

Hypoxia and Halothane Metabolism in Vivo:

Release of Inorganic Fluoride and Halothane Metabolite Binding to Cellular Constituents

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Fluoride release and covalent binding of halothane metabolites were studied in rats pretreated with phenobarbital and anesthetized with halothane in the presence of high (40 per cent) and low (7 per cent) oxygen tensions. The purpose of producing hypoxia was to promote the reductive pathways involved in the metabolism of halothane. Halothane anesthesia under hypoxic conditions caused a significant elevation in the plasma fluoride concentration. There was also a greater than threefold increase in covalent binding of ¹⁴C-halothane metabolites to microsomal lipids in hypoxic rats. The lipid/protein binding ratio in control animals averaged 0.76, while hypoxic animals had a binding ratio of 3.24. The findings demonstrate that defluorination of halothane does occur during hypoxic conditions. It is hypothesized that the products produced by this reductive metabolic pathway are also potentially more hepatotoxic than the oxidative metabolites, based upon the increased covalent binding of halothane metabolites under hypoxic conditions. (Key words: Biotransformation, halothane; Hypoxia; halothane biotransformation; Anesthetics, volatile, halothane.)

SEVERAL RECENT REPORTS on the metabolism of halothane have made it possible to focus attention on the relationship of halothane hepatotoxicity and the metabolism of the agent.^{1,2} It is recognized that both oxidative and reductive pathways are possible, and this has been substantiated by *in-vitro* studies from this laboratory, which demonstrated a clear

difference in the metabolism of halothane under aerobic versus anaerobic conditions.^{3,4}

Aerobic metabolism of halothane results in formation of inorganic chloride and trifluoroacetic acid,³⁻⁵ products generally considered nontoxic. On the other hand, anaerobic metabolism of halothane results in formation of metabolites that bind to cellular constituents,³ leading to the conclusion that reductive metabolism of halothane results in formation of more reactive metabolites.

Another important discovery made recently concerns the metabolites of halothane found in human urine. These are trifluoroacetic acid, N-trifluoroacetyl-2-amino-ethanol, and N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine.¹ The latter compound is particularly interesting because it indicates the halothane undergoes defluorination—a reaction heretofore considered not to occur.

On the basis of this information, it was decided to investigate the possibility that inorganic fluoride is released from halothane *in vivo*. This was presumed to occur via a reductive pathway, so with this in mind, we attempted to promote anaerobic metabolism *in vivo* by exposing rats to low inspired oxygen tensions during administration of halothane. The fluoride produced and the binding of halothane metabolites in these animals and in animals anesthetized in high oxygen concentrations were then compared.

Methods

ANIMALS

Male Sprague-Dawley strain rats weighing approximately 350 g were used as test animals. They were maintained on a diet of Purina Laboratory Chow *ad libitum*. Microsomal enzyme induction was produced by administration of sodium phenobarbital, 0.2 per cent solution in the drinking water, for four days.⁶ The animals

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were then returned to pure drinking water and studied 24 to 72 hours later.

The rats were divided into six groups. Group I received halothane, 0.5 per cent, with 40 per cent O₂. Group II animals were anesthetized identically, but the ambient atmosphere was maintained at 7 per cent O₂, with the remainder being N₂. Group III received 0.7 per cent halothane in the 7 per cent O₂ mixture. Group IV animals received no halothane, but were kept in the same low-oxygen atmosphere as Groups II and III. Rats in Groups V and VI were anesthetized using ¹⁴C-labeled halothane. Group V animals breathed a hypoxic mixture, and Group VI, an oxygen-enriched atmosphere.

ANESTHETIC TECHNIQUE

When unlabeled halothane was used (Groups I-IV), two to four rats were anesthetized simultaneously in a large air-tight chamber with inlet and outlet ports. Halothane was administered from a Vemtrol vaporizer and N₂ and O₂ were added as diluent gases from calibrated flowmeters set to produce the desired O₂ concentration.

Halothane-1-¹⁴C was administered in a closed circuit to rats in Groups V and VI. These animals were placed individually in a glass, air-tight 2-l chamber, which contained a receptacle for soda lime and a fuel-cell oxygen sensor (oxygen analyzer 202R, Biomarine Industries) connected to an external metering device. Two 5-mm-diameter ports plugged with rubber stoppers provided a means to inject ¹⁴C-halothane into the chamber and to add O₂ as necessary to keep the O₂ concentration at the desired level. This ranged from 7 to 14 per cent O₂ for Group V and from 25 to 60 per cent for Group VI. ¹⁴C-Halothane, 100 μ l, previously diluted with unlabeled halothane to obtain a specific activity of 1.4×10^4 dpm/ μ mol, was injected into the chamber. This dose was calculated to produce an initial concentration of approximately 1.4 per cent halothane within the chamber. No additional halothane was added; thus, the concentration decreased with time. This amount was sufficient to maintain anesthesia for 1.5 to 2 hours.

