INSULIN INDUCTION OF TYROSINE AMINOTRANSFERASE IN SYNCHRONIZED HEPATOMA CELLS

Janet Rettig Emanuel* and Thomas D. Gelehrter†

*Department of Human Genetics, Yale University New Haven, CT 06510, and †Departments of Human Genetics and Internal Medicine, University of Michigan Ann Arbor, MI 48104

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Summary: Addition of insulin to unsynchronized rat hepatoma cells, previously incubated with dexamethasone, promotes a rapid increase in tyrosine aminotransferase activity. Mitotic cells in the presence or absence of colcemid are not inducible by either insulin or dexamethasone. Inducibility by dexamethasone is restored only after the third hour of the G1 phase, presumably related to the appearance of the putative posttranscriptional repressor (Martin, D.W. et al. (1969). Proc. Nat. Acad. Sci., 63: 842-849). In contrast, inducibility by insulin is restored immediately upon passage of the synchronized cells into G1. These data suggest that insulin can induce tyrosine aminotransferase in the absence of the repressor.

In an unsynchronized population of established rat hepatoma tissue culture (HTC[1]) cells, corticosteroid hormones induce a 10-fold increase in the rate of synthesis of tyrosine aminotransferase (E.C. 2.1.1.5) by a mechanism requiring RNA synthesis (1). HTC cells (2,3,4) as well as cells derived from normal rat liver (4), are sensitive to steroid induction of TAT[1] only in the mid and late G1 and S phases of the cell cycle; enzyme synthesis is constitutive and insensitive to steroid during G2, M and early G1. Addition of insulin (5) or dialyzed bovine serum (6) to unsynchronized HTC cells, previously induced with dexamethasone in a chemically defined, serum-free medium, causes a further doubling in the amount of induced enzyme, by a mechanism which is independent of concomitant RNA synthesis. We have shown previously that corticosteroids, insulin and serum all affect different aspects of the post-transcriptional control of expression of the same enzyme (7).

In an attempt to elucidate further the differences in the modes of

[1]The abbreviations used are: HTC, hepatoma tissue culture; and TAT, tyrosine aminotransferase.

action of these inducers, we have studied the sensitivity of TAT to insulin induction in different phases of the cell cycle. Highly synchronized populations of HTC cells were obtained by mitotic selection from colcemid-treated monolayer cultures. We have observed that cells previously induced with dexamethasone show a cell-cycle phase-specific sensitivity to insulin which is different from that to dexamethasone.

METHODS

A. Cell growth and synchrony: HTC cell stocks were maintained in spinner culture as previously described (7). Cells were synchronized and induced in monolayer cultures in growth medium with half the usual serum complement, conditioned by incubation over logarithmically growing cells for 24 hours prior to use. This medium provided minimal serum induction (6) of TAT and allowed normal growth and cell cycle behavior (2,8). Under these conditions the level of TAT in steroid-induced synchronized cells was also comparable to that previously reported (2). The effective concentration of colcemid (0.25 μM)was chosen as that which produced the highest yield of mitotic cells, and yet the least multipolar cell divisions and irregular "mitotic figures"; this concentration of colcemid did not appear to alter measurable cell cycle parameters, and was far below the level necessary to block nucleoside transport in another line of hepatoma cells (9).

Monolayer cultures were established by plating 10-15 x 10^6 cells per 150 x 25 mm Falcon tissue culture plate in 30 ml of Minimal Essential Medium (Eagle) supplemented with 5% bovine and 5% fetal calf sera, and 0.02% calcium chloride. Cultures were incubated 20 hours at 37°C after which the medium was replaced and the cultures incubated for an additional 20 hours; cells were growing logarithmically and not confluent. Twenty-four hours prior to harvest of mitotic cells, the medium was removed and calcium-free half-serum growth medium added. Sixteen hours prior to harvest 0.1 μM dexamethasone was added. Colcemid was added to a final concentration of 0.25 μM at three to twelve hours prior to harvest. Detached mitotic cells were harvested by aspiration

of the medium after gently swirling the plates. Interphase and unsynchronized control populations were harvested by scraping with a rubber policeman, and all cells were collected by centrifugation at 500 x g and resuspended as described in the legends to the figures. Control cultures did not receive colcemid. Cell viability was quantitated by exclusion of trypan blue stain and direct cell count in a hemocytometer. Mitotic cells were scored after staining with 2.0% aceto-orcein as cells with tightly clumped chromatin (with or without "mitotic figures") and no visible nuclear boundary. The ratio of mitotic cells to total cells scored is expressed as "percent mitotic cells".

