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AMPLIFICATION OF MERCURY TOXICITY BY GLUTATHIONE DEPLETION IN V79 CELLS

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ABSTRACT: *The treatment of V79 cells with diethyl maleate (DEM) led to decrease in glutathione (GSH) level as increasing DEM concentration. Mercuric chloride, treated for 6 hrs with 2 ng/ml,* affected the GSH metabolizing enzymes glutathione S-transferase (GST) and glutathione peroxidase (GSP), dropping their activities to 60% and 75%, respectively, though not so much in GSH level (80%). However, the toxic effects of mercuric chloride on those enzymes and GSH level were both amplified when the Hg^{2+} treatment was combined with the preceding DEM treatment. In contrast to the rapid recovery of GSH level in the group given DEM alone, the group of combined treatment showed slow recovery of GSH level and drastic decrease in GST and GSP activities; 48% and 50% respectively. After the 30 min treatment of DEM, GSH level began to resile immediately so that it reached 86% of control in 6 hrs. The turnover rate of GSH may be considerably more rapid than those of GST and GSP in V79 cells.

Key Words: *GSH, Hg^{2+}, Diethyl maleate, V79 cells, GSH S-transferase, GSH-peroxidase*

INTRODUCTION

Glutathione (GSH) is a well-known antioxidative cellular component which is ubiquitous in nature. Several enzymes involved in GSH metabolism and recycling have been found to play important roles in detoxification of xenobiotics and free radicals.

On the other hand, mercury in the form of mercuric chloride has been widely investigated for centuries by toxicologists. The fatal systemic effects of high dose $HgCl_2$ are based on its irreversible cell injury like mitochondria disruption, lysosomal leakage and rupture of cell membrane. The involvement of GSH in mercury

toxicity as the first guard has been accepted since Clarkson (1972) elucidated the *in vivo* binding of GSH and mercurials. GSH detoxifies electrophiles such as Hg^{2+} and therefore protects protein thiols on which many cellular transport and defence mechanisms depend.

Through the last decade, researchers focused on the effects of mercuric chloride on GSH metabolizing enzymes. Chung *et al.* (1982) showed that Hg^{2+} caused the time and dose-dependent decrease in GSH level and in the activities of γ-glutamyl cysteine synthetase, GSSG reductase, γ-glutamyl transpeptidase and GSP in rat liver or kidney.

Recent studies confirmed the protective role of GSH against various harmful attacks such as irradiation and oxidative stress and tumorigenesis (Arrick *et al.*, 1982, Dethmers *et al.*, 1981, Perry *et al.*, 1992). In other words, those harmful effects could be amplified when the cellular GSH was depleted.

The purpose of this study was to examine the amplified toxicity of mercuric chloride on GSH metabolizing enzymes by GSH depletion. We used DEM as GSH depletor because its action is specific and acute (Boyland and Chasseaud, 1967).

MATERIALS AND METHODS

Chemicals

Glutathione, DEM, mercuric chloride, O-phthalaldehyde, bovine serum albumin, CDNB, NADPH, GSSG reductase, DMEM and Folin reagent were purchased from the Sigma chemical Co., St. Louis, MO. Trypsin was purchased from DIFCO. Foetal calf serum (FCS) was purchased from Commonwealth Serum Laboratories, Melbourne, Australia.

Cell Cultures

Cells were grown in Dulbeco's Modified Eagle's Media (DMEM) supplemented with 10% heat-inactivated foetal calf serum in a humidified atmosphere containing 5% CO_2. Cells were separated with solution containing 0.025% trypsin and 5.0×10^6 cells were seeded on each plate by counting with hematocytometer. Chinese hamster lung fibroblast cell line (V79) was used since the concentration of GSH and the activities of GST and GSP were determined easily. DEM was dissolved in 95% ethanol and treated up to 20 μl to make final concentrations up to 0.2 mM, right after cells were found attached to the plate. Mercuric chloride was dissolved in deionized distilled water and treated up to 40 μl to make final concentrations up to 2 ng/ml.

