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ANAEROBIC METABOLISM OF CARBON TETRACHLORIDE AND FORMATION OF CATABOLICALLY RESISTANT PHOSPHOLIPIDS

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Abstract—Anaerobic incubation of microsomes with ^{14}C-carbon tetrachloride leads to trichloromethyl radicals which, apart from other detrimental reactions, bind covalently to phospholipids. These labelled lipids are extracted, phosphatidylcholine is isolated and incubated with phospholipase A_2 from Naja naja snake venom and from porcine pancreas. Half of the covalently modified phosphatidylcholine is resistant toward phospholipase A_2. Distribution of such catabolically resistant phospholipids from the endoplasmic reticulum to other cell organelles may play an important role in the pathway leading from the primary lesion to hepatocyte necrosis.

Carbon tetrachloride, a strong hepatotoxin, is reductively metabolized by cytochrome P-450 to a trichloromethyl radical as reactive intermediate [1–3]. While this first stage is undisputed, the further sequence of requisite events leading to cell necrosis is still not defined. Among others three principal effects have been considered as most detrimental to the hepatocyte, namely depletion of pyridine nucleotides [1], covalent binding to proteins and lipids [4, 5], and lipid peroxidation [6, 7]. The latter is the most commonly accepted hypothesis, a process envisaged as continuous, self-propagating "radical-avalanche" similar to autoxidation of polyunsaturated fatty acids leading to rancidity of butter fat. This hypothesis is mainly derived from *in vitro* experiments with microsomes monitoring the formation of malonaldehyde [8].

The pathogenic responses of different cell organelles upon carbon tetrachloride metabolism evolve during several hours [9]. The protracted manner of these disturbances led to the suggestion that oxidative degradation products generated by "lipid peroxidation" are the actual cellular toxicants [7, 10, 11]. However, a contradictory observation is that *in vivo* about five molecules of carbon tetrachloride must be metabolized in order to destroy one polyunsaturated fatty acid, and, as soon as carbon tetrachloride metabolism ceases, exhalation of ethane and pentane comes to an end [12]. This indicates that degradation of polyunsaturated fatty acids does take place to a limited extent, but not as autocatalytic chain reaction and not propagated beyond the end of metabolic activation. Altogether, the hypothesis of lipid peroxidation has become questionable [13].

Strong covalent lipid binding is typical for carbon tetrachloride and distinguishes this chemical from other hepatotoxins; some authors [14, 15] consider this as the actual cause of carbon tetrachloride toxicity. Indeed, addition of trichloromethyl radicals to the double bond of oleic acid has recently been demonstrated in a reconstituted P-450 system [16]. Here we present supporting evidence for the decisive role of covalent binding to lipids and a new observation which could explain the events on the pathway to cell necrosis after carbon tetrachloride intoxication.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (420–480 *g*), receiving a standard diet (Altromin®) and water *ad libitum*, were pretreated with phenobarbital, sodium salt: one dose of 80 mg/kg intraperitoneally and subsequently 0.1% phenobarbital in the drinking water for 5 days.

Isolation of microsomes. After starvation for one day the rats were killed by decapitation. The livers were immediately removed and placed in ice-cold 0.9 % sodium chloride solution. After weighing, they were cut and homogenized in 5 vol of a buffer consisting of 0.25 moles/l sucrose, 20 mmoles/l Tris–HCl, 1 mmole/l EDTA, pH 7.4. Microsomes were prepared according to Remmer *et al.* [17] by differential centrifugation. The microsomal pellets were suspended in a buffer of 50 mmoles/l Tris–HCl, 120 mmoles/l potassium chloride, pH 7.4, recentrifuged at 100,000 *g* for 50 min, resuspended in the same buffer and stored at −80°. Microsomal protein was determined according to Lowry *et al.* [18], using bovine serum albumin as standard. Cytochrome P-450 was measured according to Omura and Sato [19] and found to be 1.8 nmoles/mg protein.

Incubation of microsomes with [^{14}C]*-carbon tetrachloride.* Anaerobic incubation of microsomes with $^{14}CCl_4$ was carried out in a 15 ml volume flask with two stop-cock connections for nitrogen-purging and

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a quick-fit connection with Teflon-laminated septum for addition of CCl_4 and NADP. 10.25 ml of an ice-cold solution consisting of 50 mmoles/l Tris–HCl, 120 mmoles/l potassium chloride, 5 mmoles/l magnesium chloride, 8 mmoles/l sodium isocitrate, 50 μg/ml isocitrate dehydrogenase, pH 7.4 and liver microsomes (3 mg/ml) was purged with nitrogen for 30 min. After addition of 10 μmoles NADP in a solution of 100 μl nitrogen-purged incubation buffer, the stop-cocks were closed and 11 nmoles $^{14}CCl_4$ (activity 1.82 Ci/moles, total 20 μCi) in a solution of 20 μl ethanol were added. Subsequently, the flask was incubated in a water bath at 37° for 60 min.

