Synergistic and additive induction of metallothionein in Chang liver cells

A possible mechanism of marked induction of metallothionein by stress

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We have studied metallothionein (MT) induction by three primary inducers; a heavy metal, a glucocorticoid and a cytokine, and by the combinations of these inducers in the cultured cells. MT-protein was synergistically induced by either a cytokine or a heavy metal with a glucocorticoid hormone and was additively induced by the combination of a cytokine and a heavy metal, but MT-mRNA levels were not completely correlated with MT-protein levels. These results suggest that posttranscriptional regulation may be involved in the synergistic induction of MT-protein. We propose a possible mechanism in which marked MT induction by stress in vivo is dependent on the combined effect of two or more inducers, because marked MT induction is not seen by an injection of a plausible dose of either a glucocorticoid hormone or a cytokine in vivo.

Synergistic induction; Additive induction; Metallothionein

1. INTRODUCTION

Metallothionein (MT) is a low molecular weight, heavy metal binding protein, which is rich in cysteine residue and thus able to bind heavy metals including zinc, copper and mercury. The physiological functions of the protein have been reported to be detoxification of heavy metals, metabolism of trace metals, scavenging of free radicals, etc. (see [1] for review).

MT is induced by heavy metals, glucocorticoid hormones [2], 12-*O*-tetradecanoylphorbol-13-acetate [3], cytokines such as interferon [4], interleukin 1 [5], epidermal growth factor (EGF) and platelet-derived growth factor [6], ultraviolet light [7] and 1α,25-dihydroxyvitamin D_3 [8] in cultured cells. In contrast, this protein is also induced in vivo by stresses including cold, heat, exercise and burn [9], mainly in the liver. MT is also induced in vivo by administrations of bacterial endotoxin [10], turpentine [11], alcohol [12], carbon tetrachloride [9] and by X-ray irradiation [13], which are thought to be stresses. However, the mechanism(s) of MT induction by these stresses are not well known. Stress increases the circulating levels of glucocorticoid hormones [14], which are primary inducers of MT, but there is no direct evidence that increased glucocorticoid hormones are mediators of MT induction in vivo [15]. We have shown that transforming growth factor-α (TGF-α) secreted by endotoxin-stimulated macrophages is involved in MT induction by endotoxin [16]. However, we have no evidence that TGF-α is the only inducer of MT by endotoxin in vivo.

Under these circumstances, we have hypothesized that MT induction by stress in vivo is dependent on two or more inducers and, therefore, we studied the combined effects of the above primary inducers on MT induction in cultured cells. In this article, we report that MT-protein is inducible synergistically or additively, depending on the combinations of inducers and that posttranscriptional regulation may be involved in the synergistic induction of MT-protein.

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Abbreviations: MT, metallothionein; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; Dex, dexamethasone

2. MATERIALS AND METHODS

2.1. *Materials*

Mouse submaxillary gland EGF (purity: >95%) was purchased from Takara Biochemicals (Kyoto, Japan). Dexamethasone (Dex; Nakarai Chemicals, Kyoto, Japan) was dissolved in 99.5% ethanol and added to cells in at least a 100 000-fold dilution with medium. Zinc chloride (Nakarai Chemicals) was dissolved at a concentration of 10 mM in 5 mM HCl. [^{35}S]cysteine and [^{35}S]dCTP were obtained from NEN research products (Boston, MA). Nick translation kit was obtained from Takara Biochemicals. The other chemicals used were analytical grade or DNA/RNA grade.

2.2. *Cell culture and MT induction*

Human Chang liver cells (ATCC CCL-13) were maintained in a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 15 mM Hepes, 20 μg/ml of penicillin, 13.5 μg/ml of ampicillin, 12 μg/ml of streptomycin and 10% (vol/vol)

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fetal bovine serum. In experiments, the cells were placed into 9.6 cm^2 and 55 cm^2 dishes for protein and mRNA analyses respectively, and cultured for at least 24 h to semi-confluency. The semi-confluent cells were cultured with the above fresh medium containing various concentrations of Zn^{2+}, Dex and EGF.

2.3. *MT-protein level analyses*

For MT-protein analyses, 5 μCi of [^{35}S]cysteine was added with the inducers. After 18 h incubation, the cell extracts were carboxymethylated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously [17]. MT levels were shown as percentages of [^{35}S]cysteine-labeled MT to the sum of [^{35}S]cysteine-labeled protein.

