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BIOCHEMICAL MECHANISM OF METALLOTHIONEIN–CARBON TETRACHLORIDE INTERACTION *IN VITRO*

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Abstract—To elucidate the mechanism underlying the protective effect of metallothionein (MT) against carbon tetrachloride (CCl_4) toxicity, *in vitro* experiments were carried out to study the interaction of metallothionein and CCl_4. Results from this study showed that incubation of Cd,Zn-MT with CCl_4 in the presence of hepatic microsomes and NADPH resulted in a time-dependent depletion of MT thiols with a concurrent reduction in the metal-binding sites of the protein. Moreover, this reaction also released Zn and Cd from MT. Results from experiments conducted to determine whether or not the CCl_4-induced decrease in MT-thiol content was due to the scavenging of CCl_4 metabolite(s) showed that the trichloromethyl radical, chloroform and phosgene as well as the products of CCl_4-induced microsomal lipid peroxidation were not directly involved. Although covalent binding of $^{14}CCl_4$ to MT was detected following incubation in the presence of a microsomal bioactivation system, it did not account for the CCl_4-induced loss of MT thiol groups for the following reasons: (i) prior oxidation of sulfhydryl groups of MT by hydrogen peroxide did not alter the binding; and (ii) anaerobiosis did not alter the extent of covalent binding but obliterated the inhibitory effect of CCl_4 on MT thiol content. Measurement of the thiol content of CCl_4-treated MT after treatment with 1,4-dithiothreitol revealed that all the thiol groups that were lost subsequent to CCl_4 treatment could be regenerated. These data suggest that CCl_4-linked oxidation of MT, rather than the covalent binding of $^{14}CCl_4$ metabolite(s), may be responsible for the CCl_4-induced loss of metal binding sites of MT with the concurrent release of Zn and Cd. However, the precise role of the metal released during the oxidation of MT in CCl_4 toxicity remains to be defined.

Metallothionein (MT) is a low molecular weight metal-binding protein important in the regulation of essential metal metabolism [1, 2], and in the detoxication of toxic metals, such as cadmium (Cd) [3, 4]. Mammalian MT contains 61 amino acids including 20 cysteines; evidence indicates that the metals are associated exclusively through thiolate bonds to all cysteine residues [5, 6]. The metals in MT are contained in two distinct, polynuclear clusters termed A and B in the presence of certain metal ions, such as zinc (Zn) and Cd. The A cluster contains 11 cysteines and binds four atoms of Zn or Cd, whereas the B cluster contains 9 cysteines and binds three atoms of Zn or Cd. All metal ions are tetrahedrally coordinated to four cysteinyl thiolate ligands [7–10].

Many studies have attributed the protective role of MT in radical-mediated toxicity to its high cysteine content [11–13]. Previous *in vivo* studies from our laboratory [14] have shown that the protective effect provided by Zn-induced hepatic MT against CCl_4 toxicity appeared to be associated with the reduction in the metal binding capacity of the protein and the loss of MT-bound Zn. This finding suggests that cysteinyl thiolates of MT may be the target of the toxic species resulting from the biotransformation of carbon tetrachloride (CCl_4).

CCl_4 is metabolized by the cytochrome P-450 monooxygenase system to the trichloromethyl radical ($CCl_3^{\cdot}$) [15] which may react with unsaturated lipids to form chloroform with the initiation of lipid peroxidation [16, 17]. Some investigators have postulated that CCl_4 causes its hepatotoxic effects by inducing lipid peroxidation [17, 18], whereas others have stressed that CCl_4 hepatotoxicity results from the covalent binding of the trichloromethyl radical to lipids and proteins [19, 20]. Reports from previous studies have also implicated the trichloromethylperoxyl radical ($CCl_3OO^{\cdot}$), formed from the reaction of $CCl_3^{\cdot}$ and oxygen, to be the more likely damaging species in aerated systems [21, 22].

The present study was undertaken to examine the biochemical basis for the protective role of MT in CCl_4 toxicity. Experiments were designed to characterize the reaction between CCl_4 or its metabolite(s) and Cd,Zn-MT-II, focusing on MT thiols as potential sites for interaction.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (300–325 g) obtained from Charles River Breeding Laboratories, Inc., Montreal, Quebec, were used throughout these studies. These animals were housed in polyethylene cages with free access to food (pelleted Purina Laboratory chow) and water. They were kept at room temperature (21–24°) and exposed to alternative cycles of 12 hr light and darkness.

