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Role of Metallothionein in Zinc(II) and Chromium(III) Mediated Tolerance to Carbon Tetrachloride Hepatotoxicity: Evidence against a Trichloromethyl Radical-Scavenging Mechanism

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The $^{\bullet}CCl_3$ radical generated during the metabolism of CCl_4 is readily spin trapped in vivo and in vitro by phenyl *N-tert*-butylnitrone (PBN) to form the stable PBN/$^{\bullet}CCl_3$ radical adduct, which can then be extracted into organic solvents and detected by ESR spectroscopy. We have used this technique to examine the proposed protective roles of Zn(II), Cr(III), and metallothionein (MT) against carbon tetrachloride toxicity in vivo. Hepatic MT, which is induced by Zn(II), has been proposed to protect against CCl_4-induced cellular damage by scavenging the free radical metabolites formed. CCl_4-induced hepatotoxicity was significantly suppressed in male Sprague-Dawley rats pretreated with a single dose of 5 mg/kg Zn(II) or Cr(III) according to standard serum assays for liver-specific enzymes, and hepatic MT was elevated after pretreatment with either Zn(II) or Cr(III). In vitro, no difference was detected in either the amount of CCl_4-derived free radical metabolites formed or the rate at which they were formed by microsomes from rats pretreated 24 h in advance with 5 mg/kg Zn(II) or Cr(III). Extraction of rat liver with 2:1 chloroform/methanol 1 h after the administration of a 0.8 mL/kg intraperitoneal or intragastric dose of CCl_4 also revealed no difference in the amount of trichloromethyl radical spin trapped in vivo following pretreatment with either Zn(II) or Cr(III). These results suggest that pretreatment with either Zn(II) or Cr(III) does not affect CCl_4 metabolism nor does the MT significantly scavenge the trichloromethyl free radical metabolite.

Introduction

The metabolism of carbon tetrachloride has been studied in some detail as a model for the hepatic metabolism of haloalkanes. It is now well established that carbon tetrachloride is metabolized by the cytochrome P450 mixed-function oxidase system in the smooth endoplasmic reticulum to the trichloromethyl ($^{\bullet}CCl_3$) free radical (*1*, *2*), particularly by the cytochrome $P450_{2E1}$ isozyme[2] (*3*, *4*). The $^{\bullet}CCl_3$ radical metabolite may then react with other cellular components directly or may combine with O_2 to form the trichloromethylperoxyl ($^{\bullet}OOCCl_3$) radical (*5*). These radical metabolites may cause damage by direct covalent binding to subcellular components or by initiation of lipid peroxidation. The initial free radical reactions also trigger a series of pathobiochemical responses within the cell which may themselves be responsible for eventual cell death (*6–9*).

Several studies have shown that the pretreatment of rats with Zn(II) protects against carbon tetrachloride toxicity (*10–13*). Among the various criteria used to assess the extent of CCl_4-induced liver damage were elevations in hepatic malonaldehyde, an index of lipid peroxidation, and in serum alanine aminotransferase and aspartate aminotransferase activities. Recently, a single intraperitoneal dose of Cr(III), but not Cr(VI), Cu(II), or Zn(II), was reported to protect both rats and mice against a lethal intragastric dose of carbon tetrachloride (*14*). This latter report was based on results where 4 out of 10 of the Cr(III)-treated rats survived 2 weeks after carbon tetrachloride administration, whereas none survived in any of the other metal-treated or control groups.

Hepatic MT,[3] which is significantly elevated upon Zn(II) administration, has been proposed to protect against cellular damage by scavenging the CCl_4-derived free radical metabolites (*12*, *13*). Even induction of hepatic MT as an inflammatory response to an intramuscular injection of turpentine in rats has been shown to coincide with reduced hepatotoxicity from carbon tetrachloride (*15*). About 30% (or 20) of the 61 amino acids which make up the MT sequence are cysteine (*16*, *17*). This high sulfhydryl content enables MT to efficiently scavenge oxy-radicals in vitro (*18*, *19*). An alternative protective mechanism of MT may be its ability to release Zn(II) for binding at sites on membrane surfaces, displacing adventitious iron and thereby inhibiting lipid peroxidation (*20*). In one report, however, it is unclear why Zn(II) pretreatment had little protective effect against a lethal dose of CCl_4 compared to Cr(III) pretreatment (*14*).

The transient $^{\bullet}CCl_3$ radical generated during the metabolism of CCl_4 is readily spin trapped in vitro and in vivo by PBN to form the stable PBN/$^{\bullet}CCl_3$ radical adduct (*21–23*). Once trapped in vivo, the radical adduct can be extracted into organic solvents and examined by ESR spectroscopy (*24*). We have used these techniques to

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[2] Also known as cytochrome $P450_j$ in rats.

[3] Abbreviations used: MT, metallothionein; PBN, phenyl *N-tert*-butylnitrone; ig, intragastric; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; SDH, sorbitol dehydrogenase.

examine the proposed protective roles of Zn(II), Cr(III), and MT against carbon tetrachloride toxicity in vivo, particularly the ability of MT to scavenge the trichloromethyl metabolite. The results suggest that metallothionein does not play a significant role in scavenging the trichloromethyl radical.