2.4. *MT-mRNA level analyses*

Cells were lysed with lysis buffer (4.0 M guanidine thiocyanate, 0.12 M 2-mercaptoethanol, 0.025 M sodium acetate; pH 6.0) 9 h after induction. The cell lysates were overlaid to CsCl buffer (5.7 M CsCl, 0.025 M sodium acetate; pH 6.0) and total RNA was spun down at 174 000 $\times g$ for 20 h. Dot blot analyses using a dot blotter (Bio-Rad) were performed as described in [18]. Total RNA (20 μg) was dotted on a GeneScreen Plus membrane (NEN research products) and hybridized with [^{35}S]dCTP-labeled phMT-II$_3$ probe, a cDNA of human MT-II$_A$, kindly provided by M. Karin, labeled by nick translation.

3. RESULTS

We have studied the combined effects of two primary inducers on MT induction in Chang liver cells. Minor MT-protein induction by Dex alone occurred in a dose-dependent manner (Fig. 1a). Zn^{2+} also induced MT in a dose-dependent manner. MT-protein levels treated with both Dex and Zn^{2+} were greater than the sum of MT-protein levels treated with the same concentrations of each inducer separately, indicating that MT-protein was synergistically inducible by Dex and Zn^{2+}. Especially, marked synergism was seen at 80 μM Zn^{2+}. Further marked induction of MT-protein was not seen with an increase in added Dex from 5 nM to 10 nM.

Similarly, synergism of MT-protein induction by EGF and Dex was observed (Fig. 1b). MT-protein was induced approximately 2-fold by either 5 ng/ml of EGF or 10 nM Dex in comparison to the control. MT-protein was induced approximately 8-fold by a combination of the same concentrations of Dex and EGF. Further MT-protein induction was seen with an increase in added Dex from 5 nM to 10 nM Dex, which differed from the synergism of Dex and Zn^{2+}. Little difference was seen among 1 ng/ml, 5 ng/ml and 10 ng/ml EGF (data for 1 ng/ml are not shown).

In contrast, the lines showing MT-protein levels treated with both Zn^{2+} and EGF were parallel to the line showing MT-protein levels treated with Zn^{2+} alone, indicating that Zn^{2+} and EGF induce MT-protein additively (Fig. 1c). Similar additive induction by either 1 ng/ml, 5 ng/ml or 10 ng/ml EGF with Zn^{2+} was observed (data for 5 ng/ml are not shown).

We compared the relationship between mRNA and protein levels of MT. MT-mRNA levels 9 h after induction were not completely correlated with MT-protein 18 h after induction (Fig. 2). Relatively high levels of MT-

