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PRIMARY INDUCTION OF METALLOTHIONEIN BY DEXAMETHASONE IN CULTURED RAT HEPATOCYTES

Michael Karin[a,b], Harvey R. Herschman[a,b,c], and David Weinstein[d]

Molecular Biology Institute[a], Dept. of Biological Chemistry[b], and Laboratory of Nuclear Medicine and Radiation Biology[c], School of Medicine, UCLA, Los Angeles CA 90024, and Division of Metabolic Disease[d], Dept. of Medicine; M-013D University of California, San Diego, La Jolla CA 92093

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SUMMARY

Dexamethasone or Zn^{++} increase the rate of synthesis of the metal-binding protein metallothionein in hepatocyte cultures. Dexamethasone induction of the capacity to synthesize metallothionein is not blocked by cycloheximide. In contrast, the dexamethasone stimulated increase in Zn^{++} uptake is inhibited by cycloheximide. Like Zn^{++}, dexamethasone is a "primary inducer" of metallothionein. The glucocorticoid induction of metallothionein in primary cultures of rat hepatocytes is not mediated through elevation of Zn^{++} uptake.

INTRODUCTION

Metallothionein's (MT's) are low molecular weight (6,000 dalton) proteins that bind the group IIa and Ia heavy metals (Zn^{++}, Cd^{++}, Hg^{++}, Cu^{+}, Ag^{+}) with high affinity. MT's are characterized by an absence of aromatic amino acids and a high (30%) cysteine content (1-3). Exposure to elevated Zn^{++} or Cd^{++} causes an increase in hepatic and kidney MT (4-8).

Hepatic MT synthesis also occurs in response to bacterial infection (9) and physiological stress (10). Stress also elevates the level of glucocorticoid hormones (11). We have demonstrated the induction of MT by both dexamethasone (dex), a synthetic glucocorticoid, in HeLa cells grown in serum-free medium (12) and by zinc (13). Etzel et al. reported that dexamethasone induces hepatic metallothionein synthesis in adrenalectomized rats (14).

We now report that dex increases the rate of MT synthesis in primary cultures of rat hepatocytes. Cycloheximide inhibition of protein synthesis during induction with either dex or zinc does not inhibit the development of the capacity to synthesize MT. In contrast, the increase in $^{65}Zn^{++}$ uptake

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in response to dex treatment is dependent on protein synthesis. Since dex induction of the capacity to synthesize MT -- presumably MT mRNA -- can occur when protein synthesis is inhibited, and consequently when no increase in Zn^{++} accumulation occurs, we conclude that dex is a "primary inducer" (15) of MT synthesis in cultured hepatocytes.

MATERIALS AND METHODS

Preparation of Hepatocytes: Hepatocytes were prepared and cultured in arginine free medium containing fetal calf serum as described by Davis et al. (16). After 18 hr, the medium was changed to arginine-free DME medium containing antibiotics, insulin and HEPES, but no serum. The cells were incubated an additional 48 hr, after which the experiments described were performed. Cells were harvested by scraping with a rubber policeman into 0.3 ml of 20 mM $(NH_4)_2CO_3$ buffer, frozen and thawed twice and disrupted by sonication with two 15 sec bursts in E/MC type USD-25 sonicator with power setting of 6. Protein was determined by a dye binding method (17).

Reduction, Alkylation, and Analysis of Cellular Proteins: To 200 µl of the cell extract were added 20 µl of 1M HCl and 20 µl of 0.25M EDTA. After 20 minutes at room temperature, 300 µl of 8M guanidine, 0.5M Tris-HCl 0.05M EDTA pH 8.5 containing 20 mM DTT were added. After 3 hours incubation at 37°C, 50 µl of 2M neutralized iodoacetic acid were added and the mixture was incubated for another 90 minutes at 37°C. The reaction was terminated by dialyzing against H_2O for 24 hours. All the samples were subjected to electrophoresis on 20% polyacrylamide, 0.1% SDS slab gels. Electrophoresis and fluorography have been described previously (18).

Quantitation by Densitometry of MT Synthesis: Fluorograms in the linear response range were scanned with an Optronics Photoscan model P-1000 digitalized densitometer, using a photometer window with a 200 µm raster. The density in the MT band was summed and normalized to the amount of radioactivity applied to each slot.

