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Oxidation of glycerophospholipids from biological membranes by reactive oxygen species: liquid chromatographic–mass spectrometric analysis of eicosanoid products

Robert C. Murphy*, Nona Khaselev, Tatsuji Nakamura, Lisa M. Hall

Department of Pediatrics, Division of Basic Sciences, National Jewish Medical and Research Center, 1400 Jackson Street, Denver, CO 80206, USA

Abstract

Peroxidation of glycerophospholipids present in cellular membranes results in the formation of a complex mixture, with many products derived from the oxidation of esterified arachidonic acid. Techniques of chromatography and mass spectrometry have facilitated the elucidation of the structure of individual components present as intact glycerophospholipids as well as the oxidized fatty acyl groups liberated from the glycerol backbone by saponification. Previously reported studies are summarized in this overview concerning those oxidized products of arachidonic acid derived from the red blood cell membrane, studied by techniques of electrospray tandem mass spectrometry developed to analyze eicosanoid products.

Keywords: Glycerophospholipids

1. Introduction

Advances in techniques of chromatography and mass spectrometry have facilitated detailed studies of the complexity of chemical reactions taking place within biological membranes as a result of the formation of active oxygen species. Polyunsaturated fatty acids esterified to glycerophospholipids (Fig. 1) that make up the cellular membrane, serve as the primary precursors of a wealth of complex products, both large and small, formed as a result of these free radical reactions. Recent interests have focused on a series of metabolites derived from arachidonic acid that have chemical structures similar to the enzymatically derived products of arachidonic acid oxidation. These include the isoprostanes [1], isoleukotrienes [2], as well as products from more complex reactions such as the levuglandins [3]. Docosahexaenoic acid has also been found to be a precursor of even more complex molecules, termed neuroprostanes [4]. Such molecules have found significant utility in serving as markers of the initiation of free radical events within tissues [5]. In the past, substantially smaller products of free radical reactions, such as pentane, malondialdehyde and 4-hydroxynonenal, were the principle marker substances analyzed. However, such low-molecular-mass products are probably the result of multiple reactions that are far removed from the initial oxidation of a polyunsaturated fatty acyl group esterified to phospholipids. The largely intact free radical product species may be more relevant targets

*Corresponding author. Tel.: +1-303-398-1849; fax: +1-303-398-1694.
E-mail address: murphyr@njc.org (R.C. Murphy)

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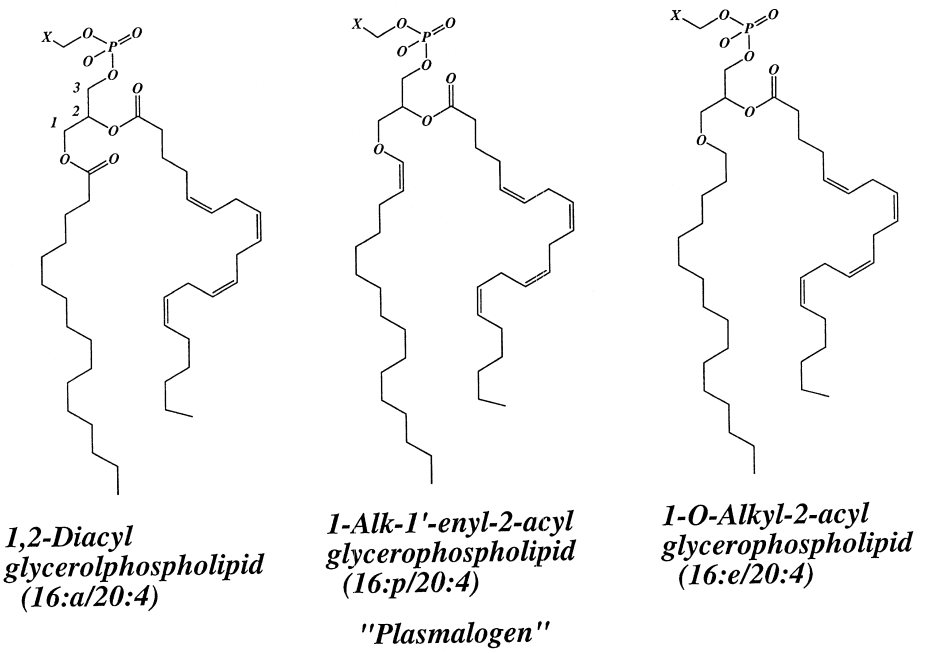

