Effects of Carbon Tetrachloride on Isolated Rat Hepatocytes

INHIBITION OF PROTEIN AND LIPOPROTEIN SECRETION

By ENRICO GRAVELA,* EMANUELE ALBANO,* MARIO U. DIANZANI,*
GIUSEPPE POLI† and TREVOR F. SLATER†

**Istituto di Patologia Generale, Corso Raffaello 30, Torino 10125, Italy, and*

†*Department of Biochemistry, Brunel University, Uxbridge UB8 3PH, Middx., U.K.*

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The effects of carbon tetrachloride on protein and lipoprotein secretion, and on lipid peroxidation, have been investigated in isolated rat hepatocytes. It was found that although the free-radical scavenger Promethazine completely suppressed the increased peroxidation produced by carbon tetrachloride, it had no effect on the inhibitory action of carbon tetrachloride on lipoprotein secretion. In consequence, the latter effect of carbon tetrachloride does not appear to be mediated through a peroxidative stage.

The fatty degeneration induced in the liver by carbon tetrachloride has been related to an impairment of protein synthesis and a consequent fall in lipoprotein formation (Robinson & Seakins, 1962). However, the strong and rapid inhibition of protein synthesis produced by many other agents results in fatty accumulation in the liver only after several hours, whereas with carbon tetrachloride fatty accumulation can be detected within the first hour of poisoning (for references see Dianzani & Gravela, 1975; Dianzani, 1978). This clear difference in time scales suggests that the earliest derangement in liver triacylglycerol metabolism induced by carbon tetrachloride is dependent on mechanisms other than an inhibition of protein synthesis. In previous work (Gravela *et al.*, 1977), with isolated rat hepatocytes whose protein was prelabelled *in vitro*, a distinction was made between an inhibition of secretion due to an impairment of protein synthesis and that produced by a derangement of the secretory process itself. In the present study we have investigated that distinction by utilizing isolated liver cell preparations to determine the effects of carbon tetrachloride on the secretion of presynthesized protein and lipoprotein.

Two major mechanisms have been proposed to account for the carbon tetrachloride-induced liver damage; they are not mutually exclusive, and experimental evidence exists in support of both possibilities. The first mechanism postulates that the damaging effects of carbon tetrachloride on liver are dependent on the peroxidative decomposition of structural membrane lipids (Recknagel, 1967; Glende *et al.*, 1976; Benedetti *et al.*, 1977). The second one postulates that the direct binding of carbon tetrachloride metabolites to the liver lipids and proteins is an important mechanism of cell injury (McLean,

1967; Cignoli & Castro, 1971; Gillette *et al.*, 1974). In the present paper we show that the carbon tetrachloride-induced inhibition of protein and lipoprotein secretion does not appear to be mediated through a peroxidative mechanism, and results from causes other than a prior block in protein synthesis.

Experimental

Materials

Collagenase (CLS, type IV) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; promethazine (*N*N α -trimethyl-10*H*-phenothiazine-10-ethanamine) and compound SKF-525A (2-diethylaminoethyl 2,2-diphenylpentanoate) were gifts of May and Baker Ltd., Dagenham, Essex, U.K., and Smith, Kline and French, Welwyn Garden City, Herts., U.K., respectively. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemical were from Sigma Chemical Co., St. Louis, MO, U.S.A., or BDH Chemicals Ltd., Poole, Dorset, U.K. Male rats of Wistar strain, 200–250 g in weight, were used. They were fed a diet (Piccioni, Brescia, Italy) devoid of any antioxidants and water *ad libitum*. The preincubation medium was Ham's F12 medium (Ham, 1965) containing 10% (v/v) horse serum. The incubation medium contained the following: 60 mM-NaCl; 40 mM-KCl; 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid] buffer, pH 7.4; 2 mM-MgSO₄; 1 mM-CaCl₂; 1 mM-NaH₂PO₄; 5 mM-glucose; 0.03 mM-cycloheximide; and 0.58 mM-amino acid mixture (for references see Poli *et al.*, 1978a). Lactate dehydrogenase was measured by using the kit procedure of Boehringer Corp. (London) Ltd., Lewes, East Sussex BN7 1LG, U.K.

