

Mechanism of Carbon Tetrachloride-Induced Hepatotoxicity. Hepatocellular Damage by Reactive Carbon Tetrachloride Metabolites

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CCl₄-induced liver damage was modeled in monolayer cultures of rat primary hepatocytes with a focus on involvement of covalent binding of CCl₄ metabolites to cell components and/or peroxidative damage as the cause of injury.

(1) Covalent binding of ¹⁴C-labeled metabolites was detected in hepatocytes immediately after exposure to CCl₄. (2) Low oxygen partial pressure increased the reductive metabolism of CCl₄ and thus covalent binding. (3) [¹⁴C]-CCl₄ was bound to lipids and to proteins throughout subcellular fractions. Binding occurred preferentially to triacylglycerols and phospholipids, with phosphatidylcholine containing the highest amount of label. (4) The lipid peroxidation potency of CCl₄ revealed subtle differences compared to other peroxidative substances, viz., ADP-Fe³⁺ and cumol hydroperoxide, respectively. (5) CCl₄, but not the other peroxidative substances, decreased the rate of triacylglycerol secretion as very low density lipoproteins. (6) The anti-oxidant vitamin E (α-tocopherol) blocked lipid peroxidation, but not covalent binding, and secretion of lipoproteins remained inhibited. (7) The radical scavenger piperonyl butoxide prevented CCl₄-induced lipid peroxidation as well as covalent binding of CCl₄ metabolites to cell components, and also restored lipoprotein metabolism.

The results confirm that covalent binding of the CCl₃* radical to cell components initiates the inhibition of lipoprotein secretion and thus steatosis, whereas reaction with oxygen, to form CCl₃-OO*, initiates lipid peroxidation. The two processes are independent of each other, and the extent to which either process occurs depends on partial oxygen pressure. The former process may result in adduct formation and, ultimately, cancer initiation, whereas the latter results in loss of calcium homeostasis and, ultimately, apoptosis and cell death.

Introduction

The hepatotoxic action of CCl₄ is closely linked to its metabolic activation to short-lived reactive intermediates (Conner *et al.*, 1986). Reductive dehalogenation of CCl₄ is catalyzed by cytochrome P450, the terminal oxidase of the hepatic mixed function oxidase system (Nogushi *et al.*, 1982). The existence of free radicals during CCl₄ metabolism has been proven by spin trapping experiments (Conner *et al.*, 1986).

CCl₄-induced cell damage can result from either covalent binding of the reactive intermediates to cellular components, or from enhanced lipid peroxidation triggered by interaction of free radical intermediates with oxygen which in turn attack unsaturated fatty acids. This gives rise to destruction of lipids, particularly unsaturated phospholipids, resulting in damage to intracellular mem-

branes and the plasma membrane (Cheeseman *et al.*, 1985). The break-down products, mostly reactive aldehydes, are found throughout the cell and will lead to further damage such as increased membrane permeability, being one indicator of impending cell death.

The course of CCl₄-induced hepatotoxicity is, to some extent, governed by the partial pressure of oxygen in tissues: low partial pressure results in predominant formation of CCl₃* and CHCl₂* radicals and covalent metabolite binding (De Groot *et al.*, 1988; Masuda and Nakamura, 1990). This affects mostly the metabolism of lipids (increased synthesis, decreased transport out of the hepatocyte) and results in steatosis, or fatty liver. High oxygen partial pressure, on the other hand, shifts CCl₄ metabolism toward formation of the CCl₃-OO* radical with consequent lipid peroxidation, essentially pushing the cell from steatosis to

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apoptosis (Kiezska and Kappus, 1980; De Groot *et al.*, 1988).

In the present communication we extend the observations on CCl_4 -induced liver damage (Boll *et al.*, 2001a, b) and report on experiments where hepatocytes were exposed to CCl_4 , and the ensuing toxicity was modified with a variety of substances targeted at different steps of the CCl_4 attack. These substances included a cytochrome P450 inducer, change in oxygen pressure, chemicals that induce lipid peroxidation without covalent binding, anti-oxidants, and a radical scavenger. Several typical parameters of CCl_4 toxicity were monitored, among them lipoprotein release and composition, covalent binding of activated metabolites to subcellular fractions, and aldehyde formation from lipid peroxidation. The results contribute to a better understanding of the mechanism of CCl_4 -induced steatosis and hepatotoxicity.

Experimental

Preparation of cultured hepatocytes, methods for lipid and lipoprotein isolation and analysis as well as general methods have been described (Boll *et al.*, 2001a, b). When indicated, animals were pre-treated with phenobarbital (100 mg i.p./kg \times day in 0.9% NaCl for 4 days).

Incubations and preparation of cell fractions

Hepatocytes (5×10^6 cells/ml) were incubated at 37 °C in 8 ml Ham's F-12 medium plus additions (Boll *et al.*, 2001a). Incubations were in the presence of 21% O_2 and 5% CO_2 (standard conditions). To simulate low oxygen pressure an atmosphere of 5% oxygen, 5% CO_2 and 90% N_2 was used, while 95% O_2 and 5% CO_2 was used to simulate high oxygen pressure. For incubations in the presence of CCl_4 see Boll *et al.*, (2001a). Incubations with labeled acetate were with 1.7 MBq [$1\text{-}^{14}\text{C}$]-acetate and 3.5 mM sodium acetate in 8 ml medium. Incubations were terminated by addition of 4 ml ice-cold 0.9% NaCl to the culture dish. Cells were then rinsed 4 times with 6 ml NaCl to remove radiolabel from the medium. [^{14}C]- CCl_4 was removed by a short vacuum treatment at 45 °C.