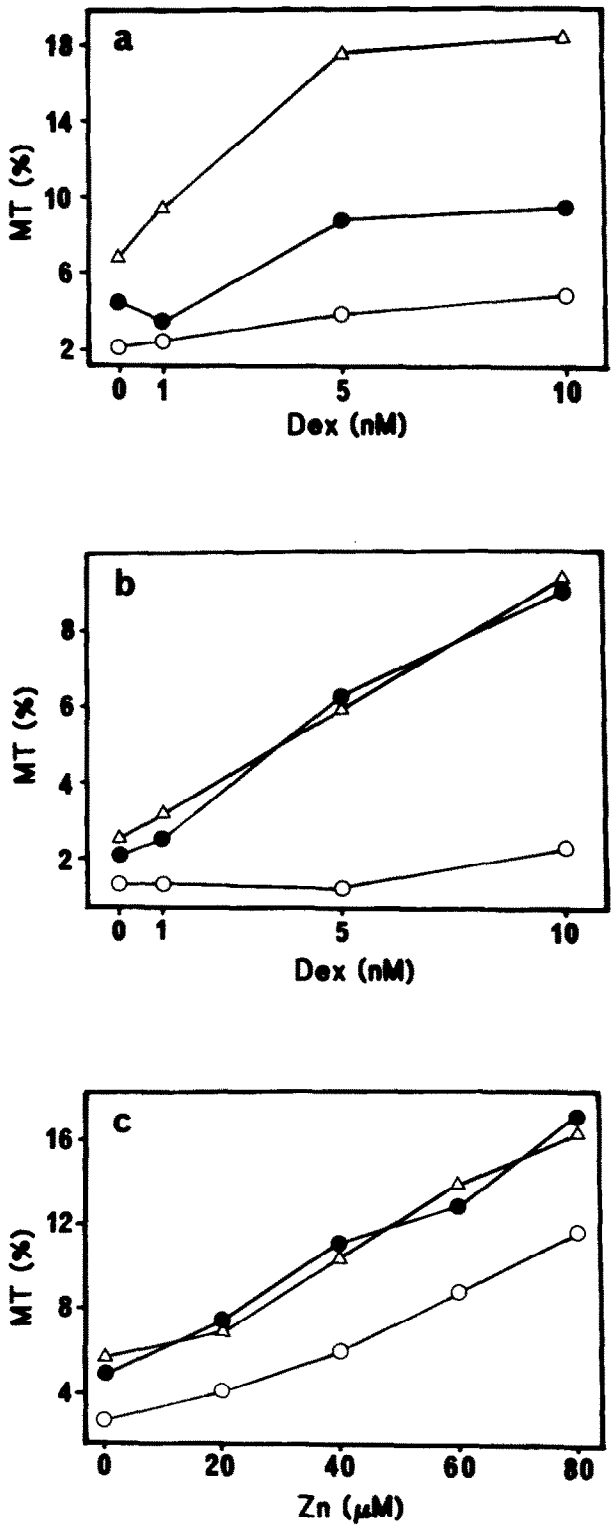

Fig. 1. The combined effects of two primary inducers on MT induction. Human Chang liver cells were treated with a, Dex and Zn^{2+} (○, 0 μM; ●, 40 μM; △, 80 μM); b, Dex and EGF (○, 0 ng/ml; ●, 5 ng/ml; △, 10 ng/ml); c, Zn^{2+} and EGF (○, 0 ng/ml; ●, 5 ng/ml; △, 10 ng/ml). After an 18 h incubation, the cell extracts were carboxymethylated and analyzed by SDS-15% polyacrylamide gel electrophoresis. MT levels were shown as percentages of [^{35}S]cysteine-labeled MT to the sum of [^{35}S]cysteine-labeled protein.

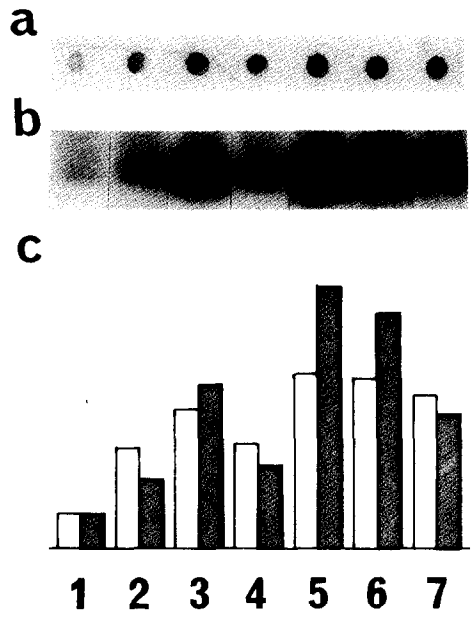

Fig. 2. mRNA and protein levels of MT induced by three inducers and their combinations. Chang liver cells were treated with 1, control; 2, 10 nM Dex; 3, 80 μM Zn^{2+}; 4, 10 ng/ml of EGF; 5, 10 nM Dex and 80 μM Zn^{2+}; 6, 80 μM Zn^{2+} and 10 ng/ml of EGF; 7, 10 nM Dex and 10 ng/ml of EGF. (a) mRNA levels 9 h after induction were analyzed by dot blot hybridizations. (b) Protein levels 18 h after induction were analyzed as shown in Fig. 1. (c) mRNA levels (open bars) and protein levels (dot bars) were shown by the relative concentrations to each control.

proteins were synthesized compared to mRNA, when induced by Zn^{2+} (Fig. 2, lanes 3, 5 and 6). The levels of MT-proteins were lower compared to mRNA, when induced by the inducers except Zn^{2+}

4. DISCUSSION

In the present study, we have demonstrated the synergistic induction of MT-protein by either a cytokine or a heavy metal with a glucocorticoid hormone and the additive induction of MT-protein by the combination of a cytokine and a heavy metal in Chang liver cells and that MT-mRNA levels were not completely correlated with MT-protein levels. These results suggest that post-transcriptional regulation may be involved in synergistic induction of MT-protein. We speculate that the inducers differ in durability of induction, degradation control of mRNA and span life of protein and that as a result of these combinations, MT-protein is induced synergistically or additively, because Richards et al. [19] reported different changes of mRNA levels at 8 h and 18 h after induction between Dex and Zn^{2+}. Further studies on posttranscriptional regulation are needed to dissolve the synergistic induction of MT-protein.

We have not studied the effects of interferon, interleukin 1 or 1α,25-dihydroxyvitamin D_3, which are reported to be primary endogenous inducers [4,5,8], because MT induction by these factors is not observed in Chang liver cells themselves [17]. Inflammatory stress increases tumor necrosis factor-α, interferon and interleukin 1 [20] and lesional stress and cold stress increase EGF [21]. Glucocorticoid hormones are well known to be increased by various stresses [14]. From these circumstances and our results, we propose a possible mechanism in which a combined effect of minor increases in circulating glucocorticoid hormones and cytokines, the doses of which are not enough to induce marked induction of MT individually, may result in the marked MT induction in the liver by stress.

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