Mechanisms responsible for carbon tetrachloride-induced perturbation of mitochondrial calcium homeostasis

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Incubation of isolated hepatocytes with CCl_4 results in early reduction of the intracellular calcium content, mostly due to loss from the mitochondrial compartment. CCl_4 treatment directly affects mitochondrial functions as indicated by the inhibition of Ca^{2+} uptake in cells permeabilized to the ion by digitonin exposure and by the reduction of intracellular ATP content in hepatocytes incubated in a glucose-free medium. Such mitochondrial damage is not caused by CCl_4-induced stimulation of lipid peroxidation since it is not prevented by α-tocopherol, used at a concentration able to inhibit completely peroxidative reactions without interfering with CCl_4 activation. All data together are in favour of a direct action of CCl_4-reactive metabolites on liver cell calcium homeostasis.

Carbon tetrachloride *Calcium homeostasis* *Mitochondria* *Isolated hepatocyte* *Lipid peroxidation*

1. INTRODUCTION

Perturbation of hepatocellular calcium homeostasis has been postulated to be one of the mechanisms responsible for causing cell death as a result of the exposure to a number of toxic compounds, including carbon tetrachloride [1,2]. In particular, one of the consequences of CCl_4 poisoning is the loss of Ca^{2+}-sequestering activity by the endoplasmic reticulum [3]. This cellular change occurs as early as 5–10 min after haloalkane addition to liver microsomal fractions or isolated hepatocytes and requires CCl_4 activation by the cytochrome P450-dependent mixed-function oxidase system [3].

A recent study by Brattin and Waller [4] has shown that, despite impairment of the microsomal calcium pump, much of the decrease in intracellular Ca^{2+} in CCl_4-treated hepatocytes is due to loss from the mitochondrial compartment.

Here, we have investigated in isolated rat hepatocytes the possible mechanisms responsible for the impairment of mitochondrial Ca^{2+} buffering activity.

Mitochondria are, in fact, generally believed not to be involved in the CCl_4 activation process and thus probably not directly affected by free radical metabolites of CCl_4. On the other hand, they might be altered by some long-lived products of lipid peroxidation able to diffuse further in the cell environment.

2. MATERIALS AND METHODS

α-Tocopherol and CCl_4 were obtained from Merck, Darmstadt, cation ionophore A23187 from Calbiochem Boehring, uncoupler protonophore CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and metallochromic dye arsenazo III from Sigma. Collagenase (grade II) and ADP (sodium salt) were from Boehringer Biochemia, Mannheim. Percoll was obtained from Pharmacia, Uppsala. $^{14}CCl_4$ (spec. act. 26.9 mCi/mmol) was purchased from Amersham International, England. All other reagents were of the highest grade of purity available and were obtained from local suppliers.

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Male Sprague-Dawley rats (200–250 g/body wt, fed ad libitum) were used. α-Tocopherol was injected intraperitoneally 15 h before killing at a dose of 100 mg/kg body wt dissolved in 1 vol. ethanol/9 vols 16% (v/v) Tween 80 in saline. Hepatocyte isolation and incubation conditions were essentially as described [5,6]. Isolated liver cell suspensions containing 3×10^6 cells/ml were incubated in the presence of 10 μl CCl_4 placed in the centre well of 50-ml Erlenmeyer flasks to give a final concentration in the incubation medium of approx. 172 μM [5]. ADP-chelated Fe^{3+} was directly added to the hepatocyte suspension to give a final concentration of 2.5 mM ADP and 100 μM Fe^{3+}.

Intracellular Ca^{2+} pools were measured by the arsenazo III assay, as described by Bellomo et al. [7] using a Hitachi-Perkin Elmer DB DW 557 spectrophotometer operating at the wavelength pair 654–685 nm. The system was calibrated by adding known amounts of $CaCl_2$ at the end of each experiment, and the result expressed as nmol Ca^{2+}/mg protein. Lipid peroxidation was measured in terms of malonaldehyde (MDA) production [5].

