Pathogenesis of Carbon Tetrachloride-Induced Hepatocyte Injury Bioactivation of CCl_4 by Cytochrome P450 and Effects on Lipid Homeostasis

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The CCl_4-induced development of liver damage was studied in monolayer cultures of primary rat hepatocytes:

(1) CCl_4 caused accumulation of triglycerides in hepatocytes following cytochrome P450 induction with β-naphthoflavone or metyrapone. Ethanol or a high dose of insulin plus triiodothyronine had the same effect. (2) CCl_4 increased the synthesis of fatty acids and triglycerides and the rate of lipid esterification. Cholesterol and phospholipid synthesis from acetate was also increased. (3) CCl_4 reduced β-oxidation of fatty acids as assessed by CO_2-release and ketone body formation. Hydrolysis of triglycerides was also reduced. (4) The content of unsaturated fatty acids in microsomal lipids was decreased by almost 50% after incubation with CCl_4, while saturated fatty acids increased slightly. (5) CCl_4 exerted a pronounced inhibitory effect on the exocytosis of macromolecules (albumin), but did not affect secretion of bile acids from hepatocytes.

Introduction

Liver cell damage is triggered by numerous impacts, arising from the external environment as well as from within the cell. Liver damage caused by drugs reveals that all cell types might be affected and that a plethora of liver injuries can be reproduced experimentally (Larrey, 2000). Carbon tetrachloride is an important model agent to study the pathogenesis of liver injury. Many chemicals cause toxicity by disrupting membrane structure in the course of polyunsaturated fatty acid peroxidation (de Groot and Noll, 1986). Fatty liver is defined by accumulation of lipids exceeding 5% of liver weight (Alpers *et al.,* 1993). This phenomenon is believed to be a result from an imbalance between hepatic fatty acid flow, triacylglycerol synthesis and excretion (Burt *et al.*, 1991). Indeed one of the earliest manifestations of CCl_4 -induced liver damage is the accumulation of fat by inhibition of triglyceride secretion (Thurman *et al.,* 1986). This is thought to be due to a disturbance of the triacylglycerol transport as very low density lipoproteins from the liver to the periphery. Chronic CCl_4 intoxication is often associated with cirrhosis and liver cancer.

In the present study, primary liver cells in monolayer culture were used instead of isolated hepatocytes because the latter have impaired functions: protein turnover is mostly catabolic, synthesis of serum proteins is low, hormonal induction of many enzymes and formation of cAMP is low. While isolated hepatocytes can be incubated for a maximum of 4h (Ichihara *et al.*, 1982), studies on lipoprotein- and albumin secretion require incubations for more than 24h. Cultured hepatocytes constitute a system that can be considered biochemically equivalent to intact liver.

CCl_4-induced hepatotoxicty involves activation of the toxicant by the microsomal cytochrome P450-dependent monooxygenase system to the trichloromethyl radical (CCl3*) which, in the presence of oxygen, is further converted to a peroxy radical (CCl_3-OO*). These radicals initiate lipid peroxidation in membranes and/or will covalently bind to macromolecules in the cell leading to degenerative processes. The function of microsomes, mitochondria and nuclei of the hepatocytes are impaired by lipid peroxidation and eventually the cells perish. Intoxication of liver cells by CCl_4 also causes an imbalance between lipid synthesis and degradation (see below). It has been shown that

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inhibition of triacylglycerol secretion requires bioactivation of CCl_4 by cytochrome P450.

The objective of the present communication was to provide further information on biochemical processes that link initial events to the resulting pathological changes, and to establish an *in vitro* model of CCl_4-induced liver damage.

Experimental

Animals

Female Sprague-Dawley rats weighing 150–200 g from our own husbandry were used throughout. They were housed in a temperature- and humidity-controlled room (25 ± 2 °C) on a 12h light and dark cycle; lights on 6 a.m. to 6 p.m. and fed a stock diet (diet # 1324, Altromin International, Lage, Germany). The animals had free access to feed and water. Animals use and care were in compliance with the NIH „Guide for the care and use of laboratory animals", publ. No 86–23 (1985). In a number of experiments animals were pretreated i.p. with phenobarbital, 100 mg/kg × day in 0.9% NaCl, for 4 days.

Hepatocytes culture and microsomal extracts

Livers were collected between 7 and 9 a.m. under ether anesthesia and rapidly chilled in ice. Hepatocytes were isolated according to Spencer and Pitot (1982) and plated on 25 cm^2 culture dishes (Gebhardt and Jung, 1982) in 8 ml Ham's F-12 medium, supplemented with fetal calf serum (10% v/v), 7.8 mM $NaHCO_3$, 10 mM HEPES, 0.1 mg/ml penicillin, 0.1 mg/ml kanamycin and 1 μM dexamethasone. Cell density at the time of plating was $5{\times}10^6$ cells/ml. Cells were incubated at 37 °C in 95% air, 5% CO_2 for 3h to allow adhesion (Spencer and Pitot, 1982), then washed once with 6 ml medium. Thereafter new media were added containing the respective additions for experimental incubations. Cell viability was assessed with the trypan blue exclusion test, and only preparations with less than 10% stainable cells were used.

