Hepatocyte Damage Induced by Carbon Tetrachloride: Inhibited Lipoprotein Secretion and Changed Lipoprotein Composition

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Changes of lipoprotein secretion and composition in response to CCl_4 treatment were studied in monolayer cultures of rat primary hepatocytes.

(1) CCl_4 decreased secretion of very low density lipoproteins (VLDL) by about 85%, while high density lipoprotein (HDL) secretion was less affected (about 40%). The effect was concentration-dependent. (2) CCl_4 significantly inhibited secretion of VLDL- and HDL-associated triglycerides and cholesterol esters. VLDL- and HDL-associated cholesterol was not affected, while secretion of phospholipids was increased. (3) Hepatocytes secreted the apolipoproteins B_{48}, B_{100}, E, C, and A-I. CCl_4 reduced secretion of apoproteins associated with VLDL by almost 20%, and by about 75% when associated with HDL. The *de novo* synthesis of apolipoproteins was attenuated by CCl_4. (4) CCl_4 caused variations in the apolipoprotein composition in VLDL and HDL.

CCl_4 intoxication of the liver affected the morphology and/or function of the lipoproteins, which drastically impaired their ability to act as transport vehicles for lipids from the liver to the circulation.

Introduction

Liver is the main target organ for CCl_4 toxicity. CCl_4 reaches maximum concentrations (1 mg/g liver) in rat liver parenchyma within 2 h after its oral administration. The structure of the endoplasmic reticulum, plasma membrane, mitochondria and Golgi apparatus of the rat liver parenchymal cell was significantly altered after 1 h following administration of a single dose of CCl_4 (review: Farber and Gerson, 1984 and references therein). Clinical investigations point to a causal relation between CCl_4-induced liver injury and long standing exposure to CCl_4.

CCl_4 induces a wide variety of toxic events in the liver. The effect of CCl_4 begins with reductive dehalogenation by cytochrome P450, producing the trichloromethyl radical (CCl_3*; Mc Cay *et al.*, 1984). Covalent binding of such metabolites to proteins and lipids leads to changes in cell structure and function. By the same token the radical may bind to DNA, causing damage there with the ultimate outcome of cancer, but these events are unrelated to fatty liver.

In the presence of oxygen the trichloromethyl radical becomes rapidly converted to a trichloromethylperoxy radical (CCl_3OO*). When this radical abstracts a hydrogen atom from an unsaturated fatty acid, carbon-centered radicals are formed (Mc Cay *et al.*, 1984) which initiate a peroxidation chain and production of reactive aldehydes (Dianzani, 1984), causing damage to lipids and, in consequence, membrane function. Vitamin E or other radical scavengers can interrupt propagation of the peroxidation process.

CCl_4-induced steatosis (fatty liver) is not the result of one single event, but of a series of episodes. One important role is played by impaired transfer of triacylglycerols as VLDL from the liver to the circulation (Poli *et al.*, 1979), and another part is due to an imbalance between lipid synthesis and degradation (Boll *et al.*, 2001).

The attack of CCl_4 and/or its metabolites on triacylglycerol transfer can occur at a variety of stages: synthesis of apolipoproteins in the endoplasmic reticulum; along the secretory pathway, i.e., assembly of apoprotein moieties; coupling with lipids on the way to the Golgi apparatus; packaging of nascent VLDL particles into secre-

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tory vesicles; the intracellular movement of the particles to the plasma membrane; and, finally, exocytosis of the lipoproteins.

In a previous study we described the characteristics of CCl_4-induced changes of *de novo* fatty acid synthesis and β-oxidation (Boll *et al.*, 2001). The present communication deals with the changes induced by CCl_4 to the composition and secretion of lipoproteins in primary liver cells. Evidence will be presented that synthesis of VLDL and HDL apoproteins is inhibited by CCl_4, thus representing a possible site of impairment of triacylglycerol movement. Covalent binding of radicals from CCl_4 metabolism to cell components (protein, lipids) is the evident primary cause of liver damage; only after extended exposure times will lipid peroxidation contribute to CCl_4 toxicity (Poli *et al.*, 1990).

