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REGULATION OF INSULIN RESPONSIVENESS IN RAT HEPATOMA CELLS

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Summary: Insulin causes a 5 to 10-fold increase in the velocity of α-aminoisobutyric acid transport and a 2 to 3-fold increase in tyrosine aminotransferase activity in dexamethasone-treated hepatoma tissue culture cells. Maximal responses occur 2-4 hours after insulin addition but then decrease to control levels by 24 hours incubation. Medium conditioned by cells incubated with insulin for 24 hours retains sufficient biologically active insulin to produce an insulin response in fresh dexamethasone-treated cells. Readdition of insulin to insulin-treated cells, however, elicits no response, indicating that the cells are insensitive to the hormone. Incubation of such unresponsive cells in the absence of insulin results in recovery of responsiveness within 2 hours. These data suggest that exposure of rat hepatoma cells to insulin causes a complete but reversible loss of sensitivity to this hormone.

Hepatoma tissue culture (HTC)[1] cells, an established line of rat hepatoma cells, respond to dexamethasone with a 5 to 10-fold increase in the cellular concentration of tyrosine aminotransferase (TAT) (E.C. 2.6.1.5) (1) and a 90% inhibition in the initial rate of α-aminoisobutyric acid (AIB) transport (2,3). Addition of insulin to HTC cells, treated with dexamethasone for 16-18 hours, results in a further 2 to 3-fold increase in TAT (4,5) and 5 to 10-fold increase in AIB transport velocity (2). Maximal transport velocity and transaminase activity occur 2 hours and 3-4 hours after insulin addition, respectively. As we show in this communication, both effects then decay despite the continued presence of insulin, and by 24 hours of incubation with insulin transaminase activity and AIB transport velocity have returned to a level similar to that in cells treated with dexamethasone alone. The present study was undertaken to explain this response of HTC cells to chronic insulin exposure

1. The abbreviations used are: HTC, hepatoma tissue culture; TAT, tyrosine aminotransferase; AIB, α-aminoisobutyric acid; MSA, multiplication stimulating activity.

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METHODS:

Cells: HTC cells were grown in spinner culture in modified Eagle's Minimal Essential Medium without antibiotics as described previously (6). Cells in the logarithmic phase of growth were harvested by centrifugation and resuspended at 0.8 - 1.0 x 10^6 cells per ml in serum-free medium containing 50 µg/ml neomycin and 0.1% BSA (IM-BSA). Incubations were carried out in suspension cultures in a gyrotory shaker water bath at 37°C. At least 95% of the cells excluded trypan blue after 50 hours incubation in serum-free medium.

TAT Assay: As described in the legends to the figures, samples of cells were removed from the suspension culture and centrifuged at 1200 x g for 2 min. The cell pellets were washed with 0.1 M potassium phosphate buffer, pH 7.6, and stored as pellets at -20°C. Assays of TAT specific activity were carried out as previously described (7). TAT specific activity is reported as munits/mg soluble protein. One munit catalyzes the formation of one nmole p-hydroxyphenylpyruvate per minute at 37°C.

AIB Transport: The initial rate of AIB transport was determined as described by Heaton and Gelehrter (5). The assay was carried out for 10 min with 0.5 mM AIB including 0.25 µCi/ml ^{14}C-AIB. Radioactivity contributed by trapped water was determined by addition of [^{3}H]-inulin 30 sec before stopping the reaction. Protein content was determined by the method of Lowry, et al., (8).

Radioimmunoassay of Insulin: Radioimmunoassay of insulin in culture medium was kindly performed by the Ligand Core Laboratory of the Michigan Diabetes Research and Training Center, according to the method of Hayashi, et. al., (9).

Materials: Insulin was a gift from Dr. R. E. Chance, Eli Lilly Company. Dexamethasone was a gift from Dr. Walter Gall of Merck and Company. α-[1-^{14}C]-aminoisobutyric acid (specific activity 45-52 mCi/mM) was purchased from New England Nuclear and [^{3}H]-inulin (approx. 800 mCi/mM) was purchased from Amersham.

