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INDUCTION OF METALLOTHIONEIN BY SIMULTANEOUS ADMINISTRATION OF CADMIUM(II) AND ZINC(II)

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SUMMARY : LLC-PK1 cells were used as a model of renal proximal tubule cells in the induction of metallothionein by cadmium(II) and zinc(II). We have found that the induction of metallothionein by either cadmium(II) or zinc(II) alone reaches a maximum which cannot be surpassed by increased amounts of the same metal. However, induction is additive when the cells are exposed to the two metals simultaneously. This indicates that the induction of metallothionein by cadmium(II) and by zinc(II) occurs by different mechanisms within these cells.

Metallothionein (MT) is a low molecular weight (~ 6-7 kD) cytosolic metal-binding protein. It has been found to bind numerous metals including Cd(II), Zn(II), Cu(I), Ni(II), Ag(II) and Pt(II) (1). Metallothionein has been found in most mammalian cell types and in a large number of non-mammalian species. The exact function of MT remains unknown although it is hypothesized that MT sequesters toxic metals such as Cd(II), preventing them from having toxic effects upon the cell. However, it has been shown that Cd-MT may itself be toxic (2).

There are several MT genes and various MT promoters (3). Metallothionein promoters can be activated and MT synthesis increased, by heavy metals, including Cd(II), Zn(II) and Cu(I) as well as steroid hormones and other physical stresses. Metallothionein is thus an inducible protein. Metallothionein is induced to different degrees in different tissues. Hepatocytes and proximal tubule cells of the kidney are the most potent sites of MT induction.

In the present study, the induction of MT by Cd(II) and Zn(II) was studied in LLC-PK1 cells, a porcine renal proximal tubule cell line (4). The two metals were administered separately and simultaneously. We have shown that induction of MT by each metal reaches a maximum at a concentration of metal slightly under the toxic range. Simultaneous administration of the two metals in the same concentration, however, shows that additive

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The following abbreviations are used: Metallothionein = MT, Alpha-Minimal Essential Medium = Alpha-MEM, Phosphate Buffered Saline = PBS.

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MT induction is achieved. No change in toxicity is seen. As the induction of MT for each metal is saturable, these results indicate that the mechanism of induction of MT in these cells is different for Cd(II) and Zn(II).

METHODS

LLC-PK1 cells were grown at 37° C, 5% CO_2 in Alpha-Minimal Essential Medium (Alpha-MEM) supplemented with 10% calf serum and 100 units/mL penicillin and 100 μg/ml streptomycin on 60 x 15 mm plates until confluence ($5x10^6$ cells). All media was obtained from Gibco. The media was changed to Alpha-MEM supplemented with 1% calf serum for overnight incubation. Cells were washed in Alpha-MEM medium before addition of the incubation medium. Incubation media consisted of the metal (cadmium acetate and/or zinc acetate) at the appropriate concentration in Alpha-MEM. The cells were incubated with this medium for 24 h. At the end of the incubation period, the cells were washed with phosphate buffered saline (PBS). 750 μL of 0.25 M sucrose was added to each plate and the cells scraped off physically by using a rubber policeman. Cells were sonicated and then centrifuged at 20000 x g to yield the cytosol as supernatant. Each experiment was done in triplicate.

Metallothionein and protein contents of the cytosol were measured. Metallothionein estimation on the cytosol was carried out using a modified version of the Ag-heme assay (5, 6), using horse kidney metallothionein (Sigma) as a standard. The Lowry method was used to estimate protein, using bovine serum albumin (Sigma) as a standard (7). Results are reported as mean +/- S.D. (n = 3).