Materials and Methods

Methods for the preparation of hepatocytes, for lipid analysis as well as general methods have been described (Boll *et al.*, 2001).

Incubations for lipoprotein secretion

Incubations of 8 ml, containing hepatocytes in a concentration of 5×10^6 cells/ml (Boll *et al.*, 2001) were made. Incubation was with 100 μM CCl_4 in arginine-deficient Dulbecco's modified Eagle's medium (DME), containing 25 mM glucose, 10 μg/ml insulin (to promote cell viability and adhesion), 1 μM dexamethasone, 0.1 mg/ml penicillin, 0.1 mg/ml kanamycin, 10 mM HEPES, 7.8 mM $NaHCO_3$, 0.4 mM L-ornithine and 10% lipoprotein-deficient serum, pH 7.4 (Melin *et al.*, 1984). Lipoprotein-deficient serum was prepared from fetal calf serum according to Goldstein *et al.* (1983). Incubations with CCl_4 were performed as described (Boll *et al.*, 2001). Cells initially cultured in Ham's F-12 medium (Boll *et al.*, 2001), were washed 3 times with DME medium prior to incubation.

In labeling experiments cells were incubated with [1-^{14}C]-acetate and/or L-[4,5-^{3}H (N)]-leucine and, after removal of the radioactive label by three washes (6 ml each) with DME medium, were incubated in label-free medium with CCl_4. For determination of the apoprotein composition cells were incubated with labeled leucine (see above) in the presence of CCl_4 and the washed label-free cells were used for apoprotein analysis (see below).

Isolation and analysis of lipoproteins

Incubations were terminated by addition of ice-cold 0.9% NaCl (1 ml/2 ml medium) and after centrifugation at 10,000×*g* for 10 min to remove cells and debris VLDL and HDL were isolated from the cell-free supernatant by polyanionic precipitation at neutral pH using sulfated polysaccharides or sodium phosphotungsstate (Burstein and Legman, 1982). This method yields selective precipitation of apolipoprotein B-containing material, viz., VLDL and HDL (Assman, 1985). Less than 5% of the total precipitated lipoproteins was LDL. Precipitates were essentially free of α-lipoproteins (Assman, 1985) and contained only traces of albumin. The efficiency of lipoprotein precipitation was about 88%.

Aliquots of the resulting VLDL and HDL pellets of the polyanionic precipitation were dissolved in NCS tissue solubilizer and radioactivity in lipids (^{14}C) and in protein (^{3}H) was measured by scintillation counting. When indicated, lipids in lipoproteins were determined after solvent extraction via thin layer chromatography (for details see Boll *et al.*, 2001). Protein, if nonradioactive, was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard and 2% deoxycholate to solubilize lipids.

Identification of apolipoproteins

VLDL and HDL fractions (see above) were dialyzed at 4 °C against 0.05% NaCl for 48 h and subsequently freeze-dried. Apoproteins were separated by SDS polyacrylamide gel electrophoresis with the modifications of Melin *et al.* (1984) to allow for a wider range of molecular weights. Gels were evaluated with an Ultrascan Laser Densitometer (LKB). Identification was based on cochromatography of protein standards and on published data (Melin *et al.*, 1984). In experiments with labeled L-leucine radioactivity was determined after solubilizing appropriate gel slices.

Statistics

The values are expressed as mean ± SEM. Statistical significance was determined by ANOVA,

followed by the Tukey-Kramer multiple comparison test.

Reagents

Dulbecco's modified Eagle's medium (DME) was obtained from ICN Biochemicals, Eschwege, Germany. [1-^{14}C]-acetate (spec. act. 2.1 GBq/mmol) and L-[4,5-^{3}H(N)] -leucine (spec. act. 1.84 TBq/mmol) were from NEN Life Sciences Products, Cologne, Germany. NCS tissue solubilizer and HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) were from Sigma, Deisenhofen, Germany and CCl_4 was a product of E. Merck, Darmstadt, Germany. All reagents were of analytical grade.

