Influence of Lipid Peroxidation on Lipoprotein Secretion by Isolated Hepatocytes[1]

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ABSTRACT

Isolated rat liver cells have been exposed to 3 different lipid peroxidation-inducing agents, CCl_4, $FeCl_3$ and cumene hydroperoxide, and the rates of malonaldehyde production and of lipoprotein secretion have been compared. Results indicate that it is possible to induce a high degree of lipid peroxidation without inducing strong changes in lipoprotein secretion. Only in CCl_4-poisoned hepatocytes is lipoprotein secretion strongly impaired. In this experimental condition, the effect of free radical scavengers, or inhibitors of lipid peroxidation, has been studied; the degree of covalent binding of CCl_4 metabolites to hepatocyte proteins, as well as the behavior of both lipid peroxidation and lipoprotein secretion, have been evaluated. Promethazine and propyl gallate prevented malonaldehyde production, but neither agent reduced covalent binding nor improved secretion. Menadione, on the contrary, besides inhibiting malonaldehyde production, decreased covalent binding and protected against the impairment of secretion. These data lead to the conclusion that covalent binding of CCl_4 metabolites, rather than lipid peroxidation products, accounts for the derangement of lipoprotein secretion in CCl_4-poisoned liver cells.

Carbon tetrachloride (CCl_4) still represents one of the most used model agents for investigating the mechanisms of liver injury. It is postulated that its toxicity is due to homolytical cleavage in the smooth endoplasmic reticulum with the production of chloride (Cl^-) and the trichloromethyl radical ($CCl_3^\cdot$) (1-3). Recent in vitro experiments have shown that $CCl_3^\cdot$ is rapidly converted, in the presence of O_2, into the much more reactive trichloromethylperoxy radical ($CCl_3O_2^\cdot$) (4,5). The cell damage (i.e., enzyme inactivation, inhibition of protein synthesis and of protein and lipoprotein secretion, fat accumulation within the liver cells) is the consequence either of covalent binding of such free radicals to liver macromolecules, or of lipid peroxidation, through hydrogen subtraction by free radicals from membrane polyunsaturated fatty acids (PUFA) (1,3,6).

With regard to lipid peroxidation, cell changes may be produced either directly, by membrane derangement, or indirectly, by production of several different reactive compounds such as lipid free radicals, lipoperoxides, lipohydroperoxides, aldehydes and others (7).

Some evidence supports the hypothesis that, in CCl_4 poisoning, changes in cell sites far from the endoplasmic reticulum are probably due to diffusible substances. These may include substances derived from the peroxidative breakdown of PUFA (7,8).

Whether the CCl_4-induced damage to membrane enzymes and to the lipoprotein secretory pathway is due to its prooxidant effect or to the covalent binding of its metabolites to cell structures is not yet completely clear.

The use of isolated hepatocytes represents a good model to further investigate the mechanism of cell damage in CCl_4 poisoning. In isolated liver cells, as well as in vivo, CCl_4 stimulates lipid peroxidation (9-11), inhibits protein synthesis and protein and lipoprotein secretion (11). Under these conditions, accumulation of fat in the cells was also seen (11).

One of the aims of studies reported in this paper was to determine whether the CCl_4-induced block in lipoprotein secretion was due to covalent binding or to lipid peroxidation. In order to check the relative influence of either covalent binding or lipid peroxidation on the secretory pathways, several criteria may be followed.

The use of free radical scavengers or of inhibitors of lipid peroxidation seems particularly useful. Previous experiments using CCl_4-poisoned hepatocytes have demonstrated that promethazine and propyl gallate strongly attenuate the peroxidative breakdown of PUFA up to aldehydic products (10,12). Furthermore, since we observed that menadione (vitamin K_3) is able to reduce CCl_4 covalent binding in liver cells, we also investigated the effect of this drug at a very early stage of derangement of lipoprotein secretion.

Other studies reported in this paper represent another approach to the problem. We used 2 experimental conditions other than CCl_4-poisoning, in which an increased lipid peroxidation occurs, i.e., the cell treatment with

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$FeCl_3$ (13), or with cumene hydroperoxide (14).

