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IN VIVO SPIN-TRAPPING OF RADICALS FORMED DURING HALOTHANE METABOLISM*

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Abstract—The results described in this paper demonstrate the formation of a free radical during the metabolism of halothane by rat liver microsomes. The radicals were stabilized by spin-trapping and assayed by electron spin resonance spectroscopy. A halothane-dependent free radical was also formed in the liver of intact animals exposed to halothane by inhalation *in vivo*.

Chemical compounds, termed spin-trapping agents, have been used recently to detect and identify reactive short-lived free radicals produced in biochemical systems. The spin-trapping compound reacts with the free radical to form a more stable radical product, i.e. a "spin-adduct", which can be detected and investigated using electron spin resonance (e.s.r.) techniques [1,2]. The use of spin-trapping agents to probe biological systems for evidence of free radical formation in specific processes is still in the developmental stage, but this method is important since it appears that certain metabolic reactions in animal tissues involve transient free radical species that cannot be observed effectively by any other technique [1]. This method has been employed to demonstrate definitively the formation of the $\cdot CCl_3$ radical from CCl_4, not only by a liver microsomal system [3], but also by the liver *in vivo* [4,5].

This report concerns the use of the spin-trapping technique to demonstrate the formation of a free radical when halothane is metabolized by liver microsomes. In addition, data are included which show that a radical with the same e.s.r. properties is produced in the liver of intact rats which have been subjected to halothane anesthesia.

MATERIALS AND METHODS

Materials. Phenyl-t-butylnitrone (PBN) was obtained from Eastman Organic Chemicals (Rochester, NY). Halothane was obtained from the Ayerst Laboratories. (New York, NY). Reagent quality $CHCl_3$ and methanol were purchased from the Fisher Scientific Co. (Pittsburgh, PA). Male, Sprague–Dawley rats, weighing between 250 and 300 g, were used in all experiments. The animals were fed a commercial laboratory rat ration. 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. The spin-trapping agent, PBN, was given at a dose of 1 ml of a 0.14 M solution in 0.02 M phosphate buffer, pH 7.4. The buffered solution of spin trap was homogenized with 1.0 ml of corn oil and administered as an emulsion by stomach tube after the rats had been fasted for 20 hr. Immediately following the administration of the PBN–buffer–corn oil emulsion, halothane was administered by inhalation at a dosage of 0.5% (v/v) in the breathing air for 2 hr, using a Foregger inhalator equipped with a Fluotec attachment. The halothane dose was checked for accuracy using a Hewlett–Packard gas–liquid chromatograph with integrator. Two types of control studies were performed. One set of control animals received the PBN–buffer–corn oil emulsion but was not exposed to halothane. Another set of controls was exposed to halothane but was not given the PBN-containing emulsion. All rats were killed 2 hr after respective treatments, and the livers were immediately removed, rapidly weighed, and homogenized directly in chloroform–methanol (2:1). The total lipids were then extracted by the method of Folch *et al.* [6]. The $CHCl_3$ layer was evaporated *in vacuo* to a volume of approximately 1 ml. The concentrated extract was either analyzed immediately in a Varian E-9 electron spin resonance spectrometer or stored at −70° for later study. The samples were gassed with N_2 for 15 min before e.s.r. analysis to eliminate any O_2 from the samples. The presence of oxygen in such samples results in peak broadening of the e.s.r. spectrum.

The e.s.r. spectrometer settings were: microwave power, 25 mW; modulation amplitude, 1 G; time constant, 10 sec; scan range 100 G; and scan time, 30 min. The spectra were recorded at room temperature, 24°. The preparation of liver subcellular fractions (nuclear + plasma membranes, mitochondria, and microsomes) from the treated rats has been described previously [7].

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RESULTS

Figure 1A shows the result of an e.s.r. analysis of a chloroform–methanol (2:1) extract of liver from a rat given halothane at a concentration of 0.5% in the inhalation air for 2 hr immediately following the oral administration of a PBN–phosphate buffer–corn oil emulsion. This reproducible signal was obtained in every experiment of this type.

Figure 1B shows the e.s.r. spectrum obtained when 20 μl of halothane was incubated in a 1 ml system containing about 2 mg microsomal protein, 0.7 ml of a 0.14 M PBN solution in 0.05 M phosphate buffer, pH 7.4, and an NADPH-generating system described previously [3], all in 0.05 M phosphate buffer, pH 7.4. The signal obtained with the *in vitro* system had the same characteristics as that obtained with the extract from the livers of animals administered halothane *in vivo*.

