Augmentation of dexamethasone induction of rat liver metallothionein by zinc

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The induction of liver metallothionein by dexamethasone in adrenalectomized rats was augmented by zinc administration. Metallothionein synthesis was increased in an additive manner with both zinc and dexamethasone compared with either treatment alone. Translational activity of polyribosomal metallothionein mRNA was also greater in zinc+dexamethasone-treated rats. Northern-blot analyses showed that dexamethasone increased these mRNA contents to a greater extent at the lower zinc dose, suggesting that the induction may be maximal at the higher zinc dose when combined with dexamethasone.

Synthesis of metallothionein has been shown to increase dramatically in response to zinc (Bremner & Davies, 1975; Richards & Cousins, 1975; Squibb *et al.*, 1977). In addition, food restriction (Bremner & Davies, 1975; Richards & Cousins, 1976), stress (Oh *et al.*, 1978), acute infection (Soboscinski *et al.*, 1978) and endotoxaemia (Etzel *et al.*, 1982) all appear to influence hepatic metallothionein. Glucocorticoids also influence hepatic metallothionein (Failla & Cousins, 1978; Etzel *et al.*, 1979; Etzel & Cousins, 1981; Hager & Palmiter, 1981). The sensitivity of metallothionein to inducers has led to a surge in interest in the control mechanisms of metallothionein synthesis, particularly since amounts of metallothionein mRNA are altered by zinc and cadmium as well as by glucocorticoids (Shapiro *et al.*, 1978; Anderson & Weser, 1978; Etzel *et al.*, 1979). Advances in recombinant DNA technology have led to the isolation and cloning of the mouse metallothionein-I gene (Durnam *et al.*, 1980). Heavy metals and glucocorticoids have been shown to increase transcription of this gene *in vivo* (Hager & Palmiter, 1981; Durnam & Palmiter, 1981). However, once the gene with flanking promoter regions was isolated and induction was attempted *in vitro*,

glucocorticoid regulation was lost in one system (Mayo *et al.*, 1982).

We are interested in the extent to which particular inducers affect the synthesis of metallothionein. Examining the complex picture of metallothionein regulation may assist in understanding the nutritional and hormonal factors involved in this segment of the metabolism of zinc. In this paper, we present evidence to show that zinc and glucocorticoids act alone and in combination to effect changes in polyribosomal metallothionein-mRNA contents and metallothionein synthesis in rat liver.

Experimental

Materials

All chemicals used were reagent grade. Glass-distilled and deionized water was used throughout. [³H]Glycine (11 Ci/mmol), [³H]lysine (40 Ci/mmol), [³H]serine (20 Ci/mmol), [³⁵S]cystine (400 Ci/mmol) and [α -³²P]dCTP (800 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Oligo(dT)-cellulose was from Collaborative Research (Waltham, MA, U.S.A.). Ficoll, activated-thiol-Sepharose 4B and Sephadex G-25 and G-75 were from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Wheat germ was provided gratis by General Mills (Vallejo, CA, U.S.A.). Nitrocellulose filters were purchased from Schleicher and Schuell (Keene, NH, U.S.A.). Dexamethasone was a gift from Merck, Sharp and Dohme Laboratories (West Point, PA, U.S.A.). Deoxyribonuclease I and

Abbreviations used: poly(A)⁺ mRNA, polyadenylated mRNA; SDS, sodium dodecyl sulphate; SSC, standard saline citrate (1 × SSC is 0.15 M-NaCl/0.015 M-trisodium citrate); cDNA, complementary DNA.

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DNA polymerase I were from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.).

Animals and treatments

Male CD-strain rats (Charles River Breeding Laboratory, Wilmington, MA, U.S.A.), weighing 150–250 g, were maintained in stainless-steel cages and fed on a commercial diet (50 p.p.m. of Zn) and tap water *ad libitum*. Adrenalectomy was performed under sodium pentobarbitol anaesthesia (60 mg/kg; intraperitoneally) at least 6 days before experimentation. After surgery, 0.9% NaCl was substituted for tap water. Rats were treated with dexamethasone (2 mg/kg) or Zn^{2+} (as ZnSO_4 , 0.5 mg or 2.0 mg) as intraperitoneal injections. Blood was collected at the time of killing, and the zinc content of diluted serum was measured by atomic absorption spectrophotometry (Etzel *et al.*, 1979).