Materials and Methods

Materials. Carbon tetrachloride (HPLC grade) and cadmium chloride (99.99%) were purchased from Aldrich (Milwaukee, WI). PBN, NADPH (tetrasodium, reduced form, type X), and bovine hemoglobin were purchased from Sigma (St. Louis, MO). [^{13}C]Carbon tetrachloride (99% minimum ^{13}C) was purchased from Isotec, Inc. (Miamisburg, OH), and [^{109}Cd]cadmium chloride (100 mCi) was purchased from Amersham (Arlington Heights, IL). Phosphate buffer, pH 7.4, prepared from a combination of the mono- and disodium salts, was incubated with preequilibrated Chelex 100 resin (Bio-Rad, Richmond, CA) to remove trace heavy metal contaminants. All other chemicals were used without further purification.

In Vivo Experiments. Male 400 ± 40 g Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) were used for all in vivo experiments and were raised on a standard chow mix (NIH Open Formula; Zeigler Brothers, Gardner, PA) with free access to both food and water. Food, but not water, was removed 24 h prior to experiments where CCl_4 was administered as an intragastric (ig) injection.

At the specified time prior to sample collection, rats were given an ip injection of either 5 mg of Zn(II) (as the sulfate dissolved in deionized water)/kg of rat body weight, 5 mg of Cr(III) (as the nitrate also in deionized water)/kg of rat body weight, or an equivalent volume (1 mL/kg) of 0.85% saline. CCl_4 (0.80 mL/kg of rat body weight[4]) was administered either ip as a 1:2 CCl_4/olive oil solution before anesthesia or ig neat after anesthesia. Controls received either pure olive oil ip or pure water ig. For in vivo spin trapping experiments, an ip dose of 70 mg/kg (as a 28 mg/mL solution) or 7 mg/kg (as a 7 mg/mL solution) of PBN was also administered 1 h prior to sample collection.

For experiments where CCl_4 was administered ip, rats were anesthetized with ether just prior to sample collection at 1, 8, and 24 h post-CCl_4 injection. Blood was withdrawn from the aorta abdominalis for serum analyses, and the liver was removed, rinsed, and frozen immediately on dry ice.

For experiments where CCl_4 was administered ig, the rats were first anesthetized with a 50–75 mg/kg ip injection of Nembutal (Abbott Laboratories), which was generally sufficient to maintain anesthesia throughout the experiment, then given an ig injection of either CCl_4 or water. One hour post-CCl_4 injection, blood was withdrawn for serum analyses and the livers were removed and either used immediately to prepare microsomes for cytochrome P450 analyses and in vitro ESR experiments or frozen on dry ice for further assays and extraction experiments. The rats were killed by exsanguination.

Microsomal Experiments. Liver microsomes were prepared as previously described (*26*). Briefly, livers were weighed, rinsed, and homogenized in ice-cold 0.1 M sodium phosphate, pH 7.4. The homogenate was centrifuged at 10000*g* for 20 min, and microsomes were separated from the resulting supernatant by centrifugation at 165000*g* for 35 min. The microsomal pellet was washed once by resuspension in ice-cold 0.1 M sodium phosphate, pH 7.4, and recentrifuged at 165000*g*. Following the final suspension in 0.1 M sodium phosphate, pH 7.4, microsomal protein concentrations were determined using the method of Lowry *et al.* (*27*). Cytochrome P450 content was measured according to the method of Omura and Sato (*28*).

Extraction of Liver. Liver tissue was extracted similar to the method of Folch *et al.* (*29*). Liver tissue (3–5 g) was homogenized directly into 5 volumes of an ice-cold 2:1 chloroform/methanol mixture. The homogenate was then washed with 0.2 mL of 0.85% saline/mL of homogenate and centrifuged for 15 min to separate the aqueous and organic phases. The organic phase was collected, dried with anhydrous sodium sulfate, and concentrated for ESR measurements by blowing a steady stream of N_2 over the solution. The final volumes were scaled to 0.25 or 1 mL/g of homogenized liver tissue from rats given 7 or 70 mg/kg PBN, respectively. This solution (0.8–1.0 mL) was transferred to a quartz ESR flat cell (Wilmad, Buena, NJ) and bubbled very gently with N_2 for 2 min to remove O_2 before the ESR spectrum was recorded.

Biochemical Assays. Hepatic MT levels were measured as the Cd-binding capacity of liver homogenate according to the procedure of Onosaka *et al.* (*30*) as described by Eaton and Toal (*31*). Cd binding was measured by the activity of the γ-emitter ^{109}Cd.

Serum activities of lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and sorbitol dehydrogenase (SDH) and the total serum concentration of bile acids were measured according to published procedures (*32–35*).

Analysis of variance (ANOVA) statistical methods were applied to the results of the biochemical assays. Two-way factorial ANOVAs were used in the zinc studies to assess the effects of experimental treatments and duration of treatment, and their interaction. One-way ANOVAs were used in the chromium studies. The natural logarithm was used as a variance stabilizer prior to analysis. Following the application of the ANOVA procedure, pairwise comparisons were made using the Ryan–Einot–Gabriel–Welsch multiple range test (*36*).