Chemicals. NADPH and 1,4-dithiothreitol (DTT) were purchased from Boehringer Manheim (Montreal, Quebec, Canada). Chelex 100 (50–100 mesh) was purchased from BioRad (Mississauga, Ontario, Canada). Cadmium chloride ($CdCl_2$) was purchased from BDH Inc. (Toronto, Ontario, Canada). All

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other chemicals used were purchased from Sigma Chemicals (St Louis, MO, U.S.A.) or Fischer Scientific (Toronto, Ontario, Canada). Radioactive $^{14}CCl_4$ was purchased from NEN Research Products, Dupont (Boston, MA, U.S.A.). Sephadex G-75 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Isolation of metallothionein. Hepatic Cd,Zn-MT-II was isolated from adult male rats pretreated with $CdCl_2$ at 18 mg/kg body weight (s.c.) in six divided doses [23]. Each molecule of the purified Cd,Zn-MT-II contained 4.20 ± 0.10 g·atom Cd and 1.90 ± 0.18 g·atom Zn as measured by atomic absorption spectrophotometry. Copper bound to MT was below the level of detection as measured by atomic absorption spectrophotometry (limit of detection 0.01 μg/mL). The thiol content of this MT was 16.97 ± 0.43 thiol residues measured following 5,5-dithiobis-(2-nitrobenzoic acid) (NbS_2) titration [23].

Microsomal preparation. Hepatic microsomes were isolated from control adult male rats as previously described [14]. The washed microsomal pellet was resuspended in 150 mM Tris buffer, pH 7.4. The microsomal suspension (approximately 14 mg/mL) was stored at 4° and used within 2 hr for the *in vitro* study.

Biochemical and chemical analyses. The maximum Cd-binding capacity of MT was measured by the Cd–heme saturation method as described by Clarke and Lui [14].

Thiol content of MT was determined following the addition of a 0.2-mL sample to a 2.8-mL solution containing 0.27 mM NbS_2, 0.10 mM EDTA, 300 mM Tris–HCl buffer, pH 8.0, 1 M guanidine hydrochloride in a total volume of 3.0 mL [23]. All solutions were deaerated and kept under nitrogen in sealed cuvettes for 1 hr. The absorbance of 420 nm was read against a blank containing an equivalent sample of control incubation mixture without MT.

Thiobarbituric acid (TBA) reacting material was taken as a measure of lipid peroxidation and measured as described by Buege and Aust [24]. The change in absorbance at 535 nm was read against a blank containing an equivalent sample from a control incubation mixture.

Metal analysis was performed by the use of a Varian Techtron model AA-475 spectrophotometer using an air/acetylene flame.

Protein determinations were estimated by the method of Lowry *et al.* [25], using crystalline bovine serum albumin (BSA) as the standard.

Reaction of CCl_4 *with* Cd,Zn-*MT-II.* The standard reaction between CCl_4 and MT-II was carried out at 37° in a 25-mL Erlenmeyer flask in a final volume of 5.0 mL incubation mixture, containing approximately 1.5 mg washed microsomal protein, 0.64 mM NADPH, 0.40 mM $NADP^+$, 0.40 mM glucose-6-phosphate, 0.2 units glucose-6-phosphate dehydrogenase, 9.0 mM $MgCl_2$ and 30 mM Tris–HCl, pH 7.4. The incubations were performed with air or nitrogen as the gas phase in a metabolic shaker oscillating at 100 cycles/min. Following a 3-min preincubation, the reactions were initiated by the addition of sufficient CCl_4 in 200 μL of methanol to give a final concentration of 10 mM in the incubation mixture. Aliquots of the incubation mixture were removed at appropriate incubation times and the reaction was terminated by boiling for 30 sec. The samples were centrifuged at 600 *g* for 15 min in a Beckman TJ-6 centrifuge, and the supernatant fractions were used for the measurement of maximum Cd-binding capacity, thiol content of MT, and lipid peroxidation. Control aerobic incubations containing hepatic microsomes and an NADPH-regenerating system in the absence of CCl_4 were included to rule out the effect contributed by oxygen radicals produced by NADPH-dependent oxidase reactions.

To determine whether the effect of CCl_4 on MT was mediated by the chloroform–phosgene metabolic pathway, chloroform ($CHCl_3$) (10 mM final concentration) instead of CCl_4 was added to the standard incubation mixture.