The reaction was stopped by cooling the flask with ice to 0°; volatile metabolites were removed by purging with nitrogen and collected in a trap cooled with liquid nitrogen.

Extraction and separation of microsomal lipids after $^{14}CCl_4$-incubation. After addition of 10 mg 2,6-di-*t*-butyl-4-methylphenol (BHT) to the incubation mixture, the lipids were extracted according to Nelson [20] with 150 ml methanol and 320 ml chloroform. This suspension was filtered through a glass fiber filter. Polar soluble substances are removed by addition of 1 g Sephadex® G-25 coarse and subsequent filtration. The solvent was evaporated and the dried lipids were dissolved in 9 ml of chloroform and stored at −20°.

Phospholipid classes were separated by thin-layer chromatography on silica gel (precoated plates Si G 60, 20 × 20 cm, Merck, Darmstadt, F.R.G.) developed with chloroform–ethanol–triethylamine–water (30 : 40 : 26 : 8, by vol.).

^{14}C-labelled phospholipid classes were detected on the plate with a TLC radioactivity scanner.

The spots were scraped from the plate and eluted with methanol. After evaporation of solvent, the phospholipids were redissolved in chloroform for storage.

Incubation of ^{14}C-labelled phosphatidylcholine with phospholipase A_2. For phospholipase treatment, the isolated labelled phosphatidylcholine was dissolved by ultrasonication in an incubation medium of 100 mmoles/l Tris–HCl, 5 mmoles/l calcium chloride, 6 mmoles/l sodium chloride, pH 7.9. The concentration of phosphatidylcholine in this medium was adjusted to 161 ± 3 nmoles phosphate per ml. Incubations were carried out at 37° and started by addition of phospholipase A_2 (32 U/ml). Aliquots of the incubation mixtures were withdrawn at 0, 10, 20, 40 and 80 min, immediately stopped by addition of EDTA (10 μmoles/ml) and 5 vol of chloroform–methanol (3 : 2, by vol). After centrifugation for phase separation, the upper aqueous layer was discarded and aliquots from the organic layer were taken for determination of phosphate and radioactivity and for thin layer chromatography. Thin layer chromatography was carried out as described above. The spots (phosphatidylcholine, lysophosphatidylcholine, free fatty acids) were detected by spraying the plate with rhodamine B, scraped from the plate, eluted with chloroform–methanol–water (5 : 5 : 1, by vol) and examined for phosphate and radioactivity.

Determination of phosphate and radioactivity. After hydrolysis of the phospholipids in 70% perchloric acid for 1 hr at 160°, phosphate was determined with malachite green by a modification of the Penney-method as described by Schmalzing *et al.* [21].

Table 1. Balance of radioactivity after anaerobic incubation of rat liver microsomes with $[^{14}C]$-carbon tetrachloride (incubation conditions as given in Materials and Methods)

	μCi	Percent
Volatile metabolites	12.8	64
Volatile metabolites in lipid extract	0.8	4
Protein-bound and polar metabolites	0.8	4
Lipid-bound metabolites	3.2	16
Losses	2.4	12
Total	20.0	100

Radioactivity was determined by liquid scintillation counting in a scintillation mixture consisting of 2 vol toluene, 1 vol Triton X-100, 5 g/l PPO, 50 mg/l POPOP. Quench correction was done by the channel ratio method.

Chemicals. $[^{14}C]$-Carbon tetrachloride (27 Ci/mole) was purchased from Amersham-Buchler, Braunschweig, F.R.G., diluted with unlabelled carbon tetrachloride to 1.82 Ci/mole and dissolved in ethanol (55 μmole CCl_4/100 μl ethanol).

Isocitrate dehydrogenase (EC 1.1.1.42) from pig heart, grade II, solution in glycerol (10 mg/ml), *ca.* 2 U/mg was purchased from Boehringer, Mannheim, F.R.G.

