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EVIDENCE AGAINST INVOLVEMENT OF CALCIUM IN CARBON TETRACHLORIDE-DEPENDENT INHIBITION OF LIPID SECRETION BY ISOLATED HEPATOCYTES*

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Abstract—Carbon tetrachloride (CCl_4)-induced inhibition of very low density lipoprotein (VLDL) secretion was studied in isolated hepatocytes. The hypothesis that inhibition of secretion is due to altered calcium homeostasis following CCl_4-dependent inhibition of endoplasmic reticulum calcium sequestration was investigated. Inhibition of VLDL secretion by CCl_4 was not dependent on extracellular calcium, since inhibition occurred when extracellular calcium was reduced to 0.1 μM. CCl_4 inhibited hepatocyte VLDL secretion more rapidly than it inhibited microsomal calcium sequestration. Further, the concentration of CCl_4 that produced half-maximal inhibition of VLDL secretion was about one-half the concentration required to produce half-maximal inhibition of microsomal calcium sequestration. The calcium ionophore A23187 did not mimic the action of CCl_4 in inhibiting VLDL secretion under conditions in which A23187 altered cellular calcium homeostasis. The results suggest that an alteration of calcium homeostasis is not involved in inhibition of VLDL secretion by carbon tetrachloride.

Carbon tetrachloride (CCl_4) has been studied extensively as a model hepatotoxin [1], and a large body of information is available concerning the initial events in the pathogenic sequence. Two immediate consequences of cleavage of the CCl_4 molecule by the mixed-function oxidase system are covalent binding of radicals (i.e. $\cdot CCl_3$) in the immediate vicinity of cytochrome P-450 and limited oxidative decomposition of endoplasmic reticulum (ER) membrane phospholipids [2]. Several more slowly developing consequences have also been well characterized, including hepatic fat accumulation [3], decreased protein synthesis [4], glycogenolysis [5], leakage of cellular enzymes into the blood stream [6], and necrosis [7]. The continuing challenge to the investigator is to discover the biochemical processes that link the initial events to the resulting pathologic changes. Because of considerations reviewed elsewhere [8], this laboratory has focused recently upon the possible role of altered calcium homeostasis as a mediating event in carbon tetrachloride-induced liver injury.

Cytosolic calcium-ion concentration is controlled by the concerted actions of three calcium-transporting systems: the mitochondria, the endoplasmic reticulum and the plasma membrane [9,10]. The relative importance of these organelles in calcium homeostasis is currently being debated [9, 11–13]. CCl_4 metabolism results in inhibition of calcium-sequestering activity of the endoplasmic reticulum *in vivo*, in microsomal suspensions, and in isolated hepatocytes [14–17]. The impact of this event upon cellular calcium-ion distribution and concentration is not yet certain, but it seems plausible that the loss of ER calcium sequestration and the accompanying release of ER stores of calcium into the cytoplasm [18] could have serious pathologic consequences for the hepatocyte.

As an initial test of this hypothesis, we investigated the rapid CCl_4-induced inhibition of very low density lipoprotein (VLDL) secretion by isolated hepatocytes [19]. The importance of cytosolic calcium levels in secretory activity is widely recognized [10, 20]. Movement of intracellular secretory vesicles containing VLDL is dependent upon the cytoskeleton [21] which, in turn, is sensitive to cytosolic calcium ion concentration [22]. The work presented here was designed to investigate the possibility that CCl_4-induced inhibition of microsomal calcium sequestration could be responsible for inhibition of VLDL secretion. The results suggest that CCl_4-induced redistribution of cellular calcium is not critical in the inhibition of VLDL secretion caused by CCl_4.

