Biochemical Pharmacology, Vol. 32, No. 10, pp. 1613–1617, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00

CARBON TETRACHLORIDE AND BROMOTRICHLOROMETHANE TOXICITY

DUAL ROLE OF COVALENT BINDING OF METABOLIC CLEAVAGE PRODUCTS AND LIPID PEROXIDATION IN DEPRESSION OF MICROSOMAL CALCIUM SEQUESTRATION*

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(*Received* 18 *July* 1982; *accepted* 12 *November* 1982)

Abstract—We have investigated the importance of covalent binding and lipid peroxidation on the depression of microsomal calcium sequestration associated with *in vitro* metabolism of $^{14}CCl_4$. Studies with $CBrCl_3$ are also reported. In aerobic systems, promethazine was used to block lipid peroxidation, measured as malondialdehyde (MDA) generation. Effects of low levels of lipid peroxidation were tested in Fe^{2+}-supplemented systems free of halogenated hydrocarbons. The results indicate that microsomal calcium sequestration can be depressed significantly by metabolism of either CCl_4 or $CBrCl_3$ in the absence of MDA generation, or by lipid peroxidation occurring in the absence of halogenated hydrocarbons.

The modern era in the study of CCl_4 hepatotoxicity began almost 30 years ago with the pioneering work of Christie and Judah [1] and of Dianzani [2]; see Ref. 3 for an earlier review. It is now generally agreed that the initial event is a reductive carbon-halogen bond cleavage catalyzed by a particular ferrous cytochrome P-450 [4, 5], to form chloride anion and trichloromethyl radical ($\cdot CCl_3$). Generation of the $\cdot CCl_3$ radical was surmised initially on indirect evidence [6, 7] and has since been detected by spin-trapping *in vitro* and *in vivo* [8–10]. The major immediate consequences of CCl_4 metabolism are covalent binding of CCl_4 carbon to microsomal lipids and proteins [11] and microsomal lipid peroxidation [12]. There has been long-standing discussion as to the relative importance of these two events with respect to unfolding of the full classical spectrum of CCl_4 hepatotoxic effects. Experimental results with isolated rat liver microsomes, as reported in this communication, bear on the latter question. By use of promethazine, which blocks lipid peroxidation but does not affect covalent binding of ^{14}C from $^{14}CCl_4$, and by means of forced microsomal lipid peroxidation in the absence of CCl_4, we were able to show that microsomal calcium sequestration is sensitive to both lipid peroxidation and covalent binding of CCl_4 carbon. Results obtained from the study of $CBrCl_3$ allowed a similar interpretation. The findings with respect to effects of covalent binding and lipid peroxidation on microsomal calcium sequestration are discussed in relation to the question of possible links between the initial events of CCl_4 and $CBrCl_3$ metabolism and the ultimate pathological consequences of these primary hepatotoxins.

* This work was supported by Grant 9-RO1-ESO1821 from the National Institute of Environmental Health Sciences, N.I.H., U.S.P.H.S.

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METHODS

Animals and materials. Animals used in this study were male Sprague–Dawley rats weighing between 160 and 400 g (Zivic–Miller Laboratories Inc., Allison Park, PA). NADP, ATP, isocitrate, and isocitric dehydrogenase (EC 1.1.1.42) were obtained from the Sigma Chemical Co., St. Louis, MO. $^{14}CCl_4$ and $^{45}Ca^{2+}$ (as aqueous $^{45}CaCl_2$) were supplied by the New England Nuclear Corp., Boston, MA, and 10-(2-dimethylaminopropyl) phenothiazine hydrochloride (promethazine) was supplied by Wyeth Laboratories, Inc., Philadelphia, PA.

