PHOSGENE: A METABOLITE OF CHLOROFORM

Lance R. Pohl, B. Bhooshan, Noel F. Whittaker* and Gopal Krishna
Laboratory of Chemical Pharmacology,
National Heart, Lung, and Blood Institute
and the
*Laboratory of Chemistry
National Institute of Arthritis, Metabolism, and Digestive Diseases
National Institutes of Health, Bethesda, Maryland 20014

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Summary

Cysteine inhibited the in vitro covalent binding of [^{14}C] chloroform, ($CHCl_3$), to microsomal protein and concomitantly trapped a reactive metabolite, presumably phosgene ($COCl_2$), as 2-oxothiazolidine-4-carboxylic acid. When the incubation was conducted in an atmosphere of [^{18}O] O_2, the trapped $COCl_2$ contained [^{18}O]. These findings suggest that the C-H bond of $CHCl_3$ is oxidized by a cytochrome P-450 monooxygenase to produce trichloromethanol, which spontaneously dehydrochlorinates to yield the toxic agent phosgene.

INTRODUCTION

Chloroform ($CHCl_3$) was widely used for medicinal and industrial purposes until it was established that this solvent produces liver tumors in mice (1) and hepatic and renal toxicity in man (2,3) and experimental animals (4-7). The results of several investigations suggest that a reactive metabolite of $CHCl_3$ is responsible for its toxicity. For example, when rats or mice are treated with [^{14}C] $CHCl_3$, the extent of hepatic necrosis parallels the amount of [^{14}C] label bound irreversibly to liver protein (5-7). Both necrosis and binding are potentiated by pretreatment of animals with phenobarbital, a known inducer of liver microsomal metabolism, and are inhibited by pretreatment with the inhibitor piperonyl butoxide. The finding that $CHCl_3$ administration decreases the level of liver glutathione in rats pretreated with phenobarbital further suggests that a reactive metabolite is produced (6,7). The results of in vitro

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studies with rat and mouse liver microsomes support the in vivo observations by establishing that [^{14}C] $CHCl_3$ is metabolized to a reactive metabolite which binds covalently to microsomal protein (5,6,8,9). This metabolic process is oxygen dependent and appears to be mediated by a cytochrome P-450, which is inducible by phenobarbital (5,8,9).

In this investigation, we report evidence that $CHCl_3$ is metabolically activated to phosgene, $COCl_2$, by liver microsomes of phenobarbital pretreated rats. The results of an [^{18}O] O_2 experiment establish that this metabolite is formed by an oxidative dechlorination mechanism.

EXPERIMENTAL

Synthesis: 2-Oxothiazolidine-4-carboxylic acid was synthesized from cysteine and $COCl_2$ following the procedure of Kaneko et al. (10). This acid was methylated with diazomethane or deutero-diazomethane, which were prepared from Diazald (Aldrich) following the directions supplied with the reagents. The products were then analyzed by gas chromatography-chemical ionization mass spectroscopy.

Preparation of microsomes: Male Sprague-Dawley rats (180-200 g) were obtained from Hormone Assay Laboratories (Chicago, Ill.). The animals were allowed free access to water and food (Purina Lab Rat Chow) and were pretreated with phenobarbital (80 mg/kg, in saline, i.p.) 72, 48 and 24 hrs before the experiment. Liver microsomes were prepared as previously described (8).

Effect of cysteine on in vitro covalent binding to microsomal protein: Each 1 ml incubation mixture contained 1 mg microsomal protein, 0.10 mM NADH, 0.20 mM NADP, 2.00 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 20 mM Tris (pH 7.4), 153 mM KCl, 1.00 mM [^{14}C] $CHCl_3$ (New England Nuclear) (1 mCi/mmole, added in 10 µl of dimethylformamide) with or without 2.0 mM cysteine. The incubation was conducted in a sealed vial at 37°C for 10 min. The reaction was stopped by the addition of methanol (4 ml) and the covalent binding of the [^{14}C] label to microsomal protein was determined as previously described (8).