MATERIALS AND METHODS

The sodium salt of [1-^{14}C]palmitic acid was purchased from ICN Pharmaceuticals, Inc., Irvine, CA. Fatty-acid-poor bovine serum albumin was from Miles Laboratories, Inc., Elkhart, IN. Collagenase (catalogue No. C0130), ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), ionophore A23187 and its vehicle, dimethyl sulfoxide, were from the Sigma Chemical Co., St. Louis, MO. $^{45}Ca^{2+}$ was obtained as aqueous $^{45}CaCl_2$ from the New England Nuclear Corp., Boston, MA.

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Male Sprague–Dawley rats (200–400 g) were supplied by Zivic–Miller Laboratories Inc., Allison Park, PA. Animals were fasted overnight before use. Hepatocyte isolation was performed as described previously [17, 19]. Hepatocytes were labeled with [1-^{14}C]palmitate, and VLDL secretion was measured as described before [19]. Briefly, hepatocytes were incubated with 0.5 mM oleate and 0.5 mM [1-^{14}C] palmitate (0.5 μCi/mmole) for 1 hr at 37°. Extracellular fatty acid was then separated from the cells by centrifugation and resuspension of the cell pellet in fresh medium. Aliquots (0.5 ml) of the washed cell suspension (60%, w/v) were added to flasks with or without 390 μM CCl_4 in equilibrium in the incubation medium. Incubations were terminated by diluting cell suspensions with ice-cold saline followed immediately by separation of the cells from the medium by centrifugation (3000 g, 1 min). VLDL was precipitated from the cell-free medium using a magnesium-phosphotungstate precipitation technique [23, 24] as described previously [19]. The radioactivity of the precipitate was measured by scinitillation counting in Unisol-Complement (Isolab, Inc., Akron, OH). Microsomes were prepared from hepatocytes by sonication and differential centrifugation, and calcium sequestration was measured, all as previously described [17].

The effect of A23187 on cell calcium content was determined by a method similar to that used by Whiting and Barrit [25]. Cells were suspended in 3 ml incubation medium at a concentration of 10% (w/v, about 10 mg protein/ml) and incubated for 40 min following the addition of 0.14 μCi $^{45}Ca^{2+}$. Composition of the incubation medium was as given for Fig. 1. At short time intervals before and after addition of A23187, aliquots of suspension (50 μl) were washed twice with 300 μl of ice-cold incubation medium. Centrifugations of 6-sec duration in an Eppendorf model 5412 centrifuge were used to quickly separate cells from wash medium. Cell pellets were dissolved in 300 μl of 1% Triton X-100, and aliquots were taken for measurement of radioactivity by scintillation spectrometry and for protein assay [26].

The effect of A23187 on cellular calcium handling was also directly assessed by measuring cytosolic free calcium ion concentrations using the method of Tsien *et al.* [27]. Quin 2/acetoxymethyl ester for this work was purchased from Lancaster Synthesis, Ltd., Lancashire, England.

RESULTS

Influx of extracellular calcium into the hepatocyte appears to be a requirement for late events in CCl_4-induced liver injury such as leakage of cytosolic enzyme into the medium and staining of the cells by trypan blue [28, 29, but also see Ref. 30]. This work prompted us to investigate whether extracellular calcium was a requirement for CCl_4-induced inhibition of VLDL secretion, which is an early response to the toxicant. The effect of CCl_4 on VLDL secretion is shown in Fig. 1. CCl_4 strongly suppressed VLDL secretion in the presence of normal (1.0 mM) or low (0.1 μM) extracellular Ca^{2+}. With cells not exposed to CCl_4 only a moderate reduction in the initial rate