Experimental procedures. Preparation of so-called EDTA-microsomes was as described previously [13]. A key feature of this procedure is the presence of 3 mM EDTA in the 0.154 M KCl homogenization medium to remove chelatable Fe^{2+}; see Ref. 14 for the role of iron in microsomal lipid peroxidation. Our basic experimental design involved an initial incubation of EDTA-microsomes under conditions which allowed either covalent binding or lipid peroxidation, or both, followed by recovery of the microsomes and a subsequent second incubation to determine Ca^{2+} sequestration capacity. Initial incubations contained EDTA-microsomes at concentrations ranging from 0.8 to 1.2 mg protein per ml of Tris–maleate buffer (0.05 M Tris, 0.05 M maleate, 0.1 M KCl, pH 7.4), along with 0.3 mM EDTA and an NADPH-generating system consisting of 100 μM NADP, 2.5 mM nicotinamide, 5 mM $MgCl_2$, 3 mM D,L-isocitrate, and 0.07 units (Sigma) of isocitric dehydrogenase per ml of the final incubation mixture. ATP at a final concentration of 5 mM was also present during the initial incubation; see Ref. 13 for

the rationale for this addition. Further details of particular experiments are in the legends to the appropriate tables. Metabolism of $^{14}CCl_4$ or $CBrCl_3$ was initiated by addition of the appropriate haloalkane dissolved in heptane; the quantity of heptane added was never more than 1 μl/ml of final incubation mixture. If the effects of lipid peroxidation alone were to be studied, $FeSO_4$ (final concentration 40 μM) was added to EDTA-microsomes in incubations exactly as above, except that no additional EDTA was added. Peroxidation was initiated by addition of $FeSO_4$. Immediately after initiation of the appropriate reaction, the experimental flasks were sealed (gas phase air) and incubated at 37° for various times up to 30 min. For reaction mixtures supplemented with $FeSO_4$, 0.3 mM EDTA was added at the end of the incubation period to prevent further lipid peroxidation. Aliquots of reaction mixtures were removed for subsequent determination of malondialdehyde (MDA) by the thiobarbituric acid (TBA) method [15]. Remaining microsomes were recovered by centrifugation at 80,000 *g* for 30 min at 4°. There was no further MDA evolution during recovery of the microsomes.

Microsomal calcium sequestration. Recovered microsomes were resuspended in imidazole–histidine buffer (30 mM imidazole, 30 mM histidine, 100 mM KCl, pH 6.8) and assayed for Ca^{2+} sequestration essentially according to Moore *et al.* [16]; see Ref. 13 for details.

Covalent binding of ^{14}C from $^{14}CCl_4$. Recovered microsomes were resuspended in imidazole–histidine buffer (see above). Aliquots of resuspended microsomes were aggregated with $CaCl_2$ [17] and recovered by filtration with low vacuum on 0.2 μm cellulose acetate filters (Metricel). The microsomes on the filter were rinsed with buffer several times and then three times with methanol, 5 ml per wash. The filters with microsomes were immersed in Scintosol and monitored for radioactivity. This procedure measures all radioactivity bound to microsomal lipids and proteins.

Other procedures. Protein was determined according to Lowry *et al.* [18]. The statistical significance of differences between means was determined with Student's two-tailed *t*-test.

RESULTS

Consequences of CCl_4 metabolism. Rat liver microsomes prepared in a medium containing EDTA (so-called EDTA-microsomes), when incubated with NADPH in the presence 0.3 mM EDTA, essentially did not peroxidize (Table 1, line 1; Table 2, line 1). Addition of 0.33 mM CCl_4 increased MDA production almost 4-fold (Table 1, line 2), and 0.64 mM CCl_4 increased production of MDA almost 5-fold (Table 2, line 2). The NADPH-dependent metabolism of 0.33 mM CCl_4 resulted in a 69.7% depression of the Ca^{2+} sequestering capacity of the microsomes (Table 1, lines 1 and 2); when the concentration of CCl_4 was increased to 0.64 mM, NADPH-dependent metabolism of CCl_4 resulted in a 92.8% depression of Ca^{2+} sequestration capacity (Table 2, lines 1 and 2). Addition of promethazine had no statistically significant effect on ^{14}C incorporation at 0.33 mM CCl_4 (Table 1, lines 2 and 4) or at 0.64 mM CCl_4 (Table 2, lines 2 and 4). The presence of promethazine, however, completely blocked MDA evolution (Table 1, line 4 and Table 2, line 4). Metabolism of CCl_4 in the presence of promethazine resulted in highly significant depressions of the capacity of the microsomes to sequester Ca^{2+} (Table 1, line 4; Table 2, line 4). CCl_4 alone (no NADPH) in the presence of promethazine marginally depressed the Ca^{2+} sequestering capacity of the microsomes, presumably due to solvent effects of the added CCl_4, since there was essentially no covalent binding of CCl_4 carbon and no lipid peroxidation (Table 1, line 3; Table 2, line 3).