Trapping the reactive metabolite with cysteine: A 45 ml incubation mixture containing cysteine was incubated in a sealed flask under the conditions outlined above for the 1 ml mixture. After 10 min the reaction was stopped by the addition of methanol (180 ml). The mixture was then centrifuged and the supernatant phase was evaporated under vacuum. The resulting residue was dissolved in water (2 ml, pH 6) and washed with ethyl acetate (3 ml, 3x). The aqueous solution was then acidified with concentrated hydrochloric acid to pH 1 and extracted four times with 3.0 ml of ethyl acetate. The combined ethyl acetate extracts of the acidic aqueous solution was then evaporated (N_2) to dryness. The residue was dissolved in methanol (100 µl) and methylated with diazomethane, which was generated from Diazald (Aldrich) following the directions given on the reagent bottle. The derivatized product was then analyzed by gas chromatography-chemical ionization mass spectrometry (GC-CIMS).

Trapping the metabolite with cysteine in an atmosphere of [^{18}O] O_2: The large scale reaction was repeated in an atmosphere of [^{18}O] O_2 (Stohler Isotope Chemicals, 99%). The actual enrichment of [^{18}O] O_2 in the reaction flask was determined by simultaneously conducting the para hydroxylation of acetanilide (1 mM) with liver microsomes in another flask (11). The experiment was performed by interconnecting the two reaction flasks and a sealed vial of [^{18}O] O_2 (100 µl) with vacuum tubing. The system was then evacuated with a vacuum pump and purged with nitrogen. This process was repeated nine more times. After a final evacuation, the seal to the [^{18}O] O_2 vial was broken and the [^{18}O] O_2 atmosphere was allowed to distribute into the two reaction flasks. Nitrogen gas was then added to bring the pressure up to approximately 1 atmosphere. The atmospheric concentration of gases in the two reaction flasks were calculated to be approximately 20% [^{18}O] O_2 and 80% N_2. The incubations were stopped after 10 min by the addition of MeOH. The trapped phosgene was isolated and analyzed as described above, while p-hydroxyacetanilide was isolated from the acetanilide reaction by following the procedure described by Hinson et al. (11). This product was then further purified and characterized by GC-CIMS.

Gas chromatography chemical ionization mass spectrometry (GC-CIMS): The GC-CIMS were performed on a Finnigan 1015 D instrument which was equipped with a chemical ionization source, a Finnigan 9500 gas chromatograph and a Model 6000 data system. The samples were injected onto a 150 cm glass column (2 mm id) which was packed with 3% OV-225 on Gas Chrom Q, 100-120 mesh. Helium was employed as the carrier gas at a flow rate of 25 ml/min. The column temperature was 175° for the $CHCl_3$ studies and 230°C for the acetanilide study. The mass spectrometer was operated at a source pressure of 1 Torr, a source temperature of 150°C, and an electron energy of 100 eV. Methane or ammonia were used as the reagent gas.

RESULTS

When cysteine was added to the incubation mixture of [^{14}C] $CHCl_3$ and liver microsomes from phenobarbital pretreated rats, it significantly inhibited the binding of the [^{14}C] label to microsomal protein (Table 1). To determine whether cysteine had produced this inhibition by trapping a reactive metabolite of chloroform, we investigated the metabolism of $CHCl_3$ in the presence of cysteine. A preliminary study indicated that one major nonvolatile radiolabeled product was produced when cysteine was added to the incubation mixture. Moreover, this metabolite was not present in the incubation mixtures that did not contain cysteine.

Since it was anticipated that phosgene, $COCl_2$, was a metabolite of $CHCl_3$, cysteine should have reacted with this intermediate to produce 2-oxothiazolidine-4-carboxylic acid (10). Reverse isotope dilution analysis of the metabolite with an authentic standard of 2-oxothiazolidine-4-

Table 1 Effect of cysteine on the in vitro irreversible binding of [^{14}C] chloroform to liver microsomal protein[a]

Incubation conditions	Irreversible binding[b] pmole/mg protein/10 min
No cysteine	2178 ± 163
Cysteine (2 mM)	1032 ± 80

[a] [^{14}C] chloroform (1 mM) was incubated with liver microsomes from phenobarbital pretreated rats in an atmosphere of air as described in the Experimental Section.