EXPERIMENTAL PROCEDURES

Animals

Male rats of the Wistar strain (Nossan, Correzzana, Milano, Italy) of 200-250 g body wt were used. They were fed a semisynthetic diet, free of any antioxidant (Piccioni, Brescia, Italy) with free access to water. All experiments started between 10:00 a.m. and 11:00 a.m.

Reagents

All chemicals were of reagent grade and were obtained from the following sources: collagenase type I, menadione, propyl gallate, amino acids, ethyleneglycol-bis-(β-amino-ethyl-ether)-N,N'-tetraacetic acid (EGTA), and N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) from Sigma Chemical Co., St. Louis, MO; promethazine-HCl from May and Baker, Dagenham, U.K.; cumene hydroperoxide from Fluka AG, Buchs, Switzerland; [U-^{14}C] palmitic acid from The Radiochemical Centre, Amersham, U.K.; other chemicals from BDH Chemicals Ltd., Poole, U.K., and Merck, Darmstadt, West Germany.

Preparation of Intact Liver Cells

The open, nonrecirculating, in situ liver perfusion technique used was essentially that described in previous works (11,12). In order to prevent loss of cell glutathione content during hepatocyte isolation, the following modifications were made, as suggested by Viña et al. (15): in the saline buffer used in the first perfusion step, 0.2 mM EGTA was included; in the cell incubation medium, the amino acid mixture was replaced by 1 mM methionine during the first 10-min incubation step.

Triglyceride Secretion from Prelabeled Hepatocytes

Hepatocytes were suspended (10^7 cells/ml) in Ham's F-12 medium containing 10% horse serum (11,12). Cell triglycerides were prelabeled by incubating 10 ml of cell suspension with 5 ml of 3 mM [^{14}C] sodium palmitate (sp act 0.33 mCi/mMol) complexed with albumin as previously reported (11,12). After 60 min incubation at 37 C, the cell suspension was diluted with 100 ml of incubation medium and then centrifuged at 400 × g for 4 min. Labeled hepatocytes were resuspended in incubation medium to 5 × 10^6 cells/ml. Aliquots of 2 ml of the suspension were poured into the main compartment of 50-ml flasks fitted with center wells and closed with screw caps.

Promethazine, propyl gallate or menadione, when indicated, were added directly to the cell suspension.

To initiate lipid peroxide formation, CCl_4 (5 μl, 86 μM final concentration) was added to the center well and allowed to diffuse in the closed system. On the other hand, when used, $FeCl_3$ or cumene hydroperoxide were added (at the different concentrations hereafter reported) directly to the cell suspension. Flasks were incubated at 37 C for 10 or 30 min. Aliquots of 2 ml of prelabeled hepatocytes were centrifuged without incubation to determine the time-zero secretion. At the end of incubation, cell suspensions were centrifuged. Triglycerides in supernatants were purified and processed for radioactivity measurements as described elsewhere (16).

Determination of Thiobarbituric Acid Reacting Materials

Malonaldehyde production was estimated by measuring the thiobarbituric acid (TBA)-reacting compounds (17). After the incubations, portions of the cell suspensions were added to 10% (w/v) trichloroacetic acid and water to give a final concentration of 5% trichloroacetic acid. After centrifugation, 1.5-ml portions of the supernatant solutions were treated with the same volume of 0.67% TBA, incubated in boiling water for 10 min and made alkaline with KOH (final concentration 0.29 M). Absorbance at 543 nm was determined with a Beckman Acta III spectrophotometer.

Determination of Covalent Binding of CCl_4 Metabolites to Membrane Proteins

Hepatocyte incubation at 37 C for 10 min was carried out with [^{14}C] CCl_4 (sp act 22 mCi/mMol) in the presence or in the absence of promethazine, propyl gallate or menadione. At the end of the incubation, the radioactivity bound to cell proteins was determined according to Rao and Recknagel (18).