Intracellular ATP was measured spectrophotometrically in acid extracts by an enzymatic method using a Boehringer Biochemia kit. For covalent binding experiments $^{14}CCl_4$ was diluted with unlabelled CCl_4 to give a specific activity of 22.5 μCi/mmol and 10 μl placed in the central well of the incubation flask as mentioned above. Hepatocyte proteins were processed as in [8] and radioactivity estimated with an LKB 1217 Rack Beta scintillation counter.

3. RESULTS AND DISCUSSION

Incubation of isolated hepatocytes in the presence of CCl_4 leads to the reduction of about 70% of the total intracellular Ca^{2+} content; this loss is due to a lowering of both microsomal and mitochondrial Ca^{2+} pools (fig.1).

Reduction of microsomal Ca^{2+} content is consistent with the reported inhibition of Ca^{2+}-sequestering activity observed in both liver microsomes treated with CCl_4 and the endoplasmic reticulum fraction [3,4] isolated from hepatocytes previously intoxicated with the haloalkane. The loss of intracellular Ca^{2+}, however, is mostly due to the reduction of the CCCP-releasable fraction, indicating that the mitochondrial Ca^{2+}-sequestering activity is also affected. This alteration occurs as early as 15 min after CCl_4 addition and is not resolved on prolonging cell incubation up to 120 min (fig.1). It is known that mitochondrial Ca^{2+} content is maintained through the concerted activity of distinct uptake and release routes [9]. The ion uptake occurs via a uniport carrier, driven by the transmembrane potential generated during coupled respiration [10]. The calcium release instead seems to involve exchange with Na^+ and H^+ [11,12]. We have investigated the mechanisms responsible for depletion of the mitochondrial Ca^{2+} pool in isolated hepatocytes preincubated for 30 min with CCl_4 and then subsequently treated with a low concentration of digitonin to make the plasma membrane permeable to extracellular calcium.

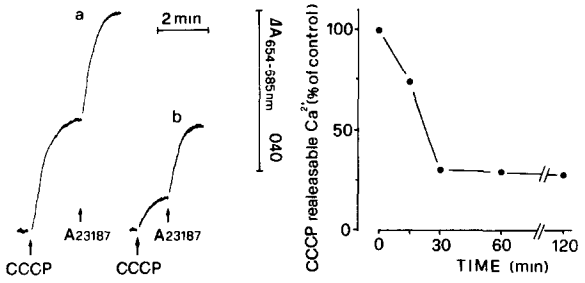

Fig.1. Alterations of intracellular Ca^{2+} compartmentation following CCl_4 metabolism in isolated hepatocytes. (Left) Typical spectrophotometric traces illustrating the titration technique employed to measure intracellular Ca^{2+} pools. (a) Control hepatocytes after 30 min incubation, (b) hepatocytes treated for 30 min with CCl_4. (Right) Time course of CCl_4-induced decrease in mitochondrial Ca^{2+} content. Each point represents the mean of 5 different experiments. The absolute value of the CCCP-releasable Ca^{2+} pool was 1.05 ± 0.15 nmol/mg protein.

As illustrated in fig.2, the amount of Ca^{2+} sequestered by the mitochondria of CCl_4-poisoned cells is approx. 75% less than that taken up in control hepatocytes. Thus CCl_4 not only induces the release of Ca^{2+} from mitochondria but also interferes with the uptake mechanisms, probably by affecting the processes which maintain the transmembrane potential. Further evidence suggesting that CCl_4 directly damages mitochondria is given by the measurement of total ATP content of isolated hepatocytes incubated in a glucose-free

