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CARBON TETRACHLORIDE-DEPENDENT INHIBITION OF LIPID SECRETION BY ISOLATED HEPATOCYTES

CHARACTERIZATION AND REQUIREMENT FOR BIOACTIVATION*

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Abstract—Secretion of lipid as very low density lipoprotein (VLDL) by isolated hepatocytes was studied in a system in which the cells were exposed to a constant concentration of carbon tetrachloride (CCl_4) throughout the duration of the incubation. Inhibition of secretion was characterized in terms of CCl_4 concentration and duration of incubation. Half-maximum inhibition of VLDL secretion occurred at 80 μM CCl_4. At 390 μM CCl_4, VLDL secretion was inhibited 40% in 2 min and was suppressed completely in 5 min. No CCl_4-dependent release of cellular glutamic oxaloacetic transaminase occurred under the conditions studied. Evidence is presented indicating that the metabolism of CCl_4 is required for the expression of CCl_4-dependent inhibition of lipid secretion. With respect to the parameters studied, the isolated hepatocyte system closely mimics the hepatic response of the intact animal to CCl_4.

The development of liver steatosis following carbon tetrachloride poisoning appears to be a consequence of the failure of movement of triglyceride as very low density lipoprotein (VLDL) from the liver to the circulation [1]. The CCl_4-poisoned liver is capable of synthesizing triglyceride from fatty acid substrate [2]. It has been concluded that this failure of triglyceride secretion is not attributable either *in vivo* or *in vitro* to an inhibition of apoprotein synthesis [3,4, and p. 99 of Ref. 5]. This implies that the locus of impairment is at one or more steps in the subsequent VLDL synthesis and secretory pathway. This pathway includes coupling of lipid and apoprotein B in the endoplasmic reticulum, packaging nascent VLDL particles into secretory vesicles, and intracellular movement of the vesicles to the plasma membrane followed by exocytosis of the lipoprotein [6]. The molecular mechanism responsible for the inhibition of triglyceride secretion by CCl_4 remains obscure. Isolated hepatocytes have proven useful in the study of this problem [4, 7, 8]. In this paper, characterization of the CCl_4-induced inhibition of lipid secretion in isolated hepatocytes is presented. Evidence is also given indicating that the bioactivation of CCl_4 at the cytochrome P-450 locus is required for inhibition of lipid secretion in the isolated hepatocyte model.

MATERIALS AND METHODS

Carbon-14 labeled carbon tetrachloride, obtained from the New England Nuclear Corp., Boston, MA, was diluted to 1 μCi/μl CCl_4 and stored at −20°. The sodium salt of [1-^{14}C]palmitic acid was purchased from ICN Pharmaceuticals, Inc., Irvine, CA. Piperonyl butoxide was obtained from Pfaltz & Bauer, Inc., Flushing, NY. Fatty-acid-poor bovine serum albumin was from Miles Laboratories, Inc., Elkhart, IN. Waymouth MB 752/1 culture medium, along with heat-inactivated horse serum, was from GIBCO, Grand Island, NY. Collagenase (catalogue No. C0130) and other chemicals were from the Sigma Chemical Co., St. Louis, MO.

Male Sprague–Dawley rats (200–400 g), from Zivic Miller Laboratories, Allison Park, PA, were fasted overnight before hepatocyte isolation. Hepatocyte isolation and incubation of hepatocyte suspensions were performed as described previously [9]. In brief, the liver was removed and perfused for 5 min with calcium-free medium, followed by 20 min of perfusion with medium containing 4 mM calcium and collagenase (0.5 to 1.0 mg/ml). Dispersed cells were collected by centrifugation and washed once with Waymouth MB 752/1 medium containing 20% (v/v) horse serum buffered at pH 7.4 with 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid]. Cells were incubated in this medium at 37° under a 95% O_2–5% CO_2 atmosphere for 20 min before use.