RESULTS AND DISCUSSION

Previous results in our laboratory showed that promethazine, a very strong antioxidant, is able to completely inhibit the production of TBA-reacting compounds (mainly malonaldehyde) induced in liver cells by CCl_4 poisoning. On the other hand, the drug, up to the concentration of 10 μM, does not protect against blockage of protein and lipoprotein secretion in hepatocytes incubated for 40 min at 37 C in the presence of 129 μM CCl_4 (11, 12).

This result suggests that the CCl_4-induced impairment of the hepatic protein and lipid secretion is mainly due to a mechanism that is different from lipid peroxidation.

In the present investigation, in order to confirm the inability of promethazine in preventing this cell damage, a lower (86 μM) concentration of CCl_4 has been used. In fact, to avoid direct effects of the scavenger on the secretion pathway, it was not possible to increase its concentration over 10 μM. Experiments to check the effect of promethazine on CCl_4-induced lipid peroxidation and on covalent binding of CCl_4 metabolites to cell proteins have been done simultaneously. In similar ways, we tested the effects of 2 other antioxidant drugs, propyl gallate and menadione, which inhibit lipid peroxidation, differing from promethazine for the site of action and the mechanism, respectively. Propyl gallate acts as antioxidant by electron donation, like promethazine, but mainly reacting at the NADPH flavoprotein level (1). Menadione, a lipophilic drug, inhibits lipid peroxidation by reducing the NADPH available to sustain it, and, under the form of semiquinone, by electron donation. (19,20).

Table 1 shows that 86 μM CCl_4 inhibits the hepatocyte release of lipoprotein triglycerides by 60% as early as 10 min after poisoning. No significant protection was detected when CCl_4 treatment was done in the presence of 10 μM promethazine. On the other hand, this scavenger completely inhibits the CCl_4-induced increase of malonaldehyde production, but it does not significantly affect CCl_4 covalent binding to cell proteins.

The evidence that the pretreatment with promethazine does not prevent the CCl_4-induced block of lipoprotein secretion is consistent with earlier studies showing that the antioxidant protects against CCl_4-induced necrosis, but has little effect on the accumulation of fat (21). All these results support the hypothesis that different mechanisms are primarily responsible for the 2 main hepatotoxic effects of CCl_4, necrosis and fatty degeneration. The 2 different CCl_4-reactive metabolites, i.e., $CCl_3O_2^{\cdot}$ and $CCl_3^{\cdot}$, respectively, might be implicated in the already mentioned types of liver injury. Indirect support to this speculation comes from recent pulse radiolysis studies on CCl_4 and promethazine interaction. This substance, in fact, reacts very quickly with the trichloromethylperoxy radical and very slowly with $CCl_3^{\cdot}$ (4,5). In other words, the first radical would initiate lipid peroxidation, whereas the $CCl_3^{\cdot}$ might be implicated in tissue changes not protected by the antioxidant, i.e., dependent on CCl_4 covalent binding to cell structures.

The following studies using propyl gallate instead of promethazine (Table 2) give similar conclusions, even if the first scavenger, at the most suitable concentration (50 μM), shows lower antioxidant activity than the second one. Another reason that neither promethazine nor propyl gallate inhibit [^{14}C]CCl_4 binding may be related to their hydrophilic nature. These 2 substances do not easily diffuse through the lipid membranes, so they could not be very effective in scavenging lipid peroxidation initi-

TABLE 1

Effect of Promethazine on Lipoprotein Secretion, Malonaldehyde Production and CCl_4-Protein Covalent Binding in CCl_4-Poisoned Hepatocytes[a]

Experimental groups	Lipoprotein secretion[b]	Malonaldehyde production[c]	[^{14}C]CCl_4-protein covalent binding[d]
Control (not treated)	2,913 ± 288	0.090 ± 0.010	–
+ Promethazine (10 μM)	2,675 ± 462[e]	0.082 ± 0.006[e]	–
CCl_4 (86 μM)	1,134 ± 473[f] (61%)	0.243 ± 0.025[f]	689 ± 38
+ Promethazine (10 μM)	1.085 ± 234[g] (59%)	0.095 ± 0.011[h]	665 ± 46[g]

[a]Cell aliquots (10^7 hepatocytes) were incubated 10 min at 37 C in the presence or in the absence of CCl_4 and/or promethazine. All data represent means of 2 experiments in triplicate ± SD.

