GLUCOCORTICOID INHIBITION OF AMINO ACID TRANSPORT IN RAT HEPATOMA CELLS

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Summary: Dexamethasone rapidly and reversibly inhibits the initial rate of transport of α-aminoisobutyric acid in rat hepatoma cells in tissue culture. Colcemid and cytochalasin B neither inhibit transport nor interfere with its inhibition by dexamethasone, arguing that microtubules and microfilaments are not involved in this hormonal effect. Continuous protein synthesis is required both for the dexamethasone inhibition of transport and for its reversal, although cycloheximide alone inhibits transport in control cells by less than 25%. A model for the dexamethasone inhibition of amino acid transport is presented suggesting that glucocorticoids either block the synthesis or enhance the degradation of a rate-limiting protein in the transport system.

Hormones modulate amino acid transport in a variety of tissues (1). Suspension cultures of HTC[1] cells, an established line of rat hepatoma cells in tissue culture, actively transport the non-metabolized amino acid α-aminoisobutyric acid (AIB) by a saturable, energy-dependent process. Dexamethasone dramatically decreases the influx of AIB (2) as well as that of the natural amino acids glycine, serine, alanine, and proline, which appear to share with AIB the A or alanine-preferring transport system (3). In order to investigate the mechanism by which glucocorticoids inhibit amino acid transport, we have used specific inhibitors to study the roles of macromolecular synthesis, and of microtubules and microfilaments, in this transport regulation.

METHODS

HTC cells (4) were grown in spinner culture without antibiotics in modified Eagle's Minimal Essential Medium for suspension culture (5). Amino acid transport was assayed by measuring the initial rate of uptake of AIB. Logarithmically-growing cells were harvested by centrifugation and resuspended to 10^6 cells/ml in IM-BSA (the same medium as above, but lacking serum and supplemented with 0.1% bovine serum albumin and 50 mg/l of neomycin (5)), and incubated in a gyrotory shaker water bath at 37° for 6 to 18 hours prior to further treatment. Cell viability as determined by trypan blue exclusion was routinely 94 to 98% after 24 to 28 hours incubation in IM-BSA. The uptake of AIB was measured in suspen-

[1]The abbreviations used are: HTC, hepatoma tissue culture; and AIB, α-aminoisobutyric acid.

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sion culture as described by Heaton and Gelehrter (5). Time of incubation was 10 minutes, and the final concentration of AIB in the mixture was 0.528 mM including 0.25 µCi [^{14}C]-AIB (specific activity 12.24 Ci/mole). Protein in potassium hydroxide-hydrolyzed pellet samples was determined by the method of Lowry et al. (6) using bovine serum albumin as standard. Transport velocity is expressed as nmoles AIB taken up per min per mg protein. Each point represents the average of duplicate assays of a single culture.

The uptake of [^{14}C]-AIB by HTC cells is linear for at least 15 minutes in both control and dexamethasone-treated cells. Variation in the rate of transport between duplicate cultures incubated in parallel and assayed in duplicate was usually less than 5%. Trapped water in cell pellets was unaffected by hormonal treatment; volume was 0.96 ±0.04 µl/10^6 cells in the control, and 0.92 ± 0.04 µl/10^6 cells in dexamethasone-treated suspensions (mean ± S.E., n = 35). Trapped extracellular radioactivity accounted for approximately 10 to 15% of the total radioactivity in control cells and approximately 50% in dexamethasone-treated cells.

Dexamethasone and actinomycin D were gifts from Merck and Company; cycloheximide was purchased from Sigma; cytochalasin B was a product of Aldrich Chemical Company; and colcemid came from GIBCO. AIB, inulin-[^{3}H(G)] (specific activity 675 mCi/g), and Aquasol were purchased from New England Nuclear.

RESULTS AND DISCUSSION

Time course and reversibility of dexamethasone inhibition of AIB transport:

Dexamethasone rapidly decreases the influx of AIB. Following a lag of approximately 30 minutes, the initial rate of transport decreases exponentially (2,7). Inhibition is half-maximal after 1.9 ± 0.1 hrs, n = 14 (see also Figures 2 and 3). Although the absolute rates of AIB transport by both control and steroid-treated HTC cells vary from one experiment to another, the degree of inhibition of uptake by 0.1 µM dexamethasone is remarkably constant; after 6 hours incubation with dexamethasone, inhibition is 77.4 ± 2.0%, while after 18 hours exposure to steroid, inhibition is 91.2 ± 1.1% and varies from 84 to 97% (n = 14).