At the end of incubations the medium was removed and cells were rinsed 3 times with 6 ml cold 0.9% NaCl. One ml homogenization buffer (100 mM sucrose, 50 mM KCl, 40 mM KH_2PO_4, 30 mM EDTA and 0.1 mM dithioerythritol, pH 7.5) was added and cells were scraped off the culture dish. The suspension was sonicated 3 × 10 sec (Branson sonifier B 12 with microtip, 50 watt) in the cold and centrifuged at 25,000 × *g* for 15 min to remove mitochondria and cell debris. The supernatant was centrifuged at 105,000 × *g* for 60 min to obtain microsomes and cytosol.

Incubations

Standard incubations were performed in Ham's F-12 medium supplemented as indicated above. For studies on albumin secretion hepatocytes were incubated in Dulbecco's modified Eagle's medium (DMEM), (fetal calf serum-free, arginine-deficient and ornithine-supplemented) which contained 25 mM glucose, 500 μU/ml insulin, 0.1 mg/ml penicillin, 0.1 mg/ml kanamycin, 10 mM HEPES, 7.8 mM $NaHCO_3$, 1 μM dexamethasone, 0.4 mM L-ornithine (pH 7.4) and supplemented with 10% lipoprotein-deficient serum. This was isolated from fetal calf serum (Goldstein *et al.*, 1983).

Incubations in the presence of CCl_4 were performed as follows: The respective concentration of CCl_4 in a 20% solution of dimethyl sulfoxide (DMSO) was added to the medium under sonication (10 sec) and then equilibrated at 37 °C for 15 min. It was then added to the culture dish which contained the hepatocytes. Control cells without CCl_4 were incubated in an equivalent volume of medium containing DMSO alone (max. 0.01%).

Lipid synthesis

Lipid synthesis was measured using (a) [1-^{14}C]-acetate or (b) [^{14}C (U)]-palmitic acid as precursors. Incubations were at 37 °C:

a) Hepatocytes were preincubated for the indicated times (up to 6h) in 8 ml Ham's F-12 medium containing 1.5 mM [1-^{14}C]-acetate (spec. act. 2.1 GBq/ mmol) and 3.5 mM sodium acetate in the absence or presence of 25 μM CCl_4.

b.) Hepatocytes were preincubated in 8 ml Ham's F-12 medium in the absence or presence of 25 μM CCl_4 (plus insulin or glucagon, see Fig. 4) for 2h. Then the medium was replaced to contain 0.05 mM [^{14}C(U)]-palmitic acid (spec. act. 31.4 GBq/mmol), and 0.05 mM sodium palmitate (time zero of experiment) and incubated for the indicated times (up to 45 min).

Incubations were terminated by adding 4 ml ice-cold 0.9% NaCl per 8 ml sample, followed by

three 4 ml washes with ice-cold 0.9% NaCl. The medium was cleared of cells by centrifugation for 5 min at 3000 × *g*.

Lipid extraction, separation and determination

Hepatocyte lipids were extracted with 3.75 ml chloroform: methanol 2:1 (v/v) per 1 ml of the incubation (see above), followed by a phase separation with 1.25 ml each of chloroform and 2 M KCl in 0.5 M KH_2PO_4, pH 7.4. After concentration and desiccation lipid classes were separated by thin layer chromatography on silica gel 60 plates (Merck # 11845) (Yagasaki *et al.*, 1984) with developing system I, chloroform: methanol: H_2O, 65: 20: 5 (v/v) for 5 min and, after drying the plates, with developing system II, n-hexane: diethyl ether: acetic acid, 80: 20: 1.5 (v/v) for 60 min. Lipids became separated in the sequence: phospholipids, monoglycerides, diglycerides, free cholesterol, free fatty acids, triglycerides and cholesterol esters. The fractions were localized by a brief exposure to iodine vapor. Then radiochromatograms were taken with a scanner (Berthold LB 2722 with a dual ratemeter integrator LB 2424). The radioactivity was scraped off the TLC plates and measured in a liquid scintillation spectrophotometer (Packard Tricarb 300) after adding 10 ml toluene scintillator.

Triglycerides, after freeze-drying and saponification were measured via glycerol determination, using triolein (30 mg/ml) as the standard.

Free cholesterol and cholesterol esters, after hydrolysis with cholesterol esterase (EC 3.1.1.13.), were determined with the CHOD-PAP method.

Phospholipid phosphorus was determined in the dry residue of the lipid extract.

Analysis of fatty acids

Lipid extracts of the cell fractions were subjected to transmethylation according to Morrison and Smith (1964). Samples of the resulting n-hexane extracts of the fatty acid methylesters were separated by gas chromatography (Varian Instruments, Model 3700) on a 6 ft. column, packed with 15% stabilized diethyleneglycol succinate polyester on 80–100 mesh Chromosorb W (acid washed) and operated at 186 °C. They were identified by relative retention times using methylmyristate as the standard. Quantitative assessment was performed by peak area integration. Hexane extracts of fatty acid methylesters from labeled lipid fractions were applied to thin layer plates (Silicagel G-60), precoated with n-undecane (15% in petrolether). The methylesters were separated with acetonitrile: acetic acid (1: 1, v/v). Radioactivity was detected with a β-scanner, scraped off the plate and counted.