Metallothionein synthesis

At 1 h before being killed, animals were injected (intraperitoneally) with 5 μCi of [^{35}S]cystine. The livers were excised and the relative rate of metallothionein synthesis was measured as described previously (McCormick *et al.*, 1981).

mRNA isolation and translation in vitro

Liver polyribosomes were isolated by Mg^{2+} precipitation (Palmiter, 1974) and the RNA was extracted by the SDS/phenol/chloroform extraction method. Poly(A) $^{+}$ mRNA was purified by methods that have been described in detail (Shapiro *et al.*, 1978). Purified poly(A) $^{+}$ mRNA was translated in a cell-free wheat-germ system containing 5 μCi of a mixture of [^3H]serine, [^3H]glycine and [^3H]lysine (Shapiro *et al.*, 1978; Shapiro & Cousins, 1980). After incubation at 25°C for 60 min, a sample was removed for determination of trichloroacetic acid-precipitable protein. ^3H -labelled metallothionein was purified from the remaining translation mixture by covalent chromatography on activated-thiol-Sepharose 4B as described by McCormick *et al.* (1981).

Preparation of cDNA probe and Northern-blot hybridization

A cloned mouse metallothionein-I cDNA was obtained from Dr. R. D. Palmiter. The cDNA clone (m₁PEH.4) contains a 380-base-pair insert at *EcoRI*/*HindIII* sites of plasmid pBR322 (Durnam *et al.*, 1980). This cDNA probe is capable of recognizing rat metallothionein coding sequences, based on hybrid-select translation. By a modification of the procedure of Ricciardi *et al.* (1979), rat poly(A) $^{+}$ RNA was hybridized to immobilized mouse metallothionein-I cDNA. The hybrid-selected mRNA was translated in a rabbit reticulo-

cyte system, and metallothionein was the only product visible by SDS/polyacrylamide-gel electrophoresis of carboxymethylated translation products. Transformation of *Escherichia coli* RR1 was performed at a plasmid-pBR322 concentration of 2 $\mu\text{g}/\text{ml}$ in 50 mM- CaCl_2 . Colonies that grew on ampicillin-containing plates were grown in large cultures containing ampicillin. After amplification with either chloramphenicol (170 $\mu\text{g}/\text{ml}$) or spectinomycin (300 $\mu\text{g}/\text{ml}$), the cells were lysed in 0.1 M-NaOH and the plasmids collected by the procedure detailed by Ish-Horowitz & Burke (1981). The purity of the plasmid preparation was confirmed by restriction-endonuclease digestion before use in hybridization studies. The cDNA was nick-translated by using [α - ^{32}P]dCTP, deoxyribonuclease and DNA polymerase I basically as described by Rigby *et al.* (1977).

Northern blots were made by the procedure of Thomas (1980). Between 10 and 20 μg of mRNA was glyoxylated in 1 M-glyoxal/50% (v/v) dimethyl sulphoxide/10 mM-sodium phosphate (pH 7.0) for 60 min at 50°C (McMaster & Carmichael, 1977) and then electrophoresed through a 1.1%-agarose gel with constant buffer recirculation. The RNA was transferred to nitrocellulose filters overnight in 20 $\times$ SSC. The filter was heated at 80°C for 2 h in a vacuum oven, placed in boiled water until it reached room temperature (approx. 2 h), and prehybridized for 4–24 h in buffer consisting of 50% formamide, 5 $\times$ SSC, 40 mM-sodium phosphate, pH 6.7, 0.05% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll and 250 μg of calf thymus DNA/ml. The ^{32}P -labelled nick-translated DNA probe (sp. radioactivity 0.5×10^8 – 2.0×10^8 c.p.m./ μg) was added after the prehybridization period and allowed to hybridize for 24–48 h. The filter was then washed four times in 2 $\times$ SSC/0.1% SDS at room temperature, followed by two washes at 50°C in 0.1 $\times$ SSC/0.1% SDS, and exposed to X-ray film (Kodak X-Omat AR) by using an intensifying screen at –70°C. After autoradiography, hybridization intensity (band intensity) was measured with a LKB soft-laser densitometer. The hybridizable polyribosomal poly(A) $^{+}$ mRNA migrated in the 9S region, as determined by comparison with ethidium bromide-stained rRNA standards.