One ml of homogenization buffer (100 mM sucrose, 50 mM KCl, 40 mM KH_2PO_4 , 30 mM

EDTA and 0.1 M dithioerythritol, pH 7.5) was added and the cells scraped from the culture dish. Cell suspensions were sonified 3 times for 10 sec each (Branson sonifier with microtip, 50 watt). The homogenate was centrifuged at $5000 \times g$ for 10 min and then the supernatant (whole cells) was fractionated by centrifugation at $25,000 \times g$ for 15 min (mitochondrial pellet) and $105,000 \times g$ for 60 min to obtain microsomes and cytosol.

Covalent binding of [^{14}C]- CCl_4 to cellular lipids and proteins

Lipid-bound radioactivity: An aliquot of the cells or each cellular fraction was subjected to lipid extraction (Boll *et al.*, 2001a). The lipid phase was evaporated under a stream of nitrogen, dried, washed with 2 ml of chloroform (to completely remove non-covalently bound [^{14}C]- CCl_4), and dried again. The residue was taken up in cyclohexane and radioactivity was determined (total lipids). Where indicated the lipid fraction was separated by thin layer chromatography (cf. Boll *et al.*, 2001a) and [^{14}C]- CCl_4 -derived radioactivity was determined in the individual lipid classes after scratching appropriate areas from the TLC plates.

Protein-bound radioactivity: Another aliquot was treated with 10% TCA as described (see protein synthesis) and labeled protein was measured. Precipitates were washed in chloroform : methanol (1:2, v/v) to remove lipid bound radioactivity.

Protein synthesis

Cells were incubated with 1.49 MBq L-[$1\text{-}^{14}\text{C}$]-leucine/8 ml medium. Incubation was terminated with 4 ml 0.9% NaCl, containing 5 mM L-leucine. After rinsing in buffer containing 5 mM L-leucine for 4 times cells were homogenized as described above. The $5000 \times g$ supernatant (whole cells) or the subcellular fractions were treated with 10% TCA in an ice-bath for 30 min. The resulting protein precipitates, after washing with TCA, were kept at 80 °C for 15 min to hydrolyze aminoacyl t-RNA's. Samples were collected on glass fiber filters (Whatman GF/C). They were washed with TCA, ethanol and diethylether (6 ml each), dissolved in 1 ml tissue solubilizer (60 °C, 60 min) and finally counted in a toluene-based scintillation cocktail.

Protein secretion

Cells were prelabeled with 74×10^4 Bq L-[4,5- $^3\text{H}(\text{N})$]-leucine/8 ml medium for 2h. After removal of radiolabel the cells were incubated and aliquots of the incubation medium were removed at different times. These were treated with 10% TCA as above. The resulting precipitates of secreted proteins were treated as described above for cellular proteins.

Distribution of microsomal protein in response to covalent binding of [^{14}C]- CCl_4

Following incubation of the hepatocytes from phenobarbital-treated animals with [^{14}C]- CCl_4 (see above) lipids were extracted from the microsomal fractions ($105,000 \times g$ pellet) (see Boll *et al.*, 2001a) and the lipid-free proteins, after lyophilization of the aqueous phase, were subjected to gel electrophoresis with 10% SDS. Gel tracks were sliced into 2 mm strips and solubilized in 2 ml 30% H_2O_2 (24 h at 55 °C; Frank and Link, 1984). Radioactivity was determined as described above.

Lipoproteins: The fractions of hepatocellular lipoproteins and medium-secreted (extracellular) lipoproteins were isolated and lipid and protein fractions were analyzed as previously described (Boll *et al.*, 2001b).

Other procedures

Separation of phospholipids: Phospholipids of the whole cell lipid fraction were further separated by two-dimensional thin layer chromatography on silica gel plates. Mobile phase for 1st dimension: chloroform: methanol: ammonia (65:35:5, v/v); for 2nd dimension: chloroform: acetone: methanol: acetic acid: H_2O (50:40:10:10:0.5, v/v). Identification was achieved using co-chromatographed phospholipid standards.

Lipid peroxidation was induced with 200 μM of either CCl_4 , cumol hydroperoxide (dissolved in DMSO) or with 3.3 mM/200 μM ADP- Fe^{3+} (Unge-mach, 1985). It was monitored by the formation of malondialdehyde (Smith *et al.*, 1982).

DNA content was determined fluorometrically according to Sterzel *et al.* (1985).

Statistics

All values are given as mean \pm SEM. Appropriate sets of data were subjected to ANOVA, and

statistical significance was determined by the Tukey-Kramer multiple comparison test.

Reagents

Ham's F-12 medium was obtained from ICN Biochemicals, Eschwege, Germany. Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone), cumol hydroperoxide and piperonyl butoxide were from Fluka, Seelze, Germany. CCl_4 and silica gel 60 F₂₅₄ plates were products of Merck, Darmstadt, Germany. α -Tocopherol acetate, phospholipid standards and tissue solubilizer were obtained from Sigma, Deisenhofen, Germany. [^{14}C]-carbon tetrachloride (spec. act. 2.25 TBq/mmol) was from Amersham Buchler, Braunschweig, Germany. [^{14}C]-acetate (spec. act. 2.1 GBq/mmol), L-[4,5- $^3\text{H}(\text{N})$]-leucine (spec. act. 1.84 TBq/mmol) and L-[1- ^{14}C]-leucine (spec. act. 1.85 GBq/mmol) were from NEN Life Sciences Products, Cologne, Germany. All reagents used were of analytical grade.