Phospholipase A_2 (EC 3.1.1.4) from porcine pancreas, suspension in ammonium sulfate solution, 9 mg protein/ml, 900 U/mg protein, was purchased from Sigma Chemical Company, St. Louis, U.S.A.

All other chemicals were purchased from commercial sources and were of reagent grade quality.

RESULTS

Anaerobic incubation of $[^{14}C]$-carbon tetrachloride leads to appearance of radioactivity in four gross fractions (Table 1). The largest portion is volatile material which consists of unmetabolized carbon tetrachloride and its first metabolite chloroform. Obviously, a small fraction of volatile material is retained in the incubation mixture in spite of nitrogen purging; this is removed from the lipid extract at the stage of solvent evaporation. Covalent binding to lipids is considerably higher than to proteins, consistent with previous results [22]. The label is distributed among all phospholipids (Table 2); highest specific binding is found in lysophosphatidylcholine, sphingomyeline and phosphatidylserine. However, phosphatidylcholine as the most abundant phospholipid carries the bulk of the label: about one third is bound to this class. Similar values have been reported by others [5] for liver microsomes from rabbits.

Incubations of the isolated labelled phosphatidylcholine with phospholipase A_2 from porcine pancreas show that half of the labelled phosphatidylcholine is resistant toward enzymic hydrolysis (Fig. 1). The label is not attached to the acyl chain in sn-1-position of the glycerol residue, as otherwise one should find significant amounts of radioactive lysophospholipids. Chemical transesterification with boron trifluoride

Table 2. Covalent binding of CCl_4-metabolites to phospholipids during anaerobic incubation of rat liver microsomes with [^{14}C]-carbon tetrachloride and NADPH for 60 min

Phospholipid	$\frac{\text{Phosphate}}{\text{mg protein}}$ [nmol]	$\frac{\text{Covalent binding}}{\text{mg protein}}$ [nmol]	$\frac{\text{Covalent binding}}{\text{Phosphate}} \times 100$ [mole %]
LPC	4	1.6	42
SM	26	4.9	18.6
PC	316	10.9	3.5
PS/LPE	24	3.6	15.3
PI	76	2.5	3.3
PE	69	2.0	2.9
Σ	515	25.5	5.0

Lipids were separated by thin layer chromatography, spots were scraped from the plate and radioactivity and phosphate were determined.

LPC, lysophosphatidylcholine, SM, sphingomyeline; PC, phosphatidylcholine; PS, phosphatidylserine; LPE, lysophosphatidylethanolamine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

in methanol or simply storage of the lecithin in methanol results in complete cleavage of the ester group and retrieval of the label in the fraction of the free fatty acids; apparently the trichloromethyl group is bound to the 2-acyl group, the position usually occupied by polyunsaturated fatty acids. The value of 15 mole % of bound carbon tetrachloride metabolites relative to the phosphate groups in the catabolically resistant lecithin indicates that these are oligomers of several phosphatidylcholine moieties, on the average six.

With phospholipase A_2 from another source, i.e. Naja naja snake venom, we found the same percentage of catabolically stable phosphatidylcholine.

DISCUSSION

Crosslinkage is probably the main reason for catabolic resistance, whereas peroxidized lipids are readily cleaved by phospholipase A_2 [23]. Mechanistically this is plausible: addition of trichloromethyl radical to the double bond of a polyunsaturated fatty acid yields a α-trichloromethyl alkyl radical which may add to a double bond of another fatty acid leading to crosslinking, apart from reactions with oxygen, endogenous radical scavengers or hydrogen donors. We are presently trying to elucidate the chemical structures of these altered lipids in order to assess the relative participation of different termination reactions. As the phenomenon of catabolic resistance can be observed with phospholipases A_2 from different sources we assume that this is a general feature, also applicable to intracellular phospholipases which have similar biochemical properties.

Phospolipases A_1 and A_2 are associated with different cell organelles [24], ensuring a constant