[b]Values are radioactivities (cpm) of lipoprotein triglycerides released from 10^7 cells prelabeled with [^{14}C]-palmitic acid. Values in parentheses are percent inhibition with respect to the corresponding control.

[c]Values are optical densities at 543 nm of thiobarbituric acid (TBA)-reacting compounds produced by 10^7 hepatocytes.

[d]Values are radioactivities (cpm) of [^{14}C]CCl^4 metabolites covalently bound to proteins of 10^7 cells.

[e]Not significant as to control group ($p > 0.05$).

[f]Significant as to control group ($p < 0.001$).

[g]Not significant as to CCl_4 group ($p > 0.05$).

[h]Significant as to CCl_4 group ($p < 0.001$).

TABLE 2

Effect of Propyl Gallate on Lipoprotein Secretion, Malonaldehyde Production and CCl_4-Protein Covalent Binding in CCl_4-Poisoned Hepatocytes[a]

Experimental groups	Lipoprotein secretion[b]	Malonaldehyde production[c]	[^{14}C]CCl_4 protein covalent binding[d]
Control (not treated)	2,030 ± 168	0.102 ± 0.015	–
+ Propyl gallate (50 μM)	1,850 ± 195[e]	0.092 ± 0.010[e]	–
CCl_4 (86 μM)	858 ± 288[f] (58%)	0.215 ± 0.024[f]	720 ± 48
+ Propyl gallate (50 μM)	923 ± 169[g] (51%)	0.153 ± 0.018[h]	696 ± 27[g]

[a]Cell aliquots (10^7 hepatocytes) were incubated 10 min at 37 C in the presence or in the absence of CCl_4 and/or propyl gallate. All data represent means of 2 experiments in triplicate ± SD.

[b]See Table 1.

[c]See Table 1.

[d]See Table 1.

[e]Not significant as to control group ($p > 0.05$).

[f]Significant as to control group ($p < 0.001$).

[g]Not significant as to CCl_4 group ($p > 0.05$).

[h]Significant as to CCl_4 group ($p < 0.003$).

ation products.

The lack of protection by the 2 scavengers against CCl_4 covalent binding is in agreement with the results obtained with liver microsomes by Cheeseman and Slater (22). In fact, in their experimental system, 100 μM promethazine or 50 μM propyl gallate inhibited by 70-80% the CCl_4-induced lipid peroxidation, producing at the same time only a very small decrease in [^{14}C] CCl_4 radioactivity bound to microsomal proteins (10-20%). These results strongly favor the assumption that covalent binding of CCl_4 metabolic products is likely to be the most important mechanism for lipoprotein secretion derangement in CCl_4-poisoned hepatocytes.

This hypothesis is strengthened by identical experiments done on CCl_4-poisoned liver cells using menadione as free radical scavenger. Table 3 shows the effects of menadione addition to liver cells just before CCl_4 poisoning. The impairment of lipoprotein secretion due to CCl_4, as well as the covalent binding of CCl_4 metabolites to hepatocyte proteins, are partly prevented by 100 μM menadione. The effectiveness of menadione in scavenging CCl_4 metabolites, probably CCl_3^*, may be related not only to the mechanism of action, but also to the lipophilic nature of the drug. It is notable that these 2 protective effects show a similar degree of intensity, whereas the stimulation of malonaldehyde production is almost completely inhibited.

