In vitro evidence for CCl_4 metabolites covalently bound to lipoprotein micelles

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CCl_4-induced impairment of the lipoprotein secretion pathway of intact rat hepatocytes was carried out using $^{14}CCl_4$ to check the possibility of binding to lipoproteins by CCl_4 metabolites. After separation of different cell suspension fractions by means of ultracentrifugation and chemical precipitation procedures, a significant amount of the radioisotope was found covalently bound to the lipid and protein components of low density lipoproteins. Suitable experiments demonstrated that the bound radioisotope was represented by CCl_4 metabolites and not by unactivated CCl_4.

Isolated hepatocyte *Carbon tetrachloride* *Trichloromethyl radical* *Covalent binding* *Lipoprotein secretion* *Fatty liver*

1. INTRODUCTION

The derangement of hepatocyte lipoprotein secretion is the generally accepted mechanism for the early onset of liver fat accumulation following CCl_4 poisoning [1–3]. The impairment of protein synthesis and of mitochondrial functions, the loss of lysosomal integrity can actually contribute only to late stages of the fatty liver due to the haloalkane [1,4]. The relative roles of the two main mechanisms of CCl_4-mediated cell injury (i.e., covalent binding of its metabolites and lipid peroxidation) in blocking lipoprotein secretion has not yet been clarified. Stimulation of lipid peroxide formation due to CCl_4 seems implicated only in maintaining the cell injury, but not in producing it [3,5,6]. However, no evidence in favour of a major role of lipid peroxidation in the onset of CCl_4-induced inhibition of lipoprotein secretion has been produced elsewhere. This report deals with the direct demonstration of CCl_4 metabolites covalently bound to low-density lipoproteins obtained from rat isolated hepatocytes poisoned with the radiolabelled compound.

2. MATERIALS AND METHODS

Male Wistar rats (Nossan, Correzzana) 200–250 g body wt, were used. They were fed on semisynthetic diet, free of any antioxidant (Piccioni, Brescia) with free access to water.

Collagenase type I was obtained from Sigma Chemicals (St Louis MD); [U-^{14}C]palmitic acid and [^{14}C]carbon tetrachloride from Radiochemical Centre (Amersham, Bucks); other chemicals were purchased from BDH Chemicals (Poole) and Merck (Darmstadt).

Intact rat liver cells were isolated by the collagenase perfusion technique described in [2,12].

2.1. *Determination of total $^{14}CCl_4$ radioactivity bound to different cell suspension fractions*

For these studies at least $1.5–2 \times 10^8$ cells were necessary. Aliquots (2 ml) of the hepatocyte suspension diluted with a defined salt solution [2] to 7×10^6 cells/ml were incubated at 37°C for 120 min in the presence of unlabelled sodium palmitate. The procedure was essentially that in [2]. After incubation,

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cells were washed and resuspended with fresh incubation medium to 7×10^6 cells/ml and 6 ml aliquots of the suspension were poured in 100 ml flasks. $^{14}CCl_4$ (spec. act. 26.9 mCi/mmol) dissolved in acetone was added directly to the cell suspension to reach ~200 μM final conc. Hepatocytes were then incubated at 37°C for 30 min in a shaking water bath. No change in hepatocyte viability, in terms of trypan blue exclusion and lactate dehydrogenase release, was present at the end of the treatment. After CCl_4 poisoning, cells and incubation medium were processed separately.

(i) *Cells:* The pellets were homogenized in 4 vol. (w/v) of 0.25 M sucrose by ultrasonic disintegration (20 μm of amplitude for 25 s). The homogenate was centrifuged at 25 000 × g for 10 min. The resulting pellet which represented cell debris plus nuclei plus mitochondria fractions was retained. The supernatant was centrifuged at 100 000 × g for 60 min. The resulting pellet was taken to represent the microsomal fraction. From the cell sap, as hereafter described, the fraction of low-density lipoproteins was obtained. Each fraction was processed for radioactivity measurements as in [2].

(ii) *Cell incubation medium:* Also in this case, the fraction represented by the low density lipoproteins was separated and processed for radioactivity measurements.