Results

Metabolic activation of CCl_4

Accumulation of fat is one consequence of the CCl_4 -induced liver damage. This damage will develop only in the presence of an intact cytochrome P450 oxygenase system (Boll *et al.*, 2001a). The cytochrome P450 inducer metyrapone was used to enhance bioactivation of CCl_4 and formation of radicals, *viz.*, CCl_3^* and CHCl_2^* . This resulted in accumulation of triglycerides in the cultured hepatocytes whereas CCl_4 alone could not increase the triglyceride content (Fig. 1, curves (1)–(4), cf. Boll *et al.*, 2001a). Piperonyl butoxide, used to scavenge radicals formed from CCl_4 , prevented triglyceride accumulation in the cells (Fig. 1, (5)). Incubation of hepatocytes with the inducer and/or radical scavenger alone had no effect (not shown).

Following [^{14}C]- CCl_4 exposure covalent ^{14}C -labeled material was rapidly bound to cell lipids, showing saturation kinetics (Fig. 2). Pretreatment of the animals for 4 days with phenobarbital to induce cytochrome P450 resulted in a ca. 20% increase of binding (Fig. 2, (1) and (2)). Not surprisingly the highest amount of radioactivity in subcellular fractions was found in lipids from microsomes ($105,000 \times g$, Fig. 2, (3)), followed by the mitochondrial fraction ($25,000 \times g$, (4)). Given

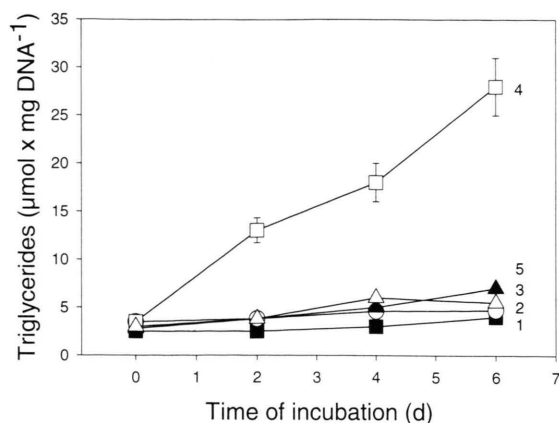


Fig. 1. CCl_4 -induced accumulation of triglycerides in cultured hepatocytes and effect of the radical scavenger piperonyl butoxide. Additions: (1) control, (no addition); (2) 0.5 mM metyrapone (inducer); (3) 200 μM CCl_4 ; (4) metyrapone and CCl_4 ; (5) metyrapone, CCl_4 and 200 μM piperonyl butoxide (scavenger). Hepatocytes, procured from phenobarbital-treated animals, were incubated with the additions as indicated. Medium plus additions were replaced after 4 h and again every 24 h thereafter. Triglycerides were determined as described (Boll *et al.*, 2001a). Means of 4 incubations; SEM in parts omitted for clarity.

the low amount of covalently-bound radioactivity in cytosol (5) this might indicate that mitochondria are also capable of metabolically activating CCl_4 . The amounts of bound radioactivity in the three subcellular fractions add up accurately to the amount found in whole cells.

Fig. 3 displays the influence of oxygen pressure on the binding of CCl_4 -derived metabolites to hepatocyte total lipids. Lowering the oxygen partial

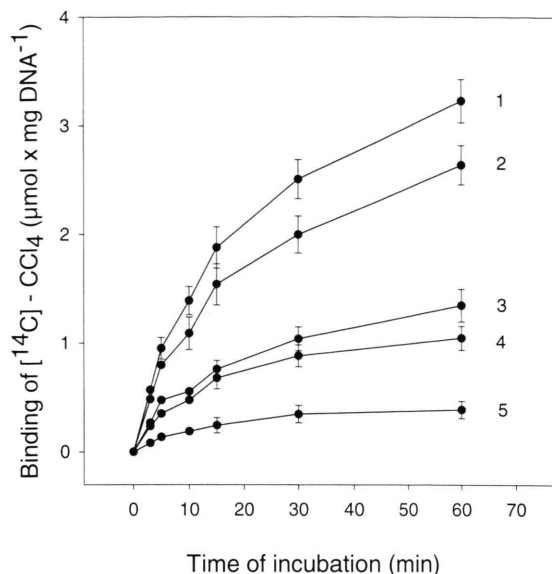


Fig. 2. Covalent binding of CCl_4 metabolites to the lipids of hepatocyte subcellular fractions. (1) whole cells from phenobarbital-treated animals; (2) whole cells; (3) 105,000 \times g pellet (microsomes); (4) 25,000 \times g pellet (mitochondria); (5) cytosol. Incubations were performed with 200 μM $[^{14}\text{C}]\text{-CCl}_4$ under standard oxygen conditions (see Methods) for the indicated times. Radioactivity in total lipids was determined in whole cells and in the cell fractions. Values are means of 4 incubations \pm SEM.

pressure gradually increased covalent binding, i.e. CCl_4 toxicity. Five percent O_2 in the atmosphere increased covalent binding of CCl_4 metabolites to cellular lipids by about 40% over regular oxygen pressure (21% O_2 , Fig. 3, curves (1) and (2)). High oxygen tension (95% O_2 , 5% CO_2 , (3)) reduced

Table I. Covalent binding of $[^{14}\text{C}]\text{-CCl}_4$ metabolites to different lipid classes (A) and to phospholipids (B).