Removal of the glucocorticoid by washing and resuspension of the cells in hormone-free medium completely reverses the inhibition of AIB transport. Removal of dexamethasone after 18 hours incubation results in recovery of control rates of transport within approximately 20 hours. Recovery begins within one hour after steroid is removed, and is half-maximal after 5.0 ± 0.7 hrs, n = 5 (Figure 1) thus the reversal of transport inhibition is much slower than the hormonal inhibition itself. Restoration of control rates of AIB transport requires concomitant protein synthesis and is completely prevented by 0.1 mM cycloheximide (Figure 1), a concentration of inhibitor which blocks amino acid incorporation into protein by more than 98% (5).

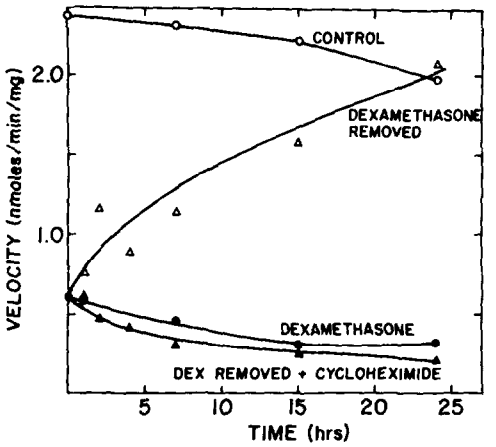

Figure 1. Reversibility of dexamethasone inhibition of AIB transport. Cells were incubated for 18 hours in IM-BSA with or without 0.1 μM dexamethasone (DEX). A portion of the dexamethasone-treated culture was centrifuged out of steroid-containing medium, washed once with IM-BSA and finally resuspended to the same volume in IM-BSA (Δ). Where cycloheximide was used, the cells were washed in IM-BSA containing 0.1 mM cycloheximide (▲). At the times indicated, AIB transport was measured as described in Methods. Because the manipulation of the cells required to wash out hormone can result in alterations of transport activity, each transport value on the "dexamethasone removed" curve was corrected for effects on transport of a "pseudo-wash", in which parallel dexamethasone-treated cultures were washed with, and resuspended in, fresh IM-BSA containing 0.1 μM dexamethasone.

Role of microtubules and microfilaments in the dexamethasone inhibition of AIB transport: We have studied the role of cytoskeletal elements in the hormonal inhibition of AIB transport using colcemid to inhibit microtubule polymerization, and cytochalasin B to cause disaggregation of microfilaments (8). Colcemid at 0.25 μM, a concentration sufficient to block HTC cells in metaphase (9), had only a modest inhibitory effect (14%) on the control rate of AIB transport and did not interfere at all with the inhibition of AIB transport by dexamethasone (data not shown), suggesting that microtubules do not play an important role in the hormonal inhibition of AIB transport.

Cytochalasin B has been reported to block several glucocorticoid-mediated effects in cultured cells (10). However, neither 21 μM, a concentration sufficient to enucleate HTC cells (11), nor 105 μM cytochalasin B inhibited AIB transport, nor did they prevent the inhibition of transport by dexamethasone, even though the higher concentration inhibited the glucocorticoid induction of tyrosine

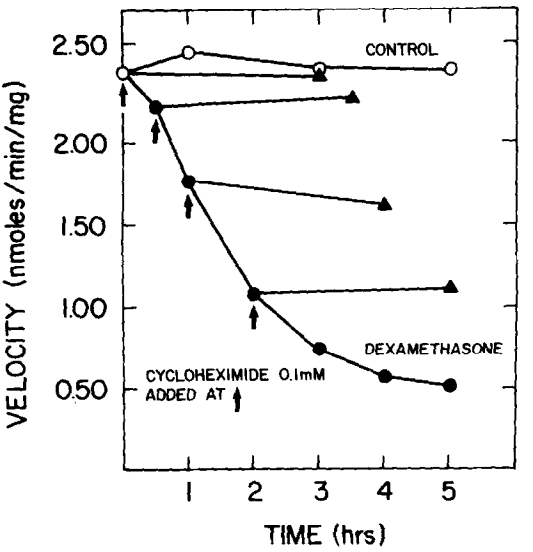

Figure 2. Requirement for continuous protein synthesis for the dexamethasone inhibition of AIB transport. Cycloheximide (0.1 mM) was added (arrows) 20 minutes before, or 30, 60, or 120 minutes after, addition of dexamethasone (0.1 μM). AIB uptake was measured three hours after each cycloheximide addition.

aminotransferase by about 50% (data not shown). These observations argue that microfilaments do not play an important role in the hormonal inhibition of AIB transport.

