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Production of 5- and 15-Hydroperoxyeicosatetraenoic Acid from Arachidonic Acid by Halothane-free Radicals Generated by UV-Irradiation

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The authors are studying the molecular details of the process that begins with hepatic metabolism of halogenated inhalation anesthetics and ends with hepatic necrosis. In previous studies they have shown that the halothane-free radical produced by UV-irradiation is identical to that produced during reductive metabolism of halothane by hepatic cytochrome P-450. In the present study, the authors have examined a mechanism by which free radicals may propagate damage in the endoplasmic reticulum of liver cells. The 1-chloro-2,2,2-trifluoroethyl free radical produced by UV-irradiation of halothane can abstract a hydrogen radical from arachidonic acid to yield 2-chloro-1,1,1-trifluoroethane and an arachidonic acid-free radical. The arachidonic acid-free radical reacts with molecular oxygen to form 5- and 15-hydroperoxyeicosatetraenoic acid. There is considerable evidence that the peroxidation process that we studied in the model system will be similar when the arachidonic acid is an acyl chain on a membrane phospholipid and the free radicals are generated metabolically. The authors suggest that these hydroperoxides may be toxic by acting as intermediates in the pathway of leukotriene production as well as by direct oxidation of membrane components. (Key words: Anesthetics, volatile: halothane. Toxicity: free radicals; leukotrienes; metabolites.)

THE KNOWLEDGE OF EICOSANOIDS, physiologically active metabolites of arachidonic acid, has progressed rapidly in recent yerars.[1,2] These potent substances are normally present in minute quantities. However, it is possible that under certain pathologic conditions their production is considerably increased. This hypothesis is substantiated by the demonstration that 5- and 15-hydroperoxyeicosatetraenoic acids (HPETE) can be formed by autoxidation and by a superoxide-mediated process.[3,4] Therefore, it is possible that their production could be increased during metabolism of xenobiotics in which intermediary free radicals are being produced.[5,6] Because arachidonic acid hydroperoxides are thought to be intermediates in the enzymatic formation of leukotrienes, a very potent family of eicosanoids[1,2,7] it is important to examine mechanisms that lead to the production, distribution, and further chemical conversion of HPETEs. In this study we used a chemical model system to produce halocarbon free radicals and examine their subsequent reaction with arachidonic acid in the presence of oxygen. We have shown that under defined conditions, 5- and 15-HPETE accumulate as the major metabolites.

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Methods

Methyl arachidonate (Applied Science Laboratories, Inc.) was purified before use by preparative HPLC on a 1 × 25 cm reverse phase C-18 column at 20° C with a 90:10 methanol:water mixture as eluting solvent. Thymol was removed from halothane by passage through a short column of aluminum oxide (Woelm N, Act. I). ^{14}C-halothane (1-^{14}C-2-bromo-2-chloro-1,1,1-trifluoroethane) was obtained from New England Nuclear and repurified by preparative gas chromatography in our laboratory.

Direct irradiations of halothane and methyl arachidonate in nitrogen-, air-, or oxygen-saturated hexane were carried out in quartz cuvettes with a long wavelength UV lamp of a Chromato-Vue model CC-20 at a distance of 8 cm. Experiments in which halothane vapor was irradiated, but methyl arachidonate was not, were conducted as follows: Fifteen milliliters of halothane was placed in a 25-ml two-neck flask through which nitrogen was bubbled for 1 h at 0° C to remove oxygen. After warming to 20° C to produce sufficient vapor of halothane in nitrogen, the gas mixture was passed through a 4-mm ID quartz tube positioned 2 cm away from a General Electric G4T4-1 ultraviolet lamp. The irradiated vapor was conducted through a glass capillary tube bent at a right angle into a solution of 10 mg purified methyl arachidonate in hexane. Oxygen was introduced into the solution via a second capillary tube. This apparatus also was used to peroxidize methyl arachidonate coated as a film on silanized glasswool in the absence of solvent. No direct irradiation by UV-light of methyl arachidonate occurred in this system as measured by lack of increased hydroperoxides in the control. Following irradiation, the hydroperoxides were isolated by HPLC on a 1 × 25 cm Lichrosorb C-18 column (Altex). Hydroperoxides were identified by their reaction with iodide/starch, by their diene absorbance, and by a change of the retention time on HPLC after reduction. Reduction of the hydroperoxides (10 μg) to alcohols was performed with a molar excess of triphenylphosphine in 1 ml wet ether at 0° C for 30 min. Reduction products were isolated by HPLC on a 0.46 × 25 cm reverse-phase Spherosorb octadecylsilyl

