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1-CHLORO-2,2,2-TRIFLUOROETHYL RADICAL: FORMATION FROM HALOTHANE BY HUMAN CYTOCHROME P-450 IN RECONSTITUTED VESICLES AND BINDING TO PHOSPHOLIPIDS

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SUMMARY. We report the first complete structural characterization of a metabolically-produced halocarbon radical bound to a phospholipid. Human cytochrome P-450 and NADPH cytochrome P-450 reductase were reconstituted into vesicles composed of dioleoylphosphatidylcholine and egg phosphatidylethanolamine. The vesicles were incubated under argon with NADPH and [^{14}C]-halothane (1-[^{14}C]-2-bromo-2-chloro-1,1,1-trifluoroethane), the dioleoylphosphatidylcholine fraction was isolated and subjected to transesterification. Separation of the resulting fatty acid methyl esters resulted in one radioactive fraction which gas chromatography-mass spectrometry revealed to be a mixture of 9- and 10-(1-chloro-2,2,2-trifluoroethyl)-stearate methyl ester.

INTRODUCTION. It has been known that halothane metabolites covalently bind to tissue components since the early autoradiography studies of Cohen and Hood (1). In 1968, Stier proposed that a 1-bromo-1-chloro-2,2,2-trifluoroethyl radical may be involved in the metabolic breakdown of halothane (2). Wood and Van Dyke demonstrated binding of metabolites containing equivalent amounts of [^{14}C] and [^{36}Cl] to a lipid extract under anaerobic conditions (3). Gandolfi *et al* demonstrated that equivalent amounts of [^{14}C] and deuterium are retained in lipid extracts following anaerobic incubation of microsomes with [^{14}C] or [^{3}H]-labeled halothane (4).

A free radical metabolite of halothane is consistent with the demonstration of increased diene conjugation following anaerobic incubations (3) and the appearance of 2-chloro-1,1-difluoroethylene and 2-chloro-1,1,1-trifluoroethane in the expired air of rabbits (5) and human patients (6) exposed to halothane.

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In previous studies of halothane metabolism, we found that when 1-[^{14}C]-halothane was incubated anaerobically with microsomes followed by extraction of the phospholipids and transesterification to form the fatty acid methyl esters an intractable mixture of halothane metabolites bound in many positions to the many possible unsaturated fatty acids was formed. It was not possible to purify a single labeled fatty acid by high pressure liquid chromatography and the mixture would overload a capillary gas chromatography column. In order to circumvent this problem, we developed a reconstituted system in which the number of possible metabolite-fatty acid adducts would be greatly reduced.

We have previously described reconstitution of human (7) and rabbit (8) cytochrome P-450 and NADPH cytochrome P-450 reductase into phospholipid vesicles. A particular advantage of this reconstituted system is that the protein and phospholipid content can be varied in a precise way. In the series of experiments reported here, anaerobic metabolism of halothane was studied in vesicles of cytochrome P-450, NADPH cytochrome P-450 reductase, and cytochrome b_5 reconstituted in a mixture of dioleoylphosphatidylcholine and egg phosphatidylethanolamine. It was anticipated that free radical or carbene metabolites formed from halothane would add to the single 9,10 double bond of the identical oleic acid chains in dioleoylphosphatidylcholine. Although egg phosphatidylethanolamine also would be a non-specific target for metabolite addition, just as microsomal lipids are, it is necessary for optimal activity in the vesicle. The phosphatidylcholine could be separated easily from egg phosphatidylethanolamine so that transesterification of the dioleoylphosphatidylcholine fraction should yield halothane metabolite adducts of methyl oleate that could be separated from methyl oleate. These few possible adducts could then be further purified and identified by gas chromatography-mass spectroscopy.