Instrumentation. ESR spectra for the microsomal experiments were recorded on a Bruker ER200D spectrometer operating at a microwave frequency of 9.77 GHz and a microwave power of 20 mW. ESR spectra for the organic extraction experiments were recorded on a Varian E-109 spectrometer operating at a microwave frequency of 9.33 GHz and a microwave power of 20 mW. Spectra were recorded using a quartz flat cell (Wilmad) in a TM_{110} microwave cavity. Other instrumental parameters are given in the figure legends. Spectra were recorded on an IBM-compatible computer interfaced with the spectrometers.

A Shimadzu UV 3000 spectrophotometer was used for the cytochrome P450 assay of Omura and Sato (*28*). All other spectrophotometric data were obtained using a Beckman DU-7 spectrophotometer. The ^{109}Cd binding to MT was determined using a Beckman Gamma 5500 radiation counter with a DP 5500 data reduction system.

Results

Effect of Zn(II) and Cr(III) Treatment on CCl_4 Hepatotoxicity. The hepatotoxicity of CCl_4 was assessed by the serum activities of LDH, ALT, and SDH. Only 1 h after an ip injection of 0.8 mL/kg CCl_4, the activities of all three hepatic enzymes were elevated in the serum (Table I). Their serum activities remained highly elevated 24 h after the CCl_4 injection, indicating significant damage to the liver. Serum activity of LDH was also significantly elevated within the first hour following the ig administration of 0.8 mL/kg CCl_4, similar to the results following ip administration, but the SDH activity remained unchanged (Table I).

Pretreatment by a single ip dose of 5 mg/kg Zn(II) 24 h prior to the injection of CCl_4 imparted significant protection against initial CCl_4-induced liver damage (Table I). Nevertheless, 24 h after the CCl_4 injection, serum activities of all three hepatic enzymes were significantly elevated over control levels. Pretreatment 24 h beforehand with an ip injection of 5 mg/kg Cr(III) instead of Zn(II) also significantly protected against hepatic damage within

[4] Or 1275 mg/kg. The oral LD_{50} for carbon tetrachloride is 2820 mg/kg for rats and 1500 mg/kg ip (*25*).

Table I. Effect of a Single Dose of Zn(II) or Cr(III) on CCl_4 Hepatotoxicity (±SE)[a]

treatment	time (h)	LDH (IU/L)	ALT (IU/L)	SDH (IU/L)	total bile acids (μmol/L)
control[b]		178 ± 36	36.8 ± 1.5	11.8 ± 0.9	26.4 ± 8.3
CCl_4[c]	1	794 ± 174[e]	58.0 ± 4.9	35.0 ± 3.9[e]	18.4 ± 4.9
CCl_4[c]	8	1468 ± 430[e]	176.3 ● 30.1[e]	322.5 ± 94.3[e]	119.2 ± 29.5[e]
CCl_4[c]	24	960 ± 146[e]	211.8 ± 96.7[e]	239.3 ± 95.8[e]	102.3 ± 30.1[e]
Zn(II)/CCl_4[c]	1	303 ± 34	46.3 ± 7.3	31.8 ± 7.8[e]	16.4 ± 1.6
Zn(II)/CCl_4[c]	8	427 ± 132[f]	64.5 ± 15.3	72.0 ± 13.8[e,f]	53.6 ± 3.6[e]
Zn(II)/CCl_4[c]	24	333 ± 27[f]	377.3 ± 156.3[e]	233.3 ± 11.7[e]	166.8 ± 24.4[e]
CCl_4[d]	1	1018 ± 277[e]		17.0 ± 6.2	23.3 ± 1.4
Cr(III)/CCl_4[d]	1	485 ± 44[e]		16.3 ± 3.2	22.0 ± 1.2

[a] The Zn(II) or Cr(III) was administered ip 24 h prior to CCl_4 administration. Serum was collected for assays at the times indicated following CCl_4 administration. [b] Rats were anesthetized with ether (N = 4). [c] 0.8 mL/kg CCl_4 administered ip. Rats were anesthetized with ether (N = 4). [d] 0.8 mL/kg CCl_4 administered ig. Rats were anesthetized with Nembutal (N = 4). [e] Values are significantly different ($p < 0.05$) from the control. [f] Values are significantly different ($p < 0.05$) from corresponding time points of CCl_4-treated animals.

Table II. Effect of a Single Dose of Zn(II) on CCl_4 Hepatotoxicity (±SE)[a]

treatment[b]	time (h)	ALT (IU/L)	SDH (IU/L)	total bile acids (μmol/L)
control		38.0 ± 3.7	13.0 ± 1.6	33.6 ± 6.5
Zn(II)[c]	1	39.4 ± 2.6[e]	21.8 ± 3.6[e]	30.9 ± 1.7
CCl_4[c]	1	104.4 ± 6.9[f]	80.6 ± 7.6[f]	50.6 ± 3.4[f]
Zn(II)/CCl_4[d]	1	65.4 ± 11.6[f]	43.0 ± 8.0[f]	33.7 ± 4.9

[a] The Zn(II) was administered ip simultaneously with CCl_4, and the serum was collected 1 h later for assays. [b] Rats were anesthetized with ether (N = 5). [c] 5 mg/kg Zn(II) or 0.8 mL/kg CCl_4 administered ip. [d] 5 mg/kg Zn(II) and 0.8 mL/kg CCl_4 administered ip simultaneously. [e] Values are significantly different ($p < 0.05$) from the CCl_4-treated and Zn(II)/CCl_4-treated groups. [f] Values are significantly different ($p < 0.05$) from all groups.

the first hour of CCl_4 treatment, similar to the effect of Zn(II) pretreatment (Table I).