[b] The results are expressed as the mean ± standard error of 5 incubations.

carboxylic acid supported this idea. In order to confirm this structural assignment, a large scale incubation was conducted to obtain enough product for GC-CIMS analysis. The results of this study are presented in Figs. 1A and 1B. The retention time and methane CIMS of the methylated standard of 2-oxothiazolidine-4-carboxylic acid (Fig. 1A) and the derivatized metabolite (Fig. 1B) were nearly identical. The ion at m/e 162 represents the protonated molecular ion (MH^+) while the fragment ions at m/e 134 and 102 corresponded to the loss of carbon monoxide (MH^+-28) and the carbomethoxy side chain (MH^+-60) respectively. These fragmentation pathways were confirmed by performing the GC-CIMS of deuterium labeled methyl 2-oxothiazolidine-4-carboxylate, which was prepared from the authentic carboxylic acid and deutero-diazomethane.

To establish that $CHCl_3$ was oxidatively metabolized to $COCl_2$, the large scale reaction was repeated in an atmosphere of [^{18}O] O_2. The retention time of methylated standard (Fig. 1C) and the derivatized metabolite (Fig. 1D) were again virtually identical. The ammonia CIMS of the authentic standard and the metabolite contain a protonated molecular ion at m/e 162 (MH^+), an ammonia addition ion at m/e 179 (M+18) and a fragment ion at m/e

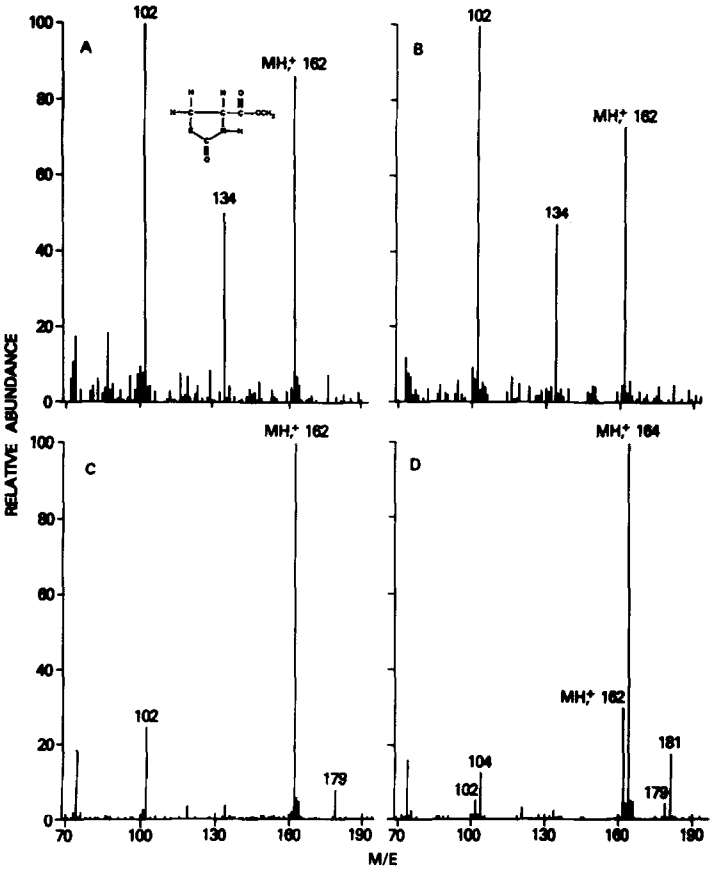

Fig. 1. Gas chromatography - Chemical ionization mass spectra (GC-CIMS). A. The methane CIMS of authentic methyl 2-oxothiazolidine-4-carboxylate. As described in the Experimental Section, this product was synthesized from 2-oxothiazolidine-4-carboxylic acid and diazomethane and analyzed by GC-CIMS, retention time 5.34 min. B. the methane CIMS of the methylated metabolite of $CHCl_3$. As described in the Experimental Section, this metabolite was isolated from the in vitro incubation mixture of $CHCl_3$ and cysteine with liver microsomes from phenobarbital-pretreated rats. The metabolite was derivatized with diazomethane and analyzed by GC-CIMS, retention time 5.34 min. C. The ammonia CIMS of authentic methyl 2-oxothiazolidine-4-carboxylate, retention time, 4.92 min. D. The ammonia CIMS of the methylated metabolite of $CHCl_3$ from the $[^{18}O]$ O_2 incubation. As described in the Experimental Section, this metabolite was isolated from the in vitro incubation of $CHCl_3$ and cysteine which was performed in an atmosphere of $[^{18}O]$ O_2. The metabolite was derivatized with diazomethane and analyzed by GC-CIMS, retention time, 4.92 min.

