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SHORT COMMUNICATIONS

Dimethyldisulfide formation during trichloromethyl radical attack on methionine

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Carbon tetrachloride hepatotoxicity has been considered to be related to trichloromethyl free radical formation and its interaction to give protein and/or lipid adducts, and/or to the initiation of a lipid peroxidation process of polyunsaturated fatty acids from cellular membranes that might occur directly or more likely after formation of $Cl_3CO_2^{\cdot}$ (trichloromethyl peroxyl) radical [1–4]. In the course of previous studies from our laboratory, we reported that chemically produced $^{\cdot}CCl_3$ (trichloromethyl radical) was able to interact with several amino acids to give adducts and with methionine to lead to the formation of volatile products of garlic-like odor [5]. In this work we analyzed the structure of these volatile productsf formed when chemically produced $^{\cdot}CCl_3$ interacts with methionine and whether enzymatically produced $^{\cdot}CCl_3$ may be able to lead to a similar end product.

Materials and methods

Chemicals. CCl_4 analytical reagent (ACS Standards) was purchased from Mallinkrodt. *dl*-Methionine ethyl ester·HCl (MetES*), *l*-methionine (Met), benzoyl peroxide and dimethyl disulfide (DDS) were Sigma Grade (Sigma Chemical Co.).

Animals and treatments. Sprague–Dawley male rats (80–100 g) were used for the experiments on phenobarbital (PB) preinduced animals (all *in vitro* experiments using liver microsomal suspensions). In those experiments, animals were injected intraperitoneally with PB once daily for 3 days at a dose of 80 mg/kg, and the animals were fasted overnight and used on the morning of day 4. The experiments on the methionine content in liver microsomal proteins were performed with Sprague–Dawley male rats (200–280 g). In these experiments, food was withdrawn 12–14 hr before CCl_4 administration. CCl_4 was given i.p. as a 20% (v/v) solution in olive oil at a dose of 5 ml/kg (1 ml of pure CCl_4/kg). Controls received an equivalent amount of olive oil. Animals were killed 6 hr after either CCl_4 or olive oil administration. Livers were rapidly removed and processed.

Procedures. The preparation of hepatic microsomal fractions and the isolation of purified microsomal protein were described previously in detail [5, 6]. The amino acid composition of the protein was performed by column chromatography by a procedure devised by Spackman *et al.* [7]. The study of the *in vitro* chemical $^{\cdot}CCl_3$ production and its interaction with Met or MetES was carried out as previously described by Villarruel and Castro [5]. A head-space aliquot of the sample (400 μl) was used for GLC analysis. Blanks without CCl_4 and without Met or MetES were run simultaneously. The interaction between enzymatically generated $^{\cdot}CCl_3$ and Met or MetES was carried out in aluminium-sealed silicone septum-stoppered glass vials of 15-ml capacity. Incubation mixtures contained liver microsomes (10.5 to 11.0 mg protein/ml); NADPH-generating system [5]; 3 mM CCl_4; 1 mM Met (or MetES) and 20 mM phosphate buffer, pH 7.4, to a final volume of 3 ml. Adequate control vials without NADPH-generating system or without CCl_4 were carried out simultaneously. After 1 hr of incubation, samples were ice-cooled and, after adding 1.8 g of anhydrous $MgSO_4$, the samples were septum-capped again for head-space analysis by GLC. Biotransformation of DDS by liver microsomes was studied by head-space analysis under experimental conditions similar to those described above but containing 1 mM DDS and excluding CCl_4 or Met (or MetES). Total ion current chromatographic (TIC) and mass spectrometric (MS) identification of head-space components was performed in a Hewlett–Packard (HP) model 5970 B mass selective detector interfaced to an HP 5890 gas chromatograph (GC). GC conditions were as follows: injection port 200°, in the splitless injection mode. Separations were carried out in a fused silica capillary column (25 m × 0.32 mm i.d.) cross-linked with methyl-silicone gum (0.52 μm film thickness) (HP No. 19091 2-112); carrier gas: helium (linear velocity 42 cm/sec, 85 kilopascals). Column temperature was maintained at 35° for 2 min and then increased to 70° at a ramp velocity of 5°/min. GLC-MS interface temperature was 280° and ion source temperature was *ca.* 200°. Scanning range in MS was from 30 to 200 atomic mass units and scanning speed was 0.96 range scans/sec. In some experiments selective ion monitoring (SIM) of mass spectra was employed to increase sensitivity. Selected masses were as follows: 46 and 31 (ethanol); 48, 47, 34 and 33 (MeSH); 118, 120, 83 and 47 (CCl_4); and 94, 79, 64 and 61 (DDS). Dwell time for all the peaks was 200 msec. Selection of masses for SIM studies and identification was made by comparison against the National Bureau of Standards Library of mass spectra.