Lipid classes	A	B	
	Lipids percent of total	Phospholipids covalent binding (nmol $\times 10^6$ cells $^{-1}$)	
Phospholipids	41.0	sphingomyelin	2.6 \pm 0.28
Triglycerides	33.2	phosphatidylcholine	5.4 \pm 0.58
Diglycerides	12.0	phosphatidylserine	0.5 \pm 0.06
Cholesterol	7.9	phosphatidylethanolamine	2.1 \pm 0.23
Cholesterol esters	3.4		
Free fatty acids	2.5		

Hepatocytes from phenobarbital-treated animals were incubated with 200 μM $[^{14}\text{C}]\text{-CCl}_4$ for 60 min (5% O_2 , 5% CO_2 and 90% N_2 , see Methods). Non-covalently bound $[^{14}\text{C}]\text{-CCl}_4$ was removed (see Methods).

(A): Total lipids were extracted from the cells and separated via thin layer chromatography (Boll *et al.*, 2001a).

(B): The phospholipid fraction of (A) was further separated by thin layer chromatography (see Methods). Values are means of 4 incubations \pm SEM.

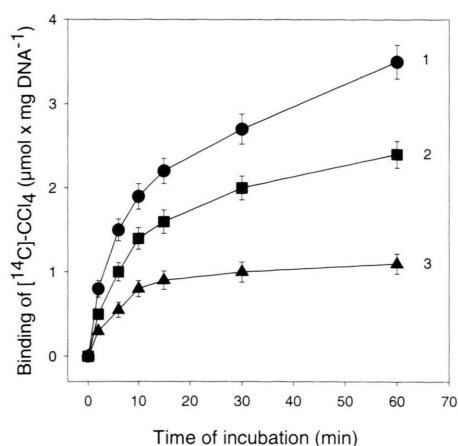


Fig. 3. Effect of different oxygen partial pressures on covalent binding of CCl_4 metabolites. Hepatocytes were incubated with $200 \mu\text{M}$ ^{14}C - CCl_4 in atmospheres containing (1) 5%, (2) 21% and (3) 95% oxygen, respectively, 5% CO_2 , and nitrogen for the balance. ^{14}C - CCl_4 -derived radioactivity was determined in total lipids. For details see Method section. Means of 3 incubations \pm SEM.

covalent binding to less than one half, lending further support to the notion that metabolic activation of CCl_4 is a reductive process.

Covalent binding of CCl_4 -metabolites to cellular lipid and protein fractions

CCl_4 is covalently bound to different classes of lipids (Table I). A low oxygen pressure was used here to increase covalent binding of CCl_4 . About 75% of the CCl_4 -derived radioactivity in cellular lipids was bound to the phospholipid and triacylglycerol fractions (Table I A). Also 8% of the lipid-bound radioactivity was associated with cholesterol, another 3 to 4% with cholesterol esters (Table I A). Within the phospholipid fraction radioactivity was predominantly bound to phosphatidylcholine (Table I B).

Hepatocytes and their subcellular fractions were investigated for the distribution of ^{14}C - CCl_4 -derived radiolabel between solvent-extractable (lipid) and TCA-precipitable material (protein) (Table II). In whole cells almost twice the amount of radioactivity was bound to lipids (a), when compared to protein (b). Of this amount more than half was associated with the $105,000 \times g$ pellet (microsomes), about one-fourth with mitochondria ($25,000 \times g$ pellet), and only about 3%

Table II. Distribution of covalently-bound ^{14}C - CCl_4 among hepatocellular fractions.

Fraction	nmol ^{14}C - $\text{CCl}_4 \times 10^6 \text{ cells}^{-1}$
Whole cells	
(a)	12.2 ± 1.5
(b)	6.6 ± 0.7
Microsomes	
(a)	7.9 ± 0.82
(b)	3.5 ± 0.36
Mitochondria	
(a)	2.8 ± 0.31
(b)	1.3 ± 0.14
Cytosol	
(a)	0.42 ± 0.05
(b)	0.15 ± 0.02
Lipoproteins (intracellular)	
(a)	1.05 ± 0.10
(b)	1.22 ± 0.13
Lipoproteins (extracellular)	
(a)	0.15 ± 0.02
(b)	0.40 ± 0.05

(a) lipid-bound radioactivity; (b) protein-bound radioactivity. Experimental conditions were the same as in Table I. For determination of lipid and protein-bound radioactivity see Method section. Means of 4 incubations \pm SEM.

with the cytosol, maintaining the 2:1 labeling ratio between lipid and protein. A significant amount of radioactivity, *viz.*, 15%, was associated with lipoproteins, with about 4 times as much label bound to the intracellular fraction than to the secreted extracellular lipoproteins (Table II). It is noteworthy that in the lipoprotein fraction the ratio of lipid- to protein-bound radiolabel was less than 1:1 and thus significantly smaller than in the other subcellular fractions.

Activated metabolites and radical intermediates of CCl_4 were bound to proteins (Table II). A separation of the proteins was attempted under the premise that covalent binding of a CCl_4 -derived di- or trichloro residue did not significantly influence the mobility of a protein in 10% SDS electrophoresis (Frank and Link, 1984). Electrophoresis of microsomal proteins from ^{14}C - CCl_4 -treated hepatocytes (see Method section) revealed that, aside of the start and the front of the gel, radioactive label was found primarily between 70 and 80 kDa, which corresponds to NADPH cytochrome P450 reductase. Further accumulation of radioactivity was observed between 47 and 52 kDa

(cytochrome P450) and around 120 kDa (oligomers of cytochrome P450) (data not shown). This is consistent with a preferential reaction of the metabolites with molecules present in their immediate vicinity at the time of bioactivation.