B. Assays: DNA synthesis was quantitated as total tritium counts per minute incorporated into 10% trichloroacetic acid precipitable material after incubation of 1 μCi [^{3}H] -methyl thymidine (SA. 19 Ci/mM; Amersham) with 5 x 10^5 cells for 60 minutes. Duplicate aliquots of incubation mixture were directly precipitated on Whatman 3MM filter discs and assayed by the method of Mans and Novelli (10). Tyrosine aminotransferase was assayed as described previously (11). Protein was measured by the method of Lowry et al. (12). Enzyme specific activity was expressed as milli-units per mg protein.

RESULTS AND DISCUSSION

Figure 1 demonstrates the difference in responsiveness to insulin between mitotic and interphase HTC cells. As reported previously (2,3,4), the TAT activity in induced mitotic HTC cells is significantly higher than that in induced unsynchronized cells. Mitotic cells previously induced with dexamethasone and incubated in the presence of colcemid and a maximally inducing concentration of insulin (4 μg/ml) exhibit virtually no increase in TAT activity above that seen in mitotic cells in the presence of colcemid alone (1-A). In contrast, unsynchronized cells (1-C) show an immediate response which is maximal about two hours after insulin addition, even when colcemid is present in the incubation medium (1-D). Interphase cells remaining after mitotic selection from colcemid-treated cultures (1-B) show a response similar to that of unsynchronized cells.

Mitotic cells collected in the absence of colcemid also show no response

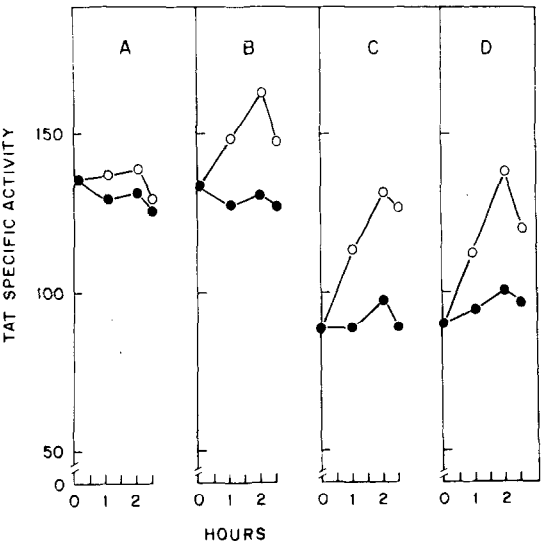

Figure 1: Insulin induction of TAT in mitotic and interphase cells. After exposure to colcemid for 12 hours as described in Methods, cells were collected and resuspended at 5 x 10^5 cells/ml in conditioned half-serum growth medium containing steroid and colcemid, and maintained as spinner cultures. A, mitotic cells; B, interphase cells remaining after mitotic selection. Parallel cultures of HTC cells were incubated under identical conditions except that they were not exposed to colcemid. C, unsynchronized cells never exposed to colcemid; D, unsynchronized cells with colcemid added at time of resuspension. Each cell population was divided and TAT activity measured on one ml aliquots of cells after 0, 1, 2 and 2 1/2 hours incubation with (○ - ○) or without (● - ●) 4 μg/ml insulin.

to insulin, and G1 cells to which colcemid has been added are fully responsive to insulin, further suggesting that the presence of colcemid is not responsible for lack of insulin induction in these mitotic cells ′8). Nor can the failure of response be explained by the high levels of TAT activity already present in steroid-induced mitotic cells, as insulin also failed to increase TAT activity in mitotic cells half-maximally induced ′80 milliunits/mg protein) with lower concentrations (0.01 μM) of dexamethasone ′8).

