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IN VIVO SPIN-TRAPPING OF TRICHLOROMETHYL RADICALS FORMED FROM CCl_4

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The spin-trapping technique has been used to detect radicals which have a half-life too brief to obtain concentration levels which can be detected directly by electron spin resonance (e.s.r.) spectroscopy. This technique not only produces a more stable derivative radical, but provides the opportunity for determining the nature of the primary radical through spectrum analysis and a computer simulation procedure. We have recently employed this technique to demonstrate definitively the production of the $\cdot CCl_3$ radical from CCl_4 by a liver microsomal system (1). The radical adduct formed by the reaction of the $\cdot CCl_3$ radical with phenyl-t-butylnitrone (PBN) is lipid-soluble and has a relatively long half-life, and can be extracted from the microsomal membrane with chloroform. Since formation of this radical in an *in vitro* system does not necessarily mean that $\cdot CCl_3$ radicals are formed in the liver *in situ*, we have investigated the feasibility of using the spin-trapping technique *in vivo*. The results described herein indicate that spin-trapping can be accomplished *in vivo*. The results also provide direct evidence for the formation of the trichloromethyl radical from CCl_4 by the liver in the intact animal.

MATERIALS AND METHODS

Male, Sprague-Dawley rats (250-300 g body wt), fed a commercial rat ration, were used in all experiments. Phenyl-t-butylnitrone and CCl_4 (spectrophotometric grade) were obtained from Eastman Organic Chemicals, Rochester, NY. Reagent quality $CHCl_3$ and methanol were purchased from the Fisher Scientific Co., Houston, TX. All e.s.r. spectra were recorded with a Varian E-9 electron spin resonance spectrometer equipped with an X-band (E-101-15) microwave bridge. CCl_4 was administered at a dosage of 120 μl/100 g body weight. The spin-trapping agent, PBN, was given at a dosage of 1 ml of a 0.14 M solution in 0.02 M phosphate buffer, pH 7.4. CCl_4 or the spin trap, or both, were homogenized with 1.0 ml of corn oil and administered by stomach tube after the rats had been fasted for 20 hr. Control animals received either the spin-trapping agent mixed with corn oil, or CCl_4 in corn

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0.5 ml samples of the lipid extracts were placed in the analysis tubes for measurement of electron spin resonance signals. (A) Electron spin resonance signal of liver extracted from rats given CCl_4 and PBN orally in corn oil. (B) Electron spin resonance signal obtained in vitro with liver microsomes, CCl_4, NADPH and PBN. The incubation system was the same as that described by Poyer et al. (1). (C) Control study showing absence of e.s.r. signal in the liver lipid extract when fresh liver, CCl_4 and PBN were added simultaneously to chloroform-methanol extracting medium. (D) Control study showing absence of e.s.r. signal in extracted liver lipid when rats were given PBN in corn oil without CCl_4. (E) Control study showing absence of e.s.r. signal in liver lipid extract when rats were given CCl_4 in corn oil without PBN.

In the present study, the signal was not observed in the lipid extract obtained when fresh liver tissue, CCl_4, and the spin-trapping agent were added simultaneously to chloroform-methanol, and the extraction procedure carried out as above (Figure 1C). In addition, administration to rats of the spin-trapping agent in corn oil without CCl_4, followed by extraction of liver lipids, yielded an extract that produced no measurable e.s.r. signal (Figure 1D). Hence, the spin-trapped radical is not formed in the rat liver in vivo in the absence of CCl_4 administration.

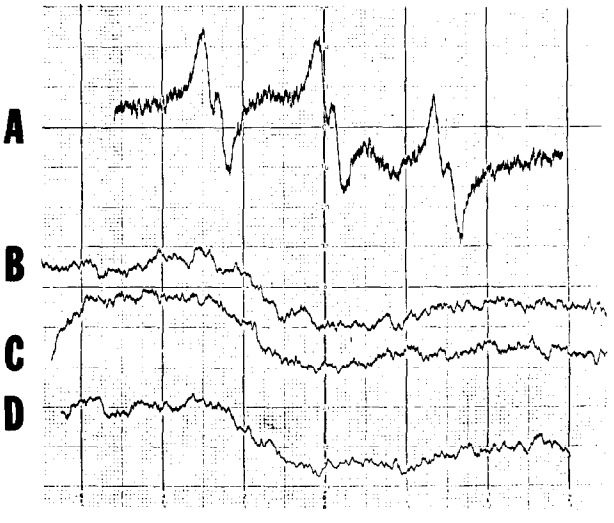

Fig. 2. Electron spin resonance studies on lipid extracts of fractions isolated from liver of rats given CCl_4 and PBN in corn oil orally. Liver (5.0 g) was homogenized and fractionated as described under Materials and Methods. The protein content per gram of liver of the fractions obtained

oil, or corn oil only. The amount of corn oil administered was constant in all studies. The treated rats were killed after 2 hr. Livers were immediately removed, rapidly weighed, and homogenized in chloroform-methanol (2:1). Total lipids were then extracted from 5.0 g of liver by the method of Folch et al. (2). The $CHCl_3$ layer was recovered at the end of the extracting procedure and the solvent removed and evaporated under nitrogen gas. The concentrated volume was either analyzed immediately in a Varian E-9 electron spin resonance spectrometer, or was stored at -20° for later study. The preparation of liver subcellular fractions (nuclear-plasma membrane fraction, mitochondria and microsomes) from the treated rats has been described previously (3). The e.s.r. spectrometer settings were microwave power, 25 mW; modulation amplitude, 1 G; time constraint, 10 sec; scan range, 100 G; and scan time, 30 min or 2 hr. The spectra were taken at room temperature, 24°.