Preparation of isolated hepatocytes and their pre-labelling

Hepatocytes were isolated by the collagenase-perfusion method described previously (Gravela *et al.*, 1977; Poli *et al.*, 1978a). Cells were suspended, after the isolation procedure, in the preincubation medium, counted with a haemocytometer and diluted with the same medium to 10^7 cells/ml. The hepatocyte viability was routinely assessed by the Trypan Blue-exclusion test (Jeejeebhoy *et al.*, 1975) and by measuring the release of lactate dehydrogenase into the suspending medium; viability ranged from 90 to 97%. In previous studies (Poli *et al.*, 1978a) we found that the Trypan Blue procedure correlated closely with the release of lactate dehydrogenase and the transaminases aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) during incubation of hepatocytes for 60 min at 37°C with various concentrations of carbon tetrachloride. Significant cell damage occurred only when the amount of carbon tetrachloride/flask exceeded 10 μ l under the conditions used in the present study (in the experiments reported here the amount of carbon tetrachloride used was 7.5 μ l/flask).

Cell protein was labelled by incubating 10 ml of hepatocytes with 20 μ Ci of L-[U- 14 C]valine (specific radioactivity 270 mCi/mmol) for 60 min at 37°C. Then incorporation of radioactivity was stopped by diluting the cell suspension with 100 ml of incubation medium containing, in addition, 10 mM unlabelled L-valine. The diluted suspension was centrifuged at 400g for 4 min. Cell triacylglycerol was labelled by incubating 10 ml of hepatocytes with 5 ml of a 14 C/ 12 C-labelled fatty acid mixture. The latter contained: 0.15 M-NaCl; 4% (w/v) albumin; 1 mM-(0.33 μ Ci/ml)-sodium oleate, -sodium palmitate and -sodium stearate; 0.1 mM(0.66 μ Ci/ml)-sodium arachidonate; 1 mM-phosphocholine. NaOH was used to adjust the pH to 7.4. Fatty acids were made to form a complex with albumin as described previously (Gravela *et al.*, 1977). After 60 min incubation at 37°C, the cell suspension was diluted with 100 ml of incubation medium and then centrifuged as above.

Protein and lipoprotein secretion from prelabelled hepatocytes

Either the [14 C]valine-labelled or the 14 C-labelled-fatty acid-labelled hepatocytes were suspended in incubation medium to give 5×10^6 cells/ml. Portions (2 ml) of the suspensions were immediately centrifuged to determine the zero-time secretion in the supernatant solutions. Portions (2 ml) of the cell suspension were placed in the main compartment of 50 ml flasks fitted with a centre well, and closed with

a screw cap. In one group of flasks, 7.5 μ l of carbon tetrachloride was added in the centre well and allowed to diffuse in the closed system. The general features of this experimental system were described previously (Poli *et al.*, 1978a). When indicated, colchicine, compound SKF-525A or promethazine were added in the main compartment of the flasks. These were incubated at 37°C; at the end of incubation cell suspensions were immediately centrifuged at 600g for 4 min. Protein and lipoprotein triacyl-

Fig. 1. Time course of protein (a) and lipoprotein (b) secretion from prelabelled hepatocytes

Symbols represent: ●, control cells; ○, carbon-tetrachloride-poisoned cells; and Δ, cells treated with 50 μ M-colchicine. Values are for radioactivity of protein (a) or lipoprotein triacylglycerol (b) released into the medium from 10^7 hepatocytes. Each point represents mean ($\pm$ s.d.) of results for three experiments; values in parentheses represent percentage inhibition.

glycerol in the supernatant solutions were purified and processed for radioactivity measurement as described elsewhere (Gravela *et al.*, 1977).

Determination of malondialdehyde

Malondialdehyde production was estimated by measuring the thiobarbituric acid-reacting compounds (Bernheim *et al.*, 1948). After the above incubation, portions of the suspensions corresponding to 2×10^6 cells were added to 10% (w/v) trichloroacetic acid and water, to give a final volume of 3 ml and a final concentration of 5% trichloroacetic acid. After centrifugation, 1.5 ml portions of the supernatant solutions were treated with the same volume of 0.67% thiobarbituric acid, incubated in boiling water for 10 min and made alkaline with KOH (final concn. 0.29 M). A_{543} was determined with a Beckman ACTA III spectrophotometer.