Results

Adrenalectomized rats were used to obtain a maximal response to dexamethasone without interference from endogenous glucocorticoids. The animals were killed 6 h after administration of zinc and dexamethasone. Zinc injections resulted in dose-related elevation of serum zinc content over control values at both concentrations tested (Table

Table 1. *Effect of dexamethasone and zinc on serum zinc concentration, relative rate of liver metallothionein synthesis and relative metallothionein mRNA content, based on translational assay*

Adrenalectomized rats were injected intraperitoneally with dexamethasone (2mg/kg) and/or Zn^{2+} (as $ZnSO_4$ at 0.5mg or 2.0mg) 6h before being killed. Metallothionein synthesis *in vivo* was measured by 1h pulse labelling with [^{35}S]cystine and gel-filtration chromatography. Polyribosomal metallothionein-mRNA activity was measured by translation in a wheat-germ system and covalent chromatography with activated-thiol-Sephadex. Means $\pm$ S.E.M. are shown. The ' ^{35}S incorporation' is that from [^{35}S]cystine into metallothionein as a percentage of that into high- M_r proteins. Values in parentheses are incorporation relative to control. The 'Relative translational activity' is the incorporation of [3H]-glycine, -lysine and -serine into metallothionein by wheat-germ translation system relative to control.

Treatment	Serum zinc ($\mu g/ml$ of serum)	^{35}S incorporation	Relative translational activity of metallothionein mRNA
Control	1.4 $\pm$ 0.1	9 $\pm$ 2 (1)	1.0
Dexamethasone	1.3 $\pm$ 0.1	23 $\pm$ 2 (2.6)	1.7 $\pm$ 0.2
Zinc (0.5mg)	2.3 $\pm$ 0.1	52 $\pm$ 3 (5.8)	2.1 $\pm$ 0.1
Zinc (0.5mg) + dexamethasone	2.2 $\pm$ 0.2	57 $\pm$ 3 (6.3)	3.5 $\pm$ 0.5
Zinc (2.0mg)	10.1 $\pm$ 0.8	75 $\pm$ 1 (8.3)	2.7 $\pm$ 0.1
Zinc (2.0mg) + dexamethasone	5.2 $\pm$ 1.0	84 $\pm$ 4 (9.3)	4.3 $\pm$ 0.5

1). A simultaneous administration of dexamethasone decreased the zinc content of the serum at the higher zinc dosage. Measurement of liver metallothionein synthesis by pulse labelling with [^{35}S]cystine indicated that zinc and dexamethasone have an additive effect in the induction of synthesis relative to other newly made proteins. Specifically, zinc administration stimulated synthesis of ^{35}S -labelled metallothionein to a greater extent than did dexamethasone (Table 1). However, dexamethasone had only a relatively small effect on metallothionein synthesis when the hormone was administered with either zinc dose. Nevertheless, metallothionein synthesis was increased 9.3-fold over control values by the combined treatment.

Translational activity *in vitro* of metallothionein mRNA from zinc + dexamethasone-treated adrenalectomized rats is shown in Table 1. Dexamethasone and the 0.5mg zinc dose yielded a comparable increase (about 3.5-fold). The combined administration of the hormone and zinc at this lower dosage resulted in greater metallothionein-mRNA translational activity than was observed in rats given the higher (2mg) dose of zinc. The combined effect of dexamethasone and the higher zinc dose was nearly additive (4.3 times greater than the control). The metallothionein-mRNA content, as measured by Northern-blot hybridization to a cDNA containing metallothionein-coding sequences, is shown in Fig. 1. Dexamethasone administration to adrenalectomized rats actually produced a slight relative decrease (30%) in metallothionein mRNA, whereas zinc treatment yielded a dose-related increase (2.8–4.4-fold) in the abundance of this mRNA in the polyribosomal mRNA pool. Simultaneous treatment with dexa-

methasone and at the lower zinc dose yielded an increase of 4.8 times that with the zinc dose alone. Dexamethasone also increased metallothionein mRNA (5.1-fold) at the higher zinc dose, but, since this dose gives a higher mRNA content, the effect of the hormone is less on a relative basis.