Enzyme assays

Activity of 7-ethoxycoumarin-O-deethylase and aldrin epoxidase was determined in the hepatocyte microsomal fraction, while activity of the other enzymes was assayed in the cytosolic fraction. Activities were determined at 30 °C.

7-Ethoxycoumarin-O-deethylase activity was measured fluorometrically (Mc Killop, 1985), following the production of 7-hydroxycoumarin. A calibration curve with 7-hydroxycoumarin (5–100 ng) was used. Specific activity was calculated as nmol 7-hydroxycoumarin formed × mg DNA^{-1} × min^{-1}. Aldrin epoxidase activity (Mc Killop, 1985) was estimated by measuring the amount of dieldrin formed and calculated as nmol dieldrin formed × mg DNA $^{-1}$ x min $^{-1}$. Activity of fatty acid synthase (EC 2.3.1.85), malic enzyme (EC 1.1.1.40) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was estimated as previously described (Boll *et al.*,1996).

Other determinations

The values are calculated on a mg DNA rather than a mg protein basis, as with longer incubation periods or addition of hormones the protein, but not DNA content of cultured hepatocyte will be affected (Sterzel *et al.*, 1985).

DNA was determined fluorometrically according to Sterzel *et al.*, (1985), using calf thymus DNA type II as the standard. To allow comparison with other reported findings from the literature it was assumed that 1 g (wet weight) of liver corresponds to 2.5 mg DNA, and that 1 g of liver protein corresponds to 15 mg of DNA (see Grunnet *et al.*, 1985).

Secretion of albumin into the medium of hepatocyte cultures was determined by immunoelectrophoresis. Total bile acids were estimated enzymatically (Mashige *et al.*,1986). Protein was determined with the method of Lowry *et al.*, (1951) using bovine serum albumin as the standard.

Lipid metabolism was determined by two methods: production of $^{14}CO_2$, measured according to Grunnet *et al.*, (1985), or by ketone body formation from [^{14}C (U)]-palmitic acid, measuring radioactivity in the aqueous phase after precipitation with 1 M perchloric acid (Mangiapane and Brindley, 1986). The amount of radioactivity recovered is the ketone body fraction.

The values are expressed as mean ± SEM. Statistical significance was determined by ANOVA, followed by the Tukey-Kramer multiple comparison test.

Reagents

Ham's F-12 medium and Dulbecco's modified Eagle's medium were obtained from ICN Biomedicals, Eschwege, Germany. Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone), β-naphthoflavone, aldrin, dieldrin, coumarin, 7-hydroxycoumarin, DMSO, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), fetal calf serum, calf thymus DNA, triolein and cholesterol monotest were obtained from Sigma- Aldrich Chemical Corp. Deisenhofen, Germany. Carbon tetrachloride was from Merck, Darmstadt, Germany. [1-^{14}C]-acetate (spec.act. 2.1 GBq/mmol), [^{14}C(U)]-palmitic acid (spec.act. 31.4 GBq/mmol) were from NEN Life Sciences Products, Cologne, Germany. ^{14}C carbon tetrachloride (spec.act. 2.25 TBq/mmol) was obtained from Amershan Buchler, Braunschweig, Germany. All reagents used were of analytical grade.

Results and Discussion

Hepatocyte viability

The integrity of cultured hepatocytes was routinely monitored by the trypan blue exclusion test, indicating necrotic cell death, by leakage of lactic dehydrogenase into the medium, and by stability of the lipogenic enzymes fatty acid synthase, malic enzyme and glucose-6-phosphate dehydrogenase. Both trypan blue staining (uptake of the dye by damaged cells) and leakage of lactic dehydrogenase remained at less than 10% after 2 days of incubation. The activities of the lipogenic enzymes were stable for at least 5 days, even in the presence of 200 μM CCl_4.

***In vitro* model of liver damage**

Ethanol and hormone-induced fat accumulation

Synthesis of triglycerides in cultured hepatocytes is regulated by insulin and glucocorticoids (Mangiapane and Brindley, 1986). Triglyceride formation could be induced with 1 μM insulin and 10 μM triiodothyronine (4–6 fold after 4 d, Fig. 1A, 3), and this was not affected by the presence of CCl_4 (not shown). Even cirrhotic liver maintains insulin sensitivity (Mion *et al.*, 1996). Monolayer cultures incubated with 50 mM ethanol (roughly corresponding to a blood alcohol concentration of 0.2% and biologically still tolerable) for 6d accumulated triglycerides to the same extent as after hormone induction (Fig. 1A, 2). Ethanol