Results and Discussion

Effect of CCl_4 on VLDL and HDL secretion

Secretion of VLDL and HDL into the medium could be detected as early as 10 min into the incubation of hepatocytes prelabeled with [1-^{14}C]-acetate (Fig. 1). 100 μM CCl_4 caused persistent inhibition of VLDL secretion, resulting in an 85% reduction after 3 h (panel A, curve 2). HDL secretion was less affected, only about 40% after 3 h (panel B, curve 2).

The effect was concentration-dependent (Fig. 2). Hepatocytes were prelabeled with [1-^{14}C]-acetate and L-[4,5-^{3}H(N)]-leucine to show the effect of CCl_4 metabolites on lipid as well as on protein secretion. Export of VLDL-associated lipid was almost completely inhibited by CCl_4 concentrations of 100 μM and above (Fig. 2A, curve 1), while secretion of the protein portion was less affected (about 50%; curve 2). HDL lipoproteins responded qualitatively in a similar fashion, but with a less pronounced inhibition, viz., about 50% and 40% for lipid and protein, respectively (Fig. 2, panel B). Since the experiment did not exceed a CCl_4 concentration of 500 μM it cannot be decided whether higher toxicant exposure would have resulted in a similar extent of inhibition of HDL-associated lipid secretion as was observed with VLDL. The concentrations of CCl_4 used to study the different toxic effects in hepatocytes were in the range 100–500 μM. Divald *et al.* (1990), determining protein- and phospholipid synthesis as a measure of functional integrity, reported that the

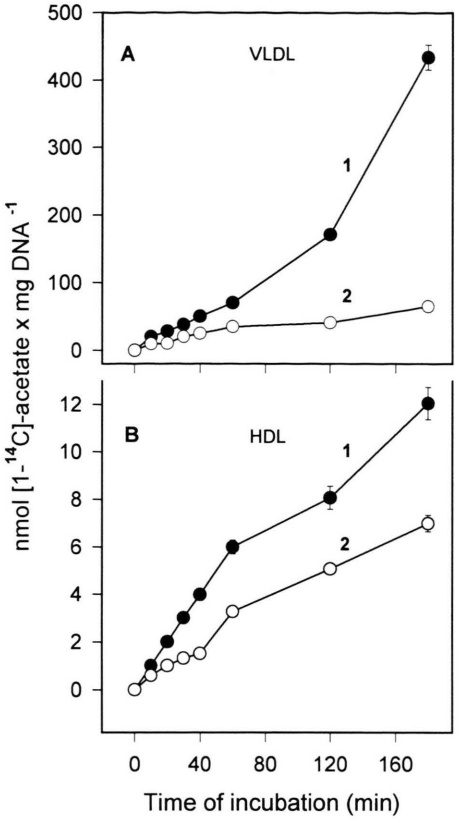

Fig. 1. Effect of CCl_4 on the secretion of hepatocyte VLDL and HDL into the medium.
Full symbols: control; open symbols: CCl_4-treated. Hepatocytes were incubated with 1.7 MBq [1-^{14}C]-acetate and 5 mM sodium acetate in 8 ml DME medium for 2 h. After 3 washings with DME medium hepatocytes were incubated (start at time zero) without (1) or with (2) 100 μM CCl_4 for the indicated times in label-free DME medium. Labeled VLDL and HDL lipids were determined in the lipoprotein fractions isolated from the medium. Mean of 4 incubations ± SEM.

maximum tolerable CCl_4 concentration was around 1 mM. Above 2 mM functional integrity became disturbed and severe toxicity was seen around 10 mM.

