicating that there is

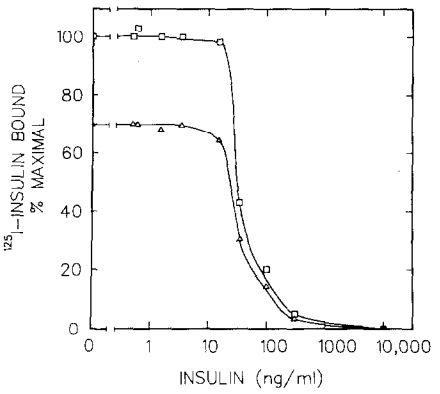


Fig. 5. The effect of insulin on insulin binding. Monolayer cultures were incubated for 18 h in serum-free medium. The medium was then replaced with either serum-free medium or serum-free medium containing 300 ng/ml insulin and the incubation continued for 24 h. The cultures were then washed and binding of [$^{125}$I]insulin was determined in the presence of increasing concentrations of unlabeled insulin. Corrections were made for nonspecific binding determined in the presence of 10 $\mu$g/ml unlabeled insulin. Each point represents the average of duplicate determinations: □, control; △, insulin-treated.

no apparent change in receptor affinity in the insulin-treated cells. Insulin-treated cells do exhibit a modest decrease ($25.0 \pm 3.4\%$, mean $\pm$SEM, $n = 4$) in binding relative to control cells. Since the receptor affinity appears to be unchanged in insulin-treated cells, this decrease in insulin binding probably represents a decrease in receptor number. This small decrease in insulin binding is not sufficient to explain the virtually complete loss of insulin responsiveness. Thus in H-35 cells desensitization to insulin action involves events at a post-receptor level.

## DISCUSSION

The proximal steps in insulin action have been extensively studied (for reviews see Kahn et al., 1981; Jacobs and Cuatrecasas, 1981), but the role of post-binding events in insulin action (Kasuga et al., 1981; Larner et al., 1982; Gorden et al., 1982) remains to be defined. Similarly, a great deal is known about insulin regulation of insulin receptors, but there are few studies on insulin regulation of post-receptor events, and the mechanism of such regulation remains to be elucidated. Insulin-induced insulin resistance in H-35 hepatoma cells provides a favorable experimental system in that it allows investigation of the regulation of insulin re-

sponsiveness at both the receptor and post-receptor level.

We report that a 24 h incubation with insulin renders H-35 hepatoma cells insensitive to further insulin action. This desensitization is accompanied by a modest (25–30%) decrease in insulin binding, which is presumably due to a decrease in receptor number since insulin does not alter the apparent affinity of binding. Insulin also causes a decrease in receptor number without change in apparent affinity in HTC (Heaton and Gelehrter, 1981) and Zajdela rat hepatoma cells (Capeau et al., 1982). It has been reported that various biological effects of insulin in H-35 cells require the occupancy of only a small fraction ($< 5\%$) of the cell's insulin receptors (Ballard et al., 1980; Hoffman et al., 1980; Iwamoto et al., 1981; Andreone et al., 1982). Thus it is highly unlikely that the small decrease in insulin binding caused by insulin could account for the virtually complete loss of biological responsiveness. Furthermore, it is unlikely that insulin's biological actions are mediated by receptors other than insulin receptors (Czech, 1982). IGF-II receptors on H-35 cells do not recognize insulin even at micromolar concentrations, and IGF-I receptors, which bind insulin with low affinity, are not present on H-35 cells (Massague et al., 1982). Therefore, insulin-induced desensitization to insulin action in H-35 cells is mediated primarily by post-receptor mechanisms.

In HTC rat hepatoma cells insulin causes a virtually complete desensitization to insulin action as measured by a failure to induce tyrosine aminotransferase or to stimulate amino acid uptake (Heaton and Gelehrter, 1980, 1981). Desensitization in HTC cells in accompanied by a 50–60% decrease in receptor number; however, receptor regulation could be dissociated from regulation of responsiveness, indicating that desensitization is occurring via post-receptor mechanisms. The work in H-35 cells reported in this paper contributes further evidence that insulin-induced desensitization to insulin action is occurring at steps distal to insulin binding. Post-receptor mechanisms mediating partial desensitization have also been postulated in 3T3-L1 cells treated with anti-insulin receptor antibodies (Grunfeld et al., 1980), and in human (Pederson et al., 1982; Bolinder et al., 1982) and rat adipocytes (Marshall and Olefsky, 1980).