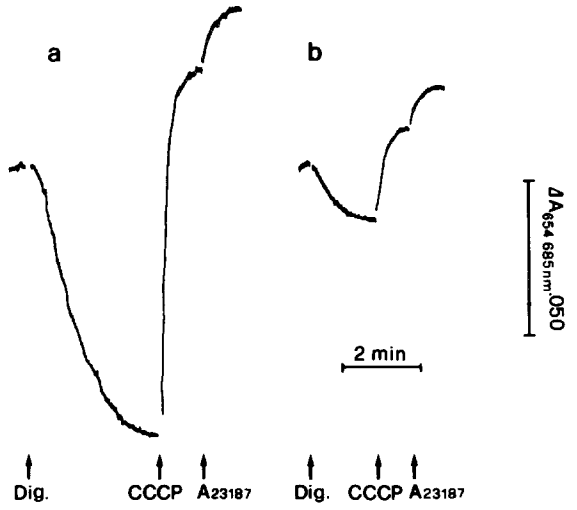

Fig.2. Alteration of mitochondrial Ca^{2+}-sequestering activity by CCl_4 metabolism in isolated hepatocytes. Control (a) or CCl_4-intoxicated (b) hepatocytes were harvested after 30 min incubation as described in section 2 for determination of intracellular Ca^{2+} pools except that the final incubation medium contained 20 μM $CaCl_2$. The absorbance changes due to Ca^{2+} sequestration by intracellular organelles were recorded upon addition of digitonin (12 μg/ml) to make permeable the plasma membrane; when Ca^{2+} uptake was complete, CCCP (10 μM) then A23187 (15 μM) were added to titrate the intracellular pools. One experiment typical of 2.

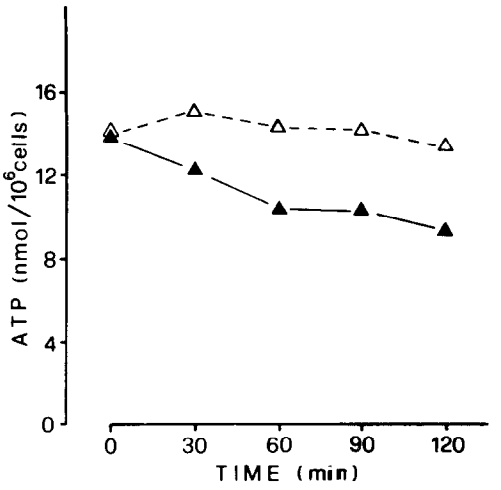

Fig.3. Decrease of intracellular ATP concentration by CCl_4 in isolated hepatocytes. Incubation conditions were essentially as described in section 2 except that glucose was omitted to minimise cytoplasmic resynthesis of ATP. At the times indicated samples were taken and processed for ATP enzymatic assay. (△) Untreated cells; (▲) CCl_4-treated cells. One experiment typical of 3.

medium, so as to reduce the cytosolic ATP resynthesis.

Fig.3 shows that the ATP content of liver cells challenged with CCl_4 is reduced concomitantly with the decrease in mitochondrial calcium.

Previous reports [13,14] have shown that CCl_4 causes an increase in total mitochondrial Ca^{2+}, as estimated by atomic absorption and radiolabelled isotope dilution, within several hours of rat intoxication. However, as pointed out by Carafoli and Tiozzo [13], the calcium accumulated in the mitochondria is in a complexed not releasable form, and is thus not measurable by the present technique. Furthermore, calcium complexation might account for at least part of the decrease in FCCP-releasable Ca^{2+} in hepatocytes. Likewise, the above authors also observed impaired Ca^{2+} uptake and alterations of oxidative phosphorylation in the mitochondria obtained from CCl_4-treated rats [13].

In isolated hepatocytes, as in whole liver, CCl_4 is activated by the mixed-function oxidase system to a free radical intermediate responsible for either covalent binding to cell macromolecules or stimulation of lipid peroxidation [12]. To investigate the possible involvement of peroxidative mechanisms in causing alterations of mitochondrial functions isolated hepatocytes have been prepared from α-tocopherol-pretreated rats [15] and subsequently intoxicated with the haloalkane. As shown in table 1 no detectable lipid peroxidation, measured MDA production, occurs in α-tocopherol-treated hepatocytes receiving CCl_4, while the haloalkane activation, indicated by the covalent binding of radioactive metabolites to cell protein, is not affected. When tested for mitochondrial calcium content and intracellular ATP concentration, α-tocopherol-treated hepatocytes are not protected against the effect of CCl_4.