In all cases, the total serum concentration of bile acids remained unchanged within the first hour following CCl_4 administration but were elevated after 24 h. Elevated levels of bile acids in serum is a clinical indication of cholestasis. Zn(II) pretreatment caused no significant protection against elevated bile acid concentrations in the serum after the first hour (Table I).

When Zn(II) and CCl_4 were administered ip simultaneously, CCl_4-induced hepatotoxicity was also diminished relative to treatment with CCl_4 alone (Table II). In this case, the Zn(II) was injected as a 20 mg/mL solution to minimize the volume of fluid entering the ip cavity. The results in Table II suggest that the protective mechanism of Zn(II) does not involve the induction of protein synthesis.

Effect of Zn(II), Cr(III), and CCl_4 on Hepatic Metallothionein Concentration. Hepatic concentrations of MT were dramatically elevated following the ip administration of either Zn(II), Cr(III), or CCl_4. As shown in Figure 1, 24 h after the ip injection of 5 mg/kg Zn(II), hepatic MT was induced 1700% over saline-treated controls according to the ^{109}Cd-binding assay. After 48 h, however, MT concentrations once more approached control levels. Similarly, Cr(III) administration ip induced MT concentrations as much as 640% after 24 h (Figure 2). CCl_4 administration ip also induced hepatic MT by 575% after 24 h (Figure 1), which may be due to an inflammatory response (*37*). In contrast, administration of CCl_4 ig did not cause a significant elevation of hepatic MT concentrations (data not shown).

Effect of Zn(II) and Cr(III) Treatment on CCl_4 Metabolism. Carbon tetrachloride is metabolized to the trichloromethyl radical by the cytochrome P450 enzymatic system (*22*). A suppression of this CCl_4-activating enzymatic system may account for the protective effects of

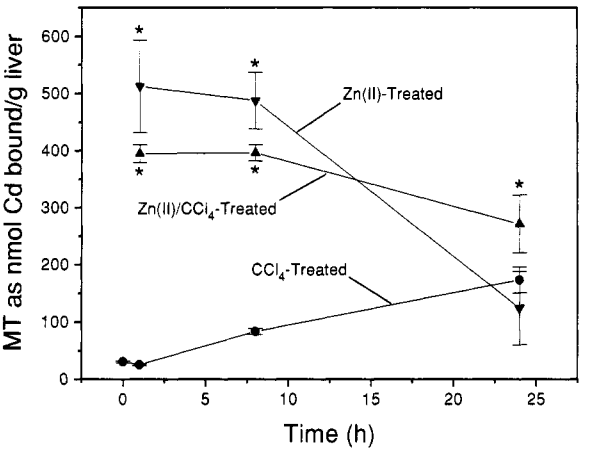

Figure 1. Effect of CCl_4 administration on hepatic MT concentrations. CCl_4 (0.8 mL/kg, ip) was administered at 0 h. Zn(II) (5 mg/kg, ip) was administered 24 h before the CCl_4. The MT concentrations (±SE, N = 4) at the times indicated were determined by the modified ^{109}Cd/hemoglobin method described by Eaton and Toal (*31*). Asterisk indicates significant difference ($p < 0.05$) from the control at 0 h.

Zn(II) or Cr(III) against CCl_4 hepatotoxicity. It has been shown previously, however, that a single dose of Zn(II) does not suppress cytochrome P450 in rats (*12, 38, 39*). In accord with these reports, no change in the hepatic cytochrome P450 concentration was observed 25 h following treatment with 5 mg/kg Zn(II) (Table III). Treatment with 5 mg/kg Cr(III) also had no significant effect on hepatic cytochrome P450 concentration (Table IV) (*14*).

The lack of a significant effect in vivo on CCl_4 metabolism by Zn(II) or Cr(III) pretreatment was also supported by in vitro spin trapping experiments using NADPH-supplemented microsomes prepared from Zn(II)- and Cr(III)-treated rats. The ESR spectrum of a 30-min incubation of NADPH, PBN, CCl_4, and microsomes from nontreated rats in 0.1 M sodium phosphate, pH 7.4, under a stream of N_2 is shown in Figure 3A. The hyperfine coupling constants obtained were a^N = 14.02 G and a_β^H = 1.60 G, with an additional coupling of a_β^{13C} = 9.64 G when $^{13}CCl_4$ was used (Figure 3B). Both parts B and C of Figure 3 demonstrate that the predominant ESR signal was derived from a CCl_4-dependent carbon-centered radical metabolite as reported previously (*22*). The small underlying, ^{13}C-independent signals in parts A and B of Figure 3 can be simulated using the parameters for lipid-derived alkyl and alkoxyl adducts of PBN previously reported (*40*). Lipid-derived radicals are presumably formed by CCl_4-initiated lipid peroxidation.