Glutathione and Enzyme Assays

Glutathione concentraion was measured by a modification of the method of Cohn and Lyle (1966). Cells were harvested by trypsin treatment and centrifugation at 2,000 g for 10 min. The pelleted cells were disrupted by sonication method in 1 ml of extraction mixture (1/1/1, v/v, 0.01 N HCl-5% trichloroacetic acid-1 mM EDTA). Protein was removed by centrifugation at 5,000 g for 10 min. To a 50 μl sample of the supernatant fraction, 1.0 ml of 0.5 M Na_2HPO_4 and 10 μl of o-phthalaldehyde in methanol(1 mg/ml) were added. The GSH value was

detected fluorometrically using Perkin-Elmer LS/3B. The excitation wavelength was 328 nm and the emission wavelength was 430 nm.

For the enzyme assays, harvested cells were disrupted by sonication in 1 ml of phosphate-buffered saline and cell debris was removed by centrifugation at 5,000 g for 10 min. The supernatant fraction was used. The activity of GST was determined by monitoring changes in formation of product at 340 nm in Beckman Du-7 spectrophotometer using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate according to the method of Habig *et al.* (1974). The enzyme activity is given as nmole product formed per mg protein per minute. The assay of GSP was conducted by a modified coupling method of Paglia and Valentine (1967). The assay medium contained enzyme source, GSSG-reductase, sodium azide (NaN_3, 1.0 mM), EDTA (3.0 mM), NADPH (0.1 mM) and potassium phosphate buffer (0.1 M, pH 7.0). The reaction was initiated by the addition of H_2O_2 (0.12 mM) and the reaction rate was measured at room temperature. The blank did not contain H_2O_2. One unit of enzyme activity (E.U.) was defined as 1 nmole NADPH oxidized per mg protein per minute. Protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. All experiments were performed on four plates for each experiment, and the data were analyzed using student T-test. The results are presented as means± S.D.

RESULT

DEM caused dose-dependent GSH depletion in V79 cells. Cellular GSH level decreased to about 20-40% of normal value after 30 min incubation with 0.2 mM DEM (Figure 1).

The levels of GSH were shown in Figure 2 by the treatment of mercuric chloride. There was not any significant decrease in GSH level by the 1 ng/ml $HgCl_2$ treatment but 2 ng/ml $HgCl_2$ treatment caused more drastic decrease in the concentra-

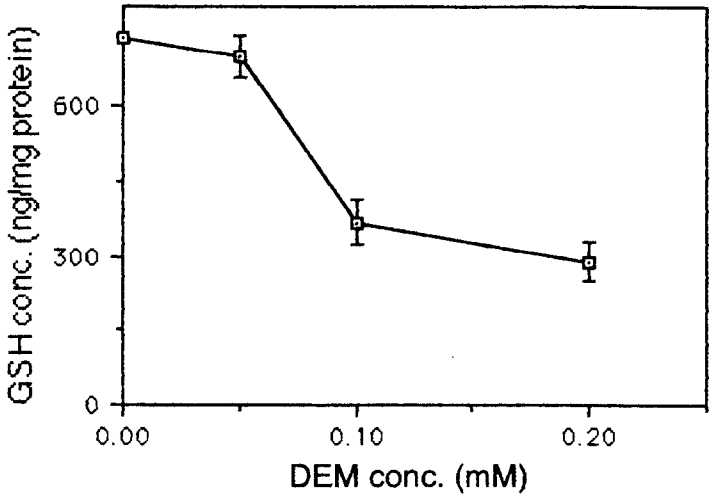

Figure 1. Decrease of GSH level by increasing concentrations of DEM. The concentrations of DEM were 0.05, 0.1 and 0.2 mM. The treated V79 cells were harvested after 30 min DEM incubation.