Rats receiving unlabeled halothane or exposed to low O₂ tension only were removed

from the large chamber after two hours. Blood samples were obtained by intracardiac puncture after the animals had been immobilized with sodium thiopental, 0.5 ml of a 2.5 per cent solution, injected intraperitoneally. Rats that received labeled halothane were kept in the small chamber for 2 hours and then removed and allowed to breathe room air for 1 hour. The animals were then sacrificed and their livers removed and homogenized for preparation of subcellular fractions as previously described.⁷

ANALYSES

Fluoride levels in plasmas from rats in Groups I, II, III, and IV were measured using a fluoride-specific electrode as described previously.⁸

Radioactivity bound to microsomal protein and lipid was determined in Groups V and VI. Using 1:1 chloroform-methanol, lipids were extracted from a microsomal suspension containing a known amount of protein (assayed by the method of Lowry *et al.*⁹). The protein was removed by centrifugation, washed with acidified methanol, and re-centrifuged. The resultant pellet was digested with NCS-solubilizer (Nuclear-Chicago Corporation) and counted in a scintillation counter after addition of Insta-Gel (Packard Instrument Company). The supernatant from the protein pellet was adjusted to 2:1 chloroform-methanol and washed with 0.1 per cent sodium chloride. The lower phase (chloroform extract) was allowed to evaporate in a hood, dissolved in modified Bray's scintillator, and counted for radioactivity.

Results

FLUORIDE PRODUCTION

The plasma fluoride concentrations for Groups I-IV are summarized in table 1. In Group I (0.5 per cent halothane, 40 per cent O₂) there was little or no fluoride production. Group II (0.5 per cent halothane, 7 per cent O₂) animals had a highly significant increase in plasma fluoride concentration compared with Group I or Group IV (7 per cent O₂; no halothane). The higher halothane concentra-

TABLE 1. Effects of Oxygen and Halothane Concentrations on Plasma Fluoride Levels

	Number of Rats	Halothane (Per Cent)	Oxygen (Per Cent)	F ⁻ (μmol/l)
Group I	6	0.5	40	1.8 ± 0.8
Group II	6	0.5	7	13.9 ± 3.5*
Group III (survived)	7	0.7	7	12.7 ± 4.5*
Group III (died)	7	0.7	7	70.4 ± 26.1†
Group IV	3	—	7	1.1 ± 0.1

Fluoride values represent mean ± SD.

* Differences from Group I values significant, $P < .001$; differences from Group IV values significant, $P < .01$.

† Differences from Group II and Group III (survived) values significant, $P < .001$.

tion to which Group III animals were exposed (0.7 per cent halothane, 7 per cent O₂) did not produce fluoride levels significantly different from those measured in Group II.

Of the Group III animals exposed to 0.7 per cent halothane, 50 per cent died, and fluoride levels in blood from those animals that died were markedly elevated above those in any other group. In a separate study, death purposely produced by severe hypoxia alone (3–4 per cent O₂) without halothane was not associated with elevation of plasma fluoride levels. Plasma fluoride levels were also determined in eight rats from Group II that were allowed to recover in room air for various periods. The plasma fluoride concentration was found to decline exponentially, with a half-life of approximately 1.5 hours.

BINDING

The bound radioactivity from rats exposed to ¹⁴C-halothane is summarized in table 2. Binding to both lipid and protein is expressed in terms of nanomoles of halothane equivalents bound per mg protein in microsomal suspension. Group V had significantly more total binding than Group VI, and this was due

entirely to the more than threefold increase in binding to the lipid fraction. Protein binding was not significantly different between the two groups. The ratio of lipid to protein binding was less than 1.0 in every animal studied in Group VI, while the ratios in Group V animals averaged more than 3.0.

In addition, a definite trend was noted among Group VI rats, in which oxygen concentrations ranged from 25 to 60 per cent. Those animals receiving the highest oxygen concentrations had the lowest amounts of lipid binding and the lowest lipid/protein binding ratios.

Discussion

Previous studies from this laboratory have demonstrated, both *in vitro* and in isolated perfused livers,^{3,7} that halothane binds irreversibly to microsomal lipids and proteins. The observation that binding was greatly enhanced during anaerobic incubations or with low liver perfusion rates led to the speculation that an intermediate produced by a reductive attack on the halothane molecule might be responsible. Under conditions where oxygen was readily available, this intermediate might

TABLE 2. Effects of Hypoxia on Binding of Halothane Metabolites

	Lipid Binding	Protein Binding	Lipid:Protein Binding Ratio	Total Binding
Group V	6.04 ± 1.58*	1.98 ± 0.42	3.24 ± 1.29†	8.02 ± 1.34†
Group VI	1.91 ± 0.71	2.56 ± 0.86	0.76 ± 0.14	4.47 ± 1.49

Binding is expressed as nanomoles of halothane equivalents bound per mg protein in the microsomal suspension (means ± SD for five animals in each group).