For study of lipid secretion, cells (10%, w/v) were incubated for 1 hr at 37° in medium containing 0.5 mM [1-^{14}C]palmitate (2 mCi/mmole), 0.5 mM oleate and 0.7% (w/v) fatty-acid-poor bovine serum albumin. This solution also contained penicillin (0.153 mg/ml) and streptomycin (0.25 mg/ml). Cells labeled in this way were collected by centrifugation (3 min, 50 *g*) and washed free of extracellular fatty acid by resuspension in incubation medium containing 60 mM NaCl, 40 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mM Na_2HPO_4, 5 mM D-glucose,

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1 mM methionine, and 50 mM HEPES, pH 7.4. Some experiments were performed in buffer containing 100 mM NaCl and 4 mM KCl, and similar results were obtained. The washed cell pellets were suspended to 60% (w/v), and aliquots (0.5 ml) were transferred into 50-ml glass Erlenmeyer flasks containing 2.5 ml of the above incubation medium which had been equilibrated at 37° for at least 40 min with various amounts of CCl_4 in 6 × 50 mm tubes in the flasks. Cell suspensions, treated as above except that the flasks were placed on ice instead of being incubated at 37°, served as zero-time controls.

Incubations were terminated by addition of 4 ml of ice-cold 0.9% NaCl. Medium was cleared of cells by centrifugation for 5 min at 3000 *g*. The cell-free medium was assayed for radioactivity associated with VLDL by a method similar to that described by Gravela *et al.* [10]. To 4.5 ml of the cell-free medium–saline mixture were added 0.1 ml rat plasma and 0.5 ml lipoprotein precipitating solution (2% phosphotungstic acid, 2 M $MgCl_2$, pH 6.3). After overnight incubation at 4°, the tubes were centrifuged at 4° for 45 min at 11,000 *g*. The supernatant fluid was removed and the sedimented material was dissolved in Unisol (Isolab, Inc., Akron, OH). Aliquots were assayed for radioactivity by scintillation spectrophotometry. Thin-layer chromatography (TLC) showed that about 90% of the radioactivity associated with the phosphotungstate precipitates was triglyceride and the remainder was probably phospholipid (data not shown). The efficiency of lipid precipitation was 95%, and this efficiency was the same regardless of whether the material was from CCl_4-treated cells or control cells. These efficiency estimations were made by scintillation counting of TLC spots from lipid extracts of cell-free media and before and after phosphotungstate precipitation as described above. For these reasons, and since 95% of triglyceride secreted by hepatocytes *in vitro* is isolated in the very low density lipoprotein fraction [11, 12], determination of phosphotungstate-precipitable radioactivity should be a good estimate of VLDL. The determination of the specific radioactivity of the labeled palmitate in the fatty acid labeling incubation allowed the phosphotungstate precipitate radioactivity to be expressed as nanomoles of palmitate converted to lipid associated with the VLDL. This quantity will be referred to as VLDL palmitate.

Measurement of covalent binding of CCl_4-carbon to hepatocyte lipids was done essentially as described by Rao and Recknagel [13]. As above, incubations were terminated by dilution of the cell suspensions with ice-cold saline followed by centrifugation (1 min, 3000 *g*) and aspiration of the medium. Total cell lipids were extracted according to Folch *et al.* [14]. To reduce carryover of non-covalently bound $^{14}CCl_4$, the lipid extract was treated in one of two ways. For the data of Fig. 5, aliquots of the lipid extract were dried in a 130° oven for 10 min. Chloroform (1 ml) was added to the residue and heating was repeated. For the data of Fig. 4, volatile material was removed by vacuum treatment at 45°. Cells that were heated at 100° for 2 min before exposure to $^{14}CCl_4$ or cells to which $^{14}CCl_4$ was added just before Folch extraction served as controls. The amount of lipid present in the $CHCl_3$ phase was measured by the method of Chiang *et al.* [15].