From the result shown in Figure 3D, it is evident that after 30 min there was little difference in CCl_4 metabolism

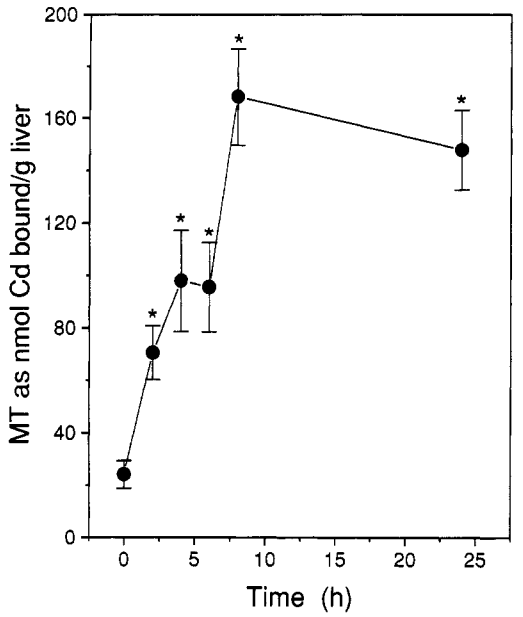

Figure 2. Induction of hepatic metallothionein (MT) following a single ip dose of 5 mg/kg Cr(III). The MT concentrations (±SE, $N = 4$) at the times indicated were determined by the modified ^{109}Cd/hemoglobin method described by Eaton and Toal (*31*). Asterisk indicates significant difference ($p < 0.05$) from the control at 0 hour.

Table III. Effect of Zn(II), CCl_4, and Zn(II)/CCl_4 Treatments on Hepatic Cytochrome P450 Concentration

treatment[a]	time (h)	cytochrome P450[b]
control		100 ± 22
Zn(II)[c]	1	107 ± 5
CCl_4[d]	1	69 ± 11
CCl_4[d]	8	57 ± 6
CCl_4[d]	24	43 ± 7[e]
Zn(II)/CCl_4[c]	1	56 ± 4
Zn(II)/CCl_4[c]	8	52 ± 7
Zn(II)/CCl_4[c]	24	44 ± 5[e]

[a] Rats anesthetized with ether. [b] Values are reported as % of control value (0.56 nmol/mg microsomal protein; ±SE, $N = 4$). [c] Zn(II) administered (5 mg/kg ip) 24 h before recorded time or CCl_4 administration (0.8 mL/kg, ip). [d] 0.8 mL/kg administered ip. [e] Values are significantly different ($p < 0.05$) from the control.

Table IV. Effect of Cr(III), CCl_4, and Cr(III)/CCl_4 Treatments on the Hepatic Cytochrome P450 Concentration

treatment[a]	time (h)	cytochrome P450[b]
control		100 ± 14
Cr(III)[c]	1	91 ± 6
CCl_4[d]	1	76 ± 5[e]
Cr(III)/CCl_4[c]	1	68 ± 8[e]

[a] Rats anesthetized with Nembutal. [b] Values are reported as % of control value (0.80 nmol/mg microsomal protein; ±SE, $N = 8$). [c] Cr(III) administered (5 mg/kg ip) 24 h before recorded time or CCl_4 administration (0.8 mL/kg, ig). [d] 0.8 mL/kg administered ig. [e] Values are significantly different ($p < 0.05$) from the control.

between microsomal suspensions from rats pretreated 24 h in advance with 5 mg/kg Zn(II) and microsomal suspensions from nontreated rats. Similar results were obtained using microsomes from rats pretreated 24 h in advance with 5 mg/kg Cr(III) (Figure 3E). Furthermore, when the microsomal incubation was 0.5 mM in Cr(III) prior to the addition of NADPH and CCl_4, the same result as that shown in Figure 3A was obtained, indicating that Cr(III) did not directly inhibit the metabolic enzymes responsible for CCl_4 metabolism.

The rate of metabolism of CCl_4 to the trichloromethyl radical was also the same for microsomal incubations from