In this study, the e.s.r. signal was not observed in the lipid extract when fresh liver tissue from a rat treated with the PBN–phosphate buffer–corn oil emulsion and 10 ml halothane was added simultaneously to the chloroform–methanol extracting mixture (Fig. 1C). Moreover, when the rats were administered a PBN–phosphate buffer–corn oil emulsion, but with no subsequent halothane inhalation (Fig. 1D), or were administered halothane by inhalation but were not administered PBN (Fig. 1E), no e.s.r. signal could be seen in the lipid extracts of the livers of those animals. The spin-trapped halothane-dependent radical, therefore, was not formed when the spin-trap was given without subsequent halothane administration, nor was it formed as an artifact of the extraction and concentration of liver lipids. The a^N splitting constant for the halothane-derived e.s.r. spectra of Fig. 1A, B, F, and G was approximately 14.6 G, and the a_β^H splitting constant for the same spectra was 2.4 to 2.5 G.

Rats were given halothane after the oral administration of the PBN–phosphate buffer–corn oil emulsion as described above. The livers were removed after a 2-hr exposure of the rats to 0.5% halothane by inhalation. The livers were homogenized in 0.15 M phosphate buffer (pH 7.4) and were fractionated at 4° into four fractions designated as containing primarily (a) nuclei + plasma membranes, (b) mitochondria, (c) microsomes, and (d) the post-microsomal supernatant (soluble) fraction. The total lipid was extracted from each of these fractions and the concentrated lipid extract was examined for an e.s.r. signal. The lipid extract from the microsomal fraction consistently showed an e.s.r. signal (Fig. 1F) and contained the major portion of the total spin-trapped radicals present in the liver homogenate and subfractions thereof. Signals observed in the other fractions were probably due to microsomal contamination.

When a halothane–PBN solution was irradiated with u.v. light, a free radical was trapped as shown in Fig. 1G which had the same splitting constants as for Fig. 1A, B, and F. The radical formed was probably either or both of those shown in Fig. 2. Both CCl_4 and $BrCCl_3$ form trichloromethyl radical adducts with PBN when irradiated with u.v. light

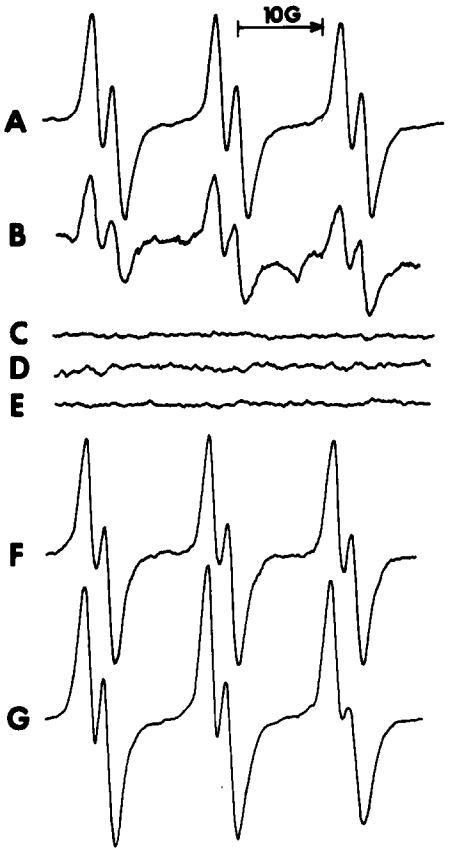


Fig. 1. Studies on the spin-trapping of the halothane radical *in vivo* and *in vitro*. (A) Electron spin resonance signal of a lipid extract from liver of a rat given halothane by inhalation and a PBN-phosphate buffer–corn oil emulsion orally prior to halothane administration. (B) Electron spin resonance signal obtained *in vitro* from liver microsomes, halothane, PBN and an NADPH-generating system. (C) Control study showing the absence of an e.s.r. signal in the concentrated liver lipid extract from an animal treated with a PBN–phosphate buffer–corn oil emulsion, and with 10.0 ml halothane added simultaneously to the chloroform-methanol extracting mixture. (D) Control study showing the absence of an e.s.r. signal in the concentrated liver lipid extract when rats were given the PBN–phosphate buffer–corn oil emulsion but were not treated with halothane in the inhalation air. (E) Control study showing the absence of an e.s.r. signal in the liver lipid extract when rats were administered halothane in the inhalation air, but not orally given PBN in the phosphate buffer–corn oil emulsion. (F) Electron spin resonance signal of concentrated lipid extract from the microsomal fraction of liver of a rat given 0.5% halothane by inhalation and a PBN–phosphate buffer–corn oil emulsion orally prior to the halothane administration. (G) Electron spin resonance signal of a solution of PBN in halothane after irradiation with u.v. light. The solution contained 14 mg PBN/ml of halothane in a quartz tube with an inside diameter of 2 mm and was bubbled with nitrogen 30 min prior to and during irradiation for 2 min with a UVS-11 mineral light 2 cm from the quartz tube. The e.s.r. spectral measurement was made 2 hr after irradiation. The a^N splitting constant for the halothane-derived e.s.r. spectra of Fig. 1A, B, F and G was approximately 14.6 G, and the a_β^H splitting constant for the same spectra was 2.4 to 2.5 G.