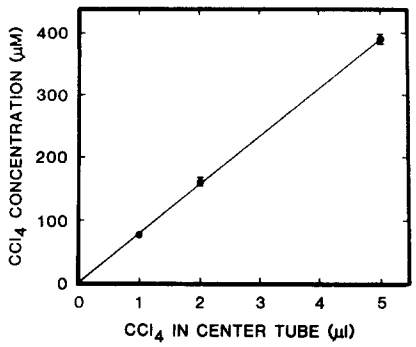

Fig. 1. Medium concentration of CCl_4 as a function of added CCl_4 volume. The indicated volumes of $^{14}CCl_4$ (1 μCi/μl) were each placed in a 6 × 50 mm test tube in a 50-ml sealed glass Erlenmeyer flask containing 2.5 ml of incubation medium (60 mM NaCl, 40 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mM Na_2HPO_4, 5 mM D-glucose, 1 mM methionine, and 50 mM HEPES, pH 7.4). After 1 hr of incubation at 37°, 0.1-ml aliquots were assayed for radioactivity. Mean values ± S.E. from four determinations are shown.

The concentration of CCl_4 in the incubation medium was determined by measuring radioactivity of medium after equilibration with $^{14}CCl_4$ placed in the center of the closed flask. As an aside, it may be noted that the value associated with 5 μl CCl_4 in Fig. 1 (390 μM) is more accurate than the value presented in an earlier report [9]. The difference is attributable to previous use of a batch of $^{14}CCl_4$ which may have been contaminated with moisture. In this report the concentrations were determined with each of two fresh lots of $^{14}CCl_4$.

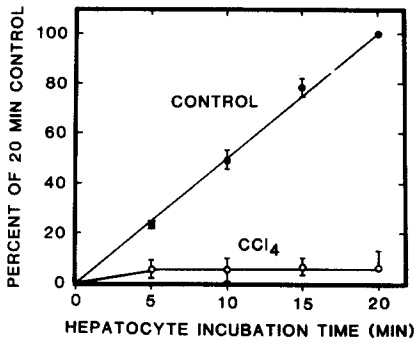

Fig. 2. Secretion of lipid by isolated hepatocytes in the presence and absence of carbon tetrachloride. Isolated hepatocytes were incubated (37°) with 0.5 mM [1-^{14}C]palmitate for 1 hr. After washing away extracellular fatty acid, the cells were concentrated to 60% (w/v), and 0.5-ml aliquots were added to flasks containing 2.5 ml of incubation medium equilibrated with 390 μM CCl_4 (open symbols) or with no added CCl_4 (closed symbols). Incubations at 37° were terminated at the indicated times, and secreted lipid was measured. To combine the results of separate experiments, data from each experiment were normalized to the secretion value of control cells at 20 min. This 100% value was 2.26 ± 0.65 (N = 4) nmoles VLDL palmitate secreted per ml of 10% cell suspension (10 mg cell protein/ml). Values shown are means ± S.E. (N = 4).

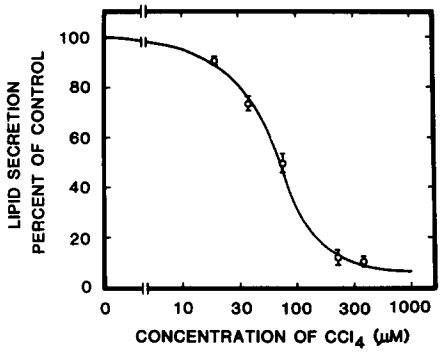

Fig. 3. Dose–inhibition relationship of medium CCl_4 concentration and lipid secretion. Volumes of CCl_4 (0.25 to 5.0 μl) were equilibrated as usual in flasks containing 2.5 ml of incubation medium to achieve a range of equilibrium CCl_4 concentrations. [1-^{14}C]Palmitate-labeled hepatocytes were added and incubated for 20 min. The amounts of VLDL palmitate secreted were measured, and the results of several experiments were combined by normalization as described in Fig. 2. Values shown are means ± S.E. (N = 3). For this series of experiments, the mean secretion value in the absence of CCl_4 was 1.14 nmoles VLDL palmitate/ml of 10% cell suspension in 20 min.

