Mechanisms of Chloroform and Carbon Tetrachloride Toxicity in Primary Cultured Mouse Hepatocytes

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Mechanisms of chloroform (CHCl₃) and carbon tetrachloride (CCl₄) toxicity to primary cultured male B6C3F1 mouse hepatocytes were investigated. The cytotoxicity of both CHCl₃ and CCl₄ was dose- and duration-dependent. Maximal hepatocyte toxicity, as determined by lactate dehydrogenase leakage into the culture medium, occurred with the highest concentrations of CHCl₃ (5 mM) and CCl₄ (2.5 mM) used and with the longest duration of treatment (20 hr). CCl₄ was approximately 16 times more toxic than CHCl₃ to the hepatocytes. The toxicity of these compounds was decreased by adding the mixed function oxidase system (MFOS) inhibitor, SKF-525A (25μM) to the cultures. The addition of diethyl maleate (0.25 mM), which depletes intracellular glutathione (GSH)-potentiated CHCl₃ and CCl₄ toxicity. The toxicity of CHCl₃ and CCl₄ could also be decreased by adding the antioxidants N,N'-diphenyl-p-phenylenediamine (DPPD) (25 μM), α-tocopherol acetate (Vitamin E) (0.1 mM), or superoxide dismutase (SOD) (100 U/mL) to the cultures. These results suggest that, in mouse hepatocytes, both CHCl₃ and CCl₄ are metabolized to toxic components by the MFOS; GSH plays a role in detoxifying those metabolites; free radicals are produced during the metabolism of CHCl₃ and CCl₄; and free radicals may be important mediators of the toxicity of these two halomethanes.

Introduction

Chloroform (CHCl₃) and carbon tetrachloride (CCl₄) are formed as by-products of sewage and drinking water chlorination and have been identified in public drinking water (1). Both compounds are toxic to mammalian liver in vivo and to isolated liver cells, with CCl₄ being more toxic than CHCl₃ (2). CCl₄ is hepatocarcinogenic in both rats and mice (3), whereas CHCl₃ has been shown to be hepatocarcinogenic only in the B6C3F1 strain of mouse when administered by corn oil gavage (4). Both compounds may be functioning as liver tumor promoters (5).

The metabolism of CCl₄ and CHCl₃ may provide clues to their relative cytotoxic potency and their capability as tumor promoters. It is becoming evident that free radicals generated during xenobiotic metabolism play a role in cellular toxicity (6), abnormal growth control (7), and tumor promotion (8). The metabolism of CCl₄ and CHCl₃ has been extensively studied in both rats and mice. The first step in CCl₄ metabolism is a one-electron reduction and homolytic cleavage catalyzed by cytochrome P450 of the mixed function oxidase system (MFOS) to yield the trichloromethyl radical (9) [Eq.(1)].

\[
CCl₄ + e^- \rightarrow \cdot CCl₃ + Cl^- \tag{1}
\]

P450 is maintained in the reduced form by reduced nicotinamide adenine dinucleotide (NADPH). The ·CCl₃ radical rapidly reacts with molecular oxygen to form the trichloromethyl peroxy radical (10) [Eq. (2)].

\[
CCl₃ + O₂ \rightarrow \cdot OOCCl₃ \tag{2}
\]

The ·OOCCl₃ radical is more electrophilic than the ·CCl₃ radical and may be more responsible for attacks on unsaturated fatty acids, leading to lipid peroxidation (10). ·CCl₃ may be more involved in covalent binding reactions of CCl₄ (10).

CHCl₃ is first metabolized by a cytochrome P450-catalyzed hydroxylation to trichloromethanol (CCl₂OH), which spontaneously dehydrochlorinates to the toxic component, phosgene (COCl₂) (11) [Eq. (3)].

\[
CHCl₃ \rightarrow [CCl₂OH] \rightarrow COCl₂ + HCl \tag{3}
\]

Phosgene can covalently bind to macromolecules if not detoxified by conjugation with glutathione (GSH), or
can be further metabolized to carbon dioxide (CO₂) by reaction with water (11). It has also been suggested that free radicals are generated during CHCl₃ metabolism and that they play a role in CHCl₃ toxicity (12, 13). Most studies, however, have failed to detect enhanced levels of free radicals or of lipid peroxidation, although no study has used the B6C3F1 strain of mouse.

