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SHORT COMMUNICATIONS

Evidence for *in vivo* covalent binding of $^{\bullet}CCl_3$ derived from CCl_4 to cholesterol of rat liver

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In a prior study of the *in vivo* binding of $^{14}CCl_4$ label to lipids of rat liver, we found a non-random distribution of radioactivity in the lipid subfractions separated by DEAE cellulose chromatography [1]. Of particular interest was the high specific activity (cpm/mg lipid) recovered in the cholesterol ester fraction. The specific activity of this fraction was similar in magnitude to that recovered as phosphatidylethanolamine and phosphatidylinositol and more than ten times greater than unesterified cholesterol.

Studies by others have provided information on the nature of the binding of CCl_4 to phospholipids [2, 3]. Gordis [2] found that label derived from both ^{14}C- and ^{36}Cl-labeled CCl_4 was recovered in the fatty acid portion of the molecule in a 1:3 ratio of ^{14}C to ^{36}Cl. He considered the labeled lipid to be the product of a $^{\bullet}CCl_3$ radical addition reaction. Benedetti *et al.* [3] isolated an abnormally migrating phospholipid fatty acid with a high specific activity of ^{14}C derived from $^{14}CCl_4$; this lipid species had a 1:1:1 molar ratio between ^{14}C label, diene conjugation formation and fatty acid content. They considered this labeled lipid species to be the product of both hydrogen abstraction and radical addition reactions by $^{\bullet}CCl_3$ intermediates and, therefore, a free radical termination product. Evidence for the involvement of free radical intermediates in the pathogenesis of CCl_4 hepatotoxicity has been reviewed recently [4].

This study was undertaken to determine if the CCl_4 derived label previously recovered in the cholesterol ester fraction is due, in part, to a trichloromethylated cholesterol free radical termination product. Addition of a polar trichloromethyl group to cholesterol could alter the role of cholesterol in moderating membrane fluidity and thus contribute to CCl_4 hepatotoxicity.

Methods

Isotopes. $^{14}CCl_4$ of 99%$^+$ purity (3.414 mCi/mmole) and $C^{36}Cl_4$ of 98%$^+$ purity (172.2 μCi/mmole) were purchased from the New England Nuclear Corp. Boston, MA, U.S.A.

In vivo *studies.* Male Sprague–Dawley rats (Charles River, Wilmington, MA, U.S.A.) were housed in stainless steel wire cages suspended over absorbent paper. Rats (250–350 g) fasted from 5:00 p.m. the evening before were given 1 mmole/kg of $^{14}CCl_4$ (250 μCi), $C^{36}Cl_4$ (62.5 μCi), or unlabeled CCl_4 in 0.5 ml of mineral oil by gavage between 9:00 and 11:00 a.m. One hour after CCl_4 administration, the rats were decapitated. The liver was rapidly removed, and the total lipids were extracted by homogenization in cold $CHCl_3$:MeOH (3:1, 100 ml/g liver). The lipid extract was filtered through Whatman No. 1 filter paper and reduced in volume under vacuum.

Cholesterol ester separation. The cholesterol ester fraction was separated from the other major lipid classes by preparative thin-layer chromatography (TLC) on 1000 μm silica gel GF plates (Anal. Tech. Inc., Newark, DE, U.S.A.) with chloroform–methanol–water (65:25:4, by vol.) as the solvent [5]. The radioactive cholesterol band, which migrated with cholesterol ester standards, was recovered by scraping and eluting the gel with chloroform, and was separated from the bulk of the cholesterol esters by chromatography on 250 μm silica gel GF plates containing 10% $AgNO_3$ with petroleum ether–ether (21:1, v/v) as the solvent [5]. Compounds with R_f values greater than the cholesterol stearate standard were recovered and analyzed by chemical ionization mass spectrometry with a Finnigan model 4000 equipped with selective ion monitoring and methane as the reagent gas.

In vitro *studies.* A mixture of 1.5 mg cholesterol and 144 μl (250 μCi) $C^{36}Cl_4$ was irradiated with 143×10^6 rads (^{60}CO γ-cell 200, Atomic Energy, Canada) and then was evaporated to dryness under nitrogen. The residue was chromatographed on a silica gel GF plate with benzene–ethyl acetate (3:1, v/v) as the solvent and showed a multicomponent mixture. The same procedure was repeated with unlabeled CCl_4, and the material thus obtained was subjected to chromatographic separations and chemical ionization mass spectrometry as described for the cholesterol ester fraction.

Autoradiography. Ultrafilm (LKB Instruments, Rockville, MD, U.S.A.) was used for autoradiography.