* Difference from Group VI value significant, $P < 0.001$.

† Difference from Group VI value significant, $P < 0.005$.

not form, or if it were produced it might be immediately oxidized by activated oxygen. The recent finding of bromochlorodifluoroethyl mercapturic acid in human urine after exposure to halothane suggests the possibility of formation of a defluorinated intermediate, such as $CF_2 = CBrCl$.⁴ Such a species would be highly reactive and could bind covalently to cell components. The finding of elevated fluoride levels *in vitro* in microsomes incubated with halothane anaerobically provided further evidence that defluorination can occur under the proper conditions.⁴

The object of this study was to determine to what extent hepatic hypoxia *in vivo* could mimic the conditions that produced increased binding *in vitro* with microsomes incubated anaerobically. While it is recognized that such low oxygen concentrations are never intentionally used clinically, hepatic hypoxia can result from other causes not always obvious to the observer. Short of measuring the P_{O_2} in hepatic venous blood, one cannot be certain that oxygen delivery to the liver is adequate. Decreased splanchnic blood flow, from whatever cause, coupled with a moderate hypoxemia during clinical anesthesia could result in an oxygen tension in hepatic tissue similar to that produced in these experimental animals. While the hypoxia produced was probably severe, Group IV rats did not lose consciousness, and a few animals from group II that were not sacrificed immediately were apparently able to recover completely.

Previous studies have shown that little or no defluorination of halothane takes place ordinarily.⁵ This study confirms that this is true under conditions where oxidative metabolism is favored. In the presence of hepatic hypoxia, however, fluoride was produced in significant amounts. When hypoxia plus the depression from halothane (Group III) was sufficient to cause death in these animals breathing spontaneously (usually after 1-1.5 hours), fluoride concentrations rose to very high levels, *i.e.*, equal to fluoride concentrations reported after three to four MAC hours of methoxyflurane anesthesia. This is mentioned only to emphasize the significance of the fluoride levels found in this study, not to suggest that fluoride production *per se* is harmful in this case. In a few animals from Group II

that were allowed to recover in room air, plasma fluoride concentrations returned to normal within six hours. This is in contrast to the prolonged elevation seen with methoxyflurane^{10,11} and lends further support to the assumption that release of fluoride from halothane stops when adequate oxygen is available.

The question whether the defluorination reaction that occurs during hypoxia is a potentially harmful pathway, *i.e.*, whether a reactive intermediate is produced, arises. This question could not be answered in a direct manner, but indirect evidence was obtained by studying the binding of ¹⁴C-halothane under similar conditions. The increase in binding during hypoxia supports the hypothesis that reductive metabolism produced a more reactive chemical species. The pattern of the binding was the same as that observed in the *in vitro* anaerobic experiments.³ The ratio of lipid to protein binding was markedly altered due to the more than threefold increase in lipid binding. This alteration in the lipid/protein binding ratio would not be expected if the increased binding were simply a dose phenomenon resulting from increased uptake of ¹⁴C-halothane in Group V animals.

The toxicologic significance of these findings remains speculative. Evidence that binding of metabolites to cellular components is responsible for the toxicity of a number of drugs is accumulating, however. The extent of covalent binding is closely correlated with toxicity of all of the halogenated benzenes.^{12,13} The same is true of the necrosis induced by carbon tetrachloride¹⁴ and by large doses of acetaminophen or furosemide.^{15,16} Pretreatments that change the severity of hepatic necrosis from these drugs cause parallel changes in the magnitude of covalent binding to hepatic macromolecules. In this study all rats were pretreated with phenobarbital in order to induce the microsomal enzymes, which has been found to enhance the metabolism of halothane.¹⁷ Previous studies in isolated perfused rat livers have shown that covalent binding of halothane metabolites to microsomes also increases with phenobarbital pretreatment.⁷ Presumably the covalent binding measured in this study is simply of greater magnitude than that which would have been observed in non-induced animals.

The finding of increased binding of halothane metabolites to microsomal lipids during hypoxia might perhaps serve as a warning and further emphasize the importance of insuring adequate oxygenation of the patient during anesthesia. Hopefully, a more accurate understanding of the metabolism of halothane will be of help in predicting which patients could develop hepatic complications postoperatively. It is noteworthy that obese patients have elevated serum fluoride levels following halothane anesthesia¹⁸—the same type of patient that is likely to have a lower than normal P_{50} ¹⁹ and also has the highest incidence of hepatic complications following exposure to this agent.²⁰ When patients are suspected of having had an inadvertent hypoxic insult to the liver intraoperatively, plasma fluoride determinations in the early postoperative period may provide a clue as to whether reductive metabolism had occurred, and alert the anesthesiologist to the possibility of hepatotoxicity.

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