2.2. *Preparation of low-density lipoprotein fraction*

Because of the small amount of lipoproteins secreted by hepatocytes incubated for a short time, the lipoprotein separation by an ultracentrifugation procedure was discarded and a procedure of chemical precipitation set up as in [8]. To 9 parts of cell sap or cell incubation medium, 1 part of 2% sodium phosphotungstate (pH 7.6) in 1 M $MgCl_2$ was added. The samples were left overnight at 4°C then the precipitates were reharvested after centrifugation at 10 000 × g for 45 min. The selectivity of the precipitation procedure for lipoproteins rich in triglycerides has again been stressed [9]. By using electrophoresis on polyacrylamide gel we also observed absence of α-lipoproteins and negligible albumin contamination in precipitates obtained and redissolved according to this method [8,9].

2.3. *Determination of $^{14}CCl_4$ radioactivity covalently bound to cell lipid and protein*

Aliquots about 1/4 of the total of the microsomal and lipoprotein fractions were processed to measure only the radioactivity covalently bound to the cell lipid or protein.

(i) *Lipid-bound radioactivity:* Lipids were extracted with chloroform–methanol mixture (2:1, v/v). The chloroform phase was then collected and dessicated. The extracted lipids were washed 5 times with $CHCl_3$ to remove $^{14}CCl_4$ not covalently bound. The radioactivity in the final dried lipid layer was eventually measured [2].

(ii) *Protein-bound radioactivity:* Proteins were precipitated with 10% trichloroacetic acid, collected by centrifugation, washed with chloroform–methanol–diethylether (2:2:1, by vol.), with acetone, again with ether. Finally, they were processed for radioactivity measurement [2]. To quantify covalent binding to lipoproteins by not activated CCl_4, aliquots of the lipoprotein fraction obtained by not radioactive hepatocyte preparations were suspended in 6 ml incubation medium then incubated for 30 min at 37°C in the presence of 200 μM $^{14}CCl_4$ and in absence of cells. The determination of radioactivity bound to lipid and protein followed the incubation.

3. RESULTS AND DISCUSSION

Carbon tetrachloride is known to exert toxic effect after its homolytical cleavage in the smooth endoplasmic reticulum with the production of the trichloromethyl radical ($CCl_3^{\cdot}$) [10]. This reactive intermediate rapidly converts to the trichloromethylperoxy radical ($CCl_3O_2^{\cdot}$) in the presence of oxygen [11]. Both physico-chemical [11] and biological studies [6,12] demonstrated that promethazine, a free radical scavenger with a very strong antioxidant activity, only scarcely reacts with $CCl_3^{\cdot}$ while it actively traps $CCl_3O_2^{\cdot}$. Since promethazine did not significantly prevent the impairment of lipoprotein secretion due to CCl_4 in spite of a complete inhibition of its prooxidant effect, we postulated that $CCl_3^{\cdot}$-covalent binding should be the mechanism initiating this specific CCl_4-induced cell derangement. We then carried out analyses of the intracellular and extracellular distribution of

Table 1

Total radioactivity recovered in different fractions of hepatocyte suspensions treated with $^{14}CCl_4$[a]

Cell suspension fractions	dpm	%
INTRACELL.		
25 000 × *g* pellet	694 967 ± 107 667	15 ± 3
100 000 × *g* pellet	2 650 684 ± 366 656	58 ± 4
Low-density lipoproteins	305 598 ± 7886	7 ± 0.5
Residual cell sap	881 104 ± 134 908	20 ± 3
EXTRACELL.		
Low-density lipoproteins	168 422 ± 4018	13 ± 1
Residual supernatant	1 110 942 ± 142 971	87 ± 1

[a] The results are expressed as the means ± SD of 3 expt in triplicate. For further details on the cell fractions, see section 2