We also studied lipoprotein secretion in hepatocytes treated with 2 other lipid peroxi-

TABLE 3

Effect of Menadione on Lipoprotein Secretion, Malonaldehyde Production and CCl_4-Protein Covalent Binding in CCl_4-Poisoned Hepatocytes[a]

Experimental groups	Lipoprotein secretion[b]	Malonaldehyde production[c]	[^{14}C]CCl_4 protein covalent binding[d]
Control (not treated)	1,878 ± 50	0.077 ± 0.010	–
+ Menadione (100 μM)	1,740 ± 29[e]	0.053 ± 0.008[f]	–
CCl_4 (86 μM)	882 ± 44[f] (53%)	0.183 ± 0.013[f]	811 ± 42
+ Menadione (100 μM)	1,270 ± 25[g] (27%)	0.083 ± 0.007[g]	566 ± 58[g]

[a]Cell aliquots (10^7 hepatocytes) were incubated 10 min at 37 C in the presence or in the absence of CCl^4 and/or menadione. All data represent means of 2 experiments in triplicate ± SD.

[b]See Table 1.

[c]See Table 1.

[d]See Table 1.

[e]Not significant as to control group ($p > 0.05$).

[f]Significant as to control group ($p < 0.003$).

[g]Significant as to CCl_4 group ($p < 0.001$).

dation stimulators, Fe^{3+} and cumene hydroperoxide. Our data indicate that, in isolated hepatocytes, it is possible to induce a high degree of lipid peroxidation without inducing strong changes in lipoprotein secretion.

Table 4 shows that 25 μM $FeCl_3$ stimulates malonaldehyde production to the same degree as 86 μM CCl_4; higher $FeCl_3$ concentrations (50-100 μM) stimulate malonaldehyde production much more than does CCl_4. Only with 100 μM $FeCl_3$ is lipoprotein secretion reduced; this reduction, however, is very low.

Experiments with cumene hydroperoxide (Table 5) further strengthen the dichotomy between degree of lipid peroxidation and degree of inhibition of lipoprotein secretion. Cumene hydroperoxide, at concentrations (100-200 μM) that are much more active than CCl_4 in stimulating malonaldehyde formation, is much less active than CCl_4 in reducing lipoprotein secretion.

These results may be interpreted either in terms of poor influence of lipid peroxidation on lipoprotein secretion (this interpretation would fit with the results obtained with free radical scavengers) or in terms of different peroxidation pathways. In other words, differences between CCl_4, $FeCl_3$ and cumene hydroperoxide stimulated lipid peroxidation may be: (a) "topographical," i.e., different cell sites are involved; (b) "chemical," i.e., different sequences, different intermediates or end-products are operative.

Regarding iron-induced lipid peroxidation, peroxidative breakdown of membrane lipids in the presence of NADPH, ADP and Fe^{3+} has

TABLE 4

Lipoprotein Secretion and Malonaldehyde Production from [^{14}C]Palmitic Acid Prelabeled Hepatocytes Treated with CCl_4 or $FeCl_3$[a]

Experimental groups	Lipoprotein secretion[b]	Malonaldehyde production[c]
Control	5,947 ± 125	0.160 ± 0.032
CCl_4 (86 μM)	1,198 ± 145[e] (80%)	0.450 ± 0.060[e]
$FeCl_3$ (25 μM)	5,806 ± 55[f]	0.481 ± 0.029[e]
$FeCl_3$ (50 μM)	5,765 ± 238[f]	0.730 ± 0.018[e]
$FeCl_3$ (100 μM)	4,654 ± 254[d] (22%)	0.770 ± 0.046[e]

[a]Cell aliquots (10^7 hepatocytes) were incubated 30 min at 37 C in the presence or in the absence of CCl_4 or $FeCl_3$. All data represent mean of 3 experiments in triplicate ± SD.

[b]See Table 1.

[c]See Table 1.

[d]Significant as to control group ($p < 0.05$).

[e]Significant as to control group ($p < 0.001$).

[f]Not significant as to control group ($p > 0.05$).