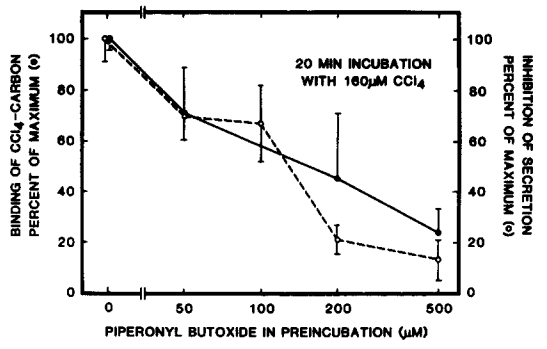

Fig. 5. Effect of piperonyl butoxide pretreatment on binding of CCl_4-carbon and CCl_4-induced inhibition of lipid secretion. Hepatocytes were preincubated with [1-^{14}C]palmitate as usual except that, in experiments where $^{14}CCl_4$ binding was measured, palmitate was not labeled. After 40 min, the suspension was divided into several portions, and piperonyl butoxide was added to achieve the final concentrations indicated. After a further 20 min at 37°, cells were recovered and washed, and 0.25-ml aliquots (60%, w/v) were added to 2.75 ml medium containing 160 μM CCl_4. After 20 min, binding of carbon from $^{14}CCl_4$ to lipids and secretion of VLDL palmitate were measured. For the experiments involving binding of CCl_4 carbon, the flasks contained 1.1 μCi $^{14}CCl_4$. Means ± S.E. are shown. For lipid secretion experiments N = 5, and average maximum inhibition of secretion was 49.5% of control. For covalent binding experiments N = 2–5, and average maximum binding was 99 cpm/mg cell lipid.

Glutamic-oxaloacetic transminase (EC 2.6.1.1) (GOT) was assayed using the method of Reitman and Frankel [16] as given in the Sigma Chemical Co. Kit No. 505. GOT release is expressed as the amount of enzyme activity present in the incubation medium after cells have been removed by centrifugation relative to the total amount of GOT activity (cells plus medium).

RESULTS

Because CCl_4 is both volatile and poorly soluble in water, exposure of cells to controlled concentrations of CCl_4 cannot be achieved simply by addition of CCl_4 to incubation media. These problems were circumvented by equilibration of medium, prior to addition of cells, with various amounts of CCl_4 added to a center chamber in a closed flask. Equilibrium was achieved at 37° within 40 min. Figure 1 shows the relationship between the amount of CCl_4 placed in the center well and the concentration in the medium before the addition of isolated hepatocytes.

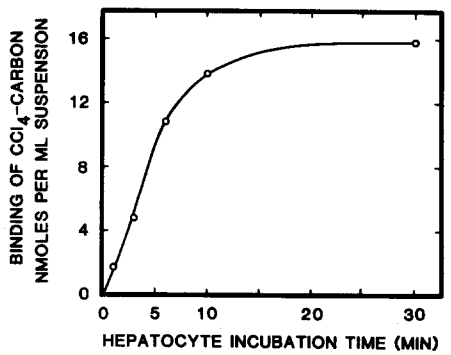

Fig. 4. Time course of covalent binding of CCl_4-carbon to lipids of isolated hepatocytes. Aliquots (2 μl) of $^{14}CCl_4$ were equilibrated with 2.5 ml of incubation medium to yield a concentration of 160 μM $^{14}CCl_4$. Aliquots (0.5 ml) of 60% (w/v) hepatocytes were added and incubated for the indicated times. Lipids were extraced, and volatile (nonbound $^{14}CCl_4$ was removed by vacuum treatment at 45°. The residue was dissolved in cyclohexane, and radioactivity was measured by scintillation spectrometry.

When isolated hepatocytes were incubated with oleate and [1-^{14}C]palmitate, cellular radioactivity reached a steady state within 1 hr (data not shown). Secretion of radioactive VLDL by cells labeled in this way was linear for at least 20 min after removal of label (Fig. 2). Severe inhibition of lipid secretion occurred when the isolated hepatocytes were added to flasks containing 390 μM CCl_4. By 5 min, secretion of VLDL from the cells had essentially ceased. Since the question of the rapidity of onset of the action of CCl_4 is of interest (see Discussion), and since VLDL secretion was inhibited completely at the 5-min point, a shorter incubation time was investigated. In three experiments conducted as in Fig. 2, it was found that, after 2 min of incubation in the presence of 390 μM CCl_4, VLDL secretion was 62 ± 4% of control.