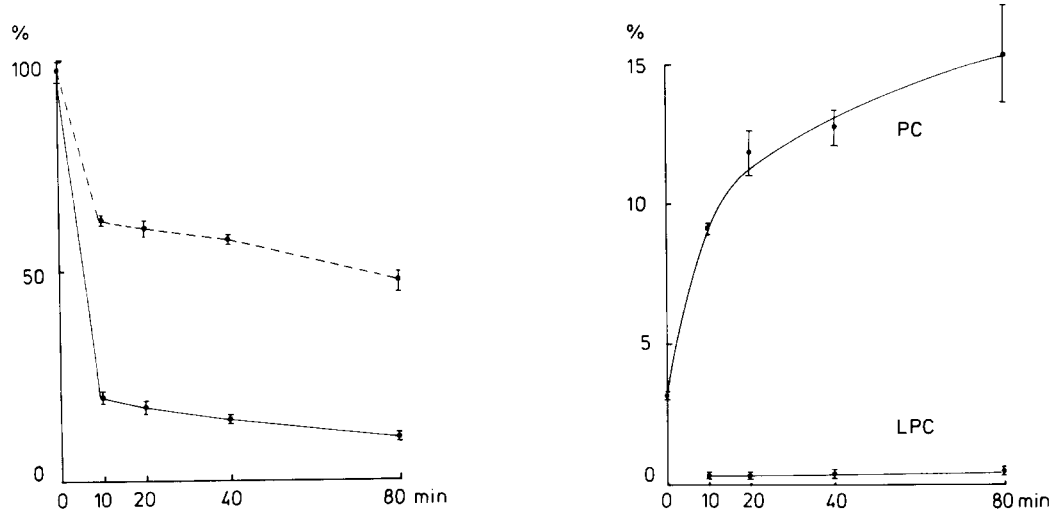

Fig. 1. Enzymic cleavage of phosphatidylcholine (PC) by phospholipase A_2 from porcine pancreas. PC was isolated from rat liver microsomes which previously had been incubated anaerobically with [^{14}C]-carbon tetrachloride and NADPH for 1 hr. After PLA_2-treatment the lipids were separated by thin layer chromatography, scraped off, and phosphate and radioactivity were determined. Left: solid line, percent of phosphate on the spot of PC, relative to total phosphate (PC + LPC); broken line, percent of radioactivity on the spot of PC relative to total radioactivity (PC + LPC + free fatty acids). Right: mole percent of covalently bound [^{14}]-carbon tetrachloride metabolites relative to the molar amounts of phosphate on the spots of phosphatidylcholine (PC) or lysophosphatidylcholine (LPC).

turnover of phospholipids; in other words, they are part of the membrane repair system of the cell. Together with specific acyl-transferases they also tailor the fatty acid composition of the furnished phospholipids to the specific pattern of each membrane.

We infer the hypothesis that an important aspect in transducing the toxic principle from the endoplasmic reticulum to other cell organelles lies in the catabolic resistance of such altered phospholipids. The terminal enzymes for phospholipid synthesis are mainly, if not exclusively, located in the endoplasmic reticulum, from whence the phospholipids are delivered to other membanes [25]. Assuming that the altered lipids are "erroneously" distributed along with the normal phospholipids to other membrane structures, the delayed effect of carbon tetrachloride on different cell organelles may be explained. If a part of the covalently altered phosphatidylcholine is resistant toward the attack of the phospholipase A_2, they are permanently incorporated into the phospholipid pool of the cell, which only could eliminate these compounds by export as triglycerides, which is inhibited, or by complete lysosomal breakdown. Incorporation of covalently modified and crosslinked phospholipids would decrease the fluidity of the lipid bilayer.

The proposed mechanism fits well to the time schedule of cellular disturbances. Transfer of phospholipids from the endoplasmic reticulum to the mitochondrial membranes lies in the range of 2–6 hr [26], to the plasmalemma about 12 hr; profound impairments of mitochondrial activities and formation of balloon cells are observed on this time scale.

Interestingly, it has been reported recently that microsomal Ca-ATPase is rapidly inhibited after metabolic activation of carbon tetrachloride [27, 28], even when lipid peroxidation is minimal [29]. The sarcoplasmic Ca-ATPase is extremely sensitive toward alterations of membrane properties which may already be effected by changes in the fatty acid pattern [30] and membrane fluidity [31]. Covalent modification of membrane lipids is probably the most important cause for the inhibition of the microsomal Ca-ATPase observed by Lowrey *et al.* [29] who reported that lipid peroxidation was minimal under their experimental conditions. If the same is true for calcium pumps in other membranes, then the breakdown of the calcium homeostasis would lead to destruction of the cell, the final pathway common to the toxicity of diverse chemicals [32].

The central aspect of the presented hypothesis is the generation of altered, catabolically resistant phospholipids. As the presented experiments deal with an artificial microsomal model, the validity of the drawn conclusions needs to be assessed by appropriate *in vivo* investigations. Also, there are other potentially detrimental reactions and pathways progressing during or shortly after generation of the trichloromethyl radical which may further interfere with cell metabolism.