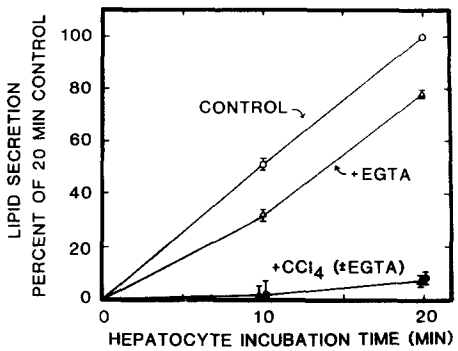

Fig. 1. Effect of extracellular Ca^{2+} on CCl_4-induced inhibition of lipid secretion by isolated hepatocytes. Cellular lipid was labeled by incubation of hepatocytes with 0.5 mM [1-^{14}C]palmitate for 1 hr. After washing away extracellular fatty acid, the cells were concentrated to 60% (w/v), and 0.5-ml aliquots were added to flasks containing 2.5 ml of incubation medium of the following composition: 60 mM NaCl, 40 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mM Na_2HPO_4, 5 mM D-glucose and 1 mM methionine, buffered at pH 7.4 with 50 mM HEPES. Triangles (△, ▲) represent flasks with medium also containing 10 mM EGTA (free Ca^{2+} approx. 0.1 μM), and circles (○, ●) represent flasks with no added EGTA. Open symbols (△, ○) indicate controls, and closed symbols (▲, ●) indicate that the medium contained 390 μM CCl_4. Incubations at 37° were terminated at the indicated times, and the amount of lipid (lipoprotein) secreted was assayed as indicated in Materials and Methods. To combine the results of separate experiments, the results of each experiment were normalized by dividing all values by the amount of lipid secreted by control cells in 20 min, and multiplying the quotient × 100. Values shown are means from two experiments.

of lipid secretion was observed with low extracellular Ca^{2+} concentration.

The time courses of CCl_4-induced inhibition of lipid secretion and of microsomal calcium sequestration are shown in Fig. 2. It is clear that VLDL secretion was inhibited more rapidly than microsomal calcium sequestration. At 5 min of exposure to CCl_4, the average lipid secretion rate was reduced by more than 80%, while the microsomal calcium sequestration of the cells was inhibited by only 20%. These curves are from separate experiments; the same results were obtained when lipid secretion and calcium sequestration were both measured in a single experiment. A comparison of the dose–inhibition relationship of lipid secretion and microsomal calcium sequestration (Fig. 3) revealed that VLDL secretion appeared more sensitive to CCl_4 than calcium sequestration. The concentration of CCl_4 yielding a 50% inhibition of the two functions differed about 2-fold.

The possibility that elevated cytosolic calcium levels might inhibit VLDL secretion was investigated by use of A23187. As a first step, it was important to assess the effects of the ionophore on hepatocyte suspensions as used in our laboratory. The effect of A23187 on cellular calcium metabolism was assessed in several ways. First, the addition of calcium ionophore after a 45-min incubation with $^{45}Ca^{2+}$ resulted in a rapid loss of radioactive calcium from the cells (Fig. 4). The net loss of calcium from the cells was

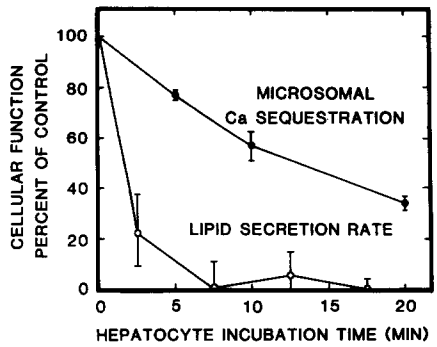

Fig. 2. Time course of CCl_4-induced inhibition of hepatocyte lipid secretion rate and of microsomal calcium sequestration. Lipid secretion (○): hepatocytes prelabeled with [1-^{14}C]palmitate were incubated in the presence or absence of 390 μM CCl_4, and lipid secretion was measured at 5-min intervals as described in Materials and Methods and Fig. 1. The average rate of lipid secretion by control cells over the duration of the experiment was constant at 0.565 nmoles VLDL palmitate/ml of 10% cell suspension in 5 min. The secretion rates of CCl_4-treated cells over 5-min intervals were calculated relative to the corresponding rates for control cells and are plotted as the means ± S.E. (N = 4) at the midpoints of the intervals. Microsomal calcium sequestration (●): Hepatocytes (0.5 ml, 60% w/v) were added to 2.5 ml of incubation medium with or without 390 μM CCl_4. At the indicated times, cells were pelleted from suspension and sonicated, microsomes were isolated by differential centrifugation, and the calcium sequestering activity of the microsomes was measured. Data are means ± S.E. of 10 min calcium uptake values relative to control uptake values for each of six separate experiments. Mean control calcium sequestration is 15.6 ± 2.16 nmoles calcium/mg microsomal protein in 10 min.