Metallothionein was isolated from confluent cells grown on Nunclon 80 cm^2 plates (approximately 15 x 10^6 cells per plate). After a 24 h incubation with the appropriate metal(s), the cells were removed by a mild trypsin treatment by which the attachments of the cells to the plates were disrupted, but the cell membranes remained intact. The trypsin digest was stopped by addition of Alpha-MEM media containing 10% calf serum. The cells were washed three times by PBS and then sonicated. Cytosol was obtained by centrifugation at 20000 x g. Metallothionein was then isolated using a Sephadex G-75 column. Cytosol was equilibrated with 1 x 10^5 dpm ^{109}Cd in 1 ml 10 mM Tris-Ac buffer, pH 7.8 at 4 °C overnight and then applied to a 200 mL Sephadex G-75 column and eluted in 10 mM Tris-Ac buffer, pH 7.8 at 4 °C. This column had been previously calibrated with purified horse kidney MT obtained from Sigma. The purified MT was then dialysed overnight at 4 °C versus H_2O and lyophilized. The identification of the MT isomers was carried out by eluting the purified MT through a DEAE Sephadex column using a stepwise gradient elution of 0.005 M Tris-HCl to 0.5 M Tris HCl buffer, pH 8.6 at 20 °C (8). This column had been previously calibrated with rabbit liver MT-1 and rabbit liver MT-2 obtained from Sigma. MT-1 was found to elute at 0.075 M Tris-HCl. MT-2 was found to elute at 0.15 M Tris-HCl. The elution of MT through this column was followed by both radioactivity and the absorbance at 254 nm.Metallothionein bound to Cd(II) or Zn(II) has a typical absorbance at this wavelength (1).

RESULTS AND DISCUSSION

The induction of metallothionein produced by Cd(II) alone is shown in Fig. 1. A maximal amount of MT induction, 4 μg per plate, corresponding to 14 μg MT/mg protein is produced by Cd(II) in the range of 15-20 μM. As seen in Fig. 2, at a concentration of 30 μM, approximately 25% of the cells did not survive the 24 h experiment. Therefore, in subsequent experiments, concentrations of Cd(II) up to 20 μM were used.

The results of the simultaneous administration of Cd(II) and Zn(II) are shown in Figs. 3 and 4. The maximal amount of metallothionein induction occurs at 15 μM Cd(II) and 200 μM Zn(II). The maximal amount of MT produced by the administration of Cd(II) alone is equivalent to 8.76 mmol MT/mol Cd(II). The maximal amount of MT produced

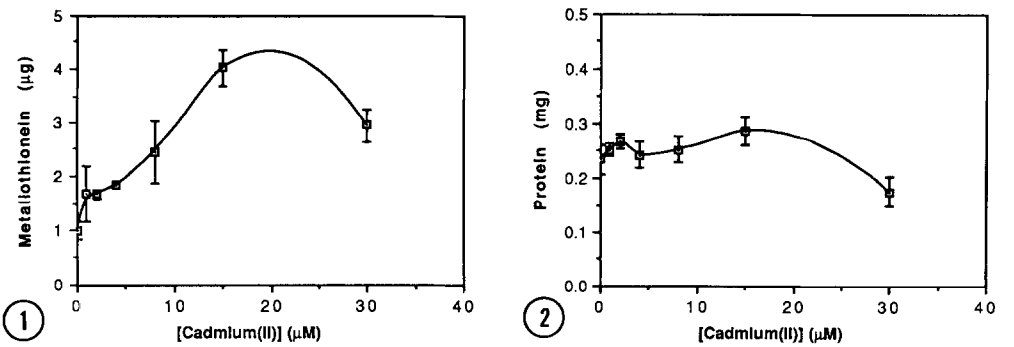


Fig. 1. Metallothionein induction in LLC-PK1 cells after 24 h incubation at 37 °C, 5% CO_2 with Cd(II). Results are expressed as µg metallothionein.

Fig. 2. Recovery of total protein in LLC-PK1 cells after 24 h incubation at 37 °C, 5% CO_2 with Cd(II). Results are expressed as mg protein.

by Zn(II) alone is equivalent to 0.885 mmol MT/mol Zn(II). Therefore, Cd(II) is ten times more efficient in the induction of MT than Zn(II). At 20 µM Cd(II), less induction of MT is observed than at 15 µM Cd(II), although the difference in toxicity between these two concentrations is negligible. Zinc(II) concentrations of 400 and 600 µM are highly toxic and give less MT induction than 200 µM. The most potent concentration of Cd(II) (15 µM) is able to induce a maximum of 14 µg MT/mg protein when administered alone. The most potent concentration of Zn (200 µM) is able to raise the level of MT to a maximum of 21 µg MT/mg protein when administered alone. The two metals administered together are able to induce almost 40 µg MT/mg protein. Figs. 5 and 6 demonstrate that the MTs induced by both Cd(II) and Zn(II) were MT-1 isomers.