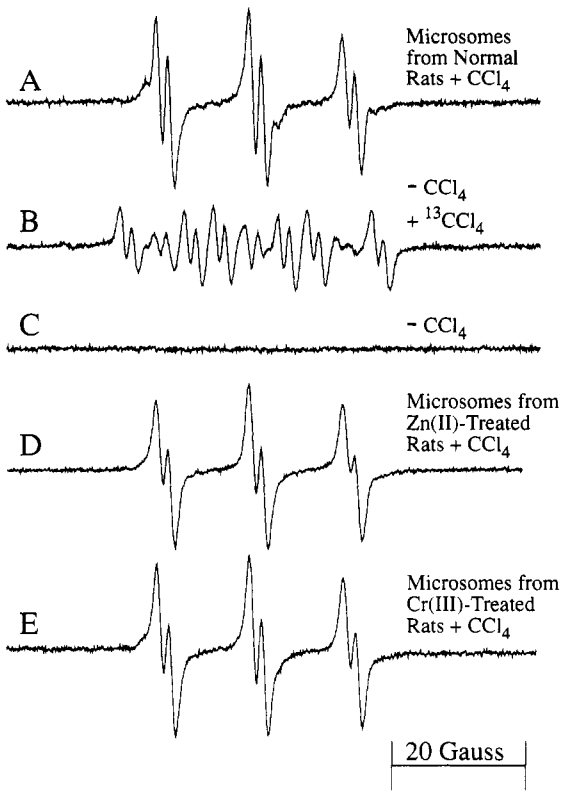

Figure 3. Effects of Zn(II) and Cr(III) pretreatment on the CCl_4-metabolizing hepatic cytochrome P450 enzyme system: (A) ESR spectrum of microsomal protein (2 mg/mL) from the liver of normal, untreated rats incubated with CCl_4 (20 μL/mL), 2 mM NADPH, and 90 mM PBN in 0.1 M sodium phosphate, pH 7.4, for 30 min under a constant stream of N_2; (B) as in A, but $^{13}CCl_4$ was used to demonstrate the identity of the PBN/$^{\bullet}CCl_3$ radical adduct (C) as in A, but without CCl_4; (D) as in A, but with microsomes prepared from rats pretreated 24 h in advance with 5 mg/kg Zn(II); (E) as in A, but with microsomes prepared from rats pretreated 24 h in advance with 5 mg/kg Cr(III). Instrumental conditions were as follows: microwave frequency, 9.77 GHz; microwave power, 20 mW; modulation amplitude, 0.25 G; time constant, 2.5 s; scan rate, 2.67 G/min.

Cr(III)-treated and nontreated rats (Figure 4). These results discount the possibility that the lack of an observed difference between parts A and D or E of Figure 3 was due to one of the substrates becoming a limiting reagent within the 30-min incubation period and, thus, disguising any differences in the efficiency of the microsomes to metabolize CCl_4.

A decrease in cytochrome P450 concentration (25–30%) was detected in microsomes prepared from the liver of rats 1 h after the ip (Table III) or ig (Table IV) administration of CCl_4. The destruction of cytochrome P450 continued with time, such that >50% was lost 24 h after the administration of CCl_4 ip (Table III). Neither Zn(II) (Table III) nor Cr(III) (Table IV) was able to prevent the CCl_4-induced destruction of cytochrome P450.

Effect of Metallothionein Induction on Radical Scavenging in Vivo during CCl_4 Metabolism. Rats were given 0.8 mL/kg CCl_4 ig and 70 mg/kg (0.40 mmol/kg) PBN ip 1 h before their livers were removed and homogenized in a 2:1 chloroform/methanol mixture to extract the PBN spin-trapped free radical metabolites formed in vivo. The results shown in Figure 5 revealed no differences in the intensities of the ESR spectra obtained from Zn(II)-treated or Cr(III)-treated and nontreated rats. Hyperfine splitting constants were $a^N = 14.27$ G and $a_\beta^H = 1.81$ G and corresponded to those for the

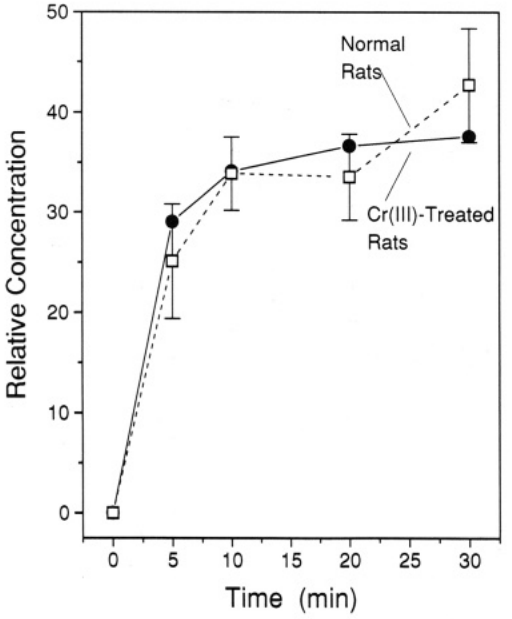

Figure 4. Rate of formation of the trichloromethyl radical adduct of PBN from CCl_4 in a microsomal incubation was monitored by ESR spectroscopy. The experimental conditions are the same as those in parts A and E of Figure 3 for microsomal incubations from untreated and Cr(III)-treated rats, respectively. For simplicity, the error bars shown (±SE, $N = 3$) are for the non-treated rat microsomal incubations only.

PBN/$^{\bullet}CCl_3$ adduct in chloroform (*22*). No ESR signals were observed when the livers of rats given only PBN ip were homogenized in 2:1 chloroform/methanol to which CCl_4 had been added, indicating that the PBN/$^{\bullet}CCl_3$ radical adduct was not a product of ex vivo metabolism (Figure 5E). Similar results to those in Figure 5 were obtained when the administered PBN spin trap was decreased 10-fold to 7 mg/kg (data not shown) or when the CCl_4 and PBN were administered ip in olive oil (Figure 6).

Discussion

Previous studies have reported protective effects by various doses of Zn(II) and Cr(III) against CCl_4-induced hepatic injury in rats and mice (*10, 11, 14*). Because of the protective effect of Zn(II) against CCl_4 hepatotoxicity, a role for MT in scavenging CCl_4-derived free radical metabolites generated in vivo had been postulated. The results of Cagen and Klaassen (*12*) demonstrate that, following $^{14}CCl_4$ administration, there was a decrease in irreversibly-bound ^{14}C-labeled metabolites to cytosolic and microsomal proteins if the rats were pretreated for 3 days with Zn(II). Significant binding of ^{14}C-labeled metabolites to induced MT was also demonstrated. Whether the binding of $^{14}CCl_4$-derived metabolites to MT in vivo represents protective scavenging or is merely a reflection of binding to an increased MT concentration is not clear. More recently, Suntres and Lui (*19*) showed that the MT thiolates are not involved in the irreversible binding of the trichloromethyl radical to MT, since its binding was not altered by prior oxidation of the thiolates with H_2O_2. This latter result suggests that MT possesses no inherent advantage over other cellular proteins in scavenging the trichloromethyl metabolite. The reversible oxidation of MT thiolates during CCl_4 metabolism in microsomal incubations was attributed instead to the trichloromethylperoxyl radical, which forms when the trichloromethyl radical reacts with O_2 (*19*).