RESULTS

Induction of Capacity to Synthesize MT Is Dependent on RNA Synthesis but Not Protein Synthesis: To demonstrate dex and Zn^{++} induction of MT synthesis in cultured hepatocytes, cells were incubated with serum-free medium for six hours in the presence of either 10^{-7}M dex or $4x10^{-5}$M Zn^{++}. The plates were washed with cysteine-free DME, then exposed for 1 hr to cysteine-free medium containing 50 µCi/ml ^{35}S-cystine. At the end of the one hour labeling period the cells were scraped into 0.3 ml of 20 mM $(NH_4)_2CO_3$ pH 8.5 buffer. Cell extracts were prepared and a 0.2 ml portion was subjected to reduction and alkylation. Samples containing approximately 14,000 cpm were applied to 20% polyacrylamide, 0.1% SDS slab gels (18) and subjected to electro-

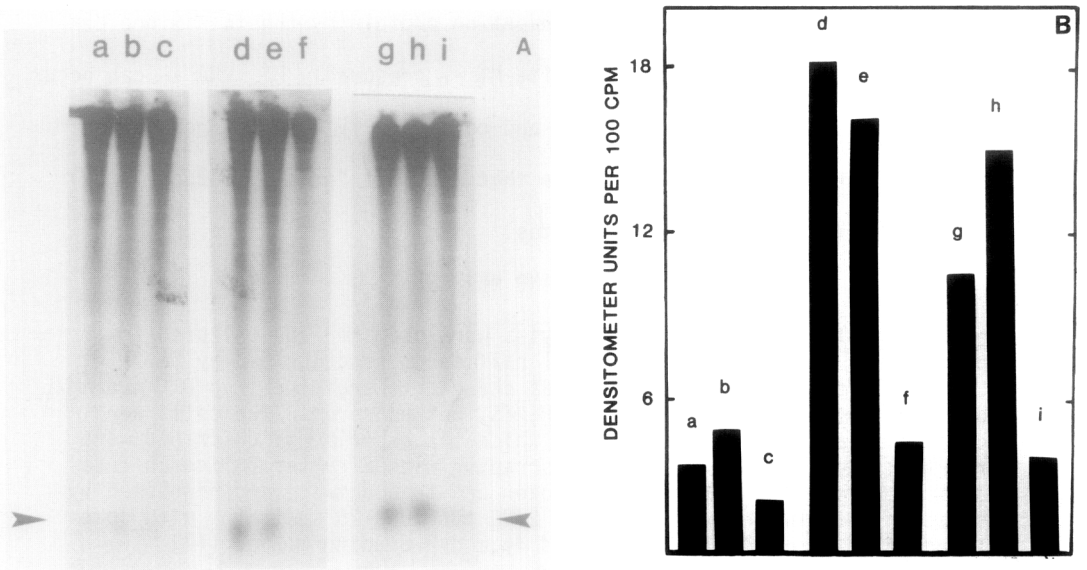

Figure 1. Dexamethasone and Zn^{++} Induction of the Capacity to Synthesize Metallothionein in Primary Hepatocyte Cultures. Panel A. Fluorograms of Polyacrylamide Gels. Hepatocytes were first incubated for six hours with (a) control medium, (b) $2x10^{-5}$M cycloheximide, (c) 1 µg/ml actinomycin D, (d) 10^{-7}M dex, (e) 10^{-7}M dex + $2x10^{-5}$M cycloheximide, (f) 10^{-7}M dex + 1 µg/ml actinomycin D, (g) $4x10^{-5}$M Zn^{++}, (h) $4x10^{-5}$M Zn^{++} + $2x10^{-5}$M cycloheximide, (i) $4x10^{-5}$M Zn^{++} + 1 µg/ml actinomycin D. The cells were then washed once with cysteine free DME in order to remove the drugs and then labeled from the sixth to the seventh hour with 50 µCi/ml of ^{35}S-cystine (NEN, 300-500 Ci/mMole) in cystine free medium. Radioactive labeling was carried out in cystine free medium containing the appropriate inducers. At the end of the 1 hour pulse, the cells were harvested, and the soluble proteins extracted as described before (16). Reduction, alkylation, and electrophoresis are described in Methods. Panel B. Quantitation of MT Synthesis by Densitometry.

phoresis. Both dex (lane d) and Zn^{++} (lane g) markedly induce MT above control values (lane a) in cultured hepatocytes (Fig. 1A).