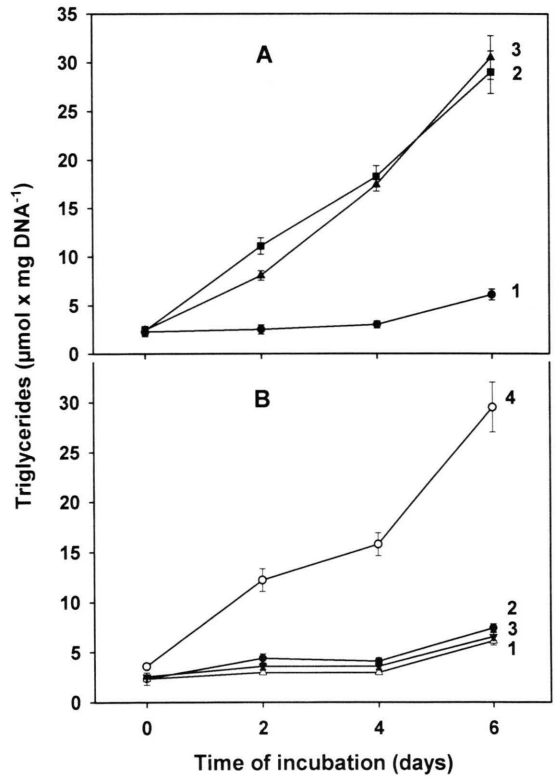

Fig. 1. Accumulation of triglycerides in cultured hepatocytes following ethanol, hormone or CCl_4 treatment.
Panel A: (1) control; (2) 50 mM ethanol; (3) 1 μM insulin and 10 μM triiodothyronine.
Panel B: (1) control; (2) 0.5 mM metyrapone; (3) 200 μM CCl_4; (4) metyrapone and CCl_4.
Basic medium: Ham's F-12 with 1 μM dexamethasone and 5% fetal calf serum. Medium plus additions was replaced after 4 h and then every 24 h. Means of 4 incubations ± SEM.

causes extensive liver damage (review: Lieber and De Carli, 1991), including accumulation of triglycerides *in vitro* (Dich *et al.*, 1983). The latter effect is thought to be due to ethanol induction of cytochrome P450 4A1, causing increases in liver fatty acid-binding protein and fatty acid esterification (Lieber, 2000). Microsomal cytochrome P450 2E1 activity in rats *in vivo* was increased 20-fold by ethanol feeding, causing enhanced lipid peroxidation (Castillo *et al.*, 1992). Histological preparations of ethanol- and hormone-treated hepatocytes, respectively, exhibited similar appearances of cytoplasmic fat droplet accumulation (not shown).

Carbon tetrachloride-induced fat accumulation

A single *in vivo* intragastric dose of CCl_4 caused zonal fatty liver in rats (Thurman *et al.*, 1986). In contrast, the intracellular lipid contents of cultured hepatocytes increased only slightly during a 4d incubation period in response to 200 μM CCl_4 (Fig. 1B, 3). There are two likely reasons for this weak response. First, CCl_4, after being activated to the CCl_3* radical by cytochrome P450, acts as a suicide inhibitor (Manno *et al.*, 1988). The inactivation is specific for cytochrome P450; although CCl_4 targets the heme prosthetic group, it does not inactivate hemoglobin *in vivo* (Manno *et al.*, 1989). Second, cytochrome P450 oxygenase activities in hepatocytes decline with extended culture periods (Wiebel and Singh, 1980; Boll *et al.*, 1999). However, when cultured hepatocytes were incubated in the presence the phenobarbital-type inducer metyrapone, 200 μM CCl_4 induced an extensive fat accumulation (Fig. 1B, 4). Metyrapone alone had no effect (Fig. 1B, 2). A 4-day incubation with either 50 mM ethanol or hormones produced the same amount of triglyceride accumulation as 200 μM CCl_4 (compare Figs. 1A and 1B). Most likely this indicates that the effector concentrations used exerted a maximum response.

Compared to the *in vivo* situation the content of microsomal cytochrome P 448/P450 oxygenases in primary hepatocytes is very low, since cultured cells apparently lack endogenous activators (Fahl *et al.*, 1979). The decline of cytochrome activities with incubation time, and the ability to induce them again, is depicted in Fig. 2. This experiment was performed with hepatocytes from rats pre-

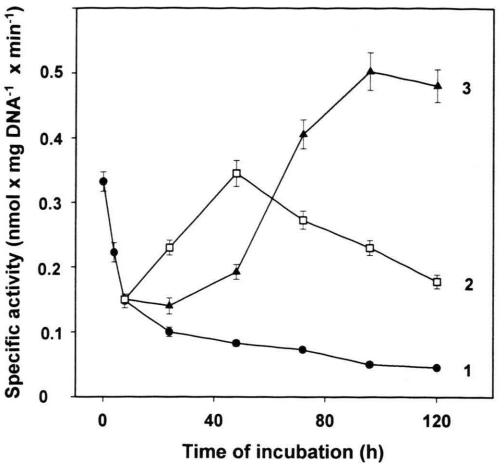

Fig. 2. 7-Ethoxycoumarin-O-deethylase activity in cultured hepatocytes.
Hepatocytes were procured from phenobarbital-treated rats. Inducers were added after 8h in Ham's F-12 medium supplemented with 1 μM dexamethasone and 5% fetal calf serum. (1) control; (2) 0.5 mM metyrapone; (3) 0.1 mM β-naphthoflavone. Means of 3 incubations ± SEM.

treated with phenobarbital (Cai and Mehendale, 1991) to assure high initial levels of cytochromes. 7-Ethoxycoumarin-O-deethylase activity, representative of P448 oxygenases and also aldrin epoxidase activity (indicative for P450 oxygenases, not shown) declined rapidly during incubation of the hepatocytes in the absence of inducers (Fig. 2, curve 1). The decline of activity was faster with aldrin epoxidase than with the coumarin deethylase, i.e. 80 and 65% after 1d, 95 and 75% after 3 days, respectively (not shown). This decrease of cytochrome activities with time apparently is not due to a loss of heme from the cultured cells, as the content of the prostethic group cytochrome P450 did not decrease with incubation (Boll *et al.*, 1999).