To determine the effect of products of CCl_4-induced microsomal lipid peroxidation of MT, incubations were carried out in the presence of 25 μM promethazine, an inhibitor of lipid peroxidation [26].

In vitro *covalent binding of* $^{14}CCl_4$ *to MT. In vitro* covalent binding of $^{14}CCl_4$-derived radioactivity to MT was determined following the reaction of MT and $^{14}CCl_4$ (sp. act. 4.5 mCi/mmol) in an incubation mixture as described in the above. Non-MT bound radioactivity was removed by treating the heat-treated incubation mixture with 80% acetone. The acetone treatment resulted in the precipitation of MT and other proteinaceous components of the incubation mixture. The precipitated pellet was then dried under nitrogen and reconstituted to original volume with incubation buffer, followed by two washes with chloroform:methanol (2:1) mixture. Then the aqueous phase was saturated with 50 ppm Cd and chromatographed on a Sephadex G-75 column (1.5 cm × 55 cm) equilibrated with 10 mM Tris–HCl buffer (pH 8.6) as previously described [14]. Eluent fractions were collected (2 mL), and radioactivity was measured by scintillation counting using a Beckman LS-6800 scintillation counter. The acetone precipitation–extraction–evaporation procedure removed 95–98% of the radioactivity in blank incubations. Moreover, this procedure did not alter the thiol content and maximum Cd-binding capacity of MT; recovery of MT was >96%.

Reduction of CCl_4*-treated MT with* 1,4-*dithiothreitol.* To determine whether or not CCl_4 caused the oxidation of cysteinyl thiolate groups of MT, heat-treated supernatant fractions from control and CCl_4-treated incubations were incubated with 5 mM 1,4-dithiothreitol (DTT) under nitrogen at room temperature for 30 min. To determine the regeneration of Cd-binding sites, the samples were then treated with 50 ppm Cd and chromatographed on a Sephadex G-75 column (as described previously). The eluent fractions corresponding to the MT peak ($V/V_o = 2$) were pooled and dried under a stream of nitrogen and then reconstituted to original volume with 10 mM Tris–HCl buffer, pH 8.0, and used for Cd and thiol determination. Recovery of MT following this procedure was >95%.

Measurement of Cd *and* Zn *release from* CCl_4*-treated MT* in vitro. To measure the amounts of Cd and Zn released from MT during its reaction with

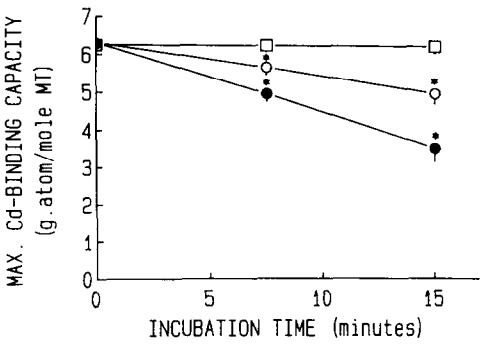

Fig. 1. Effect of CCl_4 on the maximum Cd-binding capacity of MT as a function of microsomal protein concentration. MT (30 μg/mL incubation mixture) was incubated in the absence of microsomes (□—□), or in the presence of 0.3 mg (○—○) or 1.2 mg (●—●) microsomal protein/mL incubation mixture, as described in Materials and Methods. Values are means ± SE from three experiments. Key: (*) significantly different from corresponding control values, $P < 0.05$.

CCl_4, heat-treated samples from control and CCl_4-treated incubations were treated with Chelex 100 (50 mg/mL, final concentration) for 6 min on ice to remove free metals [27]. The samples were centrifuged at 600 *g* for 15 min, and metal analyses were performed on the supernatant fractions. This procedure itself did not affect the Cd-binding capacity of Cd,Zn-MT.

Statistics. Statistical evaluation of data was performed by Student's *t*-test [28]. The level of statistical significance was chosen at $P < 0.05$.