Inhibition of lipoprotein-associated lipid export in CCl_4-intoxicated liver has been observed both *in vivo* (Barisione *et al.*, 1993) and *in vitro* (Hebbachi and Gibbons, 1999). The reasons for this event are not entirely clear. Barisione *et al.* (1993) surmised that lipid peroxidation was the fundamental mechanism of impairment of lipoprotein secretion. Certain aldehydes formed during peroxidation have been shown to achieve this effect

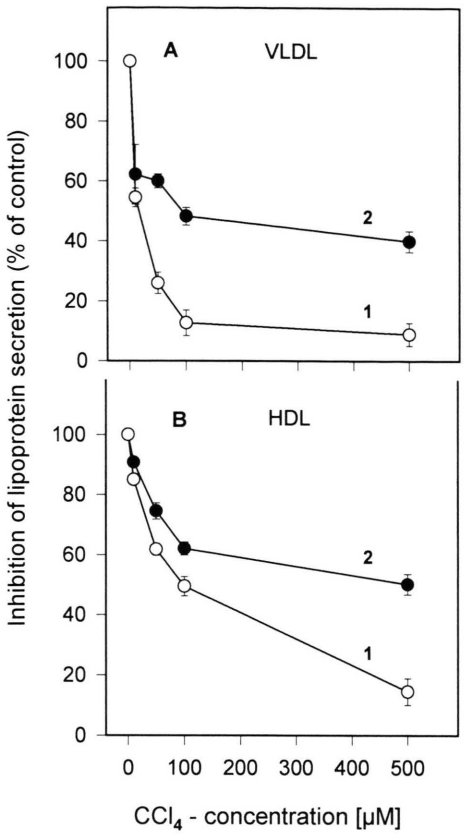

Fig. 2. Effect of CCl_4 on the secretion of the lipid and protein moieties of VLDL and HDL in hepatocytes. Full symbols: ^{3}H-labeled protein; open symbols: ^{14}C-labeled lipids. Hepatocytes were labeled for 2 h with 1.7 MBq [1-^{14}C]-acetate and 6.8 MBq L-[4,5- ^{3}H (N)]-leucine in 8 ml incubation medium. After 3 washings with DME medium different concentrations of CCl_4 were added (time zero) and incubation in label-free DME medium continued for 60 min. Radioactive label (^{14}C and ^{3}H) was then determined in the isolated VLDL and HDL fractions. Mean of 4 incubations ± SEM.

(Poli *et al.*, 1989) but Dianzani (1984) pointed out that this occurs only at extremely high levels of CCl_4 exposure. Poli *et al.* (1990), taking a close look at the time course of events in CCl_4 intoxication, found that CCl_4 intoxication can be separated into at least two phases, an early one, lasting about one hour, where covalent binding of the $CHCl_3$* radical predominated, blocking lipid secretion, and a late one where lipid peroxidation prevailed. It remains to be seen whether covalent binding mainly affects lipids, apoproteins, or other components of the transfer system, e.g., the rate-limiting microsomal triglyceride transfer protein (MTP; cf. Hebbachi and Gibbons, 1999). Bjornsson *et al.* (1998), on the other hand, reported that calcium played the central role in VLDL secretion from hepatocytes, and that lipid peroxidation had little to do with reduced lipid secretion. This view is supported by findings of Albano *et al.* (1989) who reported that Ca^{2+} sequestration was derailed by CCl_4-induced depletion of ATP levels rather than membrane damage. Since CCl_4 affected VLDL and HDL secretion differently, the general inhibition of protein synthesis induced by CCl_4 is not likely to be the immediate cause of reduced lipid export (cf. Boll *et al.*, 2001).

Effect of CCl_4 on the lipid composition of the secreted lipoproteins

CCl_4 also exerted a differential effect on the secretion of various VLDL and HDL lipid components (Table I). The four classes of VLDL- and

Table I. Effect of CCl_4 on the secretion of VLDL- and HDL-associated triglycerides, phospholipids, cholesterol and cholesterol esters.