TABLE 5

Lipoprotein Secretion and Malonaldehyde Production from [^{14}C]Palmitic Acid Prelabeled Hepatocytes Treated with CCl_4 or Cumene Hydroperoxide[a]

Experimental groups	Lipoprotein secretion[b]	Malonaldehyde production[c]
Control	5,853 ± 261	0.135 ± 0.020
CCl_4 (86 μM)	463 ± 18[e] (92%)	0.360 ± 0.020[e]
Cumene hydroperoxide (50 μM)	4,953 ± 178[d] (15%)	0.212 ± 0.017[d]
(100 μM)	3,872 ± 249[e] (34%)	0.480 ± 0.029[e]
(200 μM)	1,814 ± 220[e] (69%)	1.480 ± 0.063[e]

[a]Cell aliquots (10^7 hepatocytes) were incubated 30 min at 37 C in the presence or in the absence of CCl_4 or cumene hydroperoxide. All data represent means of 2 experiments in triplicate ± SD.

[b]See Table 1.

[c]See Table 1.

[d]Significant as to control group ($p < 0.05$).

[e]Significant as to control group ($p < 0.001$).

been observed not only in liver microsomes (23), but also in liver mitochondria (24) and lysosomes (25). This may explain why, in whole hepatocyte systems, only the cell treatment with very high Fe^{3+} concentrations induces peroxidative inactivation of the microsomal enzyme glucose-6-phosphatase (26,27), whereas, to give a similar impairment, one-half or one-third the CCl_4 concentration is sufficient (27).

Furthermore, CCl_4 and iron lipoperoxidative effects show chemical differences in terms of initiating reactions, free radical intermediates (1,28) and probably in terms of aldehydic end-products. In fact, recent analyses of the aldehydic patterns produced by CCl_4- or iron-mediated peroxidation of microsomal lipids have shown remarkable qualitative differences between the 2 treatments (Esterbauer, Cheeseman, Dianzani, Poli and Slater, manuscript in preparation).

As for lipid peroxidation stimulated by cumene hydroperoxide, several hemoproteins, including cytochrome P-450, cytochrome b_5 and cytochrome c, have been demohstrated to act as catalysts (14,29); cytochrome P-450 is 10 times more effective than the 2 other cytochromes (14). Several arguments exist against the involvement of a free radical chain reaction in the initiation step of this lipoperoxidative model system, suggesting a mechanism more like that of lipoxygenases (14). Cumene hydroperoxide seems to exert its effects mainly through the microsomal cytochrome P-450-dependent enzyme system; so, it should impair lipoprotein secretion mainly at the step of combination of phospholipid, triglyceride and lipid-carrier protein to form lipoprotein, whereas CCl_4 has been also demonstrated to affect the triglyceride transport from the liver to the plasma (11). Experiments to determine types and biological activity of aldehydes produced by cumene hydroperoxide catalyzed lipid peroxidation are now in progress.

The other possibility, that lipid peroxidation per se has little influence on liver lipoprotein secretion, and that this cell mechanism is mainly deranged by covalent binding of CCl_4 cleavage products, deserves maximal attention.

A major role of covalent binding has been proposed for some CCl_4-induced in vivo damages, such as cytochrome P-450 inactivation (30) and polyribosome dissociation (31), as well as for the reduction of aminopyrene demethylase activity and cytochrome P-450 content of isolated hepatocytes poisoned with CCl_4 (10). In addition to these data, it was observed (32) that protein synthesis in liver cell-free systems was also inhibited by $CBrCl_3$, a halogen derivative of methane that sparks lipid peroxidation in a way very similar to CCl_4 (1). The effect of $CBrCl_3$ on protein synthesis, however, seemed to be dependent on a mechanism involving free radical attack, unrelated to lipid peroxidation (32).

In conclusion, there is increasing evidence for a prominent role of covalent binding of reactive metabolites of CCl_4 in early changes induced by this haloalkane in liver cells. In other words, CCl_4-induced liver injury probably results from both covalent binding of CCl_4 metabolites and lipid peroxidation.

ADDENDUM

While this paper was in press, our attention was drawn to the work reported by Griffin, B.W. (in "Microsomes, Drug Oxidations, and Chemical Carcinogenesis," Vol. 1, pp. 319-322, Academic Press, New York, 1980). This author gives evidence for the involvement of free radical species in the reaction of cumene hydroperoxide with hemoproteins. This must be considered when examining our discussion on the mechanism of action of the compound.

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