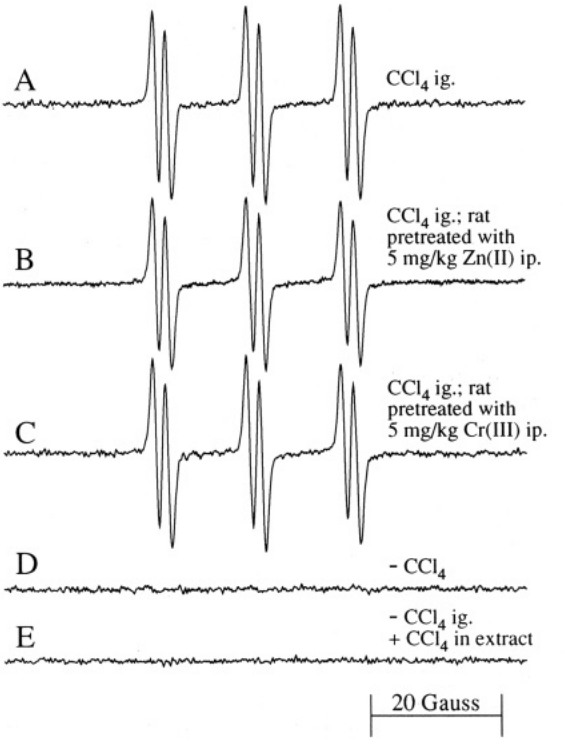

Figure 5. Spin trapping in vivo of the trichloromethyl radical metabolite of CCl_4: (A) ESR spectrum of the chloroform/methanol (2:1) extract of liver from a rat 1 h postadministration of 0.8 mL/kg CCl_4 ig and 70 mg/kg PBN ip; (B) as in A, but rat was first pretreated 24 h in advance with 5 mg/kg Zn(II) ip; (C) as in A, but rat was first pretreated 24 h in advance with 5 mg/kg Cr(III) ip; (D) ESR spectrum of the extract of liver from a rat given only 70 mg/kg PBN ip; (E) as in D, but liver was homogenized in a 2:1 chloroform/methanol mixture to which 25 μL of CCl_4/g of liver tissue had been added. Similar results (of lower intensity) were obtained in experiments where 7 mg/kg PBN was used instead of 70 mg/kg. Instrumental conditions were as follows: microwave frequency, 9.33 GHz; microwave power, 20 mW; modulation amplitude, 0.33 G; time constant, 0.25 s; scan rate, 10 G/min.

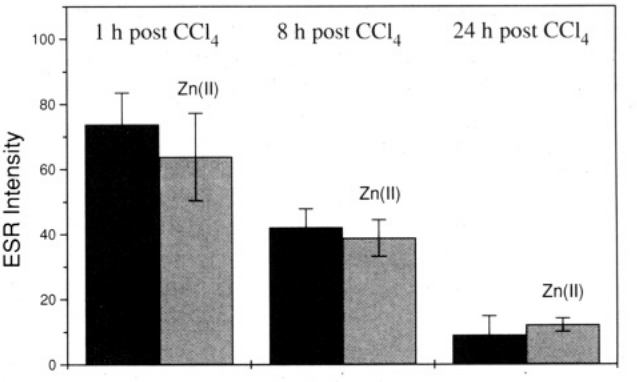

Figure 6. ESR spectral intensities in arbitrary units (±SE, $N = 4$) from the spin trapping in vivo of the trichloromethyl radical metabolite at various times post CCl_4 ip administration. Experimental conditions were the same as described for parts A and B of Figure 5 for control and Zn(II)-treated groups, respectively.

The results shown in Figures 5 and 6 demonstrate that MT does not efficiently scavenge the trichloromethyl radical in vivo. If induced MT played a significant role in scavenging the trichloromethyl radical metabolite, then it would compete with the administered PBN spin trap. The net result would be a decrease in the amount of trichloromethyl radical spin trapped by PBN and, therefore, a decrease in the amount of the PBN/$^{\bullet}CCl_3$ radical adduct observed in the liver extract. No such decrease in

radical adduct was observed, even though the hepatic MT thiolate concentrations may have been comparable to or greater than those of the PBN spin trap used. Doses of 7 and 70 mg/kg PBN were used and gave similar results. A previous report using ^{14}C-labeled PBN demonstrated that the hepatic concentration of PBN (or its metabolites) was approximately 0.3–0.5 mM 1 h postadministration of 50 mg/kg (*23*). On the other hand, MT concentration was approximately 26 and 85 μM[5] in liver tissue 24 h after the administration of Cr(III) and Zn(II), respectively, or 0.53 and 1.7 mM for the corresponding thiolate concentrations. Since the PBN radical adduct of the trichloromethylperoxyl radical is unstable and will not survive the organic extraction procedure, our spin trapping results do not allow inferences to be made about the ability of MT to scavenge this radical in vivo.