Fig. 1. Common glycerophospholipid families containing arachidonic acid esterified to the second carbon atom of glycerol (*sn*-2) found in all mammalian cells. Specific classes of phospholipids have a polar head group extending from the phosphate ester and indicated by 'X': CH_2–NH_2, ethanolamine; $CH(NH_2)COOH$, serine; $CH_2N^+(CH_3)_3$, choline, and CH_2–O–$C_6H_{11}O_6$, inositol.

to assess initiation of free radical events within cellular membranes.

In addition to the marker function served by these molecules, several compounds have been found to possess significant biological activity, including the isoprostanes [1], isoleukotrienes [2], as well as oxidatively fragmented phospholipids [6] derived from membrane precursors. These latter molecules appear to exert activity through specific receptors, including the PAF receptor [7].

We present here an integrated overview of previously reported studies [8–11] of free radical oxidation of red blood cell (RBC) membrane phospholipids in the context of the use of chromatography-based mass spectrometric techniques for complex mixture analysis. One of the most challenging aspects of studies of lipid peroxidation products has been that a large number of closely related compounds are simultaneously produced as a result of initiation of free radical reactions. These mixtures challenge the technical capabilities available to characterize even the most abundant products formed. Of particular value has been the development of electrospray ionization and tandem mass spectrometry. The combination of these techniques provides a multidimensional path for the separation of quite complex mixtures that are not obtainable by chromatographic means alone. This is not to minimize the importance of the developments taking place in chromatography and, in particular, in the development of diverse chromatographic columns, with different separation chemistries. Also, the development of microbore columns with flow-rates closely matched to flow-rates readily accepted by electrospray ionization have enabled one to maintain relatively high concentrations of minute samples in the chromatographic mobile phase, facilitating abundant positive and negative ion formation during electrospray ionization.

The complexity of membrane-derived lipid peroxidation products can be readily illustrated in the study of one polyunsaturated fatty acid, namely arachidonic acid, which is present in glycerophospholipids of the membranes of the human red blood cell. We have investigated several models of oxidation of the fatty acyl groups in RBCs and, as a result, have

generated complex mixtures of both oxidized glycerophospholipids as well as oxidized fatty acids that can be analyzed by liquid chromatography–mass spectrometry (LC–MS) and LC–tandem mass spectrometry (MS–MS) techniques. Such studies have revealed a wealth of information concerning the events taking place during free radical oxidation of the RBC membrane, including an unexpected finding of the lability of plasmalogen glycerophospholipids containing the arachidonoyl substituent at the *sn*-2 position.

2. Experimental

2.1. Materials

Eicosanoids, including epoxyeicosatetraenoic acid (ETE) and hydroxyeicosatetraenoic acid (HETE) standards, were purchased from Cayman Chemical (Ann Arbor, MI, USA), [$^{18}O_2$]12-HETE was prepared from butyrylcholine esterase and $H_2[^{18}O_2]$, as previously described [12]. Reagent-grade chemicals were obtained from Sigma (St. Louis, MO, USA), as were *tert*.-butylhydroperoxide (tBuOOH) and butylated hydroxytoluene (BHT). All solvents were of HPLC grade (Fisher, Fairlane, NJ, USA). Purified L-α-phosphatidylethanolamine (bovine brain) was purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Preparation of red blood cells and red blood cell ghosts