It is important to note that the inhibition of lipid secretion was not associated with leakage of GOT from the cells. Cells that had been incubated with or without 160 μM CCl_4 released, respectively, 7.6 ± 0.9 and 7.5 ± 0.9% (mean ± S.E.) of their total cellular GOT into the medium during a 20-min incubation. Furthermore, no stimulation of GOT release is noted with up to 570 μM CCl_4 at 1 hr of incubation [9].

The dose–inhibition relationship between CCl_4 concentration and lipid secretion is shown in Fig. 3. The concentration of CCl_4 causing 50% inhibition was about 80 μM. This concentration is lower than that reached in the blood of a rat after a 2.5 ml/kg intragastric dose of carbon tetrachloride [17].

It is well known that important features of CCl_4 hepatotoxicity, including development of fatty liver, require bioactivation of the CCl_4 molecule at the cytochrome P-450 locus of the endoplasmic reticulum. We have sought to investigate this with respect to VLDL secretion by isolated hepatocytes as a test of the similarity of the cell system and the intact animal. This has been done in two ways. First, we compared the kinetics of CCl_4 bioactivation with the kinetics of inhibition of VLDL secretion. Covalent binding of $^{14}CCl_4$-carbon to cellular lipid was used as an index of metabolic bioactivation. As shown in Fig. 4, covalent binding occurred rapidly following CCl_4 exposure, reaching half-maximum at 5 min. Similar results were obtained with 390 μM CCl_4 (J. A. Dolak: data not shown). This time course of bioactivation of CCl_4, as evidenced by the covalent binding, is consistent with the notion that the rapid inhibition of the VLDL secretion (Fig. 2) depends on CCl_4 metabolism. Second, we sought to determine whether substances which inhibit CCl_4 metabolism would attenuate the inhibition of lipid secretion by CCl_4. The free radical scavenger menadione [18] at a concentration of 1 mM reduced covalent binding by 97% during exposure to 160 μM $^{14}CCl_4$ but, unfortunately, also had a direct and pronounced inhibitory effect on triglyceride secretion in the absence of CCl_4. In our hands, SKF-525A did not produce a pronounced inhibition of covalent binding. In agreement with this, Poli *et al.* [19] found that malondialdehyde production by CCl_4-treated hepatocytes is inhibited by SKF-525A only 40%. The agent found best suited was piperonyl butoxide. This substance was quite effective in reducing covalent binding to cell lipid (Fig. 5), and it did not significantly affect VLDL secretion. For example, in three experiments, control cells secreted 0.168 ± 0.021 nmole of VLDL palmitate/mg cell protein during a 20-min incubation, and the corresponding value in cells pretreated with 500 μM piperonyl butoxide was 0.154 ± 0.004. Figure 5 shows that increasing concentrations of piperonyl butoxide produced a dose-dependent protection against CCl_4-induced inhibition of VLDL secretion. That is, the secretion rate of poisoned cells increased with respect to the non-poisoned (control) level as piperonyl butoxide concentration increased. There was a slight elevation in the percentage of total GOT released into the medium only with the highest concentration of piperonyl butoxide used (13 vs. 6% for control).

DISCUSSION

Dianzani and co-workers [4] were the first to study the isolated hepatocyte model with respect to the effect of carbon tetrachloride on lipid secretion. We have confirmed their basic finding that CCl_4 in low concentrations inhibits the secretion of VLDL from hepatocytes that had synthesized labeled VLDL during a 1-hr incubation before exposure to CCl_4 (Fig. 2). We also confirmed that at the CCl_4 concentrations and short incubations times used here, leakage of cytosolic GOT does not exceed low background levels. However, CCl_4-induced leakage of GOT does occur upon incubation for 5 hr [20]. This is consistent with the time course of GOT elevation in the blood of rats given CCl_4 [15].