Results and Discussion

Fig. 1 shows that carbon tetrachloride, as well as colchicine, induces a decrease in secretion of radioactively labelled protein and triacylglycerol from prelabelled hepatocytes. The inhibition induced by carbon tetrachloride, however, is more rapid and stronger than that given by colchicine. The latter drug is known to impair liver secretion through a specific damage to microtubules (for references, see Gravela *et al.*, 1977); thus it inhibits protein and

lipoprotein secretion to the same extent. In carbon-tetrachloride-poisoned hepatocytes, however, the triacylglycerol secretion is more severely affected than protein secretion. This fact, without ruling out the possibility of a direct impairment of microtubular function by carbon tetrachloride, suggests that either specific damage to the triacylglycerol-secreting sites of the plasma membrane occur, or other changes in steps preceding lipoprotein transport by microtubules are involved. It is noteworthy that in our experimental system the decreased secretion cannot be related to an effect of carbon tetrachloride on protein synthesis, since the latter is blocked in both control cells and cells poisoned by cycloheximide. Control experiments have shown that cycloheximide (0.03 mM) completely suppressed protein synthesis in isolated hepatocytes. The possibility that the decreased values observed in the suspending medium resulted from increased intracellular degradation of protein and/or lipid (and thereby diminishing the total available for secretion) was ruled out by 'balance-sheet' experiments. Poli *et al.* (1978a) have reported 'balance-sheet' data for amounts of triacylglycerol in hepatocytes exposed to carbon tetrachloride, and we have found that the decreased release of prelabelled protein into the medium produced by carbon tetrachloride (Table 1) is accompanied by a corresponding increased intracellular amount of labelled protein.

To try to understand more clearly the relationship between the disturbance in lipoprotein secretion

Table 1. Effects of carbon tetrachloride, promethazine and compound SKF-525A on malondialdehyde production, lactate dehydrogenase release, protein and lipid secretion from prelabelled rat liver hepatocytes

Results under (a) represent absorbance at 543 nm of the thiobarbituric acid-reacting compounds produced/h by 10^6 hepatocytes; (b) the extracellular lactate dehydrogenase activity after 40 min incubation at 37°C of 10^7 hepatocytes $\pm$ drugs and carbon tetrachloride was calculated as percentage activity in the extracellular medium compared with the total intracellular and extracellular activity determined after cell destruction produced by 0.5% Triton X-100; (c) radioactivity (c.p.m.) of protein released into the medium/40 min by 10^7 hepatocytes; (d) radioactivity (c.p.m.) of lipoprotein triacylglycerol released into the medium/40 min by 10^7 hepatocytes. Values represent means $\pm$ s.d. for two triplicate experiments; values in parentheses are percentage inhibition with respect to the corresponding controls. * $P < 0.001$, ** $P < 0.005$, *** $P < 0.05$ for tests versus control with no additional treatments. (The time of 40 min that was adopted was used as a compromise between shorter incubation time, where the effects of carbon tetrachloride are smaller, and longer incubation times, where the non-linearity of secretion and viability become significant.)

Cell treatment	(a) Malondialdehyde production	(b) Lactate dehydrogenase release (%)	(c) Protein secretion	(d) Lipid secretion
None	0.103 $\pm$ 0.009	15	37486 $\pm$ 1866	4280 $\pm$ 372
0.1 mM Compound SKF-525A	0.096 $\pm$ 0.005	16	32311 $\pm$ 1108*	3427 $\pm$ 77*
0.01 mM-Promethazine	0.084 $\pm$ 0.008**	15	36885 $\pm$ 784	3784 $\pm$ 270***
Carbon tetrachloride	0.262 $\pm$ 0.022*	16	17980 $\pm$ 288* (52%)	807 $\pm$ 51* (81%)
0.1 mM Compound SKF-525A and carbon tetrachloride	0.151 $\pm$ 0.013*	15	13817 $\pm$ 1518* (57%)	730 $\pm$ 135* (79%)
0.01 mM-Promethazine and carbon tetrachloride	0.088 $\pm$ 0.011***	15	19136 $\pm$ 380* (48%)	844 $\pm$ 98* (78%)

after exposure to carbon tetrachloride and lipid peroxidation, we have studied the effects of two agents, promethazine and SKF-525A, on both processes (Table 1). The Table shows that the free-radical scavenger promethazine and the metabolic inhibitor SKF-525A added to isolated liver cells strongly decrease the carbon tetrachloride-induced malondialdehyde production. This result confirms a previous report (Poli *et al.*, 1978b). In our experiments, promethazine completely prevents, and compound SKF-525A decreases to about 35%, the carbon tetrachloride-stimulated malondialdehyde production. Compound SKF-525A alone is slightly inhibitory towards protein and lipoprotein secretion, whereas promethazine alone is devoid of these effects on the control hepatocyte suspensions. The two substances were not protective at all on the decreases in protein and lipoprotein secretion produced by carbon tetrachloride. The lack of protection by promethazine and compound SKF-525A (in contrast with their strong effects on malonaldehyde production) suggests that the carbon tetrachloride-induced blocks in protein and lipid secretion are not mediated through the peroxidative stimulus produced by carbon tetrachloride.

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