RESULTS

As shown in Fig. 1, CCl_4 reduced the maximum Cd-binding capacity of MT as determined by the Cd–heme saturation method. The observed reductions were not due to oxygen radicals produced by NADPH-dependent oxidase reactions, since control incubation carried out in the absence of CCl_4 failed to show any effect (see Fig. 2A). It is apparent that the reduction required metabolic activation of CCl_4, since no significant decreases in binding were observed in the absence of microsomes. Furthermore, the rate of decrease in the maximum Cd-binding capacity of MT appeared to be a function of microsomal protein concentration (Fig. 1). The decrease in the maximum Cd-binding capacity of MT was linear with time during the first 15 min of incubation, but thereafter no further reduction was observed; however, addition of fresh microsomes at 15 min of incubation resulted in a further decrease in the maximum Cd-binding capacity of MT at a rate that was similar to that of the initial reaction (data not shown). This may be due to the "suicidal" effect of CCl_4 metabolites on cytochrome P-450, thereby limiting further bioactivation of CCl_4 [29, 30].

As shown in Fig. 2, the CCl_4-induced time-dependent reduction in the maximum Cd-binding capacity of MT (Fig. 2A) was accompanied by a concurrent decrease in thiol content (Fig. 2B). A significant positive linear relationship was observed between the maximum Cd-binding capacity and thiol content of MT (correlation coefficient, $r = 0.825$), suggesting that these two parameters may be related. Since CCl_4 decreased the metal binding sites on MT, its effect on MT-bound Zn and Cd was also examined. As shown in Table 1, the presence of CCl_4 resulted in the loss of Cd and Zn from MT, the loss of Zn being greater than that of Cd within the 15-min incubation.

To examine whether or not the reduction in the thiol content and maximum Cd-binding capacity of MT was mediated by certain metabolites of CCl_4, the effect of $CHCl_3$ (10 mM), an intermediary metabolite of CCl_4 in the formation of phosgene [31], was examined. Results showed that $CHCl_3$ in the presence of the microsomal activating system did not alter significantly the maximum Cd-binding capacity of MT (data not shown). Since the trichloromethyl radical is generated under both aerobic [15, 32] and anaerobic [33] conditions, the examination of the effect of CCl_4 on the thiol content of MT under nitrogen should allow us to ascertain the role of the trichloromethyl radical. As shown in Fig. 2, no apparent changes were observed under anaerobic conditions, suggesting that the trichloromethyl radical was not directly involved in the reduction of the thiol content and maximum Cd-binding capacity of MT.

Data concerning the effect of CCl_4-induced lipid peroxidation on MT are shown in Fig. 3. Carbon tetrachloride produced a time-dependent increase in microsomal lipid peroxidation as measured by the elevation of the absorbance of thiobarbituric acid reactants at 535 nm (Fig. 3A). Promethazine, which completely blocked the CCl_4-induced lipid peroxidation, did not alter the inhibitory effect of CCl_4 on the maximum Cd-binding capacity of MT (Fig. 3B).

It has been suggested that covalent binding of CCl_4 metabolites to proteins and lipids is a mechanism by which CCl_4 exerts its toxicity [19, 20]. Accordingly, the role of covalent binding of CCl_4-derived radioactivity in the inhibitory effect of CCl_4 on MT thiol content was investigated. Data presented in Table 2 show the amount of covalent binding of $^{14}CCl_4$ metabolite(s) to MT occurring under aerobic conditions. To ascertain whether or not the covalent binding of $^{14}CCl_4$ occurred at the cysteinyl thiolate groups, MT that was pretreated with hydrogen peroxide (H_2O_2) to oxidize the thiolates to disulfides was used instead of the control MT. In these experiments H_2O_2 completely depleted the thiol groups and maximum Cd-binding capacity of MT, but apparently this did not affect the covalent binding of $^{14}CCl_4$ to MT (Table 2). Moreover, anaerobiosis did not change the magnitude of covalent binding but prevented CCl_4-induced decreases in thiol content of MT, suggesting that the cysteinyl thiolate groups did not participate appreciably in the covalent binding of CCl_4 metabolites. To further investigate whether or not covalent binding of $^{14}CCl_4$-derived radioactivity to MT was sulfhydryl-specific, the binding to BSA was also examined. Although the covalently bound $^{14}CCl_4$-derived radioactivity per molecule of protein was higher in BSA than MT, no difference was