			VLDL lipids (µg × mg DNA^{-1}) 4h	12h	20h
A	Triglycerides	(a)	0.90	1.80	3.30
		(b)	0.45	0.60	0.75
B	Cholesterol	(a)	0.055	0.075	0.120
		(b)	0.025	0.062	0.085
C	Cholesterol esters	(a)	0.06	0.22	0.31
		(b)	0.05	0.07	0.11
D	Phospholipids	(a)	0.25	0.35	0.40
		(b)	0.20	0.45	0.66
			HDL lipids (µg × mg DNA^{-1}) 4h	12h	20h
E	Triglycerides	(a)	0.25	0.50	1.25
		(b)	0.15	0.20	0.35
F	Cholesterol	(a)	0.10	0.16	0.52
		(b)	0.06	0.12	0.41
G	Cholesterol esters	(a)	0.20	0.50	1.75
		(b)	0.13	0.25	0.60
H	Phospholipids	(a)	0.50	0.80	1.10
		(b)	0.40	1.20	3.30

(a): control; (b): CCl_4-treated. Hepatocytes were incubated in DME medium (start at time zero) without or with 100 µM CCl_4. At the indicated times VLDL and HDL lipids were determined in the lipoprotein fractions isolated from the medium. Values are means of 4 determinations; SEM omitted for clarity (range: 8–18% of mean).

HDL-associated lipids studied by thin layer chromatography, viz., triglycerides, phospholipids, cholesterol and cholesterol esters, were continuously secreted into the medium during a 20 h incubation (Table I). CCl_4 had essentially no effect on the secretion of cholesterol via VLDL or HDL (Table I, B and F), but markedly inhibited the secretion of triglycerides (A and E) and cholesterol esters (C and G). The effect on phospholipids was opposite: 100 μM CCl_4 increased the secretion of VLDL- associated phospholipids by about one-half after 20 h and more than doubled it in the case of HDL (D and H). Identical results were obtained when the cells had been prelabeled with ^{14}C-acetate and radioactivity in secreted VLDL and HDL lipids was determined (not shown).

A striking difference in the kinetics of the secretion of VLDL and HDL lipids emerges from Table I: secretion rate of lipids associated with HDL increased significantly between 12 h and 20 h of incubation (E-H), whereas the steep increase was not seen with the secretion of VLDL-associated lipids (A-D). Since the syntheses of VLDL and HDL are differently regulated (Mooré and Ovtracht, 1981), a toxicant like CCl_4 can affect one pathway more than the other.

The reduced export of triglycerides from CCl_4-treated hepatocytes is the primary cause of fatty liver. As explained above this may be brought forth by changes to the system that assembles and/or exports lipoproteins. Pronzato *et al.* (1989) suggest that the disturbance occurred at an early stage, viz., glycosylation of apoproteins in the Golgi apparatus. The lipid peroxidation-induced damage to glycosylation might even be the cause of the CCl_4-related general inhibition of protein synthesis.

The differential effect of CCl_4 on the various lipid classes needs further discussion. There are several ways to view the problem. First, a decrease in availability of one or more proteins, be they apoproteins or components of the assembly system, should result in a general decrease of lipid export. Yet it was found here that CCl_4 intoxication affected the four lipid portions in secreted lipoproteins in different ways (Table I). An increase in the cholesterol/phospholipid ratio in lipoproteins was shown before to result from CCl_4 exposure (Solis-Herruzo *et al.*, 1993). This was confirmed in the present investigation: the cholesterol/phospholipid ratio increased from 0.39 (2 d CCl_4 exposure) to 0.51 (4 d) and to 0.76 (6 d). Phospholipids are more susceptible to CCl_4-induced lipid peroxidation than other lipid classes, a fact that has pronounced effects on membrane structure (Morrow *et al.*, 1992) and thus may influence lipid movement across the hepatocyte plasma membrane. During normal lipoprotein metabolism phospholipids are extensively converted into triglycerides (Wiggins and Gibbons, 1996), and there is a possibility that the CCl_4-induced inhibition of lipoprotein-associated triglyceride export may be the cause of the shift to increased phospholipid export.