## ACKNOWLEDGEMENTS

We thank Dr. W.D. Wicks for the H-35 cells, and Ms. J. Worley for secretarial assistance. This work was supported by NIH grant AM-30055. N.L.K. was supported by NIH training grant AM-07367.

## REFERENCES

Amatruda, J.M., Newmeyer, H.W. and Chang, C.L. (1982) Diabetes 31, 145–148.
Andreone, T.L., Beale, E.G., Bar, R.S. and Granner, D.K. (1982) J. Biol. Chem. 257, 35–38.
Ballard, F.J., Wong, S.S.C., Knowles, S.E., Partridge, N.C., Martin, T.J., Wood, C.M. and Gunn, J.M. (1980) J. Cell. Physiol. 105, 335–346.
Blackard, W.G. and Nelson, N.C. (1971) Diabetes 20, 286–288.
Bolinder, J., Ostman, J. and Arner, P. (1982) Diabetes 31, 911–961.
Capeau, J., Flaig-Staedel, C., Beck, J.-P. and Picard, J. (1982) Endocrinology 111, 993–1000.
Czech, M.P. (1982) Cell 31, 8–10.
Deschatrette, J. and Weiss, M.C. (1974) Biochimie 56, 1603–1611.
Gelehrter, T.D. (1979) In: Glucocorticoid Hormone Action, Eds.: J.D. Baxter and G.G. Rousseau (Springer-Verlag, New York) pp. 583–591.
Gorden, P., Carpentier, J.-L., Fan, J.-Y. and Orci, L. (1982) Metabolism 31, 664–669.
Grunfeld, C., Van Obberghen, E., Karlsson, F.A. and Kahn, C.R. (1980) J. Clin. Invest. 66, 1124–1134.
Heaton, J.H. and Gelehrter, T.D. (1980) Biochem. Biophys. Res. Commun. 92, 795–802.
Heaton, J.H. and Gelehrter, T.D. (1981) J. Biol. Chem. 256, 12257–12262.
Hoffman, C., Marsh, J.W., Miller, B. and Steiner, D.F. (1980) Diabetes 29, 865–874.
Iwamoto, Y., Wang, K.Y. and Goldfine, I.D. (1981) Endocrinology 108, 44–51.
Jacobs, S. and Cuatrecasas, P. (1981) Endocr. Rev. 2, 251–263.
Kahn, C.R. (1978) Metabolism 27, 1893–1902.
Kahn, C.R., Baird, K.L., Flier, J.S., Grunfeld, C., Harmon, J.T., Harrison, L.C., Karlsson, F.A., Kasuga, M., King, G.L., Lang, U.C., Podskalny, J.M. and Van Obberghen, E. (1981) Recent Progr. Hormone Res. 37, 477–538.
Kasuga, M., Karlsson, F.A. and Kahn, C.R. (1981) Science 215, 185–187.
Koontz, J.W. and Iwahashi, M. (1981) Science 211, 947–949.
Larner, J., Cheng, K., Schwartz, C., Kikuchi, K., Tamura, S., Creacy, S., Dubler, R., Galasko, G., Pullin, C. and Katz, M. (1982) Recent Progr. Hormone Res. 38, 511–556.
Lee, K.L., Reel, J.R. and Kenney, F.T. (1970) J. Biol. Chem. 245, 5806–5812.
Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
Marshall, S. and Olefsky, J.M. (1980) J. Clin. Invest. 66, 763–772.
Massague, J., Blinderman, L.A. and Czech, M.P. (1982) J. Biol. Chem. 257, 13958–13962.
Olefsky, J.M. and Kao, M. (1982) J. Biol. Chem. 257, 8667–8673.
Pederson, O., Hjollund, E. and Sorensen, N.S. (1982) Metabolism 31, 884–895.
Pollet, R.J. and Levey, G.S. (1980) Ann. Intern. Med. 92, 663–680.
Pollet, R.J., Standaert, M.L. and Haase, B.A. (1977) J. Biol. Chem. 252, 5828–5834.
Spencer, C.J. and Gelehrter, T.D. (1974) J. Biol. Chem. 249, 577–583.
Wicks, W.D., Leichtling, B.H., Wimalasena, J. and Koontz, J. (1980) Ann. N.Y. Acad. Sci. 349, 195–209.