Consequences of $CBrCl_3$ metabolism. Experimental results with $CBrCl_3$ were in parallel with those for CCl_4. Metabolism of $CBrCl_3$ stimulated MDA production and reduced microsomal Ca^{2+}

Table 1. Rat liver microsomal metabolism of CCl_4 in the presence and absence of promethazine: effects on covalent binding of C from CCl_4, MDA production, and Ca^{2+} sequestration capacity* (experimental series 1: final concentration of CCl_4 = 0.33 mM)

Line No.	Conditions	MDA production (μg/mg protein)	C from CCl_4 bound to microsomes (nmoles C bound/mg microsomal protein)	Calcium sequestered (nmoles/mg protein)
1	NADPH, no $^{14}CCl_4$	0.03 ± 0.001		141 ± 7.6
2	NADPH + $^{14}CCl_4$	0.11 ± 0.021	0.383 ± 0.041	42 ± 6.8†
3	$^{14}CCl_4$ + promethazine	0.02 ± 0.008	Trace	104 ± 10.4‡
4	NADPH + $^{14}CCl_4$ + promethazine	0.03 ± 0.004	0.333 ± 0.042	67 ± 9.1§

* Essential conditions: rat liver EDTA-microsomes (concentration range over six experiments was 0.8 to 1.2 mg protein/ml incubation medium), an NADPH-generating system, 0.33 mM $^{14}CCl_4$ (specific activity = 8.3×10^2 cpm/nmole CCl_4), 10 μM promethazine, Tris–maleate KCl buffer, pH 7.4, 0.3 mM EDTA, and air as gas phase. Incubation was for 30 min at 37°. See Methods for further details. Calcium sequestered means Ca^{2+} taken up in 60 min, in a second incubation, by microsomes recovered from the first incubation. All data given are means ± S.E.M. (N = 6).

† Highly significantly different from line 1 ($t = 9.71$; $P < 0.001$).

‡ Significantly different from line 1 ($t = 2.87$; $0.05 > P > 0.025$).

§ Highly significantly different from line 1 ($t = 6.24$; $0.005 > P > 0.001$). Marginally significantly different from line 2 ($t = 2.20$; $0.1 > P > 0.05$). Significantly different from line 3 ($t = 2.68$; $0.05 > P > 0.025$).

Table 2. Rat liver microsomal metabolism of CCl_4 in the presence and absence of promethazine: effects of covalent binding of C from CCl_4, MDA production, and Ca^{2+} sequestration capacity* (experimental series 2: final concentration of CCl_4 = 0.64 mM)

Line No.	Conditions	MDA production (μg/mg protein)	C from CCl_4 bound to microsomes (nmoles C bound/mg microsomal protein)	Calcium sequestered (nmoles/mg protein)
1	NADPH, no $^{14}CCl_4$	0.03 ± 0.003		152 ± 9.2
2	NADPH + $^{14}CCl_4$	0.14 ± 0.003	0.567 ± 0.042	11 ± 4.7†
3	$^{14}CCl_4$ + promethazine	0.02 ± 0.003	0.077 ± 0.020	66 ± 18.0‡
4	NADPH + $^{14}CCl_4$ + promethazine	0.03 ± 0.003	0.503 ± 0.020	15 ± 1.1§

* Essentially, conditions were exactly as for Table 1, except that the final concentration of CCl_4 was 0.64 mM. All data given are means ± S.E.M. (N = 3).