To determine whether induction of the MT system by dex and Zn^{++} requires synthesis of RNA or protein intermediates, primary hepatocytes were exposed to 10^{-7}M dex or $4x10^{-5}$M Zn^{++} for 6 hours in the presence of either actinomycin D (1 µg/ml) or cycloheximide ($2x10^{-5}$M). At the end of the six hour period the drugs were washed from the plates and the cells were labeled for one hour with ^{35}S-cystine in cysteine-free medium. Synthesis of MT was analyzed by electrophoresis and fluorography. Actinomycin D caused a slight decrease in the basal level of subsequent MT synthesis (lane c) and blocked both the dex induced (lane f) and Zn^{++} induced (lane i) increase in the

Table 1. Induction by Dexamethasone of $^{65}Zn^{++}$ Uptake in Primary Hepatocyte Cultures

Condition of Incubation	A: Accumulation of $^{65}Zn^{++}$ during a six hour induction cpm/mg (% of control)	B: $^{65}Zn^{++}$ uptake during a one hour pulse after 6 hr induction cpm/mg (% of control)
Control	19,648 ± 1508 (100)	33,477 ± 2042 (100)
Control + Cycloheximide	20,066 ± 1469 (102)	32,088 ± 3379 (95)
Dexamethasone	29,325 ± 1090 (149)	48,159 ± 4966 (144)
Dexamethasone + Cycloheximide	17,740 ± 649 (97)	30,065 ± 2224 (96)

Column A: Cells were incubated either in the presence or absence of 10^{-7}M dex, with 0.5 μCi/ml of $^{65}Zn^{++}$ (final Zn^{++} concentration, 2 μM) for six hours. In each case half the cultures were incubated in the presence of $2x10^{-5}$M cycloheximide. Plates were washed twice with 2.5 ml of phosphate buffered saline (20 mM potassium phosphate, pH 7.4, .15M NaCl) and scraped with a rubber policeman into 0.5 ml of 20 mM $(NH_4)_2CO_3$ buffer, pH 8.5. Cells were frozen and thawed twice, then sonicated, and radioactivity was measured. Column B: Cells were exposed either to control medium or medium containing 10^{-7}M dex for 6 hours. In either case half the cultures contained $2x10^{-5}$M cycloheximide. At the end of six hours the media were replaced with fresh media containing 2 μCi/ml of $^{65}Zn^{++}$, and the same additions as before. Final Zn^{++} concentration was 8 μM. After one hour of $^{65}Zn^{++}$ exposure cells were harvested and radioactivity was determined. Values are the average of four plates ± standard deviations.

capacity of the cultured hepatocytes to synthesize MT. In contrast, cycloheximide inhibition of protein synthesis did not block the induction of MT-synthesizing capacity resulting from either dex exposure (lane e) or Zn^{++} exposure (lane h). The concentration of cycloheximide used ($2x10^{-5}M$) blocked 90% of ^{3}H-leucine incorporation during the six hour exposure period.

To quantitate the amounts of MT synthesized under these various conditions, a fluorogram prepared from the experiment described in Figure 1A was scanned with a densitometer. The optical density (a direct function of radioactivity, data not shown) in the MT bands from two duplicate experiments, done on separate cultures, was summed and normalized to the amount of total radioactivity applied to the gel (Figure 1B). Dex ($10^{-7}M$) increases the relative rate of MT synthesis 5.6 fold above control; Zn^{++} ($4x10^{-5}M$) increases the relative rate of MT synthesis 3.2 fold above control. The induction of MT is inhibited by 1 μg/ml of actinomycin D but not $2x10^{-5}M$ cycloheximide.

Increased Zn^{++} Uptake Is Dependent on Protein Synthesis: Dexamethasone increases the accumulation of Zn^{++} in HeLa cells (19,20) and in cultured primary hepatocytes (21,22). We have confirmed the dex induction of $^{65}Zn^{++}$ uptake by cultures of primary hepatocytes, both during a continuous six hour incubation in the presence of dex, and during a one hour exposure to $^{65}Zn^{++}$ following a six hour incubation (Table 1). Dex exposure caused a 40-50% elevation in $^{65}Zn^{++}$ uptake, both in the continuous exposure and pulse paradigms for $^{65}Zn^{++}$. However, both the increase in the amount of $^{65}Zn^{++}$ accumulated during the 6 hour period and the increase in $^{65}Zn^{++}$ taken up during the one hour exposure are inhibited by $2x10^{-5}M$ cycloheximide (Table 1).