The cytochrome P448 (3-methylcholanthrene-type) inducer β-naphthoflavone, or the cytochrome P450 (phenobarbital-type) inducer/inhibitor metyrapone (Lake and Paine, 1983) partly restored oxygenase activities in hepatocyte culture (Fig. 2). 7-Ethoxycoumarin-O-deethylase activity increased transiently in the presence of metyrapone (Fig. 2, curve 2), and more long-lasting with β-naphthoflavone (Fig. 2, curve 3). The transient effect seen with metyrapone may be due to the fact that this substance induces cytochrome P450,

but by the same token acts as an irreversible inhibitor. Another possible explanation relates to different kinetics of induction of cytochromes P450 vs. P448 (Steward *et al.*, 1985), and, of course, differences in inducing potency between the two substances. It should be noted that only phenobarbital-type cytochrome inducers affect CCl_4 toxicity, methylcholanthrene-type inducers are ineffective (Manno *et al.*, 1988)

Effect of carbon tetrachloride on lipid metabolism

Accumulation of triglycerides depends on the availability of substrates for *de novo* fatty acid synthesis, on free fatty acids, and on the enzyme activities required for synthesis. These processes are balanced in a healthy liver by the metabolism of fatty acids, proceeding either toward esterification to triglycerides, phospholipids and other fatty acids esters, or toward β-oxidation to CO_2 and formation of ketone bodies. Development of a fatty liver can be the result of excessive fatty acid and/or triglyceride synthesis, of an inhibited metabolism, or of both.

Synthesis of lipids

De novo synthesis of lipids in hepatocytes was affected by CCl_4. Prelabeling hepatocytes with [1-^{14}C]-acetate and [^{14}C (U)]-palmitic acid as precursors distinguished between synthesis and esterification of fatty acids, respectively. Acetate induced lipogenesis in hepatocyte culture at an optimal concentration of 5 mM in the medium. Higher concentrations caused no further increase, indicating either substrate saturation of a rate-limiting enzyme, or saturation of acetate transport across the cell membrane (not shown).

In untreated control cells (Fig. 3, full symbols) the incorporation of [1-^{14}C]-acetate-derived radioactivity into fatty acids proceeded linear for 4h, for 3h into diglycerides and triglycerides and for 6h into phospholipids. Incorporation of [1-^{14}C]-acetate into cholesterol and cholesterol esters exhibited a lag-phase, possibly reflecting a need of the

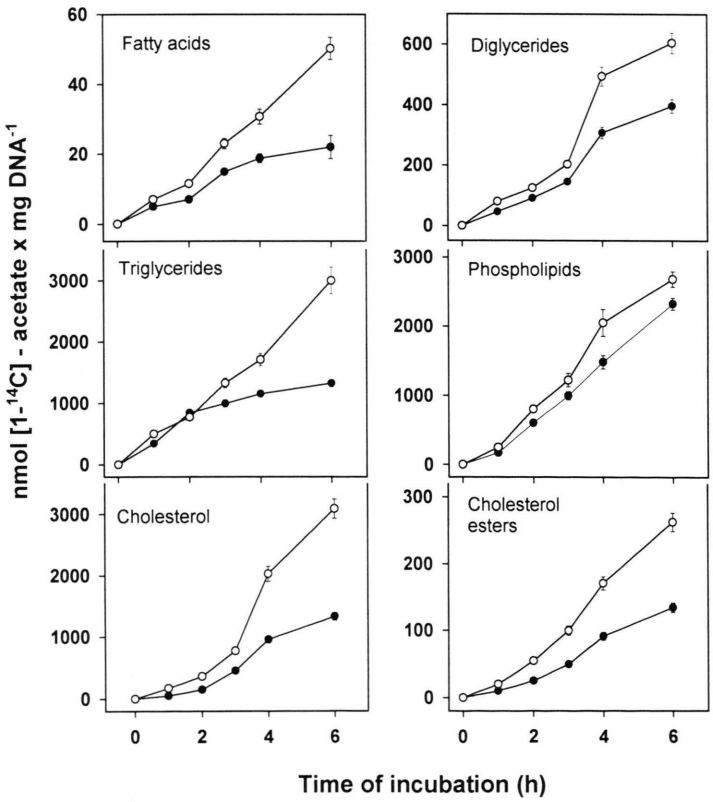

Fig. 3. Effect of CCl_4 on the incorporation of radiolabel from [1-^{14}C]-acetate into cellular lipids.
Full symbols: controls; open symbols: 25 μM CCl_4. For details on incubations and on lipid analysis see the Method section. Means of 4 incubations ± SEM.