102 (MH^+-60). In addition, the methylated metabolite contains ions at m/e 164, 181 and 104 (Fig. 1D). These ions represent the incorporation of [^{18}O] into the 2-oxo position of 2-oxothiazolidine-4-carboxylic acid. The percent incorporation of [^{18}O] into the metabolite was calculated to be 76%.

The GC-ammonia CIMS of the parahydroxylated metabolite of acetanilide, which contained ion doublets at m/e 152 and 154 (MH^+) and 109 and 111 (M-42), revealed that this product also contained nearly 76% incorporation of [^{18}O] O_2. Since it has been shown that at least 96% of the oxygen incorporated into the para position of acetanilide is derived from molecular oxygen (11), it appears that approximately 20% of the O_2 present in the atmosphere during our incubations was in the form of [^{16}O] O_2. This could have resulted from incomplete degassing or from the presence of [^{16}O] in the [^{18}O] O_2 used in this experiment.

DISCUSSION

The results of this investigation suggest that $CHCl_3$ is metabolically activated by liver microsomes of rat through an oxidative dechlorination mechanism (Fig. 2). The first step of this process involves the hydroxylation of $CHCl_3$ to trichloromethanol by a phenobarbital inducible cytochrome P-450 monooxygenase (5,8,9). This metabolic step is an anticipated reaction since the hydroxylation of aliphatic carbons is a well documented process (12). However, in this case the product would be expected to be unstable and spontaneously dehydrochlorinate to produce phosgene (13). The electrophilic $COCl_2$ could react with water to form carbon dioxide, a known metabolite of $CHCl_3$ *in vitro* (14,15) and *in vivo* (16,17) or with microsomes to yield a covalently bound product (5,6,8,9 and Table 1). The observations that cysteine inhibits the binding of [^{14}C] $CHCl_3$ to microsomal protein (Table 1) and concomitantly reacts with a metabolite of $CHCl_3$ to produce 2-oxothiazolidine-4-carboxylic acid (Fig. 1A,B), suggests the existence of $COCl_2$ in this process. Moreover, the finding that [^{18}O] O_2 is incorporated into the

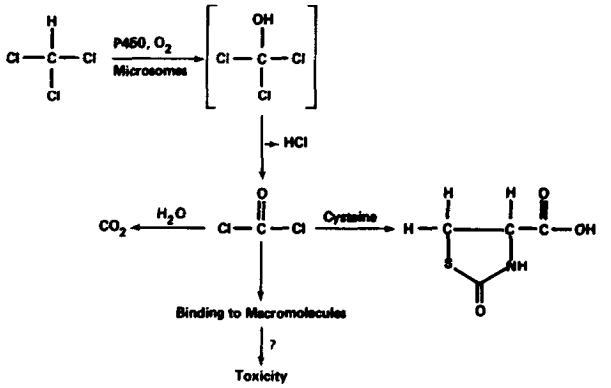

Fig. 2. Proposed oxidative dechlorination mechanism for the metabolic activation of $CHCl_3$ by liver microsomes of rat.

2-oxo position of 2-oxothiazolidine-4-carboxylic acid (Fig. 1C,D) when the incubation is conducted in an atmosphere of $[^{18}O]$ O_2 confirms the proposal that $COCl_2$ is produced by an oxidative dechlorination mechanism (Fig. 2).

Although the mechanism for the metabolic activation of $CHCl_3$ by rat liver microsomes is now more clearly understood, the toxicologic relevance of this finding remains to be elucidated. However, the previous finding in rats that pretreatment with cysteine protects against the hepatotoxicity of $CHCl_3$ (7) suggests that this mechanism is important in vivo. This process may also be responsible for the depletion of liver glutathione (6,7) and the renal toxicity (1,2,4,5) and liver tumors (1) that are produced by this compound.

The mechanism of activation suggested here may also occur with other drugs. Indeed, the results of investigations with the chlorinated antibiotic chloroamphenicol (18,19) suggest that the oxidative dehalogenation mechanism (Fig. 2) is also responsible for the metabolic activation of this compound as well as other halogenated hydrocarbons.

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