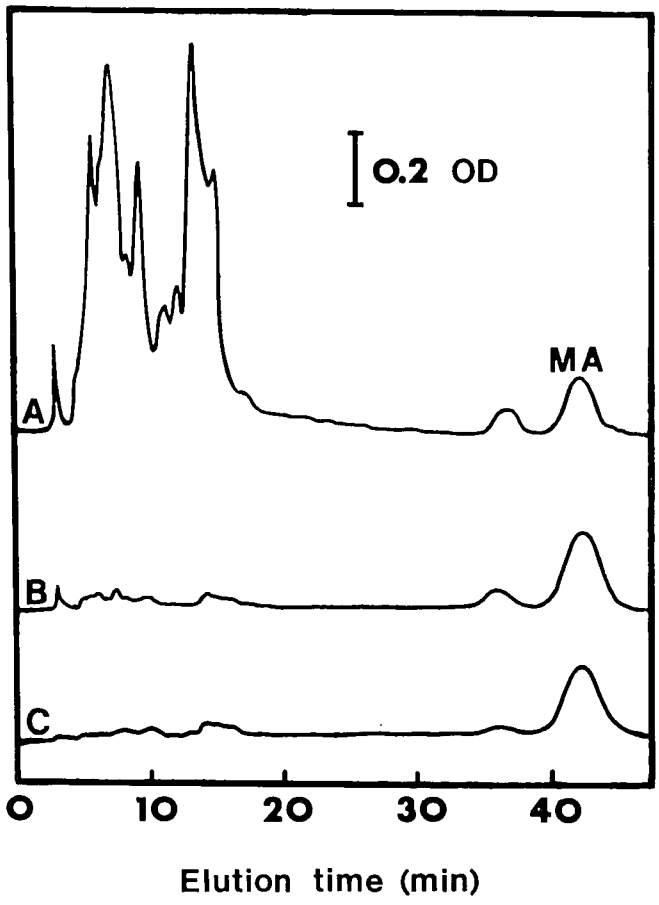

FIG. 1. Increased formation of conjugated hydroperoxides from arachidonic acid methyl ester. Trace A: UV-irradiated halothane vapor in N_2 was bubbled at 18°C through a solution of 10 mg methyl arachidonate in 5 ml hexane for 2 h at a flow rate of 180 ml/min. Oxygen was supplied through a second glass capillary at a flow rate of 60 ml/min. The unmodified methyl arachidonate (MA) was separated from the mixture of oxidized products that eluted between 5 and 18 min on a 1 × 25 cm Lichrosorb reverse-phase C-18 column with methanol/water 9:1 as an eluting solvent at a flow rate of 2.5 ml/min, monitored at 235 nm. Trace B: Same as A but no UV-irradiation. Trace C: Same as A but no halothane vapor.

(ODS) (Altex, Berkeley, California) column by elution with methanol/water, 80:20 (vol/vol) at a flow rate of 1 ml/min. The trimethylsilyl ether derivative was prepared by dissolving the purified hydroxy-eicosatetraenoic acid methyl ester in 200 μl N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane. After 10 min at 50°C the reagent was removed by a stream of nitrogen, and the final product was purified on the same ODS-column with methanol/water 90:10 (vol/vol) as a solvent. The overall yield was 54% for the silyl ether of 5-HETE. All methyl esters were extracted from methanol/water mixtures by addition of five volumes of water and two volumes of diethyl ether that had been distilled freshly over $LiAlH_4$. The ether phase was concentrated with a stream of N_2 at 20° C. All products could be stored for days in diethyl ether under nitrogen at −20° C.

Mass spectra were obtained with a Varian MAT-44 with a solid probe inlet and electron impact ionization. Absorption spectra were measured with a Cary 219.

Results

Halothane-free radicals in N_2 as carrier gas were allowed to react with methyl arachidonate in hexane in the presence of oxygen. Separation of the product mixture by HPLC on a C-18 reverse phase column produced chromatograms that were different, depending on whether halothane-free radicals were present or not. Elution of the products was monitored at 235 nm in order to detect products from methyl arachidonate that contain conjugated dienes. Trace A in figure 1 shows a complex mixture of polar products that were eluted between 4 and 20 min after injection. Traces B and C show that UV-irradiation of halothane vapor was necessary to produce these products in high yields. The yield of these products was dependent on the flow rates of the two gas phases: halothane in nitrogen and oxygen. Product formation was not linear with reaction time, but showed a delayed onset and increased formation of the most polar products at longer times. The concentration of methyl arachidonate was important; no product formation was observed under the same conditions at concentrations less than 0.1 mg/ml. Higher amounts of oxidation products were obtained when arachidonic acid was coated as a thin film on silanized glass wool and exposed to halothane radicals in the gas phase. These qualitative dependencies are indicative of auto-catalytic free radical mechanisms.