Second, at moderate doses of CCl_4, as used here, inflammatory responses result which include release of inflammatory cytokines such as tumor necrosis factor (TNF)-α (Czaja *et al.*, 1995). TNF-α was shown to inhibit lecithin: cholesteryl acyltransferase (LCAT), an enzyme that plays a key role in lipoprotein metabolism (Ly *et al.*, 1995), and thus might be partly responsible for the present observations.

Effect of CCl_4 on apolipoprotein secretion and de novo synthesis

Lipoproteins contain several apoproteins of different chemical composition (see below). The effect of CCl_4 on the secretion of the whole of the protein portions of the lipoproteins is shown in Table II A. 100 μM CCl_4 induced a significant decrease in the secretion of apoproteins of both VLDL and HDL, but to different extents: the secretion of VLDL apoprotein after 20 h was reduced 30%, while that of HDL apoprotein was reduced more than 70%.

For a study of apoprotein synthesis hepatocytes were incubated with L-[4,5-^{3}H (N)]-leucine. After removal of non-bound label the cells were exposed to CCl_4 and the appearance of ^{3}H-labeled apoproteins of VLDL and HDL in the medium was followed (Table II B). 100 μM CCl_4 attenuated the synthesis of VLDL apoprotein by 60%, and that of HDL apoprotein by about 25% after 3 h incubation.

Inhibition by CCl_4 of apolipoprotein synthesis was also observed *in vivo* (e.g., Honma and Suda, 1997), but a possible mechanism of action has not been proposed. A disturbance of the cellular

Table II. Effect of CCl_4 on (A) secretion and (B) *de novo* synthesis of apolipoproteins.

	A $\mu g \times mg\ DNA^{-1}$ VLDL		
	4h	12h	20h
Control	0.22	0.47	0.53
CCl_4-treated	0.18	0.34	0.37
	HDL		
Control	0.80	1.10	1.80
CCl_4-treated	0.40	0.50	0.50
	B nmol ^{3}H-L-leucine $\times$ mg DNA^{-1} VLDL		
	30 min	120 min	180 min
Control	1100	3150	5100
CCl_4-treated	800	1450	2000
	HDL		
Control	750	1500	1850
CCl_4-treated	700	1050	1350

A: Hepatocytes were incubated in DME medium (start at time zero) without or with 100 μM CCl_4. At the indicated times VLDL and HDL apoproteins were determined with SDS gel electrophoresis (see Method section). B: Hepatocytes were incubated for 2h in DME medium containing 9.25×10^4 Bq L-[4,5- ^{3}H(N)]-leucine / ml. After removal of radioactivity by 3 washings incubation continued (start at time zero) without or with 100 μM CCl_4. At the indicated times, ^{3}H-labeled VLDL and HDL fractions were isolated and radioactivity determined in the apoproteins (see Method section). . Values are means of 4 determinations; SEM omitted for clarity (range 6–18%).

Table III. Effect of CCl_4 on the incorporation of ^{3}H- leucine into apolipoproteins.

Apoprotein	Radioactive label (10^3 cpm $\times$ mg DNA^{-1}) 4 h		 20 h	
	Control	CCl_4	Control	CCl_4
		VLDL		
B48	0.64	0.66	2.37	1.25
B100	2.24	1.78	8.52	3.28
E	1.99	1.49	5.48	7.04
C	1.27	0.83	3.58	2.81
A-I	0.48	0.33	1.52	1.28
		HDL		
B48	0.56	0.16	2.76	0.55
B100	1.53	0.83	3.23	0.81
E	0.38	0.22	1.55	0.83
C	0.51	0.17	0.75	0.20
A-I	3.51	0.48	4.21	0.62