Venous blood was obtained from normal human volunteers and treated with sodium citrate to prevent coagulation. RBCs were isolated as previously described [11] by centrifugation techniques to remove platelet-rich plasma and the buffy coat layer containing granulocytes. The RBCs were washed three times in phosphate buffered saline (PBS) and the RBCs were resuspended to a final concentration of 2×10^8 RBC ml^{-1} in PBS. For studies involving incubation of intact RBCs, this preparation was treated with and without tBuOOH (10 m*M* final concentration) at 37°C for 90 min. After incubation, the suspension was centrifuged at 900 *g* for 10 min; the pellet was washed three times after resuspension in 10 ml of PBS prior to phospholipid extraction. Phospholipids were extracted from cell pellets following the method of Rose and Oklander [13], which employed isopropanol–chloroform mixtures to minimize problems encountered with the presence of large amounts of hemoglobin. Butylated hydroxytoluene was added to extracts (1 m*M*) to prevent further lipid peroxidation.

The preparation of RBC ghosts from isolated RBCs was carried out as previously described [8]. Briefly, isolated RBCs were centrifuged at 1700 *g* for 15 min to yield packed RBCs. Aliquots of these packed RBCs (50 μl) were placed in separate centrifuged tubes and resuspended in 3 ml of cold lysis buffer (PBS, diluted fivefold), containing 1 m*M* $MgCl_2$, 0.1 m*M* EGTA (for 1 h on ice). Following lysis, the suspension was centrifuged at 26 000 *g* for 15 min, the supernatant was discarded and the pellet was resuspended in 3 ml of water. The suspension was then passed through a 22-gauge syringe needle several times, to ensure that lysis was complete. The process of centrifugation and resuspension in distilled water was repeated four to five times to obtain fairly white RBC ghosts. The ghosts were then resuspended in 1.7-fold-concentrated PBS (4 ml), sealed by placing them in a 37°C water bath for 30 min and were stored at 4°C overnight. The ghosts were centrifuged at 1700 *g* for 15 min and then lysed with distilled water on ice, resealed, and pelleted by centrifugation immediately before incubation. After preparation, the RBC ghosts were incubated (2 ml of ghosts at 1.75 mg protein/ml ghosts) at a final concentration of 10 m*M* tBuOOH for 90 min at 37°C. Each reaction was terminated by the addition of 5 mg of BHT dissolved in 1 ml of methanol, which inhibited further oxidation prior to analysis. Lipids were extracted by the method of Bligh and Dyer [14].

For the studies of RBC ghosts and intact RBCs, phospholipid classes were purified and separated into glycerophosphoethanolamine (GPE), glycerophosphoserine (GPS) and glycerophosphocholine (GPC) lipid classes by normal-phase high-performance liquid chromatography (HPLC), essentially following a previously described protocol [8]. Since glycerophosphoinositol lipids were a minor phospholipid class in normal RBCs, this class was not collected for individual study. The normal-phase

HPLC was performed using a 5-μm silica (Licrosorb; Phenomenex, Torrence, CA) analytical column (250×4.6 mm I.D.) with a gradient of 53% solvent A (hexane–isopropanol; 3:4, v/v), which was held isocratically for 6 min and then a linear gradient to 100% solvent B (hexane–isopropanol–20 m*M* ammonium acetate, pH 6; 3:4:0.7, v/v/v) for 20 min. Slightly different HPLC conditions were used to separate the glycerophospholipid classes of the intact RBCs [9].

2.3. Saponification

Hydrolysis of the phospholipid fatty acyl groups present in the extracted and individual isolated phospholipid classes was carried out at room temperature for 1 h by the addition of 1.5 ml of 1 *M* NaOH. The reaction mixture was acidified by the addition of 50 μl of 88% formic acid and the sample was concentrated to approximately 200 μl. The sample was then diluted with 1 ml of methanol–acetonitrile (35:65, v/v) and internal standards were added to this extract for quantitative analysis.