hepatocytes to first accumulate lipogenic intermediates. The presence of 25 μM CCl_4 stimulated the incorporation of [1-^{14}C]-acetate into lipids (Fig. 3, open symbols). In most cases this effect became evident within 3 hours of exposure and doubled the radioactive label in fatty acids, triglycerides and cholesterol within 6 hours. It was less pronounced in diglycerides and cholesterol esters, and minimal in phospholipids (Fig. 3). One possible explanation for the stimulation brought about by CCl_4 is that it positively affected transport (permeability) of acetate into the liver cell, resulting in increased substrate availability.

Stimulation of lipid synthesis by CCl_4 was concentration-dependent. A significant effect was already seen with 10 μM CCl_4 (not shown), and optimum stimulation occurred with 25 μM CCl_4, as displayed in Fig. 3. Higher CCl_4 concentrations reduced stimulation, possibly due to the onset of toxicity. Fulminant toxicity, i.e., cell necrosis, occurs only at much higher CCl_4 concentrations, between 3 and 5 mM (Divald *et al.*, 1990).

Esterification of fatty acids

Another consequence of CCl_4 treatment in hepatocytes was an increase in fatty acid and cholesterol esterification, evidenced by an increase in radiolabel incorporation from [^{14}C(U)]-palmitic acid into cellular lipids (Fig. 4). A forty-five min. exposure to 25 μM CCl_4 caused an increase of radiolabel incorporation into triglycerides (A), in diglycerides (B), and in cholesterol esters (C). In contrast, incorporation into phospholipids decreased (D). Normal response and viability of the cells under the experimental conditions was demonstrated by their response to hormones (shown for triglycerides): 85 nM insulin increased the incorporation of radiolabel (curve 3 in A), while 10 nM glucagon inhibited it (curve 4 in A). The increase in esterification of fatty acids may be secondary to other CCl_4-induced effects such as inhibition of β-oxidation (see below) or decreased cellular secretion of lipids (cf. Larrey, 2000). With respect to phospholipids, Divald *et al.*, (1990) pointed out that CCl_4 decreased phospholipid synthesis in cultured hepatocytes parallel with a

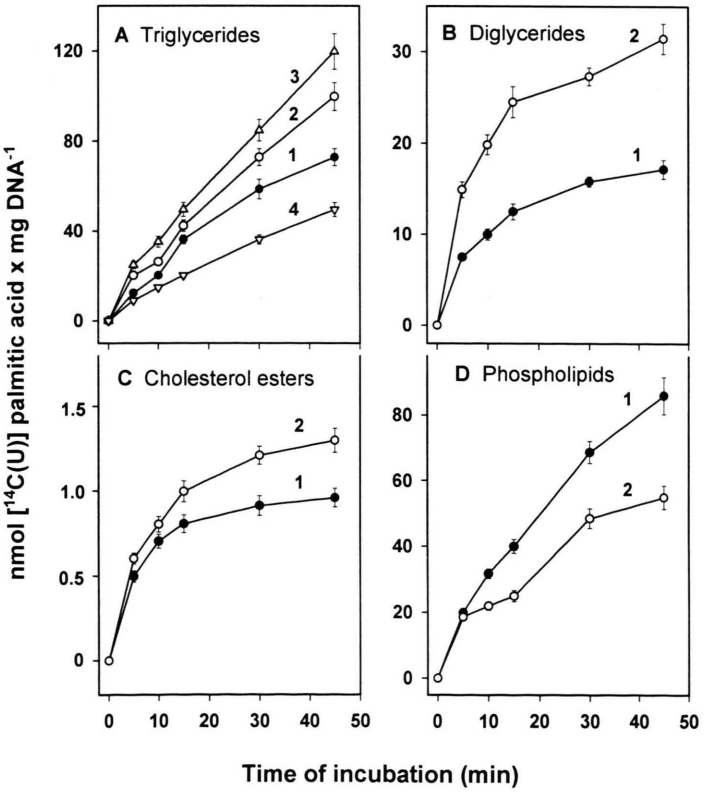

Fig. 4. Effect of CCl_4 on the incorporation of radiolabel from [^{14}C(U)]-palmitic acid into cellular lipids. (1) controls; (2) 25 μM CCl_4; (3) 85 nM insulin; (4) 10 nM glucagon, (3 and 4, triglycerides only) For details on incubations and on lipid analysis see the Method section. Means of 4 incubations ± SEM.

decrease in protein synthesis (see below) and suggested that an increase in phospholipase activity (Lamb *et al.*, 1988) may be the cause.