The CCl_4 exposure system used here allowed determination of the kinetics of CCl_4-dependent inhibition of lipid secretion. The key feature of this method was to allow CCl_4 to come to equilibrium in the incubation medium before the hepatocytes were added. When this was done, the amount of CCl_4 contained in the hepatocytes was one-half-maximal at 3 min of incubation (data not shown). In contrast, if CCl_4 is placed in the center chamber of the flask after the cells are added to the incubation medium, it takes 30 min for the amount of CCl_4 in the cells to come to a constant level [9]. With the CCl_4 equilibrium conditions as herein described, at a CCl_4 concentration of 390 μM, VLDL secretion was inhibited completely within 5 min (Fig. 2), and it was depressed 40% at 2 min (Results). This is similar to the rapid time course of CCl_4-induced inhibition of lipid secretion *in vivo* [1]. The kinetics of CCl_4 inhibition of VLDL secretion are compatible with the hypothesis that metabolism of CCl_4 is required as a first step in the inhibition of VLDL secretion, since Fig. 4 shows that binding of CCl_4 carbon to cell lipid was significant at early time points.

The role of bioactivation of hepatotoxins in liver injury has been established classically using agents that inhibit metabolic activation [5]. To gain further information concerning the role of CCl_4 metabolism in inhibition of VLDL secretion, a number of agents were studied. An effective agent must satisfy several criteria. First, the inhibition of metabolism must be pronounced to obtain clear results. Second, the inhibitory agent alone should not markedly affect the cell function being studied (VLDL secretion) or be grossly toxic. Of several agents tested (diethyl dithiocarbamate, SKF-525A, menadione), only piperonyl butoxide met all of these requirements. Piperonyl butoxide binds to cytochrome P-450 with a type I binding spectrum and a metabolite is produced. This metabolite then forms a stable complex with the cytochrome, which inactivates the enzyme. The formation of this so-called type III complex has been correlated with inhibition of the metabolism of several type I and type III substrates [21]. Once the complex has formed the inhibition is noncompetitive.

To test the effects of piperonyl butoxide on CCl_4-induced inhibition of lipid secretion, a CCl_4 concentration which would yield a significant, but submaximal, inhibition of the cell function was sought. Based on the dose-inhibition study shown in Fig. 3, 160 μM CCl_4 was chosen. When isolated hepatocytes were incubated with various concentrations of piperonyl butoxide for 20 min, a dose-dependent inhibition of CCl_4-carbon binding to cellular lipids was observed during a subsequent incubation with 160 μM CCl_4 (Fig. 5). Binding of CCl_4-carbon to cellular lipids is an index of metabolic activation of CCl_4 by the mixed-function oxidase system. The

concentration of piperonyl butoxide causing a 50% inhibition (with respect to binding) was about 100 μM. Others have found that, in general, the piperonyl butoxide IC_{50} for drug metabolism by rat microsomal preparations is about 100 μM [22].

In our study (Fig. 5), as the concentration of piperonyl butoxide IC_{50} for drug metabolism by rat gressive decrease in binding of CCl_4-carbon, and this was accompanied by a progressive attenuation of the CCl_4-dependent inhibition of VLDL secretion. This is evidence that metabolism of CCl_4 is important in the expression of the pathologic change in the *in vitro* system, just as *in vivo* [23–25]. Partial protection against CCl_4-induced inhibition of lipid secretion in the isolated hepatocyte model has been observed by others with menadione, and this, too, was associated with reduced binding of CCl_4-carbon to cell constituents [26].

The rapidity of onset of the CCl_4 effect (Fig. 2) suggests that a late step in the VLDL secretory pathway was inhibited. It takes 10–15 min for a pulse of labeled fatty acid to traverse the triglyceride secretory pathway. Since cells were incubated with free fatty acid for 1 hr before exposure to CCl_4, a pool of lipoprotein was presumed to be ready for secretion but, after only 2 min of exposure to CCl_4, appearance of labeled lipid in the medium was already significantly inhibited. If only an initial step in the pathway (i.e. coupling of lipid with the apoprotein moiety) were blocked, it seems likely that secretion would continue for the better part of 10 min before a defect would become apparent. Since the inhibition was so rapid, the implication is that, as a minimum, CCl_4 probably disrupted one or more late steps along the secretory pathway, just before release of the lipoprotein from the cell.

In conclusion, the characterization of CCl_4-induced inhibition of lipid secretion presented here further validates the similarity of the isolated hepatocyte system to the whole animal. The main significance of this work is that it provides a foundation for investigation of the biochemical events culminating in CCl_4-induced hepatic steatosis [8].

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