Role of macromolecular synthesis in the hormonal inhibition of AIB transport: Both protein and RNA synthesis are required for the dexamethasone induction of tyrosine aminotransferase (4) and of cellular adhesiveness (12) in HTC cells. Cycloheximide at 0.1 mM causes only a modest inhibition (<25%) of the uptake of AIB even after 18 to 24 hours incubation, but completely prevents the inhibition of transport by dexamethasone (Figure 2). In order to determine whether there might be an early step which is cycloheximide-sensitive (13), the experiment shown in Figure 2 was performed. Cycloheximide was added to HTC cells 20 minutes before, or 30, 60, or 120 minutes after, the addition of dexamethasone. Three hours after each addition, the initial rate of AIB uptake was measured. In each case the addition of cycloheximide completely prevented any further inhibition of AIB transport by dexamethasone, indicating a requirement for continuous protein synthesis.

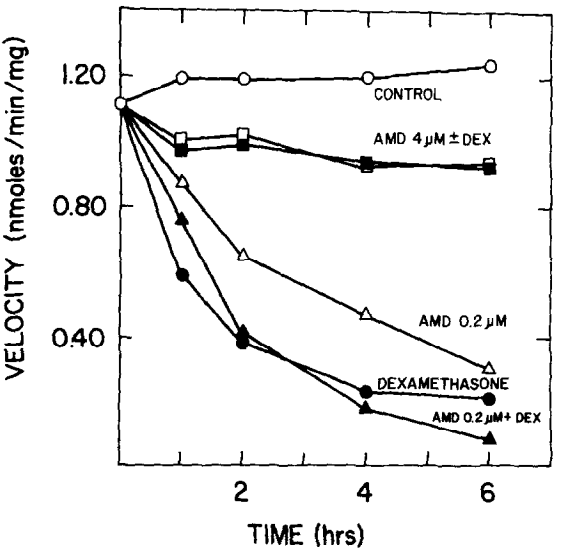

Figure 3. Effect of actinomycin D (AMD) on dexamethasone inhibition of AIB transport. Actinomycin D (0.2 or 4 μM) was added 20 minutes before dexamethasone (0.1 μM). AIB transport was measured at the times indicated.

In HTC cells, 0.25 μg/ml (0.2 μM) actinomycin D inhibits the incorporation of uridine into RNA by 90-95% and completely inhibits the induction of tyrosine aminotransferase by dexamethasone (14); at a concentration of 5 μg/ml (4 μM), it blocks uridine incorporation by more than 95% and causes "superinduction" of tyrosine aminotransferase (14,15). At a concentration of 4 μM, actinomycin D caused only a modest inhibition of AIB transport in control cells and completely prevented the dexamethasone inhibition of AIB transport (Figure 3), results similar to those obtained with cycloheximide. Curiously, the lower concentration of actinomycin D (0.2 μM) caused a striking inhibition of AIB transport in control cells (76% after 6 hours incubation) and did not apparently block the inhibitory effect of dexamethasone on AIB transport.

Interpretation of experiments utilizing inhibitors of macromolecular synthesis must be approached with caution because these inhibitors may have multiple effects on both synthesis and degradation. For example, in both HTC and H-35 rat hepatoma cells in tissue culture, cycloheximide and high concentrations of actinomycin D, but not low concentrations, slow the rate of degradation of both

total cellular protein (16,17) and of a specific protein, tyrosine aminotransferase (18,19), as well as blocking protein and RNA synthesis, respectively. We propose the following tentative model to explain our observations. We assume that the rate-limiting protein involved in AIB transport is rather labile, consistent with the observations of Heaton and Gelehrter on the reversible derepression of AIB transport in HTC cells during amino acid starvation (5). The degradation of this protein, however, appears to require concomitant protein synthesis; hence cycloheximide and high concentrations of actinomycin D have little effect on the rate of AIB transport because they block the turnover as well as the synthesis of this protein. We suggest that dexamethasone induces the synthesis of a labile protein which either decreases the rate of synthesis or increases the rate of degradation (or both) of the rate-limiting protein component of the AIB transport system. The addition of cycloheximide during the course of dexamethasone inhibition of transport would prevent both synthesis and degradation of these proteins, thus "freezing" the rate of AIB transport (Figure 2). It should be noted that an increase in the rate of degradation of the relevant transport protein would result in a more rapid change in transport rate than a comparable decrease in the rate of synthesis, since the time course of change from one steady state to another is a function only of the rate of degradation of the relevant protein (20). Because the inhibition of AIB transport by dexamethasone occurs two-and-one-half times more rapidly than its reversal after washing out steroid we favor a mechanism by which the hormone increases the degradation rate of the relevant protein.

Finally it should be noted that inhibition of protein and RNA synthesis, by cycloheximide and actinomycin D respectively, does not interfere with either the binding of glucocorticoid to the cytosol receptor of HTC cells nor the translocation of the steroid-receptor complex into the nucleus. Therefore, the inhibitory effects of cycloheximide and actinomycin D on hormonal inhibition of AIB transport cannot be explained by an interference with the uptake, binding, or translocation of steroid in these cells (21).

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