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REFERENCES

1. T. F. Slater, in *Biochemical Mechanism of Liver Injury* (Ed. T. F. Slater), p. 745. Academic Press, London (1978).
2. J. L. Poyer, P. B. McCay, E. K. Lai, E. G. Janzen and E. R. Davis, *Biochem. biophys. Res. Commun.* **94**, 1154 (1980).
3. W. Nastainczyk, H. J. Ahr and V. Ullrich, *Biochem. Pharmac.* **31**, 391 (1982).
4. M. I. Diaz Gomez, J. A. Castro, E. C. de Ferreyra, N. D'Acosta and C. R. de Castro, *Toxicol. appl. Pharmac.* **25**, 534 (1973).
5. H. Uehleke and T. Werner, *Archs Toxicol.* **34**, 289 (1975).
6. R. O. Recknagel and A. K. Ghoshal, *Lab. Invest.* **15**, 132 (1966).
7. R. O. Recknagel, E. A. Glende and A. M. Hruszkiewycz, in *Free Radicals in Biology* (Ed. W. A. Pryor), Vol. 3, p. 97. Academic Press, New York (1977).
8. F. Bernheim, M. L. C. Bernheim and K. M. Wilbur, *J. biol. Chem.* **174**, 257 (1948).
9. R. O. Recknagel, *Pharmac. Rev.* **19**, 145 (1967).
10. S. D. Aust and B. A. Svingen, in *Free Radicals in Biology* (Ed. W. A. Pryor), Vol. 5, p. 1. Academic Press, New York (1982).
11. A. Benedetti, M. Comporti and H. Esterbauer, *Biochim. biophys. Acta* **620**, 281 (1980).
12. H. Frank and H. Dürk, *Archs Toxicol.* **53**, 213 (1983).
13. J. R. Mitchell, G. B. Corcoran, C. V. Smith, H. Hughes and B. H. Lauterburg, in *Biological Reactive Intermediates-II* (Eds. R. Snyder, D. V. Parke, J. J. Kocsis, D. J. Jollow, C. G. Gibson and C. M. Witmer), p. 199. Plenum Press, New York (1982).
14. E. S. Reynolds, M. T. Moslen and R. J. Treinen, in *Oxygen and Oxy-Radicals in Chemistry and Biology* (Eds. M. A. J. Rodgers and E. L. Powers), p. 169. Academic Press, New York (1981).
15. E. G. D. de Toranzo, M. I. Diaz Gomez and J. A. Castro, *Res. Commun. chem. Path. Pharmac.* **19**, 347 (1978).
16. J. R. Trudell, B. Bösterling and A. J. Trevor, *Proc. natn. Acad. Sci. U.S.A.* **79**, 2678 (1982).
17. H. Remmer, H. Greim, J. B. Schenkman and R. W. Estabrook, *Meth. Enzym.* **10**, 703 (1967).
18. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
20. G. J. Nelson, in *Analysis of Lipids and Lipoproteins* (Ed. E. G. Perkins), p. 1. American Oil Chemists' Society, Champaign, Illinois (1975).
21. G. Schmalzing, E. Pfaff and U. Breyer-Pfaff, *Life Sci.* **29**, 371 (1981).
22. H. Frank, H. J. Haussmann, H. Remmer, *Chem. Biol. Interact.* **40**, 193 (1982).
23. A. Sevanian, R. A. Stein and J. Mead, *Lipids* **16**, 781 (1981).
24. H. van den Bosch, *Biochim biophys. Acta* **604**, 191 (1980).
25. R. M. Bell and R. A. Coleman, *Ann. Rev. Biochem.* **49**, 459 (1980).
26. F. L. Bygrave, *J. biol. Chem.* **244**, 4768 (1969).
27. L. Moore, T. Chen, H. R. Knapp and E. G. Landon, *J. biol. Chem.* **250**, 4562 (1975).
28. L. Moore, G. Davenport and E. G. Landon, *J. biol. Chem.* **251**, 1197 (1976).
29. K. Lowrey, E. A. Glende Jr. and R. O. Recknagel, *Biochem. Pharmac.* **30**, 135 (1981).
30. R. The and W. Hasselbach, *Eur. J. Biochem.* **39**, 63 (1973).
31. J. Seelig and W. Hasselbach, *Eur. J. Biochem.* **21**, 17 (1971).
32. F. A. X. Schanne, A. B. Kane, E. E. Young and J. L. Farber, *Science* **206**, 700 (1979).