RESULTS

Figure 1A shows the e.s.r. signal obtained from a chloroform-methanol (2:1) extract of liver of a rat which had been given CCl_4 orally in corn oil together with the spin-trapping agent, PBN. The signal is identical to that which is obtained when CCl_4 is incubated with liver microsomes, NADPH, and the spin-trapping agent (Figure 1B), and which was demonstrated to be the spin-trapped adduct of $\cdot CCl_3$ by computer simulation (1).

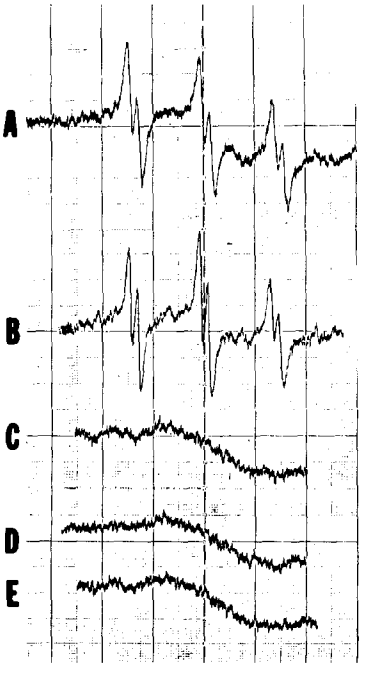

Fig. 1. Studies on the spin-trapping of the trichloromethyl radical in vitro and in vivo. Where indicated, rats were administered the compounds orally as described under Materials and Methods. After 1 hr., the total lipids were extracted from 5.0 g of liver. After removal of the solvent,

was as follows: plasma membrane-nuclei, 20 mg; mitochondria, 39 mg; microsomes, 16 mg. Total lipids were extracted from each fraction and, after solvent removal, lipid samples of 0.5 ml were analyzed in an electron spin resonance spectrometer. (A) Microsomal lipids. (B) Mitochondrial lipids. (C) Nuclei-plasma membrane fraction lipids. (D) Supernatant fraction lipids.

Rats were treated with the spin trap and CCl_4 *in vivo*. The livers were then removed, homogenized, and fractionated at 4° into three groups of organelles: (a) nuclei-plasma membrane, (b) mitochondria, and (c) microsomes. The total lipid from each subcellular fraction was extracted by the same procedure as described above. Electron spin resonance analysis of each lipid extract shows that the microsomal fraction is the only organelle which contains the spin adduct (Figure 2). No signal was detected in the aqueous phase of any of the extracted subcellular fractions.

DISCUSSION

There are two significant aspects of these results. First, the data provide strong support for the concept that the trichloromethyl radical is, in fact, a metabolic intermediate in the metabolism of CCl_4 in the rat liver *in vivo* as has been postulated (4-6), and that the formation of the radical appears to be a microsomal function since the spin-trapped radical was found to be associated with that organelle, but not with other membranous fractions at a detectable level.

The PBN adduct of the $\cdot CCl_3$ radical is very stable, and is associated with the lipid-extractable fraction just as in the case of the metabolism of CCl_4 by liver microsomes *in vitro*. There is very little likelihood that the radical adduct observed after administration of CCl_4 and PBN to intact animals is formed *in vitro* during the homogenization of the liver in the extracting solvent in view of the fact that simultaneous addition of CCl_4, PBN, and liver tissue from untreated rats to chloroform-methanol (2:1), followed by immediate homogenization, resulted in obtaining a lipid extract which did not give an e.s.r. signal.

There has been considerable speculation that a highly reactive free radical, such as the trichloromethyl radical, may be responsible for initiating the events that ultimately result in liver damage, and would explain why lipid-soluble free radical scavengers afford protection against the hepatotoxic action of CCl_4 (7-9). Other investigators have shown that both ^{14}C and ^{35}Cl bind to lipid and to protein of liver microsomes (10), but that binding of $^{14}CCl_4$ to lipid and conversion of the labeled carbon in $^{14}CCl_4$ to $^{14}CO_2$ are not inhibited by antioxidants (11). We have not determined if PBN administration itself reduces the extent of damage to the liver caused by CCl_4.

The second significant aspect of this study is that it establishes the feasibility of using a spin-trapping agent to investigate free radical phenomena in vivo. The implications are that this type of approach may be useful in obtaining more direct information on the formation of highly reactive free radicals produced during the metabolism of other compounds. To this end, we are currently applying the technique described above to other tissues known to be damaged by CCl_4 for evidence of formation of the trichloromethyl radical in vivo.

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