† Highly significantly different from line 1 ($t = 13.65$; $P < 0.01$).

‡ Marginally significantly different from line 1 ($t = 4.26$; $0.1 > P > 0.05$).

§ Highly significantly different from line 1 ($t = 14.78$; $0.005 > P > 0.001$). Not significantly different from line 2 ($t = 0.83$; $P > 0.5$). Marginally significantly different from line 3 ($t = 2.83$; $0.2 > P > 0.1$).

sequestration markedly (Table 3, line 2). Promethazine almost completely inhibited MDA production, but Ca^{2+} sequestration, following $CBrCl_3$ metabolism in the presence of promethazine, was nevertheless reduced to about 60% of the control level (Table 3, line 4). In the absence of NADPH, $CBrCl_3$ had no effect on microsomal Ca^{2+} sequestration (Table 3, line 3). In other experiments (data not shown), at a higher concentration of $CBrCl_3$ (100 μM), in the presence of NADPH, there was production of 0.34 μg MDA/mg protein, and Ca^{2+} sequestering capacity was almost completely destroyed. Promethazine completely blocked MDA production, but metabolism of $CBrCl_3$ (plus NADPH), in the presence of promethazine, nevertheless led to a 75% reduction of Ca^{2+} sequestering capacity.

Consequences of Fe^{2+}-stimulated lipid peroxidation. The capacity of liver microsomes to sequester Ca^{2+} fell precipitously as a function of MDA evolution when the latter was generated by Fe^{2+}-dependent lipid peroxidation (Table 4).

DISCUSSION

The work reported in this communication is significant in view of the long-standing discussion of the relative roles, in CCl_4 liver cell injury, of lipid peroxidation and covalent binding of products of CCl_4 metabolism. Peroxidation of lipids of the liver cell endoplasmic reticulum (ER) [12] and covalent binding of CCl_4 carbon [11] take place rapidly in the rat given CCl_4. The idea that lipid peroxidation plays a dominant role [3, 7, 19] initially emerged primarily on the strength of the argument that the process of lipid peroxidation was known to be violently destructive for biological membranes. Conversely, the covalent binding hypothesis appeared to have a major weakness because, apart from possible immediate effects on cytochrome P-450, it was not obvious how covalent binding of CCl_4 cleavage products could result in the classical display of CCl_4 hepatotoxic effects. The lipid peroxidation hypothesis appeared to be buttressed convincingly with the report from this laboratory [20–22] that aminopyrine demethylase, cytochrome P-450, and glucose-6-phosphatase (G-6-Pase) were essentially unaffected during anaerobic metabolism of $^{14}CCl_4$ by rat liver microsomes despite massive binding of CCl_4 carbon. The marked sensitivity of G-6-Pase to lipid peroxidation, and its relative insensitivity to covalent binding of CCl_4 carbon have been confirmed [23, 24]. With respect to cytochrome P-450, however, and by inference with respect to aminopyrine demethylase as well, the report [20–22] from this laboratory was clearly in error. It has been shown convincingly in three laboratories [24–27] that binding of ^{14}C from $^{14}CCl_4$, under anaerobic conditions *in vitro* (i.e. no lipid peroxidation), leads to a marked loss of cyto-

Table 3. Rat liver microsomal metabolism of $CBrCl_3$ in the presence and absence of promethazine: effects on lipid peroxidation and Ca^{2+} sequestering capacity*

Line No.	Conditions	MDA production (μg/mg protein)	Calcium sequestered (nmoles/mg protein)
1	NADPH, no $CBrCl_3$	0.030	165
2	NADPH + $CBrCl_3$	0.170	37
3	$CBrCl_3$ + promethazine	0.030	168
4	NADPH + $CBrCl_3$ + promethazine	0.045	108

* Conditions were exactly as for Table 1, except that $CBrCl_3$ at a final concentration of 20 μM was used in place of CCl_4. Data are means of two experiments.