To provide evidence that the hepatotoxicity of CHCl₃ and CCl₄ in B6C3F1 mice might be caused by free radical production during the metabolism of these compounds, the present study was undertaken. We examined whether the modifiers of hepatocyte xenobiotic metabolism, SKF-525A and diethyl maleate (DEM), and the antioxidants, α-tocopherol acetate (vitamin E), N,N'-diphenyl-p-phenylenediamine (DPPD), and superoxide dismutase (SOD), could alter the toxicity of CHCl₃ and CCl₄ to isolated B6C3F1 mouse hepatocytes. SKF-525A is an inhibitor of the MFOS, DEM decreases intracellular GSH levels, Vitamin E and DPPD are free-radical scavengers, and SOD dismutates superoxide radicals to hydrogen peroxide.

Materials and Methods

Animals

Six-month-old male B6C3F1 mice were used in this study. They were bred at the Medical College of Ohio from C3H/He sires and C57Bl/6 dams (Charles River Laboratories, Inc., Wilmington, MA). Mice were kept in plastic cages (five per cage) containing corncob bedding and given water and certified Purina Lab Chow ad libitum.

Chemicals

Leibovitz's L-15 medium and gentamicin were purchased from Grand Island Biological Co. (Grand Island, NY); fetal bovine serum, from Hyclone Laboratories (Logan, UT); CHCl₃ and CCl₄, from Aldrich Chemical Co. (Madison, WI); and DEM, α-tocopherol acetate, DPPD, SOD, dimethyl sulfoxide (DMSO), dexamethasone and glucose, from Sigma Chemical Co. (St. Louis, MO). SKF-525A was a gift from Smith, Kline and French Laboratories (Philadelphia, PA). All other reagents were purchased from Sigma Chemical Co.

Hepatocyte Isolation and Culture

Mouse hepatocytes were isolated by two-stage in situ perfusion (14). Cell viability of the isolated cells was 90%–95%, as determined by exclusion of trypan blue. Hepatocytes were cultured in L-15 medium supplemented with glucose (1 mg/mL), dexamethasone (1 µM), fetal bovine serum (10%), and gentamicin (50 µg/mL). The hepatocytes were plated at a density of 0.5 x 10⁶ cells per 60 mm dish and incubated in a humidified 100% air incubator at 37°C.

Cytotoxicity Assay

After initial plating and a 4-hr attachment period, the cultured cells were washed once and re-fed with 5 mL of fresh medium. The cultures were then treated with the test compounds. All compounds, except SOD, were dissolved in DMSO. The final concentration of DMSO in all cultures, except for untreated cultures, was 0.4%. After 2, 4, and 20 hr of treatment, aliquots of the medium were removed, filtered through Nitrex nylon mesh to remove cells, and analyzed for lactate dehydrogenase activity (LDH) on a Beckman Multistat Analyzer (Beckman Instrument Corp., Palo Alto, CA). Total LDH per culture was also determined from 0.01% Triton X-100 lysates of untreated cultures, mean of three dishes.
### Table 1. Effects of the metabolic inhibitors, SKF-525A and diethyl maleate, on chloroform toxicity in primary cultured B6C3F1 mouse hepatocytes.

| Treatments | CHCl₃ mM | Inhibitor* | 2 hr | 4 hr | 20 hr |
|------------|----------|------------|------|------|-------|
|            |          |            | 2 hr | 4 hr | 20 hr |
| 0.0        | None     |            |      |      |       |
| 0.0        | SKF-525A |            |      |      |       |
| 1.0        | None     |            |      |      |       |
| 1.0        | SKF-525A |            |      |      |       |
| 2.5        | None     |            |      |      |       |
| 2.5        | SKF-525A |            |      |      |       |
| 5.0        | None     |            |      |      |       |
| 5.0        | SKF-525A |            |      |      |       |
| 5.0        | DEM      |            |      |      |       |

*Inhibitor concentrations were 25 μM for SKF-525A and 0.25 μM for diethyl maleate (DEM).

*Mean ± SD (n = 3).

*Significant difference versus the corresponding CHCl₃-only treatment.

### Table 2. Effects of metabolic inhibitors, SKF-525A and diethyl maleate, on carbon tetrachloride toxicity in primary cultured B6C3F1 mouse hepatocytes.

| Treatments | CCl₄ mM | Inhibitor* | 2 hr | 4 hr | 20 hr |
|------------|---------|------------|------|------|-------|
|            |         |            | 2 hr | 4 hr | 20 hr |
| 0.0        | None    |            |      |      |       |
| 0.0        | SKF-525A|            |      |      |       |
| 1.0        | None    |            |      |      |       |
| 1.0        | SKF-525A|            |      |      |       |
| 2.5        | None    |            |      |      |       |
| 2.5        | SKF-525A|            |      |      |       |
| 5.0        | None    |            |      |      |       |
| 5.0        | SKF-525A|            |      |      |       |
| 5.0        | DEM     |            |      |      |       |

*Inhibitor concentrations were 25 μM for SKF-525A and 0.25 μM for diethyl maleate (DEM).