MATERIALS AND METHODS. Human livers were obtained from Stanford Heart Transplantation Program donors within 15 minutes after cessation of blood flow. Human cytochrome P-450-HA-2 was purified as previously described (7). NADPH cytochrome P-450 reductase was purified from microsomes of phenobarbital-pretreated rabbits (8) to a specific activity toward cytochrome C of 40

μM/mg/min measured at 30 degrees in 0.3M potassium phosphate buffer pH 7.5 with 20% glycerol (9). Cytochrome b_5 was purified from phenobarbital-treated rabbit microsomes as previously described (10) to a purity of over 90%. Reconstitution of the three purified proteins was achieved by a modification of the slow dialysis method (7,8). One ml of a solution of 9.2 mg dioleoylphosphatidylcholine and 4.6 mg egg phosphatidylethanolamine and 190 mg sodium cholate in 0.3M potassium phosphate buffer pH 7.5 was added to 11.5 ml of a solution of 1.4 mg human cytochrome P-450-HA-2, 1.1 mg rabbit cytochrome P-450 reductase, and 0.24 mg cytochrome b_5 in 0.3M potassium phosphate buffer pH 7.5 containing 20% glycerol and 0.5% sodium cholate and stirred overnight at 4° under argon to allow complete formation of mixed micelles. After dialysis against a 20 mM potassium phosphate buffer pH 7.5 with 0.5 mM dithiothreitol, 0.5 mM EDTA, and 20% glycerol for four days at 4° over 90% of cytochrome P-450 was recovered in the vesicles and no cytochrome P-420 was detectable. Suspensions of reconstituted vesicles containing only NADPH cytochrome P-450 reductase in the phospholipid mixture or only the pure phospholipid mixture were prepared exactly as described above for control purposes. Before incubation with halothane, the vesicle preparations were deoxygenated by a stream of argon and the addition of 5 mM β-D-glucose, 50 μg/ml glucose oxidase, and 10 μg/ml thymol-free catalase for one hour at 20°. Then an NADPH-generating system was added through a septum to yield a final concentration of 0.5 mM NADP, 5 mM D-glucose-6-phosphate, and 1 I.U./ml of glucose-6-phosphate dehydrogenase. Finally, 1 μl of halothane (specific activity 1.3 μCi/μl) was added and the mixture was stirred for one hour at 30°. The phospholipids were extracted with 10 ml of chloroform:methanol (2:1, v/v), evaporated and subjected to high pressure liquid chromatography on a 1 x 25 cm Si-100 column with hexane:isopropanol:water. The dioleoylphosphatidylcholine fraction was transesterified with 2 ml of BCl_3:MeOH for two hours at 45°. The resulting methyl esters were separated on a reverse phase C-18 column with MeOH:H_2O (96:4, v/v). The single radioactive fraction was subjected to rechromatography on a C-8 reverse phase column eluting with MeOH:H_2O (90:10). The resulting single radioactive peak was free of interfering non-radioactive peaks as determined by absorption at 208 nm and refractive index. This fraction was subjected to gas chromatography on a fused silica OV-17 capillary column programmed from 50-190° at 10°/min coupled directly to a Varian CH-7 mass spectrometer. Mass spectra were measured with an ionization energy of 20 e.v.

RESULTS AND DISCUSSION. Significant covalent binding of radioactive metabolites to phospholipids was observed following incubation of reconstituted vesicles with [^{14}C]-halothane under argon; the phosphatidylethanolamine fraction contained 16,100 dpm and the dioleoylphosphatidylcholine fraction contained 10,600 dpm. Following transesterification of the dioleoylphosphatidylcholine fraction and separation of the methyl esters on a C-18 reverse phase column, a single peak of radioactivity (8,200 dpm) was eluted at 23 minutes under conditions where methyl oleate eluted at 26 minutes. This radioactive fraction was repurified on a C-8 column and then applied to an OV-17 fused silica capillary column that was directly coupled to a Varian CH-7 mass spectrometer. A mass spectrum was measured with a molecular ion of m/e