Results

Interaction of $^{\cdot}CCl_3$ with methionine (Met) or methionine ethyl ester (MetES). The GLC/TIC analysis of the volatile products arising from chemically generated $^{\cdot}CCl_3$ interaction with Met revealed the presence of five peaks (Fig. 1). Peaks 1 and 4 corresponded to ethanol and CCl_4 present in the reaction mixture. Peak 2 was identified by GLC/MS as ethyl chloride which is likely to result from $^{\cdot}C_2H_5$ and $^{\cdot}Cl$ radicals formed when ethanol and CCl_4 were heated in the presence of benzoyl peroxide catalyst (reaction blank). Peak 3 was identified as chloroform by GLC/MS and would result from $^{\cdot}CCl_3$ hydrogen atom abstraction from medium. Peak 5 was identified by GLC/MS as DDS (see Fig. 1). The same five peaks were observed when MetES was used instead of Met. In anaerobic incubation mixtures containing liver microsomes, NADPH and CCl_4, a peak identified as $CHCl_3$ was observed. Very little $CHCl_3$ was formed in contrast when NADPH was omitted. Addition of Met to incubation mixtures where CCl_4, NADPH and microsomes were present did not result in any GLC/TIC detectable peak of DDS under the experimental conditions employed. However, when MetES was used in the incubation mixtures in place of Met, a peak identified by MS as DDS was evident. An additional peak corresponding to MeSH was also observable by GLC/SIM. Neither DDS nor MeSH was observed when CCl_4, NADPH or MetES was excluded from the incubation mixtures. The GLC/SIM head-space analysis of anaerobic incubation mixtures containing liver

* Abbreviations: Met, *l*-methionine; MetES, *dl*-methionine ethyl ester·HCl; DDS, dimethyldisulfide; MeSH, methyl mercaptan; MS, mass spectrometry; TIC, total ion current; and SIM, selective ion monitoring.