*Mean ± SD (n = 3).

*Significant difference versus the corresponding CCl₄-only treatment.

### Table 3. Effects of the antioxidants DPPD, vitamin E, and superoxide dismutase on chloroform toxicity in primary cultured B6C3F1 mouse hepatocytes.

| Treatments | CHCl₃ mM | Antioxidant* | 2 hr | 4 hr | 20 hr |
|------------|---------|--------------|------|------|-------|
|            |         |              | 2 hr | 4 hr | 20 hr |
| 0.0        | None    |              |      |      |       |
| 0.0        | DPPD    |              |      |      |       |
| 0.0        | Vitamin E |            |      |      |       |
| 0.0        | SOD     |              |      |      |       |
| 1.0        | None    |              |      |      |       |
| 1.0        | DPPD    |              |      |      |       |
| 1.0        | Vitamin E |            |      |      |       |
| 1.0        | SOD     |              |      |      |       |
| 2.5        | None    |              |      |      |       |
| 2.5        | DPPD    |              |      |      |       |
| 2.5        | Vitamin E |            |      |      |       |
| 2.5        | SOD     |              |      |      |       |
| 5.0        | None    |              |      |      |       |
| 5.0        | DPPD    |              |      |      |       |
| 5.0        | Vitamin E |            |      |      |       |
| 5.0        | SOD     |              |      |      |       |

*Antioxidant concentrations were 25 μM for DPPD, 0.1 mM for vitamin E, and 100 U/mL for SOD.

*Mean ± SD (n = 3).

*Significant difference versus the corresponding CHCl₃-only treatment.
Table 4. Effects of the antioxidants DPPD, vitamin E, and superoxide dismutase on carbon tetrachloride toxicity in primary cultured B6C3F1 mouse hepatocytes.

| Treatments | CC14, mM | Antioxidant* | 2 hr | 4 hr | 20 hr |
|------------|----------|--------------|------|------|-------|
| 0.0        | None     | 8.0 ± 1.0*   | 8.9 ± 1.5 | 14.8 ± 1.0 |       |
| 0.0        | DPPD     | 5.7 ± 1.5    | 6.2 ± 1.4 | 12.3 ± 1.3 |       |
| 0.0        | Vitamin E| 7.7 ± 0.6    | 6.9 ± 0.2 | 13.8 ± 0.8 |       |
| 0.0        | SOD      | 7.5 ± 0.6    | 9.9 ± 0.8 | 14.8 ± 1.1 |       |
| 1.0        | None     | 53.0 ± 4.8   | 49.3 ± 3.5 | 73.6 ± 2.2 |       |
| 1.0        | DPPD     | 28.5 ± 3.6*  | 30.9 ± 3.7* | 47.6 ± 4.4* |       |
| 1.0        | Vitamin E| 40.4 ± 3.6*  | 34.8 ± 3.9* | 60.1 ± 2.3* |       |
| 1.0        | SOD      | 36.9 ± 2.2*  | 37.7 ± 2.5* | 58.3 ± 4.6* |       |
| 2.5        | None     | 65.5 ± 1.6   | 66.8 ± 2.8 | 76.4 ± 5.0 |       |
| 2.5        | DPPD     | 44.2 ± 4.6*  | 50.0 ± 7.0* | 61.0 ± 5.9* |       |
| 2.5        | Vitamin E| 45.2 ± 5.2*  | 40.6 ± 5.7* | 62.7 ± 3.9* |       |
| 2.5        | SOD      | 42.9 ± 3.0*  | 41.9 ± 2.1* | 60.0 ± 1.4* |       |

*Antioxidant concentrations were 25 μM for DPPD, 0.1 mM for vitamin E, and 100 U/mL for SOD.

Results

The toxicity of CHCl₃ to isolated B6C3F1 mouse hepatocytes was dependent on dose and treatment duration (Fig. 1). Little increase in LDH leakage was evident in cultures treated with less than 1.0 mM CHCl₃. CC14 toxicity was also dependent on dose and treatment duration (Fig. 2). As with CHCl₃, little difference in LDH release was evident between the 2-hr and 4-hr sampling times, and LDH release was greater after 20 hr of treatment. CC14 was approximately 16 times more toxic than CHCl₃ to the hepatocytes. Percent total LDH activities of 50% occurred with approximately 0.25 mM CC14 and 4.0 mM CHCl₃ at the 20-hr sampling time (Figs. 1 and 2).