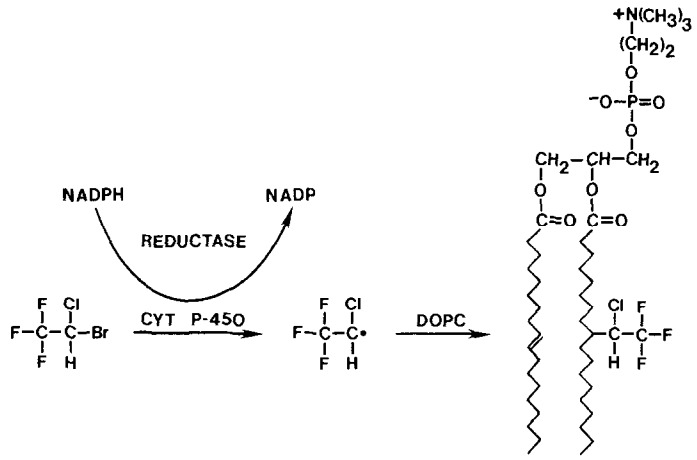

Figure 1: Halothane incubated with vesicles containing human cytochrome P-450 and NADPH cytochrome P-450 reductase and NADPH under an argon atmosphere is reduced to a 1-chloro-2,2,2-trifluoroethyl radical. This radical is released from the cytochrome and adds to the double bond in dioleoylphosphatidylcholine in the surrounding phospholipid bilayer. Transesterification of the dioleoylphosphatidylcholine and purification of the single [^{14}C]-containing adduct followed by gas chromatography-mass spectrometry revealed a mixture of 9- and 10-(1-chloro-2,2,2-trifluoroethyl)-stearate methyl esters.

414. This molecular weight is consistent with addition of a CF_3CHCl radical to the double bond of oleic acid followed by abstraction of a hydrogen radical from a neighbouring donor (Figure 1). The mass spectrum has a doublet at m/e 414 and 416 with a 3:1 intensity ratio that is also consistent with a structure containing one chlorine atom in that the natural abundance ratio of [^{35}Cl] and [^{37}Cl] is 3:1. The mass spectrum has fragment ions at m/e 383 corresponding to loss of CH_3O and at m/e 297 corresponding to the loss of the CF_3CHCl group.

Formation of a trifluoroethyl carbene on cytochrome P-450 following anaerobic incubation with halothane has been postulated as the complex responsible for an absorption peak at 470 nm (11,12). Incubation of the reconstituted human cytochrome P-450 vesicles with halothane as above also produced an absorption peak near 470 nm. It is likely that a cyclopropane product resulting from addition of 2,2,2-trifluoroethyl carbene to the 9,10-double bond of oleic acid would be stable under the conditions of extraction and

transesterification employed in this study. However, this addition product was not observed. The lack of the product of addition of a carbene to a double bond in our study suggests that either the cytochrome P-450-carbene complex is so stable that it does not dissociate once formed; that the lifetime of the carbene is too short to diffuse to a double bond; or that the peak in absorption at 470 nm is not due to a carbene complex.

CONCLUSION. The difficulty we experienced in our attempt to isolate a metabolite bound to a single fatty acid chain in a microsomal incubation may explain why it has not been previously possible to characterize the structure of a halocarbon radical produced by cytochrome P-450 and bound to a phospholipid. In contrast, the use of dioleoylphosphatidylcholine in a reconstituted phospholipid vesicle system provided a single target for radical binding that resulted in isolation of a mixture of a 9- or 10- double bond addition product. The structure of this product suggests that cytochrome P-450 is able to form a free radical from a halocarbon by one electron reduction. Evidence was not found for the 2,2,2-trifluoroethyl carbene that would result from two electron reduction. The transient fatty acid free radical formed following the first step of 1-chloro-2,2,2-trifluoroethyl radical addition to a double bond (Figure 1) is an adequate explanation for the subsequent steps of diene conjugation and lipoperoxidation that are associated with halocarbon metabolism.

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