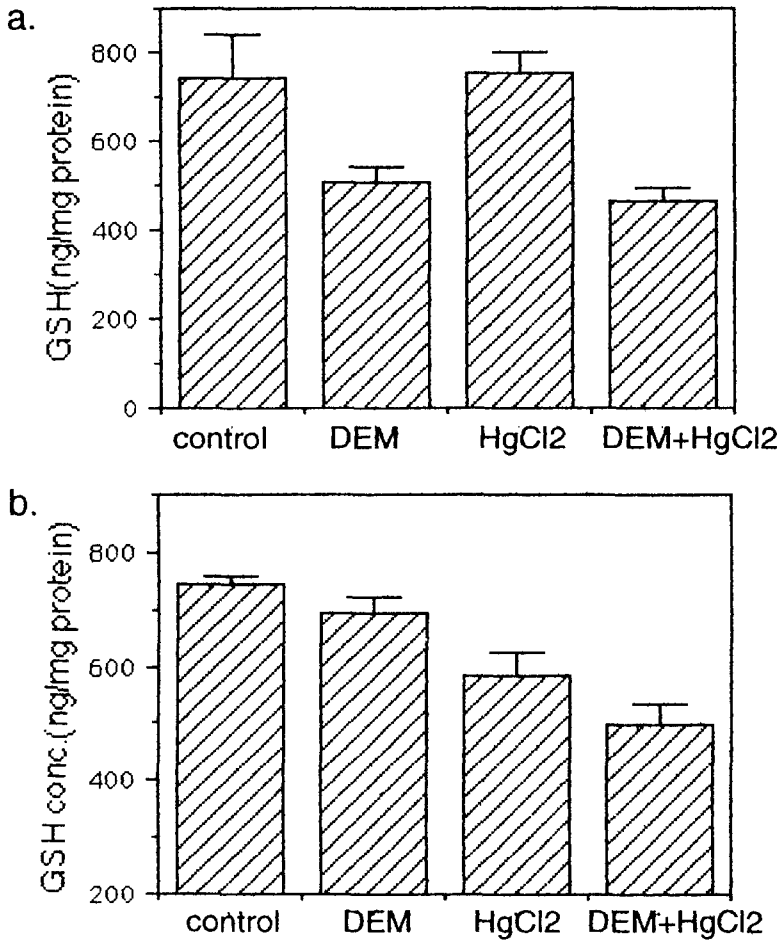

Figure 2. The effect of combined treatment of DEM and $HgCl_2$ on GSH content.
Right after the incubation with 0.2 mM DEM for 30 min (at 37℃) , media with or without DEM was replaced by new media containing $HgCl_2$ for third and fourth group.
Control and second group were replenished with new media without mercuric chloride.
a.; 1 ng/m*l* of $HgCl_2$ treatment. b.; 2 ng/m*l* of $HgCl_2$ treatment.

tion of GSH. The treatment of $HgCl_2$ had more effect on the enzyme activities of GST and GSP (Table 1, Table 2) than on the GSH level. The activities of the enzymes were not much influenced by the higher concentration of $HgCl_2$ (2 ng/m*l*) compared with the 1 ng/m*l* of $HgCl_2$ treatment. The combined treatment of $HgCl_2$ and DEM showed more effect on the level of GSH and the activities of the enzymes than the single treatment alone. It should be noted that the treatment of DEM (0.2 mM) showed the significant decrease in the activities of both enzymes and the level of GSH, but the effects of DEM on the activities of both enzymes were less than that of $HgCl_2$.

The combined effects of DEM and mercuric chloride were obvious on the amplification of decreases in the enzyme activities (Table 2). The activity of GST was reduced to 48.5% by the combined treatment. It outstood the results of single

Table 1. Effects of 0.2 mM DEM followed by 1 ng/ml $HgCl_2$ on GST and GSP activities.