Table 4. Loss of Ca^{2+} sequestering capacity as a function of Fe^{2+}-dependent microsomal lipid peroxidation*

Length of incubation (min)	MDA production (μg/mg protein)	Microsomal Ca^{2+} uptake (% of control)
0.5	0.07 ± 0.04	95.3 ± 3.5
1.0	0.13 ± 0.02	86.7 ± 6.5
1.5	0.19 ± 0.07	58.0 ± 24
2.0	0.41 ± 0.24	17.0 ± 14
3.0	1.15 ± 0.61	5.8 ± 6.2
4.0	1.86 ± 0.35	0.8 ± 0.3

* Conditions: rat liver microsomes (EDTA-microsomes) were incubated at 37°. The incubation conditions and the NADPH-generating system were exactly as for Table 1, except that no EDTA was added, and 40 μM $FeSO_4$ was added in place of CCl_4. Lipid peroxidation was stopped at the times indicated by addition of EDTA to a final concentration of 300 μM. The microsomes were recovered by centrifugation and assayed for Ca^{2+} sequestration. Average calcium uptake of control, non-peroxidized microsomes was 114 ± 7.4 nmoles Ca^{2+} taken up/mg microsomal protein. Data in the table are mean values for three experiments ± S.E.M.

chrome P-450.* The idea that loss of cytochrome P-450 in the CCl_4-poisoned animal can be due to covalent binding of CCl_4 cleavage products has long been held by J. Castro and his coworkers [28–33]. That part of the argument favoring covalent binding, which rested on failure to find conjugated dienes in microsomal lipids of the CCl_4-poisoned mouse (A/J strain), has been greatly weakened by the report [34] that, within 1 hr after CCl_4 administration to A/J strain mice, unmistakable evidence of peroxidative decomposition of lipids of the liver cell ER could be found. It thus appears solidly established that covalent binding of CCl_4 cleavage products and lipid peroxidation both take place in the livers of either rats or mice given CCl_4. Since cytochrome P-450 is known to be sensitive to lipid peroxidation in the absence of halogenated hydrocarbon metabolism [22], and to covalent binding of CCl_4 carbon in the absence of lipid peroxidation [24–27] both of these immediate consequences of CCl_4 metabolism may contribute to the CCl_4-dependent loss of cytochrome P-450 *in vivo*. This point has been made recently by Fander *et al.* [35], who said ". . . we would like to suggest that both covalent ^{14}C incorporation from $^{14}CCl_4$ and lipid peroxidation are causally involved in the destruction of cytochrome P-450. The extent of their relative contribution depends on the experimental condition."

This resolution of a long-standing debate, although gratifying, does not solve the problem of how the initial events of CCl_4 metabolism are transmuted into the pathology of triglyceride (TG) accumulation, polyribosomal disaggregation, depression of protein synthesis, cell membrane breakdown and eventual death of the cell. The work reported in this paper has a particular bearing on this point. For example, the fatty liver of CCl_4 toxicity probably depends on an interference in the intracellular traffic of very low density lipoprotein (VLDL) [36], and it has been shown that TG secretion by isolated hepatocytes can be strongly inhibited by CCl_4 metabolism in the absence of lipid peroxidation [37]. If, as seems likely, the intracellular movement and ultimate secretion of VLDL depend on the structural and functional integrity of those components of the cytoskeleton subserving intracellular motion, cytoplasmic streaming, etc., then initial events connected with and emerging from covalent binding of CCl_4 cleavage products must be able to affect the cellular machinery necessary for VLDL secretion. On theoretical grounds, because of their high reactivity, direct attack on such cellular machinery by radicals such as $\cdot CCl_3$ or $\cdot OOCCl_3$ seems unlikely. It has been shown, for example, that polyribosomal dissociation and depression of protein synthesis are evident in rat liver 15 min after $^{14}CCl_4$ administration, but no ^{14}C label was incorporated into ribosomal or polyribosomal fractions [36]. Even more convincing, when CCl_4 and the spin trap phenyl-*t*-butylnitrone (PBN) were administered to rats intragastrically, the $\cdot CCl_3$ radical adduct of PBN was detected after 2 hr only in microsomal lipids [9]. It seems crystal clear that an intermediate pathological process of some sort must intervene between generation of the $\cdot CCl_3$ radical at the cytochrome P-450 locus and pathological involvement at distant loci. Disturbed hepatocellular Ca^{2+} homeostasis may be the critical link.