Effect of CCl_4 on lipid peroxidation

In order to elucidate the respective shares of covalent metabolite binding and lipid peroxidation in hepatocellular damage several substances were used which are known to cause lipid peroxidation

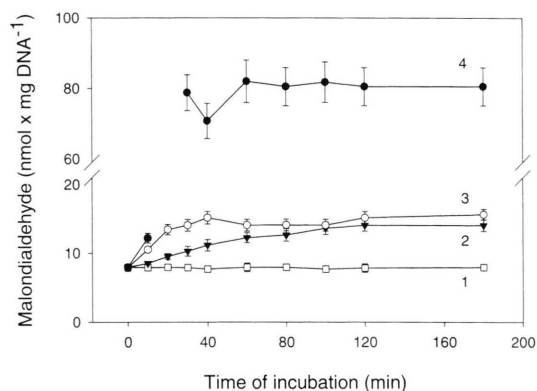


Fig. 4. Formation of malondialdehyde in hepatocytes treated with various lipid peroxidating agents. Hepatocytes were incubated in the presence of $200\ \mu\text{M}$ of the different agents (see Methods): (1) control (no addition); (2) cumol hydroperoxide; (3) CCl_4 ; (4) ADP-Fe^{3+} . At the indicated times malondialdehyde was determined. Means of 4 incubations \pm SEM.

in the absence of covalent binding, *viz.* ADP-Fe^{3+} and cumol hydroperoxide. Their effects were compared to those brought forth by the CCl_4 -derived peroxidation starter radical $\text{CCl}_3\text{-OO}^*$ (Fig. 4). Lipid peroxidation which CCl_4 caused in a concentration-dependent manner was estimated via malondialdehyde formation (not shown). On a molar basis, *i.e.*, at $200\ \mu\text{M}$, the lipid peroxidation potency of CCl_4 (Fig. 4, (3)) was somewhat higher than that of cumol hydroperoxide (2), but significantly lower than that of ADP-Fe^{3+} (4). This emerged also from the kinetics of the three reactions: cumol hydroperoxide reached the maximum of malondialdehyde formation after 2 hours, CCl_4 after 40 min, and ADP-Fe^{3+} already after 30 min. In addition, $200\ \mu\text{M}$ ADP-Fe^{3+} produced 5 times the amount of malondialdehyde than CCl_4 did (note the split y-axis in Fig. 4). Viability of the hepatocytes (trypan blue staining, leakage of lactic dehydrogenase, lipogenic activities: Boll *et al.*, 2001a) was not affected by the peroxidative substances.

A major difference in the actions of CCl_4 and the other two peroxidative substances, respectively, became evident in their effects on lipoproteins. CCl_4 decreased secretion of both very low density lipoproteins (VLDL) (-53%) and high density lipoproteins (HDL) (-28%) (Table III; see also Boll *et al.*, 2001b). The effects of ADP-Fe^{3+} and cumol hydroperoxide, respectively, were minor in comparison. Incorporation experiments

Table III. Effect of $200\ \mu\text{M}$ CCl_4 , ADP-Fe^{3+} or cumol hydroperoxide on (A) secretion of lipoproteins, (B) protein synthesis and (C) protein secretion.

	A		B	C
	Lipoprotein secretion VLDL	HDL	Protein synthesis	Protein secretion
CCl_4	46.7 ± 5.6	71.2 ± 6.6	62.0 ± 5.4	76.8 ± 5.1
ADP-Fe^{3+}	85.4 ± 7.2	90.4 ± 8.1	88.2 ± 7.6	96.0 ± 8.8
Cumol hydroperoxide	79.3 ± 4.8	92.4 ± 7.9	81.4 ± 8.8	93.1 ± 7.7

Values are given as percent of controls. Controls (100 percent) refer to the experimental procedures of A, B and C in the absence of the respective compound (CCl_4 , ADP-Fe^{3+} or cumol hydroperoxide).

(A) Cells were incubated with $1.5\ \text{mM}$ $[1\text{-}^{14}\text{C}]\text{-acetate}$ for 2 h (see Methods). After removal of radiolabel incubation (start at time zero) was in label-free medium for 60 min in the presence of the respective compound. Then radioactivity in the lipid fractions of secreted VLDL and HDL lipoproteins was determined in the medium (see Boll *et al.*, 2001b). Controls: VLDL: $75\ \text{nmol} \times \text{mg DNA}^{-1}$; HDL: $6.2\ \text{nmol} \times \text{mg DNA}^{-1}$.

(B) Cells were incubated with the respective compound for 60 min. Then protein synthesis was determined after a 30 min incubation with $1.5\ \text{mM}$ $\text{L}\text{-}[1\text{-}^{14}\text{C}]\text{-leucine}$ (see Methods). Control: $124\ \text{nmol } ^{14}\text{C-leucine} \times \text{mg DNA}^{-1}$.

(C) Cells were incubated for 2 h with $\text{L}\text{-}[4,5\text{-}^3\text{H}(\text{N})]\text{-leucine}$. After removal of radioactive label incubation continued for 60 min in the presence of the respective compound. Then labeled protein, secreted into the medium, was determined. For details see Method section. Control: $5.3\ \text{nmol } ^3\text{H-leucine} \times \text{mg DNA}^{-1}$. Values are means of 3 incubations \pm SEM.

with labeled leucine revealed that neither cellular protein synthesis nor secretion of serum proteins were affected by the peroxidative substances ADP-Fe³⁺ or cumol hydroperoxide but CCl₄ decreased both pathways (synthesis: -38%; secretion: -23%; Table III). It was thus possible to produce a high degree of lipid peroxidation, but in the absence of covalently bound metabolites lipoprotein secretion was not significantly affected.