	Control	DEM	$HgCl_2$	DEM+$HgCl_2$
GST (EU)	6.98± 0.75	5.64± 0.22*	3.94± 0.50*	3.47± 0.41*
%	100	80.8	56.4	49.7
GSP (EU)	38.01± 1.94	30.43± 3.52*	28.90± 0.37	27.93± 4.63*
%	100	80.1	76.0	73.5

Each value represents the mean± S.D.
The asterisk (*) indicates $p \leq 0.05$ when compared with the control values. The E.U. of GST is defined as 1 nmole GSH conjugated/min/mg protein. The E.U. of GSP is defined as 1 nmole NADPH oxidized/min/mg protein.

Table 2. Effects of 0.2 mM DEM followed by 2 ng/ml $HgCl_2$ on GST and GSP activities.

	Control	DEM	$HgCl_2$	DEM+$HgCl_2$
GST (EU)	7.07± 0.19	5.01± 0.17*	4.32± 0.57*	3.43± 0.30
%	100	70.9	61.1	48.5
GSP (EU)	33.52± 4.26	26.20± 3.22*	25.07± 5.71*	17.27± 6.23*
%	100	78.2	74.8	51.5

Each value represents the mean± S.D.
The asterisk (*) indicates $P \leq 0.05$ when compared with the control values.

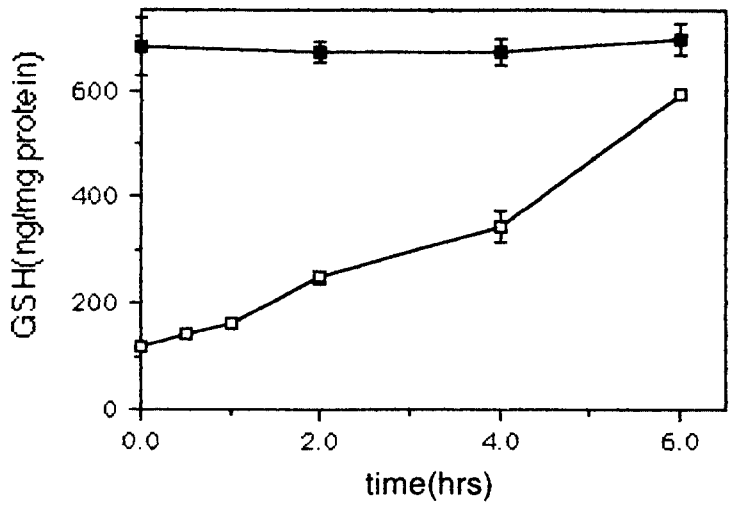

Figure 3. The recovery of GSH content along with time after the treatment of DEM.
After 30 min incubation with 0.2 mM DEM, the media was replaced by normal media. GSH contents were dtermined at the hours above. Empty squares (—□—) indicate the group treated with DEM and filled squares (—■—) show control.

treatments; the activity of GST was significantly reduced to 70.9% and 61.1%, by the treatments of 0.2 mM of DEM and 2 ng/ml of Hg^{2+}, respectively. The activity of GSP showed a similar result with that of GST. The reductions of the enzyme activity by combined treatment, by 0.2 mM of DEM and by 2 ng/ml of $HgCl_2$ were

Table 3. Protein contents of control and treatment groups.

	Control	DEM	$HgCl_2$	DEM+$HgCl_2$
Protein content (mg/ml)	2.25± 0.02	2.40± 0.08	2.31± 0.03	2.36± 0.04

The concentrations of DEM and $HgCl_2$ were 0.2 mM and 2 ng/ml, respectively. Each value represents the mean± S.D.

51.1%, 78.2% and 74.8%, respectively.