It has been proposed that the expression of tyrosine aminotransferase is regulated by a post-transcriptional repressor of the enzyme specific mRNA (1), which is produced only during the steroid inducible phases of the cell cycle (13). Inducing steroids are thought to antagonize the action of the putative repressor, stablizing the TAT mRNA and allowing it to accumulate. On the basis of studies with metabolic inhibitors, the insulin mediated induc-

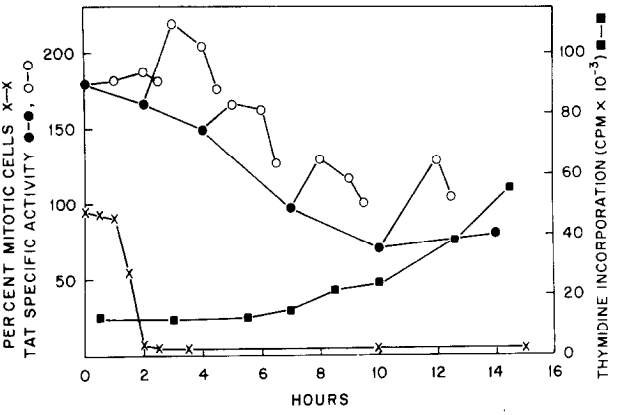

Figure 2: Insulin induction of TAT during the cell cycle. Mitotic cells were collected after 12 hours incubation with colcemid, resuspended at 5 x 10^5 cells/ml in conditioned half-serum growth medium containing steroid but no colcemid, and maintained as spinner cultures. Percent mitotic cells, X——X, and [^{3}H]-thymidine incorporation into DNA, ■ - ■, were monitored as markers of cell cycle phase as described in Methods. At 0, 2, 4, 7, 10 hours after collection of cells, TAT activity was measured and insulin (4 μg/ml) added to subcultures. TAT activity was measured 1, 2, 2 1/2 hours after insulin addition. ● - ●, dexamethasone only; ○ - ○ dexamethasone plus insulin.

tion of TAT synthesis, in contrast, appears to represent an increased efficiency of utilization of existing cytoplasmic mRNA for TAT (5,14,15).

The action of insulin in steroid induced HTC cells relative to the cell cycle and thus to the putative presence or absence of post-transcriptional repressor is shown in Figure 2. There is tight synchrony of cells passing through mitosis into G1 as measured by the decrease in percent mitotic cells (Fig. 2), and a sharp increase in RNA and protein synthesis that parallels this transition (8); DNA synthesis begins about 10 hours later. As previously reported (2-4), the high steroid induced activity of TAT in mitotic cells persists into the early part of G1, with a decline to a lower level in late G1 and S, similar to the level observed in unsynchronized populations. Mitotic cells are unresponsive to insulin induction; but sensitivity to insulin is acquired immediately upon passage into G1, and is maintained well into S phase. Thus, there is a period early in G1 in which TAT synthesis is sensitive to induction by insulin, but not by dexamethasone. Therefore, it appears that insulin induction of TAT is independent of the presence of the postulated

post-transcriptional repressor. This conclusion is also supported by the observation that insulin further increases TAT activity in cells already "superinduced" with high concentrations of actinomycin D, a condition in which repressor is thought to be absent (16).

Two basic mechanisms might be proposed for the lack of response to insulin seen in mitotic cells: (a) alteration in the protein synthetic machinery of mitotic cells, (b) alteration in the cell surface such that insulin receptors are not stimulated. Although total protein synthesis is greatly diminished in mitotic HTC cells, there is in fact an increase in the differential rate of TAT synthesis (3). Thus, TAT mRNA is translated in mitotic cells. Fan and Penman (17) have demonstrated that the reduced rate of total protein synthesis in mitotic cells is due to a block in the rate of initiation; elongation is not altered. Since insulin may increase the rate of protein synthesis by increasing the rate of initiation of mRNA for TAT (5) and total protein (18), this inhibition might negate the stimulation of TAT synthesis by insulin.

The cellular response to insulin appears to be mediated by specific receptors on the cell surface (19-21). Consequently, the magnitude of the response is a function both of the concentration of available receptors and of insulin. The receptor has been shown to be a glycoprotein which can be recognized selectively by wheat germ agglutinin (22,23). Since the glycoprotein composition of the cell membrane (24) as well as agglutinability by plant lectins (25,26) is dependent on the cell cycle phase, it is possible that availability of the insulin receptor is limiting in mitotic cells. Against this interpretation is the preliminary finding that the affinity of insulin binding to HTC cells is the same in mitotic and interphase cells (27).

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