Reversed-phase HPLC of oxidized fatty acyl groups was carried out in a 5-μm ODS (100 Å; Prodigy, Phenomenex, Torrence, CA, USA) analytical column (250×1.0 mm I.D.). The mobile phase consisted of a gradient from 20% 6.5 m*M* ammonium acetate (pH 5.7) programmed to 100% solvent B (methanol–acetonitrile; 35:65, v/v) over a 15-min period at a mobile phase flow-rate of 50 μl/min.

2.4. Oxidation and solid-phase extraction of bovine brain GPE

Bovine brain GPE lipids (10 μmol) were suspended in 6.5 ml of 50 m*M* PBS (pH 7.4) by vortex-mixing and sonication. This suspension was then treated with H_2O_2 and $CuCl_2$ at final concentrations of 70 m*M* and 100 μ*M*, respectively. This mixture was then gently shaken at 37°C for a time period up to 3 h. During the course of this oxidation, the absorbance at 235 nm was monitored at 30 min intervals. Incubation was stopped at 4°C and with the addition of 1 vol. of $CHCl_3$ containing 5 mg/ml BHT. The lipids were then extracted according to the method of Bligh and Dyer [14].

Further separation of the extracted lipids was carried out by drying an aliquot of the extract and suspending the dried lipid extract in 10 ml of distilled water. The extract suspension was then loaded onto a C-18 reversed-phase 40 μm silica Sep Pak cartridge (solid-phase extraction) that had been preconditioned with 10 ml of methanol and 10 ml of distilled water. After addition of the sample, the excess salts were eluted with water and the phospholipid fraction eluted with 10 ml of 80% methanol followed by a second elution of 10 ml of 90% methanol and 10 ml of pure methanol. The organic-phase extracts were taken to dryness, then redissolved in 1 ml of chloroform–methanol (2:1, v/v) prior to direct analysis by electrospray tandem mass spectrometry.

2.5. Mass spectrometry

Mass spectrometric analysis was performed on Sciex API-III$^+$ (PE-Sciex, Toronto, Canada) operated in the negative ion mode with an orifice potential of −55 V (except as noted) and a collisional offset potential of 11 eV for those experiments involving collision-induced decomposition. The curtain gas flow was set to 1.2 l/min, the nebulizer pressure was set to 40 p.s.i. (1 p.s.i.=6894.76 Pa) and the ion spray voltage was set at −3500 V. A high purity air (zero air) generator (Packard Instruments, Downers Grove, IL, USA) was used to supply nebulizing gas and to reduce the glow discharge at the ion spray needle. Argon was used in the second quadrupole collision cell at a pressure equivalent to 240×10^{13} molecules/cm^2 for collision-induced decomposition reactions.

3. Results

The normal-phase separation of phospholipids present in the RBC membrane (ghosts or intact RBCs) is illustrated in Fig. 2A, which shows the elution of the arachidonate-containing phospholipids during the 40-min HPLC elution. Arachidonic acid is present not only as cholesterol esters (neutral lipids), but also in several individual phospholipid molecular

