Cadmium, an Effector in the Synthesis of Thionein

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Cadmium can elicit the synthesis of thionein in liver cells independent of tissue-organ interactions. The metal diffuses across the plasma membrane and is partitioned between subcellular components in a time dependent manner such that thionein synthesis responds to levels of nonspecifically and specifically bound cytoplasmic metal. Cadmium appears to function at the transcriptional level, and the metal may act to increase the pool of specific m-RNA's.

Introduction

Cadmium is among a group of closely related elements (Cu, Zn, Cd, and Hg) which have been isolated in animals in association with sulfur-rich metal-binding proteins (*1–7*). Animals respond to short-term sublethal levels of the metals by producing increased quantities of these specific cytoplasmic proteins known collectively as the metallothioneins (*5–13*). Neither the mechanisms which produce and regulate this response nor the precise role metals play in mediating it are clear. Cadmium is among the most toxic elements in the group (*14, 15*) and is also very effective in eliciting the production of metallothionein in liver (*11, 13*). An understanding of the mechanisms which enable organisms to respond to heavy metal exposures, and in particular to cadmium, is essential for evaluating the effects of environmental pollution by heavy metals.

We have previously suggested that the synthesis of thionein may be regulated in liver by cadmium acting as an extracellular effector (*16–20*), according to a model which can be summarized as follows. The metal induces thionein in the absence of synergistic and antagonistic effects between different tissues and organs. Cadmium enters liver cells by diffusing across the plasma membrane, and is partitioned between cytoplasmic and nuclear components such that the uptake and disappearance of cadmium from nuclei can be correlated with the appearance of cytoplasmic Cd-thionein. Cadmium appears to act at the transcriptional level in liver, and cadmium may function to increase the pool of translatable thionein m-RNA's.

We believe that there is a growing body of evidence to support these points and a summary of some of the evidence follows. However, we wish to stress that it is not yet known whether cadmium functions directly, or in association with other molecules or ions.

Materials and Methods

Adult male Sprague-Dawley rats weighing 250–300 g were housed in galvanized steel cages, had free access to Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.) and glass-distilled water. Analytical-grade cadmium chloride and other metal salts were from Baker Chemical Co. (Phillipsburg, N. J.). Male Jax mice of the H/J strain were housed in polypropylene cages and were further treated as described earlier (*20*). ^{115}Cd (0.132 mCi/mg) and [^{35}S]-cystine (92 mCi/mg) were from New England Nuclear Corp. (Boston, Mass.); actinomycin D, and cycloheximide were from Sigma Chemical Co. (St. Louis, Mo.).

The isolation of cadmium thionein and the measurements of cadmium and ^{35}S-cysteine were carried out as described earlier (*19, 20*). The methods for cell isolation (rat liver and mouse tumor) as well as their treatment with cadmium, preparation of thionein, and its measurement have been fully described (*19, 20*). The isolation and translation of

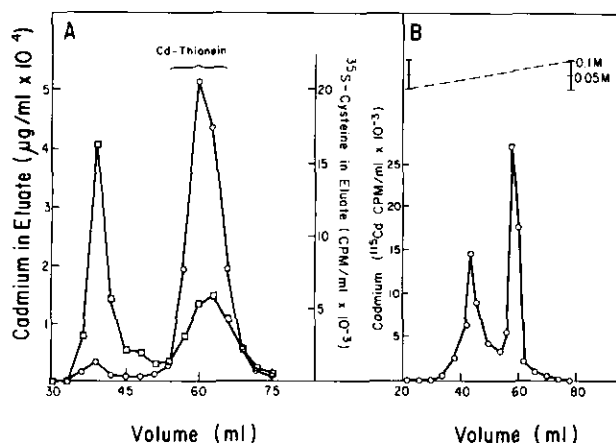* Department of Biological Sciences, University of New Orleans, New Orleans, Louisiana 70122.