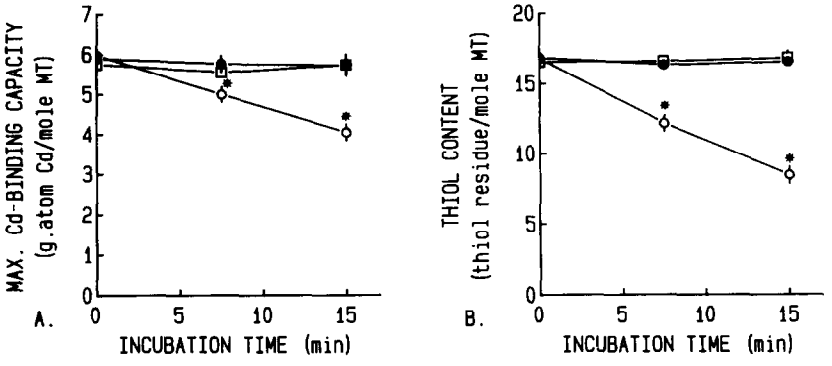

Fig. 2. Effect of CCl_4 on the maximum Cd-binding capacity (A) and thiol content (B) of MT (30 μg/mL incubation mixture) under air (○—○) or nitrogen (●—●). Control incubations (□—□) were performed under air in the absence of CCl_4. Incubations were carried out as described in Materials and Methods. Values are means ± SE from three experiments. Key: (*) significantly different from corresponding control values, $P < 0.05$.

Table 1. CCl_4-mediated release of Cd and Zn from MT*

Incubation condition	Treatment	Cd (g·atom metal/mol MT) (0-min incubation)	Zn (g·atom metal/mol MT) (0-min incubation)	Cd (g·atom metal/mol MT) (15-min incubation)	Zn (g·atom metal/mol MT) (15-min incubation)
Air		4.10 ± 0.10	2.00 ± 0.14	3.95 ± 0.20	1.87 ± 0.15
Air	CCl_4	4.05 ± 0.15	2.00 ± 0.10	3.55 ± 0.25†	0.90 ± 0.20†
Nitrogen	CCl_4	4.10 ± 0.05	1.95 ± 0.10	4.00 ± 0.15	1.90 ± 0.10

* CCl_4-induced metal release from MT was measured with Chelex 100 as described in Materials and Methods (30 μg MT/mL of incubation mixture). Values are means ± SE from three experiments.
† Significantly different from corresponding time zero values, $P < 0.05$.

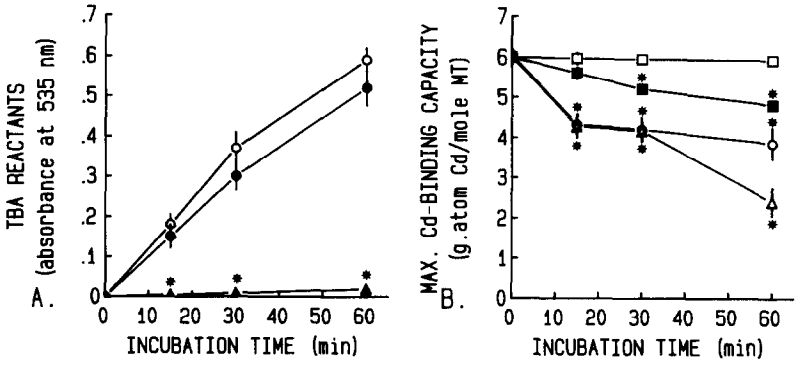

Fig. 3. Influence of promethazine on the CCl_4-mediated changes in microsomal lipid peroxidation (A) and maximum Cd-binding capacity of MT (B) (30 μg MT/mL incubation mixture). Incubations were carried out as described in Materials and Methods. (A) TBA reactants were measured at different times following the incubation of microsomes with 10 mM CCl_4 alone (●—●) and in the presence of MT (○—○) and MT plus 25 μM promethazine (▲—▲). Microsomal lipid peroxidation was not detected in the presence of MT or promethazine alone. (B) The effect of microsomal lipid peroxidation on the maximum Cd-binding capacity of MT was measured in the absence of CCl_4 (□—□) and in the presence of 25 μM promethazine (■—■) or 10 mM CCl_4 (○—○) plus 25 μM promethazine (△—△). Values are means ± SE from three experiments. Key: (*) significantly different from corresponding control values, $P < 0.05$.

apparent in the binding per unit weight of the two proteins, reflecting perhaps the approximately 10-fold difference in their molecular weight (Table 2).