Further indications that peroxidative reactions are not involved in causing mitochondrial damage derive from experiments where lipid peroxidation is induced in hepatocytes by ADP-iron. Under these conditions no decrease in mitochondrial Ca^{2+} content is observed (fig.4). In addition, when the plasma membranes of ADP-iron-treated cells

Table 1

Effect of α-tocopherol pretreatment on CCl_4 activation, stimulation of lipid peroxidation and derangement of mitochondrial functions in isolated hepatocytes

Treatments	Covalent binding (nmol CCl_4/mg protein)	MDA production (nmol/10^6 cells)	CCCP-releasable Ca^{2+} (nmol/mg protein)	Intracellular ATP content (nmol/10^6 cells)
Cells from untreated rats				
Control	–	0.40 ± 0.08	1.05	13.47 ± 0.82
+ CCl_4	0.73 ± 0.08	1.80 ± 0.16	0.28	9.70 ± 1.08
Cells from α-tocopherol-pretreated rats				
Control	–	0.20 ± 0.05	1.20	13.90 ± 1.11
+ CCl_4	0.80 ± 0.09	0.33 ± 0.04	0.31	10.72 ± 0.96

Isolated hepatocytes from untreated and α-tocopherol pretreated rats were incubated for 60 min at 37°C with or without CCl_4 as described in section 2. The values are means of 2–4 experiments ± SD

are made permeable by digitonin, calcium uptake comparable to that of untreated cells occurs (fig.4).

The possibility that some of the early effects of CCl_4 intoxication involve mitochondria was suggested by the some studies of Dianzani [16] and Artizzu et al. [17]. These authors observed mitochondrial swelling in the liver of rats as early as

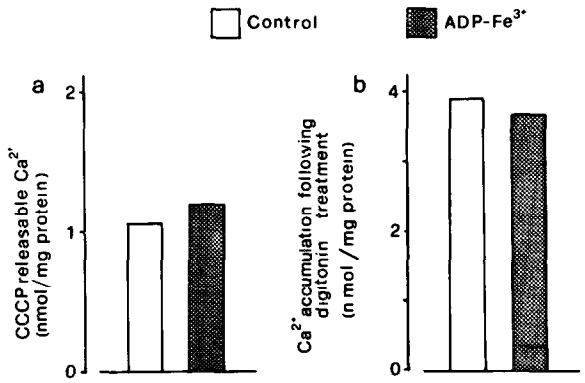

Fig.4. ADP-iron induced lipid peroxidation does not affect the ability of hepatocyte mitochondria to take up and retain Ca^{2+}. Mitochondrial Ca^{2+} content (measured as CCCP-releasable Ca^{2+}) and Ca^{2+} accumulated after digitonin treatment were measured in control and ADP-iron-treated (30 min) hepatocytes as described in section 2. One experiment typical of 2.

30–60 min after CCl_4 intoxication in vivo. Also, uncoupling of oxidative phosphorylation and decrease in ATP content were observed in organelle preparations exposed in vitro to the same CCl_4 concentration as found in the liver 30 min after administration of the haloalkane [17].

The present results indicate that the decrease in mitochondrial calcium uptake and ATP content occurs shortly after exposure of hepatocytes to CCl_4 and is not mediated by the stimulation of lipid peroxidation, appearing rather as a consequence of attack by free radical metabolites. Thus, 2 possibilities exist, i.e. that free radicals might diffuse from their microsomal site of production to the mitochondria or alternatively that mitochondria themselves are able to activate CCl_4. Levy and Brabec [18] and De Castro et al. [19] have recently given some evidence in favour of this latter possibility.

Concerning the significance of the impairment of calcium homeostasis as a mechanism of cell death in the case of CCl_4 poisoning, in contrast to what has been found with other toxic compounds [1], alterations in the intracellular Ca^{2+} distribution do not result in an early loss of cell viability or in morphological alterations of the isolated hepatocytes [5]. This observation, however, does not exclude a possible later contribution of this cellular change to CCl_4-induced liver cell necrosis.

Such a consideration is based on the fact that in an almost identical experimental model in which CCl_4 is substituted by iron, i.e. lipid peroxidation without the occurrence of haloalkylation, the eventual hepatocyte damage is definitively lower [20].

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