Oxidation of fatty acids

Hepatocellular β-oxidation of the fatty acids was also affected by CCl_4, as indicated by changes in the release of $^{14}CO_2$. Cells were exposed for 150 min to CCl_4, followed by [^{14}C(U)]-palmitic acid. 25 μM CCl_4 in the medium resulted in a 40% inhibition of $^{14}CO_2$ release after 45 min (Fig. 5A). Recovery of radioactivity as $^{14}CO_2$ was 4–6% of the total oxidation products of the hepatocytes. Lieber (2000) points out that alcohol induction of cytochrome P450 results, among others, in increased β-oxidation. Thus, the CCl_4-induced inactivation of cytochrome P450 might be the origin of reduced β-oxidation observed here.

A stoichiometric by-product of β-oxidation are ketone bodies that can be obtained from an acid extract of hepatocytes incubated with radiolabeled palmitate. Their rate of formation was not linear with time of incubation (Fig. 5B), probably reflecting further oxidative metabolism. CCl_4 exposure (25 μM) inhibited the formation of ketone bodies to the same extent as CO_2 release, i.e., about 40% (Fig. 5B).

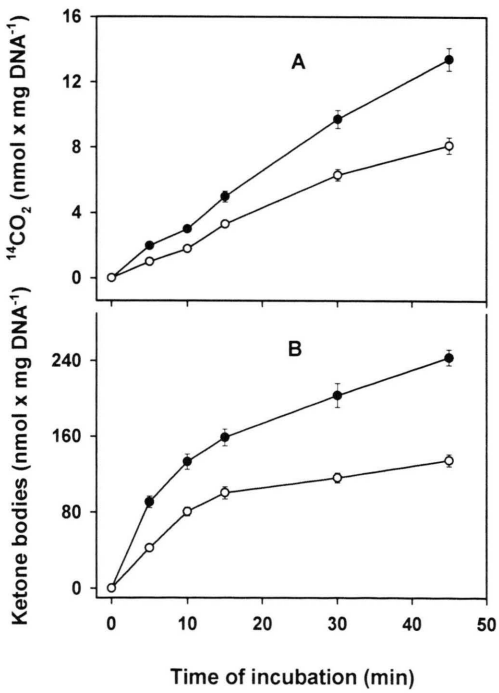

Fig. 5. Effect of CCl_4 on β-oxidation of [^{14}C(U)]-palmitic acid in cultured hepatocytes.
Panel A: $^{14}CO_2$ formation; Panel B: ketone body formation. Full symbols: controls; open symbols: 25 μM CCl_4. For incubations, $^{14}CO_2$ and ketone body determination see Method section. Means of 4 incubations ± SEM.

In hepatocytes incubated with radiolabeled fatty acids, the amounts of radiolabel in acid-soluble extracts and $^{14}CO_2$ released from the hepatocytes can serve as an index of triglyceride hydrolysis (lipolysis). Accordingly, in the present study lipolysis was reduced 18 and 27% after 30 and 60 min of incubation, respectively, following exposure to 25 μM CCl_4 (details not shown). This suggested that CCl_4 intoxication leads to an imbalance of lipid metabolism, i.e. decreased degradation and increased synthesis (with the exception of phospholipids, see above) resulting in an increased triglyceride content.

At this time it is not entirely clear how CCl_4 causes this wide range of in part opposing effects. Inhibition of protein synthesis has been mentioned, but according to Clawson (1989) the most likely explanation of these, and other effects is the severe derailment of Ca^{2+} sequestration brought about in hepatocytes by CCl_4. This seems to be closely related to a disturbance of membrane function caused by CCl_4, in part via lipid peroxidation, in part by direct solvent effect (cf. Clawson, 1989). CCl_4-induced lipid peroxidation per se does not cause death of hepatocytes in vitro (Johnston and Kroening, 1998).

CCl_4-induced alterations of hepatocyte fatty acid composition

Rat hepatocytes contain a variety of saturated and unsaturated fatty acids (Table I, controls) that may be located in membranes, particularly the Golgi apparatus, or may be part of the lipoprotein transportation system. Metabolic activation and subsequent covalent binding of ^{14}C-CCl_4 metabolites to these fatty acids affected predominantly unsaturated fatty acids: 70% of the bound radioactivity was identified as adducts to unsaturated fatty acids, with oleic acid (18:1) and docosahexaneic acid (22:6) being affected most (Table II). The covalent binding of CCl_4 metabolites to unsaturated fatty acids (Castillo *et al.*, 1992) affects the permeability and characteristics of both the plasma

Table I. Fatty acid content of hepatocyte microsomal lipids and effect of CCl_4.

Fatty acid	Fatty acid content (μmol × mg protein $^{-1}$) Control	CCl_4-treated
C14: 0	1.5 ± 0.16	1.6 ± 0.11
C16: 0	323.6 ± 23.8	348.8 ± 27.1
C18: 0	583.6 ± 89.4	625.1 ± 80.5
C16: 1	2.2 ± 0.12	1.6 ± 0.40
C18: 1	25.7 ± 1.7	*11.1 ± 1.14
C18: 2	360.7 ± 30.3	*235.1 ± 21.7
C18: 3	1.2 ± 0.14	*0.5 ± 0.05
C20: 4	3.5 ± 0.57	*0.7 ± 0.07
C22: 5	11.0 ± 1.63	*4.1 ± 0.7
C22: 6	126.0 ± 21.6	*28.8 ± 2.66
sum sat.	908.7	975.5
sum unsat.	530.3	281.9

Hepatocytes from phenobarbital-treated animals were incubated in Ham's F-12 medium without (control) or with 200 μM CCl_4 for 2 h. Lipids were extracted from the microsomal fractions and, after transformation to methylesters, composition was determined by gas chromatography (see Method section). Values marked with * are significant ($p < 0.05$) of controls. Means of 3 incubations ± SEM.