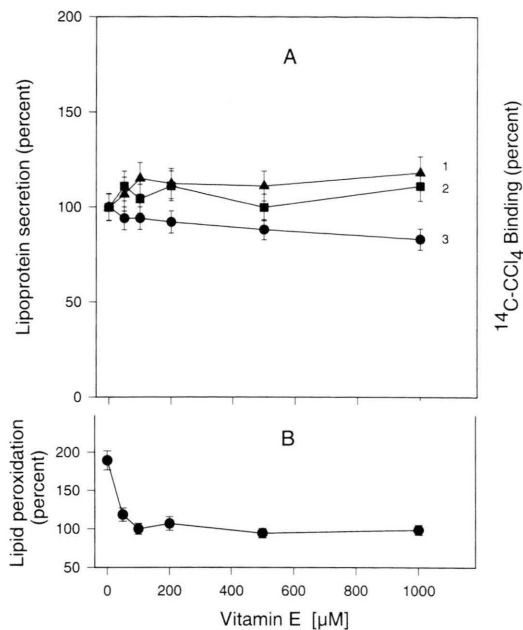


Fig. 5. Effects of vitamin E on CCl₄-induced toxicity in hepatocytes.

Panel A: (1) HDL; (2) VLDL; (3) Covalent binding. 100 percent refers to (1) and (2) the extent of inhibition of lipoprotein secretion (Boll *et al.*, 2001b) and to (3) the extent of covalent binding (see (2) in Fig. 2) caused by 200 μM CCl₄ within 30 min in the absence of α-tocopherol.

Panel B: The zero value refers to the induction of peroxidation over control (100%) by 200 μM CCl₄ within 30 min (see also (1) and (3) in Fig. 4) in the absence of α-tocopherol.

Hepatocytes were incubated with 1.5 mM [1-¹⁴C]-acetate and 3.5 mM sodium acetate for 2 h. This was followed by incubation for 30 min with α-tocopherol as indicated. After removal of radiolabel the cells were incubated with 200 μM CCl₄ for another 30 min. Then radioactivity of secreted VLDL and HDL lipids and malondialdehyde was determined. For determination of covalent binding cells were incubated with unlabeled sodium acetate followed by α-tocopherol treatment as above. Then the cells were incubated with 200 μM [1-¹⁴C]-CCl₄ and covalent binding of labeled CCl₄ to lipid fraction was determined after 30 min. Values are means of 4 incubations ± SEM.

Effect of antioxidants and radical scavengers on CCl₄-induced cellular damage

Dietary supplementation with vitamin E will protect against CCl₄-induced chronic liver disease (Parola *et al.*, 1992). Vitamin E, at concentrations up to 1 mM, had little effect on the CCl₄-related inhibition of lipoprotein secretion from hepatocytes or on covalent binding of ¹⁴C-labeled CCl₄ metabolites (Fig. 5 A). However, the CCl₄-induced lipid peroxidation, i.e. about 100% over control in the presence of 200 μM CCl₄ (Fig. 5 B, see also Fig. 4)), was completely prevented by as little as 100 μM α-tocopherol (Fig. 5 B).

In contrast to anti-oxidants, piperonyl butoxide, a free radical scavenger and cytochrome P450 ligand, was able to abolish all effects of CCl₄ in cultured hepatocytes. Increasing concentrations of piperonyl butoxide suppressed covalent binding of activated CCl₄ metabolites (Fig. 6 A). It also released the CCl₄-induced blockage of VLDL and HDL secretion (Fig. 6 A). CCl₄-induced lipid peroxidation was also prevented completely by piperonyl butoxide in a concentration-dependent man-

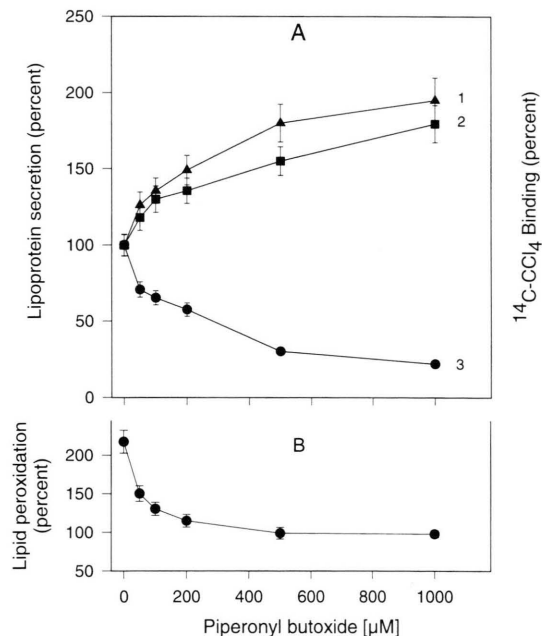


Fig. 6. Effect of piperonyl butoxide on CCl₄-induced toxicity in hepatocytes.

Experimental conditions are as in Fig. 5, except piperonyl butoxide was used instead of α-tocopherol. Values are means of 4 incubations ± SEM.

ner (Fig. 6 B). Thus, the process of lipoprotein secretion inhibition is independent from lipid peroxidation.