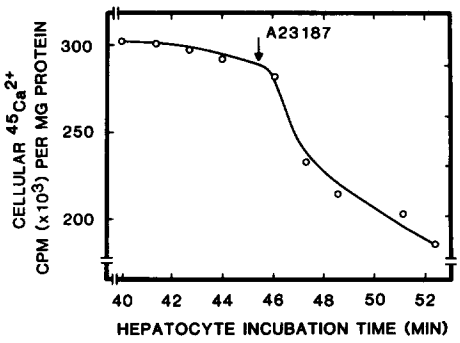

Fig. 4. Effect of A23187 on cellular $^{45}Ca^{2+}$ content. Hepatocytes (10%, w/v) were incubated at 37° for 40 min in the presence of 3.1 μCi $^{45}Ca^{2+}$/ml. See Fig. 1 for composition of incubation medium. At the indicated times, aliquots (50 μl) of the suspension were assayed for cellular radioactivity as described in Materials and Methods. At the arrow, A23187 was added to yield a final ionophore concentration of 5 μM. Data from one representative experiment are shown.

confirmed in an experiment in which total cell calcium was measured by atomic absorption spectroscopy (R. L. Waller: data not shown). The A23187-dependent loss of cell calcium is believed to be associated with a rise in cytosolic calcium (see below). Second, the effect of A23187 on cytosolic calcium-ion concentration was measured with the fluorescent metallochrome quin 2 [27]. A typical result (Fig. 5) shows the increase in quin 2 fluorescence upon A23187 addition. The initial jump in fluorescence was due to the fluorescence of the ionophore itself. The subsequent elevation in

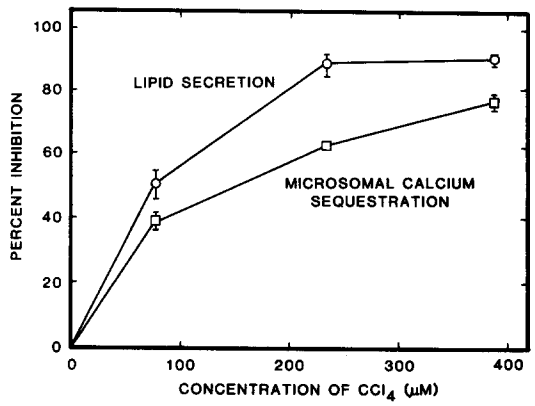

Fig. 3. Carbon tetrachloride dose–inhibition relationship of microsomal calcium sequestration and lipid secretion. Hepatocytes were incubated in the usual medium for 20 min in the presence of 0, 77, 234, or 390 μM CCl_4. Cells for microsomal calcium sequestration study were not preincubated with radioactive fatty acid. Lipid secreted into the medium as lipoprotein was assayed as described in Materials and Methods. Following the incubation, hepatocytes were sonicated, and microsomes obtained by differential centrifugation were assayed for calcium sequestering activity. Data shown are means ± S.E.: lipid secretion (○) N = 3; microsomal calcium sequestration (□), N = 3.