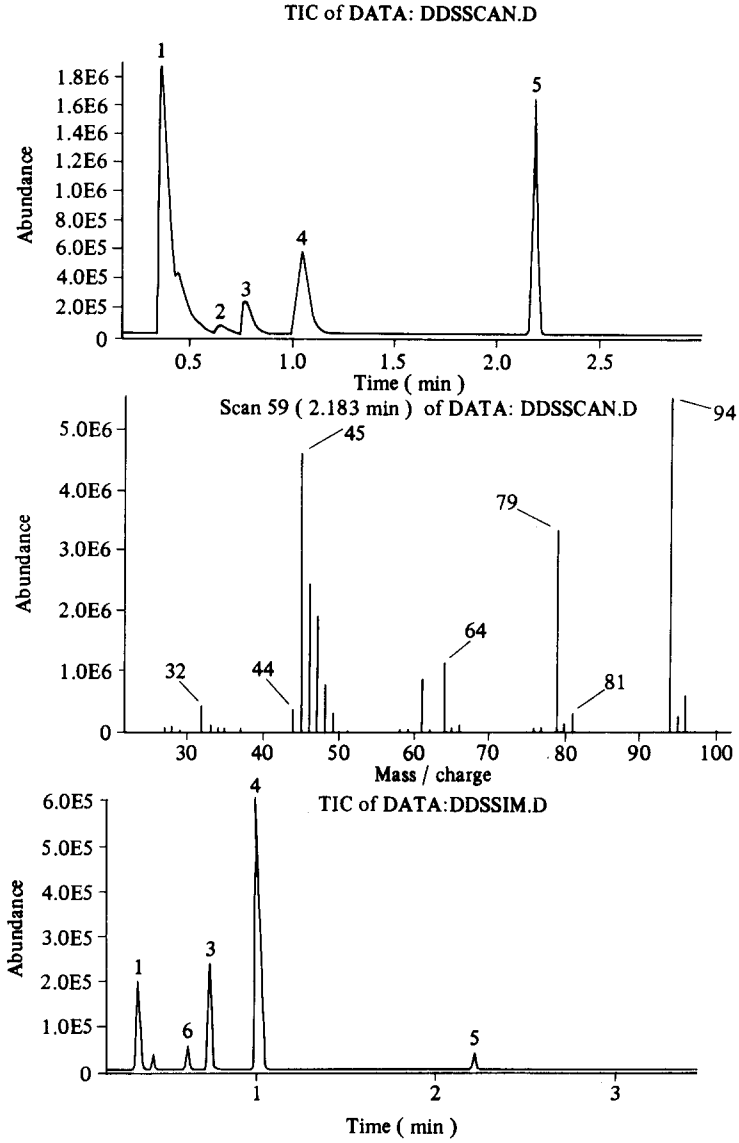

Fig. 1. Identification of volatile reaction products arising from $^{\cdot}CCl_3$ attack on Met or MetES. Top panel: GLC/MS (scan mode) obtained from the head-space analysis of reaction products formed when chemically generated $^{\cdot}CCl_3$ interacts with Met or MetES. Peak 1, ethanol; 2, ethyl chloride; 3, $CHCl_3$; 4, CCl_4; 5, DDS. In peaks 2 and 3, the expected isotopic ratios for chlorine containing ions were observed. Center panel: Mass spectra of peak 5. Comparing it against the MS library and a pure standard revealed that it corresponded to DDS. Bottom panel: GLC/MS (SIM mode) obtained from the head-space analysis of reaction products formed when enzymatically generated $^{\cdot}CCl_3$ interacts with MetES under a N_2 atmosphere. Peak 6, MeSH. See Materials and Methods for details. Abundance is expressed as total ion current signal. E indicates use of exponential nomenclature, e.g. 1.8E6 is equivalent to 1,800,000.

microsomes, NADPH and DDS showed that the system degraded DDS. Degradation was accompanied by the formation of products which were identified by GLC/MS as MeSH and H_2S. A part of the DDS anaerobic degradation was heat sensitive, but a relevant fraction of it was not. Both heat-sensitive and heat-resistant anaerobic DDS metabolizing activity of rat liver microsomes partially required NADPH presence, but there was also a fraction that did not. Degradation of DDS under aerobic conditions was more intense than under anaerobic conditions. NADPH was able to stimulate slightly both the heat-sensitive and the heat-resistant aerobic metabolizing activity of rat liver microsomes. Most of the aerobic ability of liver microsomes to degrade DDS was heat insensitive (in either the presence or absence of NADPH) (Table 1).

Methionine content in liver microsomal protein from control and CCl_4-treated animals. There was no significant difference in the Met content of liver microsomal protein of control and CCl_4-treated animals (6 hr of poisoning) (controls = 440.93 ± 20.53; treated = 434.13 ±

Table 1. DDS degradation by liver microsomes from phenobarbital-preinduced rats*

Experimental condition	DDS degradation ($\times 10^{-8}$ mol/mg protein)
Nitrogen	
NADPH	7.1 ± 0.2
No NADPH	6.3 ± 0.4†
Heated + NADPH	5.0 ± 0.3†‡
Heated, no NADPH	3.4 ± 0.3†‡§
Air	
NADPH	9.0 ± 0.3
No NADPH	7.3 ± 0.5†
Heated + NADPH	8.2 ± 0.4†‡
Heated, no NADPH	5.6 ± 0.8†‡§

* Liver microsomes from phenobarbital-preinduced animals were used (microsomal protein, 10.5 to 11.0 mg/ml). The incubations contained an NADPH-generating system when indicated. Heated microsomes were obtained by heating microsomal suspensions at 100° for 5 min. The final concentration of DDS was 1 mM. GLC/MS/SIM headspace analysis of reaction mixture was made after a 1-hr incubation at 37°. Values are means ± SD, N = 3.

† $P < 0.05$ when compared to unheated NADPH control.

‡ $P < 0.05$ when compared to unheated counterpart.

§ $P < 0.05$ when compared to heated + NADPH.

31.57 nmol/mg microsomal protein; values were obtained using three animals per group; $P > 0.05$) (Student's *t*-test).

Discussion

The studies described here revealed that chemically or enzymatically generated $^{\cdot}CCl_3$ interacts under appropriate experimental conditions with Met and/or MetES to produce DDS. DDS may result from $^{\cdot}CCl_3$ attack on the RCH_2—SCH_3 bond in Met or MetES to abstract a hydrogen and release $^{\cdot}SCH_3$ radical followed by dimerization and $CHCl_3$ formation. The proposed reaction mechanism takes into consideration the fact that under these experimental conditions no adducts are formed [4] and that $CHCl_3$ is produced. In anaerobic enzymatic incubation mixtures we also found MeSH and H_2S formation which would result from enzymatic and non-enzymatic pathways of liver microsomal reductive degradation. One interesting observation was that only MetES but not Met led to detectable amounts of DDS in enzymatic incubation mixtures producing $^{\cdot}CCl_3$ and that endogenous Met present in microsomal protein did not seem to interact. These results might be explained assuming that the lipid soluble MetES has more chance to have access to cytochrome P-450 active sites and be close to the place where the highly reactive $^{\cdot}CCl_3$ is known to be produced [1–4]. Cytochrome P-450 Met residues [8] (or other Met present in microsomal protein), on the other hand, have relatively fixed positions in the proteins that may not be close enough to the site of $^{\cdot}CCl_3$ production to be attacked and lead to measurable amounts of DDS. In support of this assumption is the fact that we did not observe a significant decrease in Met content in liver microsomal protein from 6-hr CCl_4 poisoned rats. These results suggest that, if the interactions described here between $^{\cdot}CCl_3$ and Met are biologically significant, they should involve only a minor fraction of the total Met pool.

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