FIGURE 1. Cadmium-thionein from rat liver cells. (A) Cell suspensions were exposed for 15 min to 1.0 μg Cd/ml (labeled, or unlabeled for the cysteine experiment), and were then incubated as described (19) for 6 hr with or without ³⁵S-cysteine: (□) ³⁵S-cysteine, (0) ¹¹⁵Cd. The resulting preparations were chromatographed on Sephadex G-50. (B) The cadmium-thionein (¹¹⁵Cd-labeled) fraction from the above experiment was collected and treated as described (17), and then fractionated on DEAE-cellulose.

polysomes from control and cadmium-treated rats were carried out essentially as described by Squibbs and Cousins (21).

Results and Discussion

Cadmium Appears to Function in Thionein Induction in the Absence of Tissue–Organ Interactions

Synthesis of Cd-thionein occurs readily *in vivo* in response to whole body exposure to the metal. Isolated cells in culture retain this ability independently of other tissue-organ interactions, as shown for different cell types in several laboratories (19, 20, 22–24). Our laboratory has studied isolated rat liver cells and mouse hepatoma cells in primary cultures. When rat liver cells were incubated for 15 min in Earle's solution containing 1 μg Cd/ml, a Cd-thionein fraction was isolated from the lysate following heat treatment and gel chromatography (Fig. 1A). This thionein fraction from gel filtration was separated into two subfractions (Cd-thionein 1 and Cd-thionein 2) by DEAE cellulose ion exchange chromatography (Fig. 1B). These fractions isolated from rat liver single cells were similar to cadmium thionein obtained from *in vivo* studies (17).

We have also observed Cd-thionein synthesis in primary cultures of tumor cells. Cells derived from mouse hepatomas, upon exposure to cadmium (0.25 μg/ml), incorporated the metal into a fraction iden-

tified as Cd-thionein by gel filtration and ion-exchange chromatography (Fig. 2A, B). The appearance of Cd-thionein in tumor cells was accompanied by the incorporation of radioactive cysteine into the protein (Fig. 2C), suggesting *de novo* synthesis of thionein in tumor cells following cadmium exposure. Thus, cells derived from a mouse hepatoma retained the capacity of liver cells to synthesize thionein. Webb and co-workers, working with pig liver and kidney cells (22, 23), and Enger, working with cultured Chinese hamster ovary cells (24), have also reported synthesis of metallothionein *in vitro* in response to cadmium. Therefore, it seems clear that cadmium can function independent of secondary tissue–organ interactions in the thionein induction process.

Cadmium Enters Cells and Is Partitioned between Subcellular Components

In isolated cells exposed to 1 μg Cd/ml, cadmium uptake did not vary significantly with time of incubation; however, if the cadmium concentrations were varied metal uptake increased linearly with increasing cadmium concentrations (19). These observations suggested that the cells absorbed the metal by diffusion, an equilibrium was established fairly rapidly after exposure, and the higher the concentration to which cells were exposed, the more metal they absorbed (19).

Once the metal is inside the cell, it is thought that cadmium is distributed among subcellular components in a time dependent fashion as described below. Initially, after an acute cadmium exposure (20 μmole/kg), cadmium binds nonspecifically to high molecular weight proteins; this type of binding decreases with time and at 24 hr is less than 5%. In contrast, the level of specifically bound metal increases progressively, and at 24 hr more than 90% is associated with Cd-thionein. Cadmium enters the nucleus following cell exposure to the metal, establishes an equilibrium, and decreases thereafter as the amount of thionein-bound metal in the cytoplasm increases. Thus, there is a strong correlation between the appearance and binding of the metal to thionein, and the uptake and disappearance of cad-

Table 1. Cadmium uptake in nuclei.