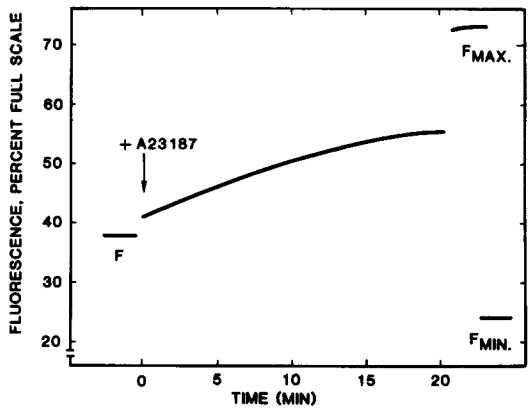

Fig. 5. Effect of A23187 on fluorescence of quin 2 loaded hepatocytes. Hepatocytes (0.5%, w/v) were incubated with 126 μM quin 2-acetoxymethyl ester for 15 min. The cells were then resuspended in medium without quin 2-acetoxymethyl ester and incubated for at least 15 min more. Finally, cells were resuspended in the usual incubation medium (0.5%, w/v) and placed in a cuvette (thermostated at 37°) in an Aminco–Bowman spectrophotofluorometer. Fluorescence (F) was monitored at emission wavelength 492 nm (excitation wavelength 339 nm). At the arrow, A23187 was added to yield a final concentration of 5 μM. F_{max} was determined by adding digitonin (50 μM), and F_{min} was obtained upon subsequent addition of EGTA (19 mM). The trace shown is representative of eight separate experiments.

Table 1. Lack of effect of A23187 on lipid secretion by isolated hepatocytes*

Additions		VLDL palmitate secreted (nmoles/ml suspension in 20 min)					
A23187	$CaCl_2$	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Mean ± S.E.
None	1 mM	1.28	2.36	1.29	1.59	2.51	1.81 ± 0.26
5.0 nM	1 mM	3.19	2.73				
0.5 μM	1 mM	2.93	2.43				
5.0 μM	1 mM	2.04	2.15	2.37	1.21		1.94 ± 0.25
5.0 μM	10 mM				1.37	2.60	

*[^{14}C]Palmitate-labeled hepatocytes were incubated at 37° for 20 min with the indicated additions. The amount of lipid secreted into the medium was determined by measurement of radioactivity in lipoproteins precipitated from cell-free medium as described in Materials and Methods.

fluorescence reflected an increase in cytosolic calcium-ion concentration. This increase may actually be an underestimate of the effect of the ionophore, since intracellular quin 2 acts as a calcium buffer as well as an indicator. In a series of experiments, doses of A23187 ranging from 0.1 to 5.0 μM all increased cytosolic calcium-ion concentration about 3-fold, from 185 ± 60 nM (N = 6) to 474 ± 107 nM (N = 8, $P < 0.01$, paired Student's t-test).

If CCl_4 were to inhibit VLDL secretion through a mechanism involving an increase in cytosolic calcium-ion concentration, then A23187 would mimic this inhibition. The effect of several concentrations of A23187 in the presence of various extracellular calcium concentrations on VLDL secretion from prelabeled hepatocytes is shown in Table 1. No significant inhibition of the secretory activity was seen under any of the conditions studied. Also, in two experiments, the addition of EGTA (10 mM) and A23187 (5 μM) together did not inhibit VLDL secretion compared to control. The rate of lipid secretion under the most extreme conditions tested (50 μM A23187 and 10 mM calcium) is shown in Fig. 6. Again, the ionophore did not have a significant effect on VLDL secretion. There was no stimulation of glutamic oxaloacetic transaminase release from the cells during a 20-min incubation with A23187 (data not shown).

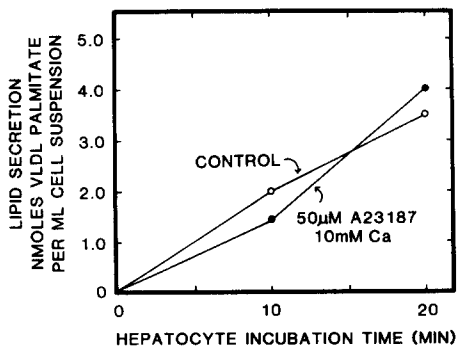

Fig. 6. Lack of effect of A23187 on VLDL secretion by isolated hepatocytes. Hepatocytes prelabeled with [1-^{14}C] palmitate as described in Materials and Methods were added to incubation medium of the composition listed in Fig. 1, containing 1 mM calcium (○), or containing 10 mM calcium and 50 μM A23187 (●). Incubations were terminated at the indicated times, and VLDL palmitate secreted by the hepatocytes was assayed as described in Materials and Methods. Data from one hepatocyte preparation are shown.