Table II. Covalent binding of $^{14}CCl_4$ to hepatocyte fatty acids.

Fatty acid	Percent of total fatty acids
C22: 6	29.1
C18: 1	24.6
C18: 0	15.6
C16: 0	11.7
C22: 5	10.0
C18: 2	6.6
C16: 1	2.4

Hepatocytes from phenobarbital-treated rats were incubated in Ham's F-12 medium in presence of 200 μM $^{14}C-CCl_4$ (spec.act. 2.25 TBq/mmol) for 1h. Extracted lipids were subjected to transmethylation. Separation and detection of the fatty acid methylester adducts was as described in the Method section. Values are means of 3 incubations ± SEM.

membrane and of the endoplasmic reticulum (Fulceri *et al.*, 1984), with subsequent effects on cellular homeostasis.

The overall effect of CCl_4 was a minor increase in saturated fatty acid and an almost 50% loss of unsaturated fatty acids (Table I). It is likely that the increase in saturation of fatty acids was due to adduct formation, but the much larger decrease in unsaturated fatty acids remains to be explained, particularly in the light of concomitantly decreased β-oxidation.

Other targets of carbon tetrachloride toxicity in hepatocytes

So far it has been demonstrated that CCl_4 affects lipids in the hepatocytes, and thus membrane characteristics. However, exocytosis of macromolecules such as lipoproteins or serum proteins is fundamentally different from membrane transport of much smaller molecules such as bile acids. Isolated hepatocytes secrete a number of proteins like albumin, fibrinogen or transferrin (Tanaka *et al.*, 1978), a process that can be stimulated by hormones. CCl_4 can affect this process.

Albumin secretion

For the initial 4 hours of incubation there was little difference in the release of albumin between

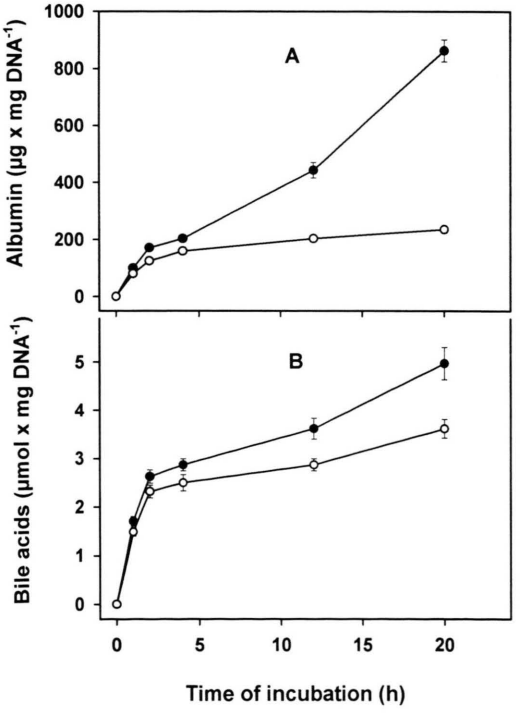

Fig. 6. Effect of CCl_4 on secretion of albumin and bile acids from cultured hepatocytes.
Panel A: albumin secretion; panel B: bile acid secretion. Full symbols: control; open symbols: 200 μM CCl_4. Hepatocytes in panel A were incubated in DMEM medium containing 10% lipoprotein-deficient serum. Hepatocytes in panel B were incubated in serum-free Ham's F-12 medium. Means of 3 incubations ± SEM.

controls and hepatocytes treated with 200 μM CCl_4 (Fig. 6, panel A). During the following 16h of observation, however, control cells continued to secrete albumin at a rate of about 44 μg/mg DNA/h (Fig. 6A, full symbols), while CCl_4-treated cell essentially ceased to release albumin (Fig. 6A, open symbols). The reason for this observation may be twofold: first, CCl_4 is well-known to cause a general reduction of protein synthesis which is thought to be due to a defect in methylation of ribosomal RNA in the 2′-O-ribose position (Clawson *et al.*, 1987); and second, changes in membrane fluidity may have affected the process of secretion itself (cf. Larrey, 2000).

Bile acid secretion

Liver cells in suspension are able to synthesize and secrete bile acids (Anwer *et al.*, 1975). This process worked very effectively for about for 2h at a rate of 1.1 ± 0.39 μmol bile acids/mg DNA/h (Fig. 6B), which is within the physiological range. Thereafter, maybe in response to feed-back regulation of synthesis, secretion from control hepatocytes continued at about one tenth the previous rate (Fig. 6B, full symbols), and at an only slightly lower rate in CCl_4-treated cells (Fig. 6B, open symbols). Thus, hepatic secretion of smaller molecules such as bile acids appeared to be little affected by CCl_4 toxicity.

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