Intracellular Location of Dex Stimulated Zn^{++}: To determine the intracellular location of the $^{65}Zn^{++}$ taken up after six hours exposure to dex, cells were exposed to inducer in the presence of 0.5 μCi/ml $^{65}Zn^{++}$ and lysed. The supernatants were analyzed by chromatography on Sephadex G-75 columns (Fig. 2). Three $^{65}Zn^{++}$ containing peaks are observed. The first peak (fractions 15-22) is $^{65}Zn^{++}$ bound to high molecular weight proteins,

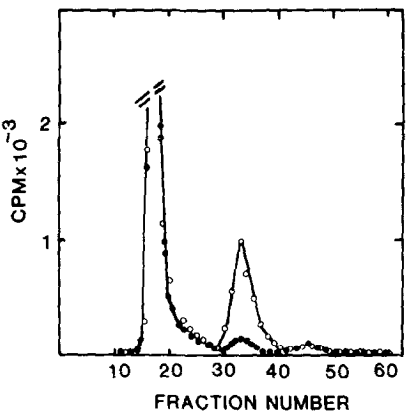

Figure 2. Gel Filtration Chromatography of Cytosol Fractions from Primary Hepatocytes Exposed to $^{65}Zn^{++}$ in Either Control Medium or Medium Containing 10^{-7}M Dex. Cells were incubated for six hours with 0.5 µCi/ml $^{65}Zn^{++}$. Plates were washed and cells were harvested and broken as described in Table 1. The broken cell suspensions were centrifuged for 5 min. in a Beckman microfuge B. The soluble fraction was analyzed by chromatography on G-75 Sephadex column (1.1x57 cm) equilibrated and eluted with 20 mM $(NH_4)_2CO_3$ buffer. One ml fractions were collected and analyzed for radioactivity. Data are expressed as cpm/mg protein applied to the column.

the second (fractions 30-40) is $^{65}Zn^{++}$ bound to MT and the third small peak is either free $^{65}Zn^{++}$ or $^{65}Zn^{++}$ bound to low molecular weight compounds. The only difference between control and dex treated cells is in the amount of $^{65}Zn^{++}$ bound to MT. Dexamethasone treated cells have more $^{65}Zn^{++}$ bound to MT than control cells.

DISCUSSION

The induction of the capacity to synthesize MT in response to dex in the presence of cycloheximide suggests that dex, like Zn^{++}, is a "primary inducer"(15) of MT in cultured hepatocytes. However, Failla and Cousins, in their study of dexamethasone-treated hepatocytes suggest that "... the zinc content of cells must be sufficiently elevated before metallothionein will be synthesized" (22). We have confirmed the report of Failla and Cousins (22) that dexamethasone is able to stimulate Zn^{++} uptake by cultures of primary hepatocytes (Table 1). Our data, like those of Failla and Cousins are similar to the observations made by Cox (23), who showed a number of years ago that dex induction of Zn^{++} uptake occurred in HeLa cells. Cox

also showed that the dex stimulation of Zn^{++} uptake could be blocked by cycloheximide. We also find that, in primary hepatocyte cultures, inhibition of protein synthesis blocks the dex induction of Zn^{++} uptake (Table 1). In contrast, the dex induced capacity to stimulate MT synthesis is independent of protein synthesis (Figure 1). Thus, dex induction of the capacity to synthesize MT occurs in the absence of elevated Zn^{++} transport.

Our data suggest that (i) dex directly induces the synthesis of MT-mRNA in hepatocyte cultures in a "primary induction" response (15) independent of increased Zn^{++} transport, (ii) the increased levels of MT-mRNA lead to an elevation in MT synthesis, (iii) the increased synthesis of an intracellular Zn^{++} binding protein (metallothionein) leads to increased Zn^{++} uptake by the hepatocytes, and (iv) the increased Zn^{++} is bound in the cell by MT.

The hepatic induction of MT synthesis by stress has been attributed to Zn^{++} mobilization and a proposed transient elevation of "free" Zn^{++}, leading to MT induction (10). Circulating Zn^{++} levels drop in stressed animals (9,10), presumably as a consequence of Zn^{++} sequestration by the induced MT. Our observation that glucocorticoid hormones are primary inducers of MT synthesis suggests an alternative explanation: stress may induce elevated glucocorticoid levels, leading to hepatic MT synthesis and consequent plasma Zn^{++} depletion. We suggest steroid hormone modulation of MT synthesis may be a vital controlling element in Zn^{++} homeostasis.

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