Perhaps the most compelling evidence for the lack of a protective role for MT in scavenging any CCl_4-derived free radical metabolite is the protective effect of Zn(II) only 1 h after its administration (Table II). Significant induction of MT synthesis cannot occur within 1 h following Zn(II) administration, yet 5 mg/kg Zn(II) provided protection against CCl_4-induced hepatotoxicity when administered either simultaneously with or 24 h prior to the CCl_4. Therefore, these results suggest that the scavenging by MT of any trichloromethylperoxyl radical which may have formed in vivo is not significant insofar as the protective mechanism of Zn(II) toward CCl_4-induced hepatotoxicity is concerned.

Protection against CCl_4-induced hepatotoxicity is apparently not due to the suppression of the cytochrome P450 enzymes at the metal ion dose used (*12, 14, 38, 39*). That the metabolism of CCl_4 in vivo is not affected by a single ip dose of Zn(II) or Cr(III) is confirmed by the extraction experiments in Figures 5 and 6. These results demonstrate that comparable amounts of the trichloromethyl radical can be spin trapped in vivo from the hepatic activation of CCl_4 with or without Zn(II) or Cr(III) pretreatment. The possibility of ex vivo radical generation is excluded by the lack of an observed signal in the extract of a liver from a control rat to which CCl_4 was added externally (Figure 4E of ref 24). Furthermore, the rate of formation of the trichloromethyl radical in vitro was the same in NADPH-supplemented liver microsomal incubations from Zn(II)-treated or Cr(III)-treated rats. The external addition of Cr(III) to NADPH-supplemented microsomal incubations from normal rats showed no inhibitory effects, indicating that Cr(III) neither reacts with the trichloromethyl radical formed, as had been suggested previously (*14*), nor directly inhibits the cytochrome P450 mixed-function oxidase system. All these results strongly suggest that the activation of CCl_4 in vivo to its free radical metabolite is unaffected by pretreatment with a single dose of 5 mg/kg of either Zn(II) or Cr(III).

The initial activation of CCl_4 to its free radical metabolites is now believed to be only indirectly responsible for the eventual death of the hepatocyte by triggering a series of cellular pathobiochemical responses (*6–9*). One of the earliest cellular responses to CCl_4 intoxication is the dramatic increase in overall intracellular calcium levels (*41*), accompanied by fluctuations in the abilities of various organelles to sequester or release calcium (*6*). Cellular viability is extremely sensitive to intracellular calcium levels, since calcium is a regulator of many fundamental subcellular processes. For example, a recent report links the resultant elevation in calcium from CCl_4 toxicity to the release of toxic eicosanoids and cytokines by Kupffer cells, which may be responsible for the death of the hepatocyte (*42*). When female Sprague-Dawley rats were first administered $GdCl_3$ to kill the Kupffer cells, the incidence of CCl_4-induced liver damage was significantly reduced (*42*). Nevertheless, since the effect of $GdCl_3$ administration on MT induction was not reported in that study, the involvement of MT cannot be ruled out.

Zinc has long been known to be an antagonist to cellular calcium (*43*). For example, zinc is known to inhibit calmodulin functions (*43*), and a recent report demonstrates its inhibitory effects on Ca/Mg-dependent endonuclease, an enzyme involved in DNA fragmentation contributing to apoptosis (*44*). Zinc and calcium may even play regulatory roles toward each other in their transport across cellular membranes (*45, 46*). More recently, evidence for the stimulation of MT mRNA synthesis and an increase in MT-bound zinc as a result of an increase in intracellular calcium concentration has been reported (*47*). Therefore, it is quite plausible that one of the mechanisms for the reported protective effect of Zn(II) against CCl_4 hepatotoxicity is related to a moderation in the intracellular Ca(II) fluctuations following CCl_4 intoxication or a modulation of the consequential events following a rise in intracellular Ca(II). Certainly, the protective effect of Zn(II) against CCl_4-induced hepatotoxicity only 1 h following its administration is supportive of this proposed mechanism.

Another possible protective mechanism of Zn(II) during CCl_4 intoxication is its ability to bind and stabilize membranes against lipid peroxidation and disintegration. Thomas *et al.* (*20*) have reported that either Zn(II) or Cd(II) alone mimicked the protective effects of Cd/Zn–MT against iron-driven lipid peroxidation in erythrocyte ghosts and were much more effective than dithiothreitol at a concentration comparable to the MT thiolates. They concluded that it is the ability of MT to release Cd(II) and Zn(II) (as a result of MT thiolate oxidation) and the binding of these metal ions to the membrane surfaces, perhaps displacing iron, which offer the greatest protection against lipid peroxidation. Zn(II) has also been reported to protect liposomes and micelles from iron-induced lipid peroxidation (*48*) and cell membranes against damage from hemolytic viruses, toxins and other cytotoxic agents (*49*).

In conclusion, we find no evidence that Zn(II)-induced MT protects against CCl_4 hepatotoxicity in vivo by preferentially scavenging the trichloromethyl (or trichloromethylperoxyl) radical formed. Instead, we provide evidence for a more rudimentary role for Zn(II) in protecting against CCl_4-induced hepatotoxicity other than its ability to induce MT.

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[5] MT concentration was estimated from the assumption that 6 g-atom of Cd is bound per mole of protein (*16*).

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