DISCUSSION

A redistribution of intracellular calcium, subsequent to the impairment of calcium sequestration by the endoplasmic reticulum, has been hypothesized to be important in linking the initial events of CCl_4 metabolism in the endoplasmic reticulum to the spreading of pathological consequences throughout the cell [8, 14, 17]. A key aspect of this hypothesis is that an early redistribution of calcium should be the mediator of various pathologic changes and that this calcium redistribution should be distinct from the late calcium influx leading to cell death [31, 32]. For example, the early inhibition of VLDL secretion by CCl_4 was not dependent upon an influx of calcium through the plasma membrane (Fig. 1).

The data reported here constitute an initial test of the calcium redistribution hypothesis with regard to one cell function, VLDL secretion. We compared the time-course of the CCl_4-dependent inhibition of VLDL secretion with the time-course of CCl_4-dependent inhibition of hepatocyte microsomal calcium sequestration (Fig. 2). In contrast to the extremely rapid depression of VLDL secretion (half-maximal depression at about 2 min), the depression of microsomal calcium sequestration was slower (half-maximal depression at about 13 min). As a first approximation, the data (Fig. 2) do not support the view that loss of microsomal calcium sequestering capacity is casual with respect to inhibition of VLDL secretion. However, the possibility remains that a small decrease in calcium sequestration activity may be sufficient to cause a large impairment in lipid secretion in the initial minutes of CCl_4 exposure. Because of this uncertainty, we turned to another test of the calcium redistribution hypothesis.

A prediction of the hypothesis with respect to VLDL secretion is that any agent which significantly increases cytosolic calcium-ion concentration should also inhibit lipid secretion. The calcium ionophore A23187 was used to alter intracellular calcium levels. The effects on cellular calcium were assessed in two ways. First, A23187 induced a rapid efflux of $^{45}Ca^{2+}$ from cells (Fig. 4). Net loss of calcium from the cells was confirmed by atomic absorption spectrometry. This effect has been observed previously [25, 33, 34] and has been interpreted as being consistent with

an increase in cytosolic calcium-ion concentration subsequent to movement of calcium down its concentration gradient from inside the mitochondria and ER to the cytoplasm [33, 35]. Although the ionophore may increase flux of calcium into the cell through the plasma membrane, this is apparently not the predominating effect [33]. Second, the ionophore has been shown to produce significant elevations of cytosolic calcium ion when measured by spectrophotometric or fluorometric methods [13, 27], and this is confirmed here in the isolated hepatocyte system (Fig. 5). Surprisingly, treatment of cells with 0.5 nM to 50 μM A23187 did not result in any significant impairment of VLDL secretion (Table 1, Fig. 6). Likewise, incubation of hepatocytes with A23187 and EGTA (a maneuver which should deplete cells of Ca^{2+}) did not inhibit VLDL secretion. Additionally, preliminary studies have indicated that VLDL secretion is not inhibited in the presence of sufficient digitonin to cause rapid elevation of cytosolic calcium-ion concentration to levels 3- to 10-fold higher than control (data not shown). Thus, major changes in intracellular calcium do not seem to be sufficient to inhibit VLDL secretion. The explanation of this unexpected finding could relate to the constitutive, substrate-limited nature of VLDL secretion, as opposed to secretory processes which are stimulated by agonists binding to receptors at the cell membrane. While these results cast doubt on a role for calcium redistribution in CCl_4-dependent inhibition of lipid secretion, the possibility remains that toxigenic intracellular calcium redistribution may be involved in other CCl_4-induced pathologic events.

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