In figure 1, the polar products are well separated by HPLC from unchanged methyl arachidonate (MA) and from some small amounts of nonpolar dienes that eluted at 37 min. The entire mixture of polar products that eluted between 5 and 20 min was extracted into ether and separated into single products on a reverse phase ODS column. Figure 2 shows such a separation. Several of those products that eluted between 10 and 35 min as well as all of those that eluted between 55 and 75 min gave a positive starch–iodine reaction for hydroperoxides. The two diene hydroperoxides of methyl arachidonate that eluted at 58 and 74 min in figure 2 were extracted individually into ether and reduced to the corresponding alcohols with triphenylphosphine. The elution position of the fraction that had eluted at 58 min was little changed after reduction such that co-chromatography of equal amounts of the reduced and unreduced compound resulted in only a wider peak. Reduction of the fraction that had eluted at 74 min increased the retention time

such that cochromatography of aliquots of the reduced and unreduced forms resulted in a doublet with a separation of 1 min in methanol/water 80:20. The individual hydroxy-derivatives were trimethylsilylated and repurified on the same column in methanol/water 90:10. Mass spectrometry of the two main products allowed their identification as the methyl ester and silyl ether derivatives of 15- and 5-HPETE. Because the peroxidation is nonenzymic, it will produce a racemic mixture of the R and S hydroperoxides. Therefore, only half of the 5-HPETE that is formed is 5-S-HPETE that could lead to other biologically active molecules. It is very likely that the peaks between the 5- and 15-HPETE in figure 2 represent the 8, 9, 11, and 12 isomers. Thus, this technique yields microgram quantities of these various isomers.

The mass spectra of the trimethylsilyl ether derivatives are shown in figure 3. We suggest the following rationalization of the major fragment ions in the mass spectrum of the 15-Me_3Si ether derivative of methyl arachidonate: m/e 391 [M-15:loss of $^{\bullet}CH_3$]; 375 [M-31:loss of $^{\bullet}OCH_3$]; 335 [M-71:loss of $CH_3(CH_2)_3CH_2^{\bullet}$]; 316 [M-90:loss of $(CH_3)_3SiOH$]. The following fragment ions result from charge retention on the siloxy: m/e 279 [fission between C-6 and C-7]; 225 [fission between C-10 and C-11]; 173 [fission between C-14 and C-15].

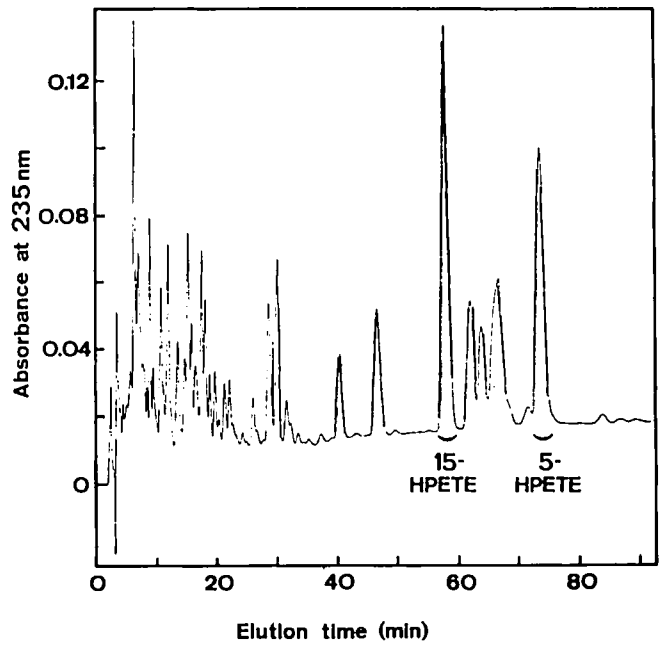

FIG. 2. Rechromatography of that portion of the oxidized product mixture that eluted between 5 and 18 min in fig. 1A on a 0.46 × 25 cm reverse-phase Spherosorb ODS column by elution with methanol/water 8:2. The two main products were the 5- and 15-hydroperoxyeicosatetraenoic acids (HPETE).