Discussion

The results presented in this paper give us some further indication of the intricacies of the induction of metallothionein synthesis. Particularly interesting is the differential effect of dexamethasone in adrenalectomized and non-adrenalectomized animals. In these experiments, we measured the relative amounts of polyribosomal metallothionein mRNA as compared with total polyribosomal poly(A) $^+$ mRNA. It is widely accepted that dexamethasone causes increases in metallothionein-gene transcription (Hager & Palmiter, 1981; Mayo & Palmiter, 1981). Those experiments were performed with either non-adrenalectomized mice or isolated cell lines. In adrenalectomized rats, we found that dexamethasone alone did not enhance the proportion of polyribosomal metallothionein mRNA over controls (Fig. 1). However, non-adrenalectomized rats produced an increased amount of metallothionein mRNA in response to dexamethasone (results not shown). Therefore, we conclude that adrenalectomy, by eliminating endogenous adrenal corticosteroids, alters the induction process.

Other evidence suggests that adrenalectomy affects the quality and quantity of rat liver polyribosomal poly(A) $^+$ mRNA (Bajwa *et al.*, 1982). Approx. 7000 out of 27000 diverse sequences of an average length of 2000 nucleotides

Fig. 1. Northern-blot analysis performed with polyribosomal poly(A)⁺ RNA prepared from rats treated as described in the Experimental section and Table 1

The RNA was size-fractionated in a 1.1%-agarose gel (anode at bottom) and transferred to a nitrocellulose filter. After hybridization to a ³²P-labelled nick-translated cDNA to mouse metallothionein-I, the filter was washed and exposed to X-ray film. The film was evaluated by densitometry and the relative intensities of the detected bands were compared. The control band-intensity value was assigned a value of 1.0 (lane at left). The treatments (from the left) were control, dexamethasone, zinc (2mg), zinc (0.5mg), dexamethasone + zinc (2mg) and dexamethasone + zinc (0.5mg), with relative band intensities of 1.0, 0.7, 4.4, 2.8, 5.1 and 4.8 respectively. Migration of ethidium bromide-stained yeast rRNA standards is shown at the left.

are no longer present in polyribosomes after adrenalectomy. A small proportion of the remaining sequences either increase or decrease in abundance. In other words, adrenalectomy causes changes in the abundances of mRNA sequences. Furthermore, dexamethasone administration returns the mRNA profile to normal values within 6h. These findings suggest a redistribution of mRNA species after adrenalectomy, with the metallothionein sequence becoming relatively more abundant. This is consistent with comparisons of metallothionein-mRNA contents of normal rats with those from adrenalectomized rats, i.e.

untreated normal rats have less polyribosomal metallothionein mRNA than do untreated adrenalectomized rats. Therefore adrenal corticosteroids appear important for the steady-state amounts of a large number of polyribosomal mRNA species. The fact that metallothionein mRNA is not lost after adrenalectomy indicates that glucocorticoids are not responsible for maintaining basal amounts of this mRNA.

Dexamethasone administration to adrenalectomized rats led to a decrease in the relative abundance of metallothionein mRNA. However, this does not suggest that the amount of this mRNA has also decreased, but rather that there was a return to normal amounts. Whether there is an actual increase in the absolute amount of cellular metallothionein mRNA in response to dexamethasone is not measurable within the boundaries of this experimental design. However, data from the [³⁵S]cysteine-pulse-labelling experiments and translational activity of isolated mRNA indicate that there is more metallothionein synthesis in response to dexamethasone. This is in agreement with our previous findings (Etzel *et al.*, 1979). The apparent disparity between metallothionein synthesis and polyribosomal mRNA amounts in adrenalectomized animals and in adrenalectomized animals treated with dexamethasone may indicate some translational control. The presence of glucocorticoids may alleviate translational inhibition. Because glucocorticoids affect the amounts of many polyribosomal mRNA species, it is not unlikely that they influence many cellular processes. Moreover, zinc and dexamethasone administration appears to elevate synergistically mRNA contents in adrenalectomized rats. This may be expected, since the metallothionein promoter is responsive to both stimuli (Pavlakakis & Hamer, 1983).

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