Hepatocytes were incubated in DME medium with L-[4,5-^{3}H(N)]-leucine (spec. act. 1.84 TBq/mmol) (1 μCi/ml incubation) in the absence (control) or in the presence of 100 μM CCl_4 for 4 and 20 h. Apoproteins were isolated from the lipoprotein fractions of the medium and separated by SDS gel electrophoresis as described in the Method section. Mean of 3 incubations. SEM omitted for clarity (range 7–16% of mean).

transport system involving the microtubuli appears conceivable. The composition of VLDL secreted into the medium from untreated hepatocytes was identical to that of intracellular lipoproteins secreted from the Golgi apparatus (unpublished data) and it could well be that the changes caused by CCl_4 originate during the biosynthesis and packing of VLDL components within the cell, or the export from the cell.

Effect of CCl_4 on the apoprotein composition of the secreted lipoproteins

CCl_4 intoxication, in addition to its effect on the lipid portions of the hepatocyte (see Table I), also caused changes in the apoprotein composition of VLDL and HDL. Primary hepatocytes continuously secreted apolipoproteins B_{48}, B_{100}, E, C and A-I into the incubation medium, as shown by ^{3}H-leucine prelabeling of proteins (Table III, controls at 4 and 20 h). Exposure to 100 μM CCl_4 within 4 h reduced the incorporation of label into individual apoproteins of VLDL 20–35% with the exception of B_{48} where incorporation was not reduced (Table III). After 20 h incubation with CCl_4 incorporation of labeled leucine was reduced, most significantly in apo-B_{100} (minus 62%), but increased 28% in apo-E. On the other hand in HDL apoproteins CCl_4 had a more uniform action, significantly reducing incorporation of label into all apoproteins after 4 h. Here the effect by CCl_4 was more intense than in VLDL apoproteins (Table III). The extent of reduction of incorporation after 20 h of exposure was between 45% (apo-E) and more than 80% (apo-A-I). With the exception of apo-E there had been an increase in effect of CCl_4 in HDL apoproteins between 4 and 20 hr.

The effect of CCl_4 on the synthesis of apolipoproteins varied between the four groups studied (apo-A, B, C and E). This points to differences in the regulation of their expression. Indeed Panduro *et al.* (1990) have shown that induction of liver cirrhosis revealed differences in the expression of apo-A and apo-E which they discussed in terms of apo-A synthesis being regulated at the post-transcriptional level, whereas apo-E synthesis is mostly

regulated at the transcriptional level. The exact mechanism of attack, however, has not been elucidated.

There is a constant number of apo-B $_{100}$ molecules per VLDL particle (Redgrave and Carlson, 1979). CCl_4 exposure for 20 h reduced apo-B_{100} by about 60%, and triglyceride secretion by 75% (Table I), suggesting a decrease in the size of secreted lipoprotein particles. As mentioned above a CCl_4-induced change in MTP availability could explain this effect, as MTP is the rate-limiting factor in the assembly and secretion of apo-B-containing lipoproteins (Jamil *et al.*, 1998).

In summary, it is evident that due to either lipid peroxidation or covalent binding of haloalkyl radicals in CCl_4-intoxicated cells the lipoproteins apparently acquire an irregular chemical composition and shape and, therefore, their ability to act as lipid transporters (packing) is impaired. The CCl_4-induced disturbance of lipoprotein secretion and alterations in their composition cannot be explained by the general inhibition of protein synthesis observed following CCl_4 intoxication. Differential effects on both the compositions of the lipid and the apoprotein portions, and the fact that their syntheses are regulated differently, suggest that the attack occurs at specific, as yet unidentified sites. The work of Dianzani (1984) indicates that the attack on lipoprotein secretion occurs early during CCl_4 toxicity, viz., covalent binding of the CCl_3* radical to cellular components, and that the subsequent lipid peroxidation-related damage may kill the cell, but does not contribute to steatosis.

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