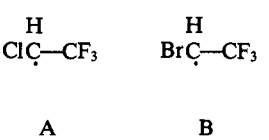
[3]. Irradiation of a PBN solution without halothane resulted in no detectable radical adduct formation.

DISCUSSION

Several anesthetics, such as halothane, used in surgical and other clinical procedures are halogenated hydrocarbons, and they are presumed to be metabolized *in vivo* by the NADPH-dependent drug-metabolizing system [8,9] in the endoplasmic reticulum of the liver. CCl_4 has been shown to be converted to trichloromethyl radicals by this same system [4,5]. Numerous attempts have been made to determine a cause and effect relationship between halothane anesthesia and "halothane hepatitis" since reports of post-operative halothane-induced hepatitis began appearing in 1958, the year that the anesthetic was released for clinical use [10]. Although the metabolic formation of a free radical associated with halothane metabolism has not been demonstrated previously, it has been postulated [11,12], and it is possible that the formation of a free radical of halothane itself is responsible for the liver damage sometimes observed following the clinical use of halothane.

Because it has been observed that liver microsomes can form trichloromethyl radicals from both CCl_4 and $BrCCl_3$ [3], it is reasonable to anticipate that the halothane-derived free radical trapped and observed here will be found to form from the abstraction of a bromine or a chlorine atom to produce either or both of the free radical species shown in Fig. 2.

Since it has been shown that the e.s.r. signal due to the PBN-trapped trichloromethyl radical could be observed in a lipid extract from the microsomes of liver of rats given, orally, an emulsion containing the spin-trap PBN in phosphate buffer and CCl_4 in corn oil [4], a similar study was done to determine if a free radical could be trapped and detected in a similar way if halothane was administered to rats by inhalation after the oral administration of PBN. A halothane-dependent radical was trapped and observed as described in Results. The control experiments shown in Fig. 1 indicate that it is unlikely that the PBN radical adduct was formed during the extraction and concentration of the liver lipid sample. Rather, the radical must have formed during the metabolism of halothane in the liver and was trapped as a stable radical by reaction with PBN. The radical that was formed *in vivo* appears to have been identical to that formed by the metabolism of halothane by liver microsomes and was presumably, a product of the drug-metabolizing system in the liver of the intact animal. This is consistent with the observation that nearly all of the spin-trapped radical was found in the microsomal fraction of the liver of animals administered CCl_4 and PBN *in vivo*.



A B

Fig. 2. Proposed structures for the halothane-derived free radical(s).

A quantitative estimation of the amount of halothane-derived free radicals trapped by PNB after a 2-hr exposure of rats to 0.5% halothane, and subsequent extraction of the rat liver, was made by the method described by Ayscough [13] and used similarly by Floyd *et al.* [14], employing the nitroxide free radical standard, 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy, and using the e.s.r. spectrum of Fig. 1A. The calculated amount of halothane-derived free radicals in the extracted liver was 0.38 pmole/g of liver. This corresponds to about 19×10^{-12} moles/mg of microsomal protein, or a concentration between 100 and 120×10^{-9} M in the liver endoplasmic reticulum.

The data provide support for the hypothesis that halothane can be converted *in vivo* in the endoplasmic reticulum of the liver to a free radical product. Although no relationship between the formation of this radical and the etiology of halothane hepatitis has been established, the similarity of this reaction to that of CCl_4 in the liver suggests that, under some undefined conditions, a halothane radical associated with halothane metabolism may be toxic.

The techniques described here indicate that other halogenated anesthetics and halogenated hydrocarbons should be investigated to determine if they also form free radicals in the liver and other organs when administered to animals through inhalation.

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