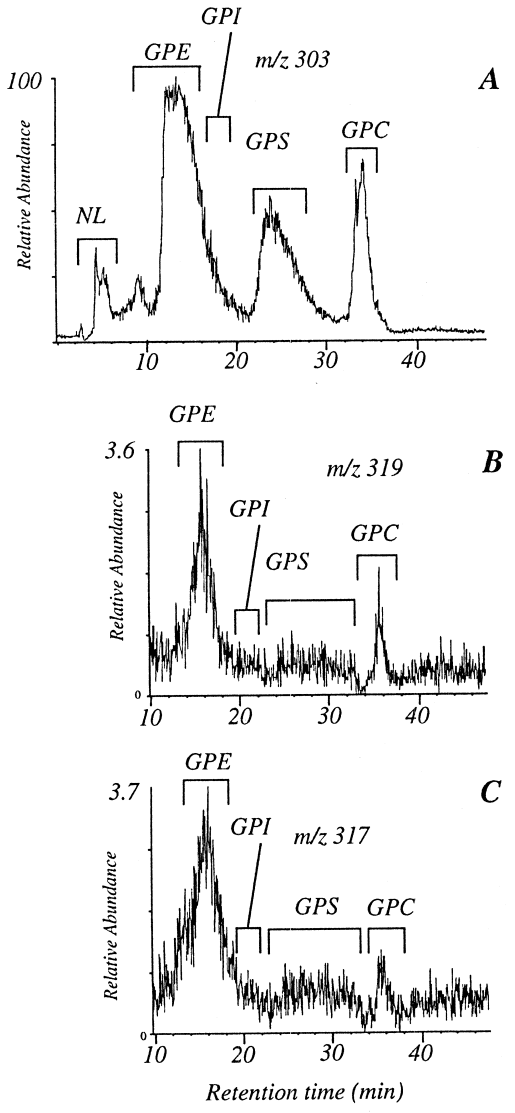

Fig. 2. LC–MS and ion chromatograms of phospholipids extracted from red blood cell ghost membranes and separated by NP-HPLC prior to electrospray mass spectrometry. A high orifice potential, −110 V, was employed to induce decomposition in the ion source region. The extracted ions from a control sample (A) for m/z 303 corresponded to arachidonic acid (20:4) esterified to phospholipids. Extracted ions (B) from oxidized red blood cell ghosts (10 m*M* tBuOOH) for m/z 319 corresponded to monooxygenated arachidonic acid, and extracted ions from oxidized RBC ghosts for m/z 317 corresponding to (C) arachidonate after the addition of two oxygen atoms and the loss of water. Individual phospholipid classes containing these fatty acyl groups are indicated as follows: NL, neutral lipids; GPE, glycerophosphoethanolamine lipids; GPS, glycerophosphoserine lipids; GPC, glycerophosphocholine lipids.

species within each class, causing the HPLC peaks to appear quite broad. The separation of glycerophosphoinositol lipids containing arachidonic acid was not complete from certain GPE molecular species. The tandem mass spectrometer was used to specifically detect those phospholipids eluting from the HPLC column that contained arachidonic acid, by forming the carboxylate anion of arachidonic acid (m/z 303) during electrospray ionization, as indicated in Fig. 3. This was done by elevating the skimmer voltage to over −110 V and, thus, providing relatively high energy to the ions for collision-induced dissociation reactions in the high pressure region of the electrospray interface. Thus, a fatty acid-specific profile could be obtained for the elution of those glycerophospholipid classes containing arachidonic acid (Fig. 2A). The same strategy was used to analyze the phospholipids (Fig. 2B and C) extracted from the RBC ghosts following treatment with tBuOOH, but monitoring the formation of monooxygenated arachidonic acid (m/z 319) formed during collisional activation of all glycerophospholipid ions, as shown in Fig. 3 during electrospray ionization. These results suggested that the abundance of the monooxygenated arachidonic acid fatty acyl groups present in oxidized RBC ghosts closely followed the distribution of arachidonic acid in the precursor glycerophospholipid classes. Similar results were obtained for the analysis of oxidized linoleic acid (m/z 297, data not shown) as well as arachidonic acid formed by the addition of two oxygen atoms and loss of water (m/z 317) from hydroperoxides (Fig. 2C).

A different distribution of oxidized arachidonate within a phospholipid class was obtained when a detailed analysis of the individual molecular species present in normal and oxidized RBC ghosts was performed. For this experiment, the phospholipid classes were initially separated by NP-HPLC (Fig. 2) and each phospholipid class was separately analyzed by tandem mass spectrometry. This precursor ion strategy involved sequentially isolating each molecular ion by the first quadrupole mass analyzer of the individual molecular species formed during electrospray ionization, then collisionally activating each molecular anion in the second quadrupole region to form the ion m/z 303, corresponding to arachidonate, which was specifically detected by the third quad-