Discussion

CCl_4 in the form of the above-mentioned radicals can bind to both cellular lipids and proteins. Apparently this is an essential step in blocking of lipoprotein secretion (Dianzani and Poli, 1984). CCl_4 -induced liver damage progresses through a series of steps that contribute to various extents to the ultimate damage: reductive dehalogenation, covalent binding of resulting radicals; inhibition of protein synthesis (in particular, apolipoprotein synthesis), assembly, packaging and release of VLDL and HDL, fat accumulation; formation of $\text{CCl}_3\text{-OO}^*$ radicals, lipid peroxidation, membrane damage, loss of Ca^{2+} sequestration, apoptosis; fibrosis (Clawson, 1989; Boll *et al.*, 2001a, b). Serious damage will develop only in the presence of an induced cytochrome P450 oxygenase system, i.e., when bioactivation of CCl_4 occurs; the stronger the induction, the more damage results. Radicals apparently play a pivotal role in this process, as demonstrated here by the protection afforded by anti-oxidants and radical scavengers.

Low oxygen partial pressure favored reductive CCl_4 metabolism (Fig. 3), and high oxygen pressure protected against CCl_4 -induced liver injury both *in vivo* and *in vitro* (Kiezcka and Kappus, 1980; De Groot *et al.*, 1988). Evidently oxygen, possibly with the aid of an oxidase or oxygenase, has a higher affinity for CCl_3^* radicals than the hydrogen substituents on surrounding biomolecules (Link *et al.*, 1984). The result on one side is a decrease in the generation of free radical intermediates (Burk *et al.*, 1984; De Groot *et al.*, 1988), but on the other side formation of $\text{CCl}_3\text{-OO}^*$ radicals that initiate lipid peroxidation. This is a somewhat simplified representation of the events, as De Groot *et al.* (1988) have shown that there exists a more complicated relationship between oxygen partial pressure and CCl_4 toxicity. Covalently modified and possibly cross-linked phospholipids are resistant to the action of phospholipase A and can decrease the fluidity of the lipid bilayers (Frank and Link, 1984).

Damage to membrane integrity will affect calcium homeostasis, thought to be the cause of

CCl_4 -induced liver necrosis. Ca^{2+} was proposed as a toxic messenger (Nicotera *et al.*, 1992). Irreversible membrane damage, loss of Ca^{2+} sequestration in hepatocytes, leakage of K^+ and entry of Na^+ may be the critical events in the initiation of cell death (Ozaki and Masuda, 1993). It was also suggested that Ca^{2+} channel blockers are able to limit hepatocellular damage arising from the destructive action of CCl_4 on Ca^{2+} channels (Deakin *et al.*, 1991). Radical formation and lipid peroxidation are the predominant cellular mechanisms involved in the development of fatty liver caused by CCl_4 exposure as well as excessive alcohol intake (Tribble *et al.*, 1987; for a review see Lieber, 2000). There are numerous agents known to ameliorate or prevent alcoholic liver disease, both natural and synthetic, which were also investigated for their potential beneficial effect in connection with CCl_4 . The effectiveness of such agents, and knowledge of the step in disease development they interrupt, are very helpful to better understand the mechanism of toxicity of CCl_4 .

S-adenosyl-methionine, a popular nutritional supplement, elevated glutathione levels and reduced CCl_4 -induced liver damage (Gasso *et al.*, 1996). The effect is most likely related to lipoprotein formation: phosphatidylethanolamine methylation is a crucial step in VLDL secretion (Nishimaki-Mogami *et al.*, 1996), and exogenous supply of S-adenosyl-methionine restored the methylation process suppressed by liver disease (Muriel *et al.*, 1994).

Several plant products were found to protect from liver damage. Silymarin, a bioflavonoid from milk thistle, was effective in preventing CCl_4 -induced liver injury (Letteron *et al.*, 1990) as was colchicine, the toxin from crocus (Mourelle *et al.*, 1988). Both are cytochrome P450 inhibitors, and both have anti-oxidant properties. Another natural substance is hepatic stimulator substance (HSS), a 12.8 kDa peptide growth factor typically isolated from fetal liver. It protected liver against acute failure induced by CCl_4 due to an anti-oxidant effect that reduced lipid peroxidation (Mao Hua *et al.*, 1993). No cytochrome P450 inhibition is associated with HSS.

Colchicine and HSS have several properties in common to suggest that their liver-protective action uses a similar mechanism of action. Both, by their as yet unexplained anti-oxidant effect, pre-

vented lipid peroxidation and stabilized membranes (Mao Hua *et al.*, 1993; Martinez *et al.*, 1995). Lipid peroxidation and the subsequent intracellular membrane damage disturbed Ca^{2+} sequestration and gave rise to CCl_4 -induced liver injury (Clawson 1989). This sequence of events may well explain how HSS and colchicine can protect from any kind of lipid peroxidation-related cell damage.

Furthermore, both colchicine and HSS are mitogens, and both are credited with anti-inflammatory action (Yao *et al.*, 1992; Lieber, 1997). This raises the possibility of a participation of inflammatory cytokines, in particular, tumor necrosis factor (TNF)- α . This cytokine is involved in CCl_4 -induced cell damage (Czaja *et al.*, 1995; see also discussion in Boll *et al.*, 2001b). Available evidence suggests strongly that both colchicine and HSS exert an anti-inflammatory action by inhibiting the synthesis or action of inflammatory cytokines (Yao *et al.* 1992; Lieber, 1997).