Data presented in Table 3 show the effect of treatment of CCl_4-treated MT with the reducing agent DTT on the thiol content and maximum Cd-binding capacity of MT. Incubation of control MT with DTT resulted in increases in the thiol content

Table 2. Covalent binding of $^{14}CCl_4$ and the effect of CCl_4 on the thiol content and maximum Cd-binding capacity*

Incubation condition†	Covalent binding		Max. Cd-binding capacity‡ (g·atom/mol MT)	Thiol content§ (thiol residue/mol MT
	μmol CCl_4/mg MT or BSA	mol CCl_4/mol MT or BSA		
MT (control)				
Time zero	ND‖	ND	6.12 ± 0.30	16.00 ± 0.70
Omit NADPH	ND	ND	6.00 ± 0.25	16.00 ± 0.45
Omit microsomes	ND	ND	6.15 ± 0.15	16.10 ± 0.30
MT				
Air	1.65 ± 0.16	10.00 ± 1.10	3.67 ± 0.40¶	10.80 ± 0.80¶
N_2	1.70 ± 0.19	10.30 ± 1.25	5.99 ± 0.20	15.57 ± 0.60
H_2O_2-treated MT	1.52 ± 0.20	9.21 ± 1.40	ND	0.64 ± 0.10¶
BSA	1.80 ± 0.28	107.00 ± 18.70**		

* Values are means ± SE from three experiments.

† Incubations were carried out as described in Materials and Methods, and a single 30-min incubation period was used unless otherwise stated (20 μg MT or BSA/mL of incubation).

‡ Estimated by the Cd–heme saturation method.

§ Estimated by titration with NbS_2.

‖ Not detected.

¶ Significantly different from corresponding control values, $P < 0.05$.

** Significantly different from the binding to MT under air atmosphere, $P < 0.05$.

Table 3. 1,4-Dithiothreitol-dependent regeneration of thiol content and maximum Cd-binding capacity of CCl_4-treated MT*

Pre-incubation with CCl_4†	DTT treatment	Thiol content (thiol residue/mol MT) (0-min incubation)	Max. Cd-binding capacity (g·atom/mol MT) (0-min incubation)	Thiol content (thiol residue/mol MT) (15-min incubation)	Max. Cd-binding capacity (g·atom/mol MT) (15-min incubation)
– Air	–	16.20 ± 0.40	5.90 ± 0.20	16.20 ± 0.30	5.90 ± 0.15
	+	19.00 ± 0.80	6.70 ± 0.30	19.10 ± 0.55	6.73 ± 0.25
+ Air	–	16.35 ± 0.35	5.93 ± 0.15	10.12 ± 0.90‡	3.57 ± 0.40‡
	+	18.88 ± 0.20	6.60 ± 0.25	18.50 ± 0.75§	6.30 ± 0.25§
+ N_2	–	16.30 ± 0.25	5.98 ± 0.20	16.28 ± 0.40	5.96 ± 0.15
	+	18.95 ± 0.40	6.68 ± 0.25	18.75 ± 0.55	6.48 ± 0.40

* Thiol content was estimated by NbS_2 titration. Maximum Cd-binding capacity was estimated by the Cd-heme saturation method. Values are means ± SE from three experiments.
† Incubations were carried out in the absence (–) or presence (+) of CCl_4 as described in Materials and Methods (30 μg MT/mL of incubation mixture).
‡ Significantly different from corresponding time zero values, $P < 0.05$.
§ Significantly different from corresponding non-DTT-treated samples, $P < 0.05$.

and maximum Cd-binding capacity of MT, presumably due to the restoration of thiolate groups that were oxidized during the initial isolation and purification steps. Incubation of CCl_4-treated MT with DTT restored its thiol content and maximum Cd-binding capacity to control MT levels.

DISCUSSION

The data obtained in the present study show that treatment of hepatic Cd,Zn-MT-II with CCl_4 *in vitro* resulted in reduction in the thiol content of MT (Fig. 2B). Since all thiolate groups are known to participate in the metal-binding of MT [5, 6], the reduction in the thiol groups of MT may explain the decreases in the maximum Cd-binding capacity of MT (Fig. 2A) and the release of Cd and Zn from MT (Table 1). Our *in vitro* data also corroborate with our previous observation of the loss of Zn from Zn-induced hepatic MT and the reduction in the maximum Cd-binding capacity of the protein following the administration of CCl_4 to Zn pretreated rats *in vivo* [14]. However, the *in vitro* effects on the metal binding of MT are not unique to CCl_4. Previous *in vitro* studies have shown that oxygen reactive species formed during radiolysis [11] or other forms of oxidative stress [12, 34] react with MT, resulting in the oxidation of the protein thiols to disulfides with the subsequent loss of MT-bound metals. It is known that alkylation of the cysteinyl thiolate groups on MT by methyl bromide [35], iodoacetamide [36], or mercuribenzoate [37] *in vitro* also result in the release of MT-bound metals.