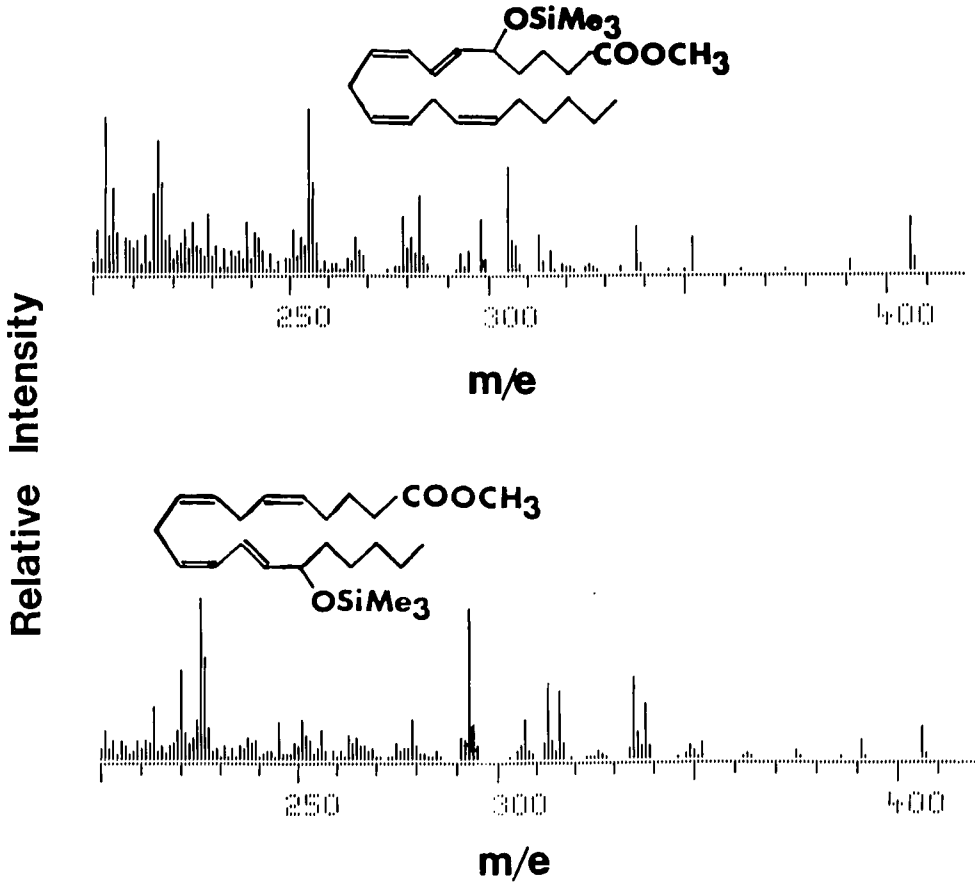

FIG. 3. Mass spectra of the purified methyl ester-trimethylsilyl ether derivatives of 5- and 15-hydroxyeicosatetraenoic acids.

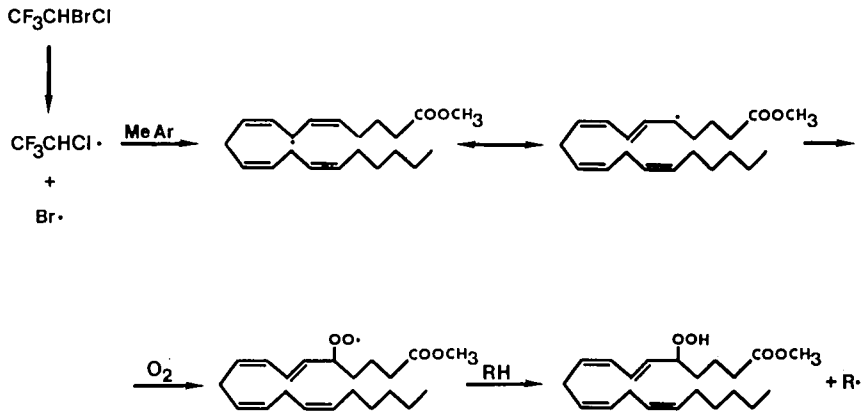

FIG. 4. Possible mechanism for the formation of 5-HPETE by free radical induced oxidation.