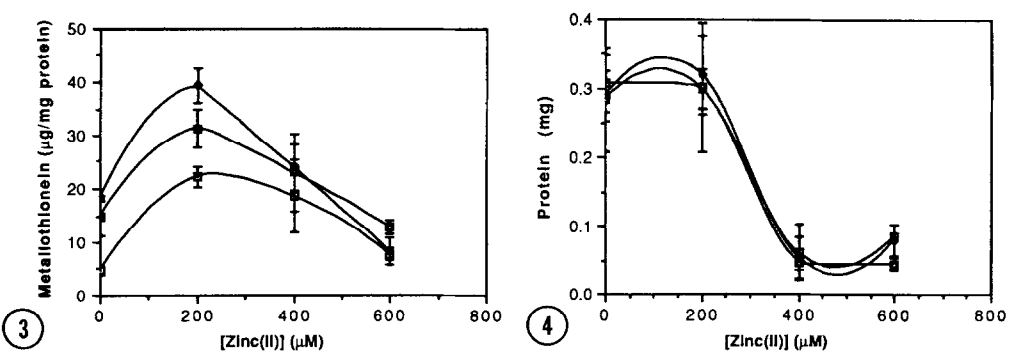
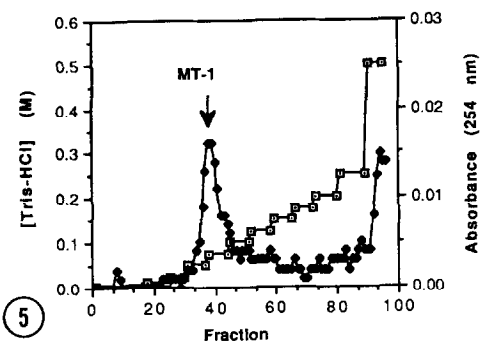


Fig. 3. Metallothionein induction in LLC-PK1 cells after 24 h incubation at 37 °C, 5% CO_2 with Zn(II) with or without Cd(II). [Cd(II)] = 0 µM (□), [Cd(II)] = 15 µM (◆), [Cd(II)] = 20 µM (■). Results are expressed as µg MT/mg total protein.

Fig. 4. Recovery of total protein in LLC-PK1 cells after 24 h incubation at 37 °C, 5% CO_2 with Zn(II) with or without Cd(II). [Cd(II)] = 0 µM (□), [Cd(II)] = 15 µM (◆), [Cd(II)] = 20 µM (■). Results are expressed as mg protein.



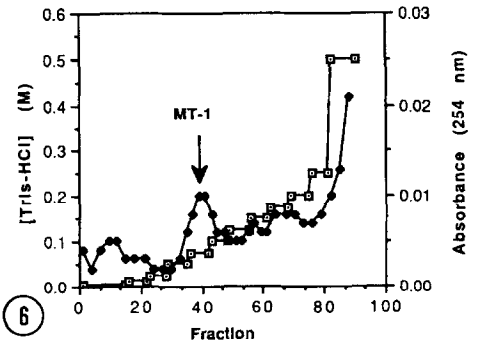


Fig. 5. Elution of Cd(II) induced metallothionein through DEAE Sephadex, pH 8.6, 20°C. [Tris-HCl] (□), Absorbance (254 nm) (◆).

Fig. 6. Elution of Zn(II) induced metallothionein through DEAE Sephadex, pH 8.6, 20°C. [Tris-HCl] (□), Absorbance(254 nm) (◆).

Toxicity due to each metal is not affected by the simultaneous administration of the other metal (Fig. 4). Therefore, the increased MT induction upon simultaneous administration of Cd(II) and Zn(II) cannot be explained by increased cell survival.

There are at least two possible mechanisms to explain these results. We hypothesize that the induction of metallothionein by these two metals occurs through different mechanisms, both of which are saturable. This may occur by the binding of the metals to two or more different MT promoter binding proteins. These proteins may interact with two different MT promoters to activate MT transcription. Alternatively, there still exists the possibility of interaction between these two metals in the induction of metallothionein. Cadmium(II) may bind to one site on a promoter binding protein and Zn(II) may bind to a second site. The binding of one metal may cause approximately half-maximal induction of MT transcription and the binding of both metals would be required for full activation of the promoter.

In conclusion, we have determined that there are likely at least two possible mechanisms of metallothionein induction by Cd(II) and Zn(II) in LLC-PK1 cells. Cadmium(II) is ten times as efficient as Zn(II) in the induction of metallothionein in these cells.

Recently, others have begun to look at the induction of metallothionein using two or more inducers (9, 10) This type of experiment promises to give much more insight into the complicated mechanisms of metallothionein induction.

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