CCl_4 metabolites by incubating liver cell suspensions with radiolabelled CCl_4. One of the aims of this study was to check the possibility of CCl_4 metabolites binding to lipoprotein micelles. The total radioactivity recovered in the different cell suspension fractions is reported in table 1. With regard to the intracellular fractions examined, the highest percentage of radioactivity was found in that corresponding to microsomes (100 000 × *g* pellet), the activation site of CCl_4. Lower amounts of radioisotope were recovered in the 25 000 × *g* pellet, in which nuclei, mitochondria and cell debris are represented, and in the so called 'residual' cell sap, that is the cytosol after chemical precipitation from it of low-density lipoproteins. The most notable result was the reproducible recovery of radioactivity bound both to intracellular and extracellular (i.e., already secreted) lipoproteins. The specificity of the method for separating these two fractions was discussed above (section 2). Since the intracellular lipoprotein fraction presumably represents both native and mature lipoprotein micelles, the obtained results strongly support the hypothesis that CCl_3 covalent binding cannot only damage the structure and consequently the secretion of already synthetized lipoproteins but also induces derangement of lipoprotein assembly itself. As far as the microsomal and the two lipoprotein fractions are concerned, experiments were performed to distinguish ^{14}C-radioactivity bound to protein and lipid from that unbound, (table 2). About 70–80% and 50% of total radioactivity, recovered in the two intracellular and in the extracellular fractions were covalently bound, while most of the residual radioisotope was probably just solved. The lipid–protein binding ratio (4:1) found in the microsomal fraction corresponded to that obtained in studies on the whole animal [13]. The higher protein binding recovered in the intracellular lipoprotein fraction compared to the extracellular one could indicate a major involvement of the apoprotein in the secretion derangement. To evaluate how much unmetabolized CCl_4 and not its meta-

Table 2

Total radioactivity and its relative percent bound to lipid or protein of different cell fractions after hepatocyte treatment with $^{14}CCl_4$[a]

Cell suspension fractions	Recovered radioactivity (dmp)		
	Total	Lipid-bound	Protein-bound
1st EXP.			
100 000 × *g* pellet	2 611 478 ± 271 112	1 775 805 ± 121 009 (68%)	496 180 ± 27 223 (19%)
Intracellular lipoproteins	295 054 ± 12 039	108 022 ± 3665 (36%)	95 420 ± 6137 (32%)
Extracellular lipoproteins	157 540 ± 5067	54 096 ± 2770 (34%)	19 273 ± 1414 (12%)
2nd EXP.			
100 000 × *g* pellet	2 120 302 ± 311 755	1 253 981 ± 202 079 (59%)	282 942 ± 21 810 (13%)
Intracellular lipoproteins	303 030 ± 5174	153 294 ± 9010 (50%)	105 580 ± 3817 (34%)
Extracellular lipoproteins	168 037 ± 11 089	63 240 ± 5712 (37%)	29 533 ± 2735 (17%)

[a] For each experiment the mean ± SD of 3 values is shown. For further details see section 2

Table 3

Treatment of isolated low density lipoproteins with $^{14}CCl_4$[a]

Experimental samples	Added radioactivity	Recovered radioactivity (dpm)	
		Lipid-bound	Protein-bound
1st EXP.			
Intracellular lipoproteins	2289156 ± 8274	7172 ± 610 (0.15%)	1514 ± 54 (0.03%)
Extracellular lipoproteins	2289156 ± 8274	14879 ± 1367 (0.65%)	2746 ± 210 (0.10%)
2nd EXP.			
Intracellular lipoproteins	2341463 ± 2304	6980 ± 279 (0.30%)	454 ± 56 (0.02%)
Extracellular lipoproteins	2341463 ± 2304	16416 ± 580 (0.70%)	2420 ± 117 (0.10%)

[a] See table 2

bolites would bind to lipoproteins, aliquots of this fraction isolated from unlabelled cells were incubated with $^{14}CCl_4$ under the reported conditions, but without a source for CCl_4 activation. With regard to lipid binding, only about 0.2% and 0.7% of the total added radioactivity was bound to intra- and extracellular lipoproteins, respectively (table 3). Protein-bound radioisotope was in any case <0.05–0.1% of total added amount.

In conclusion, even if the involvement of lipid peroxidation cannot be excluded, strong evidence has been obtained which supports a major role of covalent binding in CCl_4-induced early derangement of the liver lipoprotein secretion pathway. Several observations [10–13] allow us to believe $CCl_3^{\cdot}$ to be the reactive species involved in the binding.

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