RESULTS

Fig. 1A illustrates the time course of insulin induction of TAT in dexamethasone-treated HTC cells. In this experiment incubation of cells for 17 hours with 0.1 µM dexamethasone increased TAT activity 6-fold. Addition of 4 µg/ml insulin resulted in a further 2-fold increase in TAT specific activity at 3 hours, after which the TAT activity decreased so that by 24 hours of exposure to insulin, TAT specific activity was nearly identical to that of cells incubated with dexamethasone alone.

A similar phenomenon is seen when one examines insulin enhancement of AIB transport. In the experiment shown in Fig. 1B, dexamethasone inhibited AIB transport by 82% after 18 hours incubation. The addition of insulin (4 µg/ml) to dexamethasone-treated cells stimulated transport nearly 8-fold.

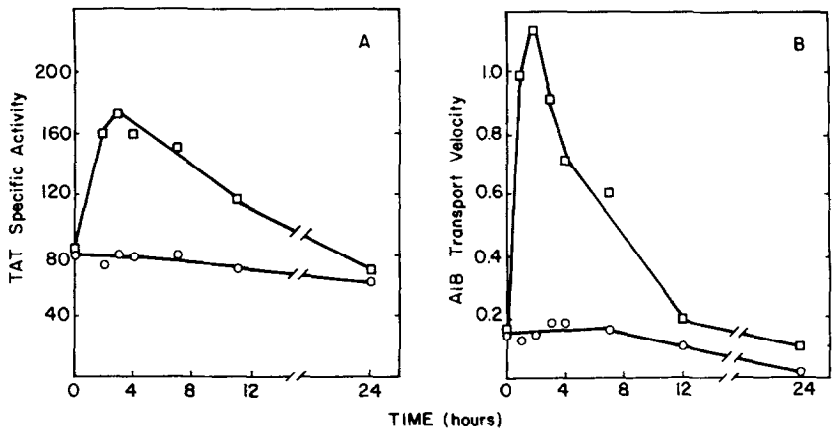

Fig. 1. Time course of insulin stimulation of TAT activity and AIB transport. HTC cells were incubated in suspension culture in IM-BSA containing 0.1 μM dexamethasone for 17 hours. The cells were then divided into two portions, and to one insulin was added to a final concentration of 4 μg/ml. The other portion was used as control. At the times indicated samples were taken for assay of TAT (Part A) or for assay of initial rates of AIB transport (Part B).

(○) dexamethasone alone
(□) dexamethasone + insulin at 0 time

Insulin-stimulated transport is maximal after 2 hours incubation and then decreases. Again, after 24 hours in insulin-containing medium, these cells transport AIB at a rate similar to that of cells incubated with dexamethasone alone.

Since liver cells degrade insulin, it is possible that the loss of insulin response is due to degradation of the insulin in the medium. Three lines of evidence argue against this explanation.

Radioimmunoassays were performed to determine the concentration of insulin in the medium after 0-24 hours in incubation with cells. The results of a representative experiment are shown in Table I. After 4 hours incubation, a time at which the rate of amino acid transport has decreased (Fig. 1), little insulin has been degraded. At 12 and 24 hours, however, the insulin concentration is significantly decreased. We have shown previously that a maximal insulin response is routinely achieved with an insulin concentration of 1 μg/ml and can be obtained with concentrations as low as 200 ng/ml. Half-maximal response is seen at 35 ng/ml insulin (10). Therefore, at 12 hours of incubation the remaining 1.2 μg/ml insulin should be sufficient to produce a maximal

TABLE I

Degradation of Insulin by HTC Cells

A	B	C	D
Time of Incubation	Insulin Concentration ng/ml	TAT Induction (Percent Increase)	AIB Transport (Percent Increase)
0	3950	124	917
2	3820	122	917
4	3890	106	896
6	2840	103	754
12	1200	104	708
24	84	51	333

HTC cells were incubated for 17 hours with 0.1 μM dexamethasone. The culture was then divided into two portions, and to one insulin was added to a final concentration of 4 μg/ml. At the times indicated after insulin addition (Column A), a portion of the culture was centrifuged to remove cells. The insulin concentration of the medium was determined by radioimmunoassay (Column B). This conditioned medium was also tested for its insulin action by measuring TAT activity (Column C) and AIB transport velocity (Column D) in fresh dexamethasone-treated cells incubated in the conditioned medium for 4 or 2 hours, respectively.

response. Even at 24 hours sufficient immunoreactive insulin (84 ng/ml) remains in the medium to elicit at least a partial response.