One contradictionary feature was found in the results of combined treatment experiments. In contrast to the result of GSH depletion shown in Figure 1, GSH values were surprisingly high after treatment of DEM alone. This inconsistency arose from the experimental procedure of letting the group stand in normal media during the 6 hrs of HgCl2 treatment. That period was chosen because the toxic effect of $HgCl_2$ was shown in other experiments at 6 hrs after the treatment in CHO cells (Byun *et al.*, 1991). It implicates the GSH resynthesis was stimulated by the feedback of GSH depletion. The rate and pattern of GSH recovery were shown in Figure 3. The initial value of GSH after the treatment of DEM was 17.6% of control value. The control value of GSH did not change for 6 hrs of the cell culture. The level of GSH began to climb steadily at 1 hr after the treatment almost to 30% of control value, and the recovery of GSH was accelerated more thereafter and reached 86% of control value at 6 hrs. The concentrations of protein were shown in Table 3. There were no differences in protein concentrations among the control, the treatment of DEM, the treatment of Hg^{2+} and the combined treatment of both. No cell death was observed by the treatment during the experimental period (data not shown). This result suggests that the concentrations of DEM and $HgCl_2$ would be mildly toxic.

DISCUSSION

The treatment of both Hg^{2+} and DEM could decrease more the concentration of GSH than that of single treatment alone but showed the decreasing trend in the activities of GST and GSP compared with those of Hg^{2+} or DEM alone. These results demonstrated that the depletion of GSH in V79 cells could increase the toxic effct of Hg^{2+} on the glutathione metabolism. Mercury might cause to decrease the level of GSH by direct interaction with GSH or to elevate the permeability of membrane by interaction with sulfhydryl groups of membrane proteins. Recently it has been shown that mercury and other metal ions can interact with sulfhydryl groups of the proteins in cell membrane and the interaction is responsible for increased cation permeability in renal proximal tubules(Kone *et al.*, 1990). In our experiment, the content of protein was not altered by the treatment. This indicates that the mercury toxicity can be more specific inhibition related to the enzymes of GSH metabolism rather than that of general protein depletion.

Mercury toxicity can be partly explained that the synthesis of GSH is repressed by the inhibition of GSSG-reductase and γ-glutamylcysteine synthetase, the rate-limiting enzyme of GSH *de novo* synthesis (Meister, 1976, Chung and Maines,

1982). Since GSSG-reductase is a disulfide enzyme, Chung and Maines (1982) have demonstrated that Hg^{2+}, both *in vivo* and *in vitro*, inhibited the GSSG-reductase. The decreased level of GSH by Hg^{2+} can be related to depressing the activities of γ-glutamylcysteine synthetase, GSSG-reductase and other enzymes of GSH metabolism. The concentration of Hg^{2+} has not affected the concentration of protein which could possibly mean a direct action of the metal ion on the activity and/or turnover of the enzyme protein rather than membrane damage related to protein loss.

Some researchers suggest that GSH has the versatility of defence mechanisms against electrophilic attacks of free radical xenobiotics and further tumor development (Babson and Reed, 1981, Bailey *et al.*, 1992). Another evidence of the protection of early stages, which GSH occupies, is that GSH turnover rate is closely related with the cellular capacity of detoxification (Lauterburg *et al.*, 1980, Reed *et al.*, 1983). V79 cells seem to have considerable rate of GSH turnover according our experimental data and this cells from lung fibroblast can be exposed to toxic chemicals so that the cells need more creditable defence mechanism.

DEM is an αβ-unsaturated compound and its own spatial feature enables it to bind covalently and enzymatically with GSH (Boyland and Chasseaud, 1967) or other sulfhydryl groups. Recently, it was suspected to have some side effects more than GSH depletion; it appears to interact with mixed function oxidase system (Anders, 1978). In our experiment, DEM reduced the activities of GST and GSP which were accompanied with the depletion of GSH. It was not possible to detect the activity of γ-glutamylcysteine synthetase and GSSG-reductase or other enzymes related to GSH metabolism in V79 cells except above two enzymes. It is conceivable that DEM not only decreases the level of GSH but also interferes several enzymes of GSH metabolism including GST and GSP.

Further research is needed to clarify how mercury reduces the level of GSH and the activities of its related enzymes and to investigate the mechanisms suggested so far such as increasing membrane permeability, interacting with GSH or other sulfhydryl groups.

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