Results and discussion

Preliminary autoradiographic studies were done in order to verify that the cholesterol ester fraction separated from total lipids by TLC contained label derived from CCl_4. Autoradiographs of representative preparative TLC separations of animals given ^{14}C- or ^{36}Cl-labeled CCL_4 showed label in the rapidly moving band which migrated with cholesterol ester standards. Autoradiographs of the TLC separation of the γ-irradiated $C^{36}Cl_4$–cholesterol mixture also showed most of the radioactivity similarly located in the rapidly moving band which migrated with cholesterol ester standards. Further efforts to use autoradiography to localize the ^{14}C- or ^{36}Cl-labeled lipid species on the $AgNO_3$-containing TLC plates proved unsatisfactory due to the low level of radioactivity compared to the high background with $AgNO_3$-containing plates.

Our working hypothesis was that the formation of a stable trichloromethylated cholesterol would be a two-stage process: an abstraction of a labile hydrogen atom from cholesterol by $^{\bullet}CCl_3$ and then addition of $^{\bullet}CCl_3$ at this site. Since chlorine exists in a 3:1 ratio of two isotopic forms (^{35}Cl and ^{37}Cl), the most abundant forms of the anticipated product between $^{\bullet}CCl_3$ and a cholesterol free radical should have molecular weights of 502 and 504. With methane chemical ionization, ions are formed by the interaction of the product with H^+ (M + 1) and with C_2H_5 (M + 29) [6]. In addition, we anticipated an ion from the product after loss of $^{\bullet}CCl_3$ plus $H^{\bullet}$ or (M + 1 − $CHCl_3$).

Methane chemical ionization mass spectra of isolated lipid material from animals given $C^{36}Cl_4$ yielded ions consistent with the quasi molecular ions sought, namely *m/z* 534 (M + 29) and 506 (M + 1). Numerous other unidentified ions in the low mass range were also seen. Because of the small amount of material available, we analyzed the other specimens by the more sensitive selective ion monitoring mass spectra. Table 1 summarizes the results obtained from selective ion monitoring of the lipid material from animals given $^{14}CCl_4$ or unlabeled CCl_4 and the γ-irradiated CCl_4 plus cholesterol. Each showed peaks con-

Table 1. Methane chemical ionization mass spectra*

Treatment	Ion sought	Ion observed [m/z (relative abundance)]
+ $^{14}CCl_4$ *in vivo*	M + 1	507 (78%)
	M + 1	505 (100%)
	M + 1 − $^{14}CHCl_3$	385 (78%)
+ CCl_4 *in vivo*	M + 1	505 (100%)
	M + 1	503 (53%)
	M + 1 − $CHCl_3$	385 (95%)
+ γ-Irradiated CCl_4 plus cholesterol	M + 1	505 (100%)
	M + 1	503 (42%)
	M + 1 − $CHCl_3$	385 (67%)

* Finnigan 4000 quadruple mass spectrometer, operated in chemical ionization mode with methane at 0.5 torr and ionization source temperature 100°. The chosen diagnostic ions were monitored in the selective ion monitoring mode.

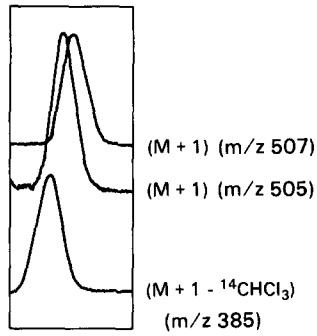

Fig. 1. Selective ion monitoring profile of ^{14}C-trichloromethylated cholesterol.

sistent with the three ions sought. A typical selective ion monitoring spectrum from an animal given $^{14}CCl_4$ is presented in Fig. 1. These results are indicative of the formation of trichloromethylated cholesterol *in vitro* and *in vivo*.

Further studies to determine the specific activity and to definitively characterize the structure and stereochemistry of the observed trichloromethyl cholesterol product are in progress. Chemical studies by others [7, 8] on the interaction of cholesterol with several types of free radicals indicate that the C-7 is the most vulnerable site for free radical interaction.

In summary, we have presented the first chemical evidence, based on methane ionization mass spectrometry, for *in vivo* binding of a $^{\cdot}CCl_3$ radical metabolite to cholesterol.

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Inhibition of hepatic γ-glutamyl-cysteine synthetase by chloroform

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Centrilobular liver necrosis induced by chloroform is related to glutathione (GSH) depletion in hepatic parenchymal cells [1]. These effects can be studied in isolated hepatocytes from phenobarbital-pretreated rats, and in a previous report we showed that chloroform-metabolizing cells rapidly lost GSH and eventually lysed [2]. It was also