Incubation treatment		¹¹⁵Cd nuclei uptake, DNA	
		20 hr after Cd challenge	24 hr after Cd challenge
Nuclei	Cytoplasm		
I Control	Experimental	40	37
II Experimental	Control	1087	915
III Experimental	Experimental	1192	—

mium from the nucleus. It is possible for metal contamination to arise from isolation procedures such that the observed cytoplasmic and nuclear metal levels may not correspond to endogenous levels. This possibility was investigated in the following experiment: homogenates from control (no cadmium) and experimental (10 μCi ^{115}Cd/5 μmole of Cd) animals were subjected to a 700g centrifugation, and the resulting cytoplasmic and crude nuclear pellets were removed, divided into several equal aliquots, and reconstituted. Control nuclei were combined with experimental cytoplasm, experimental nuclei with control cytoplasm, and experimental nuclei with experimental cytoplasm. Samples were incubated at 0–4°C for 1 hr with stirring prior to isolation of purified nuclei, and an analysis of the nuclei is given in Table 1.

Little or no metal entered control nuclei from the experimental (Cd-thionein containing) cytoplasm, and neither did metal "leak" from experimental nuclei upon exposure to control cytoplasm. It appears that 20 and 24 hr after an acute cadmium exposure the subcellular partitioning of cadmium is a measure of endogenous levels of metal and not an artifact of the system.

Action of Cadmium in Isolated Hepatocytes

In an isolated single cell system the chemical environment, and in particular the amount of metal to which the cells are ultimately exposed, are under the control of the experimenter. Independent studies on the kinetics of induction and the appear-

ance of Cd-thionein in whole animals have shown that maximum thionein synthesis, after an acute cadmium exposure, appears to be preceded by a lag period of about 2 hr and to continue for approximately 8 hr. When a similar time study was performed with isolated cells exposed to 1 μg of Cd/ml during the preincubation, the cells incorporated cadmium into thionein continuously up to last time measured (6-8 hr). We believe the increase between 0 and 2 hr was due to thionein synthesis and not only to zinc-displacement in thionein (19).

In protein synthesis inhibitor experiments in which variables of concentration and time of exposure were controlled, cycloheximide and actinomycin D were added at either zero time (the end of cadmium exposure), or two hours thereafter. Under these conditions, cycloheximide stopped thionein synthesis at zero and 2 hr (Fig. 3). When actinomycin D was added at time zero, it also prevented any apparent increase in thionein. However, if actinomycin was added 2 hr after thionein synthesis had started, it was only partly effective in inhibiting thionein synthesis. It is probable that enough mRNA's were synthesized between 0-2 hr to maintain a limited synthesis of thionein after inhibition of transcription.

Cell-Free Homologous Polysomal System for Cd-Thionein Induction

If cadmium is an effector in the synthesis of Cd-thionein, it should be possible to isolate polysomes from control and from cadmium-treated (experi-
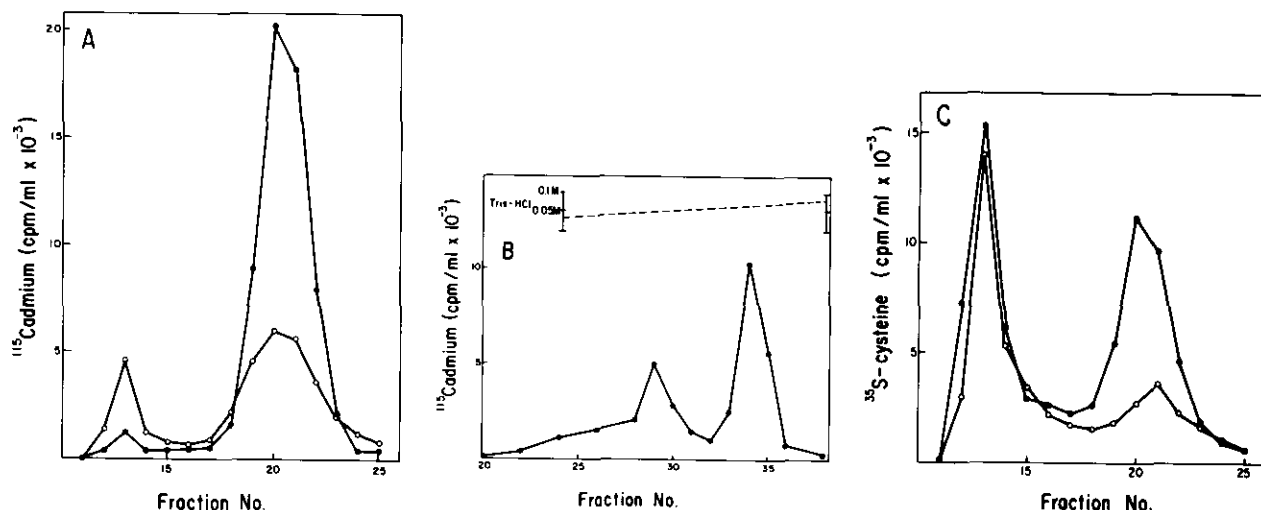