The level of cytosolic free Ca^{2+} appears to play a regulatory role in all aspects of intracellular motion including secretion of hormones and enzymes, cytokinesis, and cytoplasmic streaming [38]. The level

* The discrepancy between our earlier results [20–22] and the later work [24–27] is no doubt due to the presence of CCl_4 in our controls. Dithionite-reduced cytochrome P-450 evidently catalyzed cleavage of CCl_4 in our controls, leading to anaerobic binding of CCl_4 cleavage products and losses of cytochrome P-450 more or less equivalent to losses of cytochrome P-450 in microsomes incubated anaerobically with NADPH and $^{14}CCl_4$. Since losses in both cases were essentially equal, and since cytochrome P-450 was known to be sensitive to lipid peroxidation in the absence of CCl_4, the erroneous conclusion was drawn that loss of cytochrome P-450 depended obligatorily on lipid peroxidation and not on covalently bound CCl_4 carbon (E. A. Glende, Jr. and R. O. Recknagel).

of cytoplasmic free Ca^{2+} probably plays a regulatory role in the intracellular traffic of VLDL and ultimate secretion of TG by liver cells. This line of reasoning allows for the emergence of a simple and, hopefully, a testable hypothesis, as follows. Carbon-halogen bond cleavage for the primary hepatotoxins, CCl_4 and $CBrCl_3$, is confined to the cytochrome P-450 locus. The most immediate consequences are covalent binding of cleavage products and lipid peroxidation, which rapidly depress the capacity of the ER to sequester Ca^{2+}. A physiologically unacceptable rise in cytosolic free Ca^{2+} results, with pathological consequences. This hypothesis envisages rising cytosolic free Ca^{2+} levels as a kind of toxicological second messenger. The hypothesis does not require migration of metabolic cleavage products of CCl_4 or $CBrCl_3$ to any intracellular sites other than cytochrome P-450 and those elements of the ER necessary for Ca^{2+} sequestration. Since microsomal Ca^{2+} sequestration is compromised by covalent binding of CCl_4 carbon in the absence of lipid peroxidation (Tables 1 and 2), the hypothesis allows for the expression of CCl_4 toxicity in the absence of lipid peroxidation. Since microsomal Ca^{2+} sequestration is sensitive to both covalent binding and lipid peroxidation (Table 4), the hypothesis affords a basis for understanding why administration of various antioxidants to the whole animal given CCl_4 has never afforded more than partial protection against CCl_4 hepatotoxic effects.

The work presented in this paper, along with closely related data recently published [13, 39, 40], represents an open invitation to consider disturbed hepatocellular Ca^{2+} homeostasis as a possible component in the chain of causality standing between the metabolism of CCl_4 and $CBrCl_3$ and the ultimate consequences of their toxicological action. The recently published method of Tsien *et al.* [41], employing the fluorescent indicator "quin 2", used in conjunction with isolated hepatocytes may provide a method for testing that part of the hypothesis requiring that CCl_4 and $CBrCl_3$ metabolism should result in a rise in concentration of cytosolic free calcium.

Acknowledgement—We acknowledge the contribution to this work of Dr Karen Lowrey, who independently repeated and confirmed the results relative to the action of promethazine.

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