The mechanism(s) underlying the observed destruction of CCl_4 on MT thiols is not clear. The data in the present work suggest that this effect on MT required metabolic activation of CCl_4 (Fig. 1) which is consistent with the findings of the involvement of CCl_4 reactive intermediates in mediation of its toxicity [16–20]. Electrophiles and other chemically-reactive intermediates react with nucleophilic sites on endogenous and exogenous compounds [38]; however, several of the known toxic metabolites of CCl_4, such as trichloromethyl radical, chloroform and phosgene, do not appear to be directly responsible for the CCl_4-induced depletion of MT thiolate groups. Nevertheless, the requirement for aerobic conditions in the induction of damage to MT would suggest the involvement of the highly reactive trichloromethylperoxyl radicals, which are generated from trichloromethyl radicals in the presence of oxygen [21, 22].

Covalent binding of CCl_4-derived metabolites to tissue macromolecules has been implicated as a possible mechanism for CCl_4 to exert its toxicity [19, 20]. On the basis of the observed covalent binding of $^{14}CCl_4$-derived radiolabel to MT, Cagen and Klaassen [13] postulated that the protective effect provided by Zn-induced hepatic MT against $^{14}CCl_4$ toxicity was due to the scavenging of $^{14}CCl_4$ metabolites by MT thiolate groups. Although covalent binding of $^{14}CCl_4$ to MT was detected in the *in vitro* study (Table 2), it does not account for the decreases in the reduced sulfhydryl content for the following reasons: (i) prior oxidation of the sulfhydryl groups of Cd,Zn-MT-II by H_2O_2 did not alter the extent of

covalent binding of $^{14}CCl_4$ to this MT; (ii) anaerobiosis did not alter the covalent binding of $^{14}CCl_4$ to MT but obliterated the effect of CCl_4 on the maximum Cd-binding capacity and the total thiol content of MT; and (iii) the extent of covalent binding per unit weight of protein to MT and to BSA (a high molecular weight protein containing a single free sulfhydryl group) was similar, suggesting that covalent binding of $^{14}CCl_4$ metabolite(s) to MT was not sulfhydryl-specific.

The ability of CCl_4 to induce microsomal lipid peroxidation is well established [16–18], and this mechanism has also been implicated in its hepatotoxicity [17, 18]. Carbon tetrachloride-induced lipid peroxidation has been shown to generate a variety of degradative products originating from membrane lipids, and these chemically-reactive "secondary messengers" [17] are known to be reactive toward sulfhydryl compounds [39]. As shown in Fig. 3, the CCl_4-induced reduction in the maximum Cd-binding capacity or thiol content of MT was not mediated by products of CCl_4-induced lipid peroxidation, as promethazine which was effective in inhibiting CCl_4-induced microsomal lipid peroxidation, as demonstrated in Fig. 3A and other previous studies [26], did not modify the action of CCl_4 on MT.

The regeneration of the thiol content as well as the maximum Cd-binding capacity of CCl_4-treated MT provides evidence supporting the CCl_4-linked oxidation of MT. Although the mechanism underlying the CCl_4-linked oxidation of MT thiolate groups is not known, it has been reported that protein and non-protein thiol groups are readily oxidized by many free radicals and the thiyl ($RS^{\cdot}$) radical produced may dimerize, resulting in disulfide formation [40]. Since the loss of MT thiols does not appear to be due to lipid peroxidation, and since it did not occur under anaerobic conditions, it is likely that the trichloromethyl peroxyl radicals are involved in the oxidation of MT sulfhydryl groups.

In summary, our data suggest that CCl_4-induced oxidation of MT rather than the covalent binding of CCl_4 metabolite(s) is responsible for the CCl_4-induced loss of metal binding sites of Cd,Zn-MT with the concurrent release of Zn and Cd. The precise role of the metal released during the oxidation of MT in CCl_4 toxicity, however, remains to be clarified.

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