FIGURE 2. Cadmium thionein from mouse tumor cells. (A) Isolated tumor cells prepared as described (20) were exposed to 0.25 μg Cd/ml and incubated for 6 hr. Cadmium thionein assays were carried out after cadmium exposure, or 6 hr thereafter, on Sephadex G-50. (B) DEAE-cellulose chromatography of the 6 hr thionein sample was carried out as described for 1B. (C) Tumor cells were treated with unlabeled cadmium and then with ^{35}S-cysteine as described earlier (20). After incubation, control (o), and experimental (●) preparations were assayed for thionein by gel filtration.

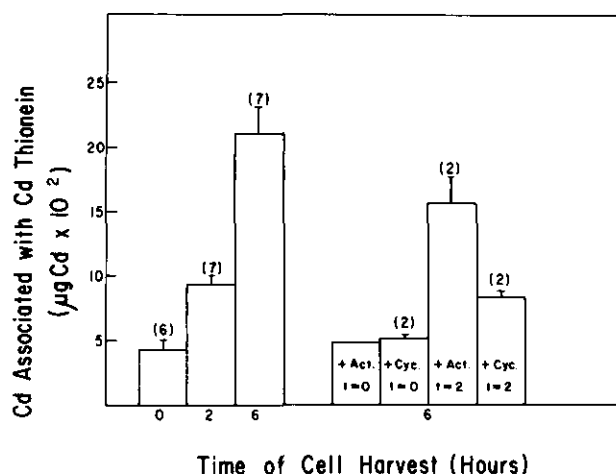**Time of Cell Harvest (Hours)**

FIGURE 3. Effects of actinomycin-D and cycloheximide on cadmium-thionein synthesis. Rat liver cell suspensions (50 mg/ml) were incubated for 15 min in Earle's salt solution containing 1 μg of radioactively labeled (^{115}Cd) cadmium per ml. Cells were incubated in Eagle's medium and, at the times indicated, harvested for cadmium-thionein isolation. Actionomycin D and cycloheximide were added at the beginning ($t = 0$), or 2 hr ($t = 2$) after incubation had started to a final concentration of 5 μg per ml. The bars, solid bracketed lines, and the numbers in parentheses represent the mean ± SEM (n), respectively.

mental) animals, and show in a cell-free homologous system that cadmium-thionein synthesis is regulated by changes in the pool of translatable thionein mRNA, as reported by Squibb and Cousins for Zn-thionein (21). Experiments carried out in our laboratory over the past months have not conclusively demonstrated differences between control and experimental polysomal activity in a cell-free translation system. Cherian and Goyer (25) have also noted the failure to develop successfully *in vitro* systems to study the biosynthesis of metallothionein. Thus, to date, *in vitro* translation experiments have not confirmed the *in vivo* effects of cadmium on RNA metabolism (18, 24, 26, 27), and in particular the stimulated synthesis of messenger RNA's following cadmium exposure (24).

The detailed mechanisms which regulate cadmium-thionein synthesis are unknown at the present time; however, it is clear that in rat liver (1) they involve controls at the transcriptional level, (2) these controls are responsive to the levels of nonspecifically bound cadmium in the cell, (3) synthesis of thionein decreases as the cytoplasmic levels of this protein sequester intracellular cadmium. An understanding of the regulatory processes of thionein synthesis and degradation is needed to assess how excessive doses of heavy metals can cause permanent damage, or even death, in organisms.

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