The addition of SKF-525A (25 μM) to CHCl₃- or CC14-treated cultures could be shown to significantly reduce LDH release below that of cultures treated only with CHCl₃ or CC14 (Tables 1 and 2). This effect was evident only at the 2-hr and 4-hr sampling times. At 20 hr, SKF-525A was cytotoxic by itself and either increased or had no effect on LDH release in CHCl₃- and CC14-treated cultures. Adding DEM (0.25 mM) to CHCl₃- or CC14-treated cultures significantly increased LDH release above CHCl₃- or CC14-only treated cultures (Tables 1 and 2). This effect was evident at 2, 4, and 20 hr. DEM was not toxic by itself.

The antioxidants, DPPD (25 μM), vitamin E (0.1 mM), and SOD (100 U/mL), when added to CHCl₃- and CC14-treated cultures, significantly reduced hepatocyte toxicity below that of cultures treated with CHCl₃ or CC14 alone (Tables 3 and 4). With the high dose of CHCl₃ (5 mM), vitamin E best prevented cytotoxicity compared to SOD and DPPD (Table 3). No differences in antioxidant protection were evident with 2.5 mM CHCl₃. DPPD provided the greatest protection against low-dose CHCl₃ (1 mM) toxicity. Cytotoxicity of the low CC14 dose (1.0 mM) was best prevented by DPPD, whereas no differences between the antioxidants were apparent with the high CC14 dose (2.5 mM) (Table 4).

Discussion

The results of this study indicate that CHCl₃ and CC14 are cytotoxic to primary cultured B6C3F1 mouse hepatocytes. Both compounds exhibited dose- and time-dependent effects. CC14 was approximately 16 times more toxic than CHCl₃. Previous studies have also indicated that CC14 is more hepatotoxic than CHCl₃ (2, 3).

CHCl₃- and CC14-induced cytotoxicity was decreased by the simultaneous treatment of SKF-525A, an inhibitor of mammalian MFOS. These results substantiate previous studies that indicated both CHCl₃ and CC14 are metabolized in hepatocytes by the MFOS to hepatotoxic metabolites (9, 11). In the case of CC14, the initial reaction is a reduction and homolytic cleavage of CC14 to the trichloromethyl radical (·CCl₃). This radical may react directly with cellular macromolecules or may react with oxygen to form the trichloromethylperoxyl radical (·OOCCL₃), which may then attack lipids more readily than CCl₄ (10). In any event, the initiation of free radicals and attack on membrane lipids ultimately can lead to lipid peroxidation chain reactions and cell death (10).

CHCl₃ is thought to be first hydroxylated by a P-450 reaction to trichloromethanol (CCl₃OH), which dehy-
drochlorinates spontaneously to phosgene (COCl₂) (11). Phosgene is thought to covalently bind cellular macromolecules and to be the ultimate cytotoxic component (11). However, two reports (12, 13) have suggested that CHCl₃ is metabolized to free radicals and that it induces lipid peroxidation.

The results of the present study suggest that free radical production is an important mechanism of both CCl₄ and CHCl₃ toxicity in B6C3F1 mouse hepatocytes. The antioxidants SOD, DPPD, and vitamin E all significantly reduced the toxicity of CCl₄ and CHCl₃ to these cells. Similarly, depletion of cellular glutathione (GSH) (with DEM) increased CCl₄ and CHCl₃ toxicity to the hepatocytes. GSH functions both as an antioxidant and in the conjugation of xenobiotic metabolites (18).

Thus, the results of this initial study indicate that both CCl₄ and CHCl₃ are metabolized in B6C3F1 mouse hepatocytes by the MFOS, that cellular GSH is important in the detoxification of CCl₄ and CHCl₃ metabolites and/or induced free radicals, and that the cytotoxicity of both compounds might be partly mediated by free radical production. Previous studies have demonstrated the role of free radicals and lipid peroxidation in CCl₄-mediated hepatotoxicity. However, evidence that CHCl₃-induced hepatotoxicity is mediated by free radicals has been less substantiated. The results of the present study provide additional evidence that, in mouse hepatocytes, free radical production may be an important mechanism of CHCl₃-induced toxicity.

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