The mass spectrum of the 5-Me_3Si ether derivative of methyl arachidonate could be interpreted in an analogous way: m/e 406 (M); 391 [M-15]; 375 [M-31]; 316 [M-90]; 305 [M-101:loss of $^{\cdot}CH_2$-$(CH_2)_2$-$COOCH_3$]; 255 [M-151:loss of $^{\cdot}CH_2$-$(CH{=}CH$-$CH_2)_2$-$(CH_2)_3$-CH_3]; 215 [305-90]; 203 [$(CH_3)_3C$-$SiO^+{=}CH$-$(CH_2)_3$-$COOCH_3$]. The mass spectra are in qualitative agreement with those obtained with products from hydroxyl radical-induced oxidation of arachidonic acid.[4] The Me_3Si ether derivative of 5-hydroxy-6,8,11,14-eicosatetraenoic acid methyl ester that was obtained as a product after adding arachidonic acid to a suspension of peritoneal neutrophils[8] gave a mass spectrum similar to the one in figure 3, top.

Direct irradiation of a solution of thymol-free halothane and arachidonic acid at various concentrations in hexane resulted in very complex product mixtures. In a previous study on spin trapping of halothane radicals,[9] we also found separation of the site of production of radicals and the site of their subsequent reaction to be necessary for a study of only radical-induced reactions.

Discussion

Halothane-free radical-induced oxidation of methyl arachidonate results in the formation of 5- and 15-HPETE as the main products. It is significant that these two products are the main constituents in the product mixture and that they are sufficiently stable under conditions of continued oxidation to accumulate. In studies of the autoxidation of arachidonic acid, the hydrogen atoms at positions 7, 10, and 13 have been shown to be particularly susceptible to hydrogen transfer because of the stabilized pentadienyl radical intermediate that is formed. A free radical-induced formation of arachidonic acid hydroperoxides also has been shown in another *in vitro* system, co-oxidation of arachidonic by a superoxide radical anion generating system.[10] Our experiments suggest that halocarbon radicals are also capable of abstracting a hydrogen radical from these activate methylene positions. Figure 4 illustrates a mechanism that would lead to the formation of 5-HPETE and leave a free radical for further propagation.

The finding that in a chemical model system 5- and 15-HPETE can be accumulated during halocarbon free radical-induced oxidation may be an important link in understanding lipid peroxidation during metabolism and subsequent toxicity. Free radicals have been suggested to be involved in lipid peroxidation reactions in the endoplasmic reticulum.[11] However, in most studies chemical changes in the phospholipids were followed by the appearance of late oxidation and breakdown products of lipids such as malondialdehyde. Most of these products may be of no physiologic relevance at the low concentrations at which they occur. However, if 5-hydroperoxy-arachidonic acid chains are formed from phospholipids of the endoplasmic reticulum during halocarbon metabolism, then cleavage of the phospholipids by phospholipase A_2[12] will release 5-hydroperoxyeicosatetraenoic acid into the cell. In the case of 5-hydroperoxytetraenoic acid, it is also a precursor for enzymic conversion to leukotrienes. The generation of leukotrienes in the liver could trigger an inflammatory response involving changes in microcirculation and cell permeability as well as activation of lysosomes and macrophages.

Increased production of fatty acid hydroperoxides during halocarbon metabolism also may induce liver necrosis by a direct mechanism. Fatty acid hydroperoxides may be toxic to cells through inactivation of important proteins by oxidation of sulfhydryl groups to disulfides or by initiating cross-linking and autoxidation of membrane phospholipids. The oxygen gradient across a liver lobule ranges from 100 μM (74 mmHg) at the portal vein to as low as 1 μM (0.74 mmHg) at the hepatic vein.[13] Hypoxic cells exposed to oxygen concentrations less than 30 μM (22 mmHg) have large alterations in their NADPH/$NADP^+$, NADH/NAD^+, and ATP/ADP ratios.[13] In these cells the increased consumption of glutathione during reduction of hydroperoxides by glutathione peroxidase may result in a substantial alteration in the intracellular redox potential.[14] It has been shown

that reductive metabolism of halothane will occur when the oxygen concentration is below 50 μM.[15] Therefore, there is a range of intracellular oxygen concentration, possibly near 5–10 μM, in which production of halothane radicals occurs by reductive metabolism,[6] oxygen is sufficient to form hydroperoxides, and a cell has a reduced ability to destroy hydroperoxides. We recently have shown that hepatic cytochrome P-450 catalyzes the conversion of 5-S-HPETE to leukotriene B_4 *in vitro*.[16] If this highly biologically active molecule also is produced in the liver *in vivo*, it may be a step in the initiation of liver necrosis.

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