The following experiment established that the insulin remaining in the medium is biologically potent. Dexamethasone-treated HTC cells were incubated with 4 μg/ml insulin and at times from 0-24 hours the cells were removed from a portion of the culture by centrifugation. The conditioned medium was then tested for its ability to induce TAT and to stimulate AIB transport in fresh dexamethasone-treated cells. The results are shown in Table 1. Conditioned medium from incubations of up to 12 hours evoke a nearly maximal response, whereas conditioned medium from a 24-hour incubation is less potent. This is consistent with the insulin concentration as determined by radioimmunoassay. After 12 hours incubation, when the insulin effect has significantly decreased (Fig. 1), sufficient biologically active hormone remains to produce a nearly maximal response. Even after 24 hours incubation, when the insulin response is totally lost, the medium is capable of producing at least a partial response Thus the decay of the insulin response observed in Fig. 1 is not due simply to degradation of insulin.

Thirdly, we examined the ability of insulin-treated cells to respond to additional hormone. As illustrated in Fig. 2, readdition of 4 μg/ml insulin to cells which have been incubated with insulin for 24-26 hours does not elicit a response, indicating that these cells have become completely unresponsive to insulin. Cells incubated in dexamethasone-containing, insulin-free medium for the same time period show a full response to added insulin, indicating that cells in serum-free medium for that period of time are viable and responsive. The data are consistent with the hypothesis that exposure of HTC cells to insulin causes a complete loss of responsiveness to this hormone.

Insulin-induced insensitivity to insulin in HTC cells is reversible, as shown in Fig. 3. Cells rendered insulin-insensitive by a 24 hour incubation with the hormone, were centrifuged out of the medium and washed to remove insulin. The cells were then resuspended in dexamethasone-containing medium and incubated at 37°C. Immediately after ('0' time), and 2 hours after the wash procedure 4 μg/ml insulin was again added to a portion of the cells. Within 2 hours, cells in insulin-free medium have recovered full responsiveness. When insulin was added immediately after the wash procedure, there was a short lag and a blunted response to insulin. Since the wash procedure takes about 20 min and since recovery is rapid, this may reflect a partial recovery. The lag is not caused by the wash procedure itself as dexamethasone-treated cells, washed immediately before insulin addition, exhibit a normal response to insulin (results not shown). A more rapid wash (5 min) can be accomplished in monolayer cultures. Cells in monolayer, which have been desensitized to insulin by a 24 hour incubation with the hormone, do not respond to insulin which is readded immediately after the wash procedure (data not shown).

DISCUSSION:

HTC cells display specific membrane receptors for insulin and for the insulin-like growth factor, multiplication stimulating activity (MSA). Since insulin does not bind to the MSA receptor, even at concentrations as high as