Dietary supplementation with the anti-oxidant vitamin E will protect against CCl_4 -induced chronic liver, oxidative stress and membrane damage due to lipid peroxidation (Parola *et al.*, 1992). The present investigation confirmed these findings (cf. Fig. 5 B). On the other hand vitamin E deficiency potentiated liver fibrosis induced by CCl_4 (Seifert *et al.*, 1994). The protective effect manifested itself in reduced formation of aldehyde-peroxidation products (Dianzani, 1984; Dianzani and Poli, 1984). ESR studies revealed that vitamin E was able to scavenge $\text{CCl}_3\text{-OO}^*$ radicals, but had no effect on the formation of CCl_3^* radicals and thus could not prevent haloacylation (Willson, 1985). On a molecular basis α -tocopherol was shown to reduce binding of the transcription factor NF- κB in CCl_4 -induced liver damage. Production of free radicals and oxidative stress were shown to enhance NF- κB expression, which in turn initiated the synthesis of cytotoxic cytokines that caused liver injury (Liu *et al.*, 1995). There are striking parallels to the above-mentioned potential interactions of colchicine and HSS with TNF- α .

Dianzani and Poli (1984) suggested that the aldehydes formed during peroxidation block the synthesis of proteins and secretion of serum proteins but pointed out that this occurred only at extremely high levels of CCl_4 exposure. Indeed, in the present investigation, inducing lipid peroxida-

tion alone with ADP-Fe^{3+} or cumol hydroperoxide, without covalent metabolite binding, there was no effect on either protein synthesis or secretion to any notable extent (Table III).

Cumol hydroperoxide displayed a peroxidation potency similar to that of CCl_4 (Fig. 4), but like ADP-Fe^{3+} had little effect on lipoprotein secretion (Table III). It was suggested that cumol hydroperoxide had some effect at the step of packing phospholipids and triglycerides with the lipid carrier proteins of the lipoproteins (Dianzani and Poli, 1985), but it did not affect exocytosis of secretory proteins. Thus, lipid oxidation alone was not the cause of CCl_4 -induced liver damage, but there was a quantitative correlation between covalent binding of activated metabolites and inhibition of VLDL and HDL secretion (Boll *et al.*, 2001b).

Increasing concentrations of the radical scavenger piperonyl butoxide prevented lipid peroxidation as well as haloalkylation, restoring the inhibition of VLDL and HDL secretion (Fig. 6 A, B). Thus, an important outcome of this series of experiments is that CCl_4 -induced inhibition of lipoprotein secretion, and thus liver steatosis is for the most part a consequence of covalent binding of CCl_4 -metabolites to cell constituents, but not of lipid peroxidation.

Both intracellular apoproteins and the lipid portions of lipoproteins were labeled to a greater extent than extracellular ones, with intra/extracellular labeling ratios ranging from 3 to 6 (Table II). This suggested that covalent modification of lipoproteins was the first step in the process of blocking lipoprotein secretion. Dianzani (1984) reported that haloalkylated apoproteins were not galactosylated in the Golgi apparatus. CCl_4 intoxication led to early damage of the Golgi apparatus and CCl_4 -induced reduction of the activities of glucosyl- and galactosyl transferases (Marinari *et al.*, 1985). Galactosylation was considered essential for the intracellular maturation process of lipoprotein particles to be secreted.

The data presented here indicate that bioactivation by cytochrome P450 is required for the toxicity of CCl_4 to unfold (Fig. 1). The process is most effective in the absence of oxygen (Fig. 3), and covalent binding affects particulate cell subfractions (membranes, microsomes) more than soluble ones (Table II). Lipids are more easily attacked by the activated metabolites than protein (Table II), and

within the lipid fraction phospholipids are the prime targets (Table I). CCl₄-induced lipid peroxidation can be mimicked by other peroxidative compounds such as ADP-Fe³⁺ or cumol hydroperoxide (Fig. 4), but since the latter do not affect lipoprotein synthesis or secretion (Table III), lipid peroxidation does not contribute to the strong inhibition of lipoprotein metabolism caused by CCl₄. Antioxidants such as α -tocopherol (vitamin E) completely suppress lipid peroxidation, but cannot restore the inhibition of lipoprotein secretion (Fig. 5). The radical scavenger piperonyl butoxide, however, suppresses lipid peroxidation and covalent binding of activated metabolites, and restores lipoprotein metabolism (Fig. 6). Therefore it appears that covalent binding of activated metabolites from the reductive dehalogenation is the major cause of CCl₄-induced fatty liver, while lipid peroxidation may exacerbate the damage through subsequent impairment of membrane function.

The current view of CCl₄ toxicity does not consider a specific „receptor“ to explain the multiple actions of the toxicant. At very high doses it elicits

typical general solvent toxicity, *viz.*, general anesthesia, respiratory arrest and thus lethality. This acute toxicity is not immediately related to the liver. Medium to high doses, on the other hand, cause wide-spread liver necrosis that can cause death within a few days. This may be due to a combination of factors such as the thorough inhibition of protein synthesis, the severe derailment of intracellular Ca²⁺ sequestration, and the effect on membrane integrity. At lower doses inflammatory responses prevail. These responses are related to inflammatory cytokines, in particular, tumor necrosis factor (TNF)- α (Czaja *et al.*, 1995). Healthy hepatocytes are insensitive to TNF- α action, but become sensitive once protein and RNA synthesis are inhibited (Kull and Cuatrecasas, 1981). In addition to adduct formation resulting from reactive metabolites of CCl₄, the inflammatory processes may contribute to CCl₄-induced carcinogenesis. The present study contributes to the understanding of effects observed in the mid to lower dose range of CCl₄ toxicity, where disturbance of lipid homeostasis prevails (Boll *et al.*, 2001a).

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