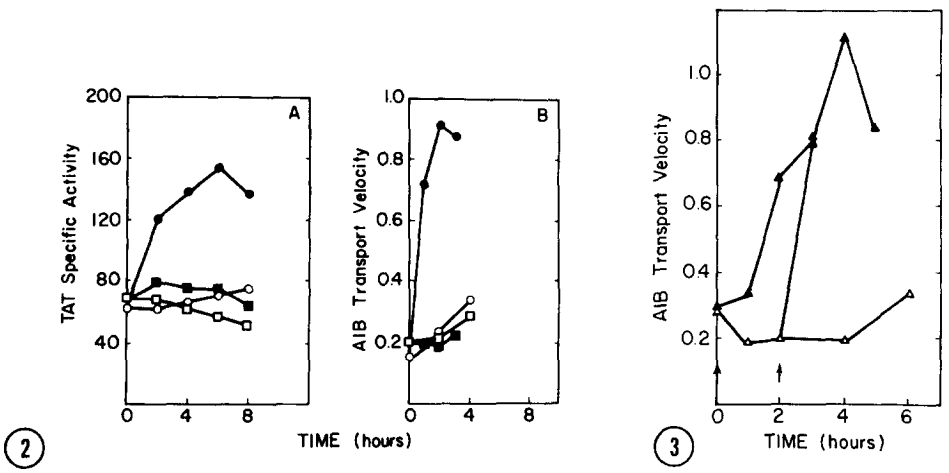

Fig. 2. Insulin responsiveness in HTC cells after chronic exposure to insulin. HTC cells were incubated in suspension culture in IM-BSA containing 0.1 μM dexamethasone for 17 hours. The cells were then divided into two portions, and to one insulin was added to a final concentration of 4 μg/ml. The other portion was used as control. Incubation continued for 24 to 26 hours, at which time each culture was again divided into two portions. To one of each pair was added 4 μg/ml fresh insulin. In this figure '0' time represents a total of 41-43 hours preincubation. At the times indicated samples were taken for assay of TAT (Part A) or AIB transport (Part B).

(○) dexamethasone alone for total incubation
(□) dexamethasone 17 hours + insulin for 24-26 hours
(●) dexamethasone 41-43 hours + insulin at '0' time
(■) dexamethasone 17 hours + insulin for 24-26 hours + insulin again at '0' time

Fig. 3. Recovery of insulin responsiveness. HTC cells were incubated in suspension culture in IM-BSA containing 0.1 μM dexamethasone for 17 hours. Insulin was then added to a final concentration of 4 μg/ml and incubation continued for 24 1/2 hours. The medium was then removed by centrifugation and cell pellets were washed 3 times by resuspension in IM-BSA containing dexamethasone but not insulin. Washed cell pellets were resuspended in IM-BSA containing 0.1 μM dexamethasone. Immediately ('0' time) and after 2 hours of incubation, fresh insulin (4 μg/ml) was added to a portion of the cell culture. Samples were taken at the times indicated for assay of AIB transport velocity.

(△) washed cells incubated in IM-BSA with dexamethasone
(▲) fresh insulin added to 0 or 2 hours

10 μg/ml, and does bind its own receptor with an affinity consistent with its biological potency, it is likely that this hormone exerts its biological effect via the insulin receptor (10). Regulation of responsiveness to this hormone could, therefore, be accomplished by modulation of the insulin receptor (11).

There is considerable evidence that insulin can regulate the apparent concentration and/or affinity of its own receptors (12,13). In IM-9 lymphocytes

insulin causes a time-and concentration-dependent decrease in the apparent number of insulin receptors (14). These cells, however, have no known biological response to insulin and, thus, the significance of down-regulation of receptors in this system is unknown. Chronic exposure to physiological levels of insulin results in a decreased number of insulin receptors in human fibroblasts (15) and in primary cultures of rat hepatocytes (16,17) also. In both systems maximal down-regulation has usually been a 50-60% decrease in receptor number. It is doubtful that such a decrease could cause the complete loss of response to high concentrations of insulin that we observe. Similarly, it is unlikely that a decrease in receptor affinity for insulin, as reported in adipocytes (18), could explain the total loss of responsiveness described here.

Regulation of hormone responsiveness could also occur by regulation at a step distal to the receptor. For example, high concentrations of opiates can decrease cellular responsiveness to these agents without decreasing the concentration of opiate receptors (19). Karlsson, et. al. have recently reported that anti-insulin-receptor antibody-induced insulin resistance in 3T3-L1 fatty fibroblasts is apparently caused at an early post-receptor step rather than by a change in insulin binding (20).

We report here an experimental system in which long-term insulin exposure causes a complete but reversible loss of insulin responsiveness as assessed by both enzyme induction and stimulation of amino acid transport. HTC cells provide a unique model system in which to study the regulation of both the biological response to insulin and insulin binding to its receptor. This system may allow the elucidation of the mechanism by which insulin regulates cellular responsiveness to itself.

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