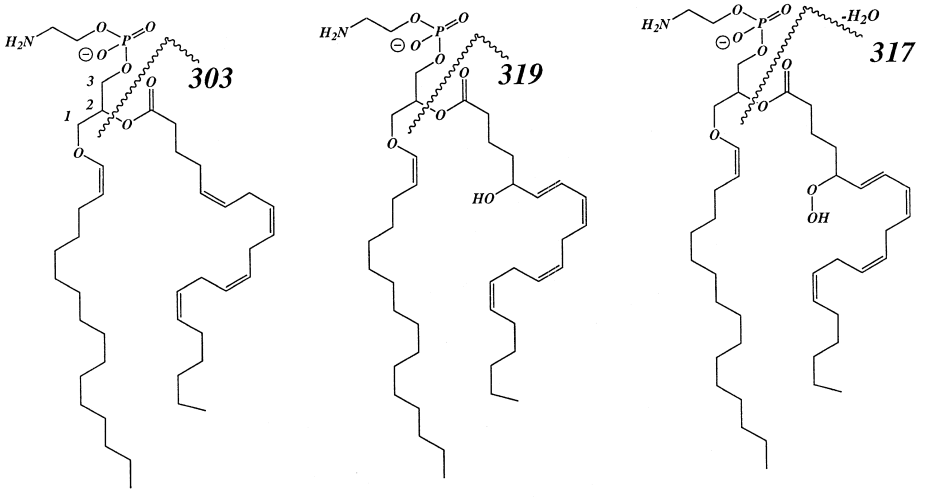

Fig. 3. Specific molecular anions from species of 16:0p/20:4-GPE (plasmalogen) and oxidized analogs formed by electrospray ionization. Fatty acyl product ions obtained following collisional activation of $[M-H]^-$ (MS–MS) are indicated.

rupole mass analyzer. Using this precursor ion scanning strategy [15], it was possible to readily identify the most abundant molecular species of GPE and GPC lipids containing arachidonate (Fig. 4A), for example, the most abundant molecular species containing arachidonate esterified at the *sn*-2 position were found to be 16:0p/20:4 (*m/z* 722.5), 16:0a/20:4 (*m/z* 738.5), 18:0p/20:4 (*m/z* 750.5) and 18:0a/20:4-GPE (*m/z* 765.5). When the precursor molecular species for *m/z* 319, corresponding to monooxygenated arachidonate, were analyzed in those RBCs exposed to tBuOOH, a very different molecular species precursor pool was identified (Fig. 4B). In fact, the most abundant phospholipid molecular species containing *m/z* 319 corresponded to a diacyl species and the plasmalogen-containing molecular species that contain a vinyl ether substituent at the *sn*-1 position were substantially less abundant. This experiment revealed that, in spite of the fact that abundant arachidonate-containing molecular species were observed in GPE or GPC phospholipids as plasmalogens as previously reported [16,17], those molecules that remained as intact oxidized phospholipids after exposure to reactive oxygen species were primarily diacyl molecular species. This finding was also true for those molecular species that retained hydroperoxides at the *sn*-2 position. Thus, it appeared that, in a complex mixture of phospholipids, the plasmalogen species that can, under normal conditions, represent the largest reservoir of arachidonic acid were not proportionally surviving the oxidation protocol.

In order to further investigate this phenomenon, purified GPE lipids from bovine brain were subjected to oxidative stress using Fenton-like reaction conditions (Cu^{++}/H_2O_2) [10]. An example of the reversed-phase HPLC separation of glycerophospholipids is shown in Fig. 5 for a mixture of glycerophosphocholine molecular species. Reversed-phase HPLC separated the glycerophospholipids based on the hydrophobic character of the two radyl groups at *sn*-1 and *sn*-2, with the polar head substituent at *sn*-3 having little influence on the retention time. Nonetheless, detection of the separated components eluting from the HPLC column is somewhat problematic in that the most common

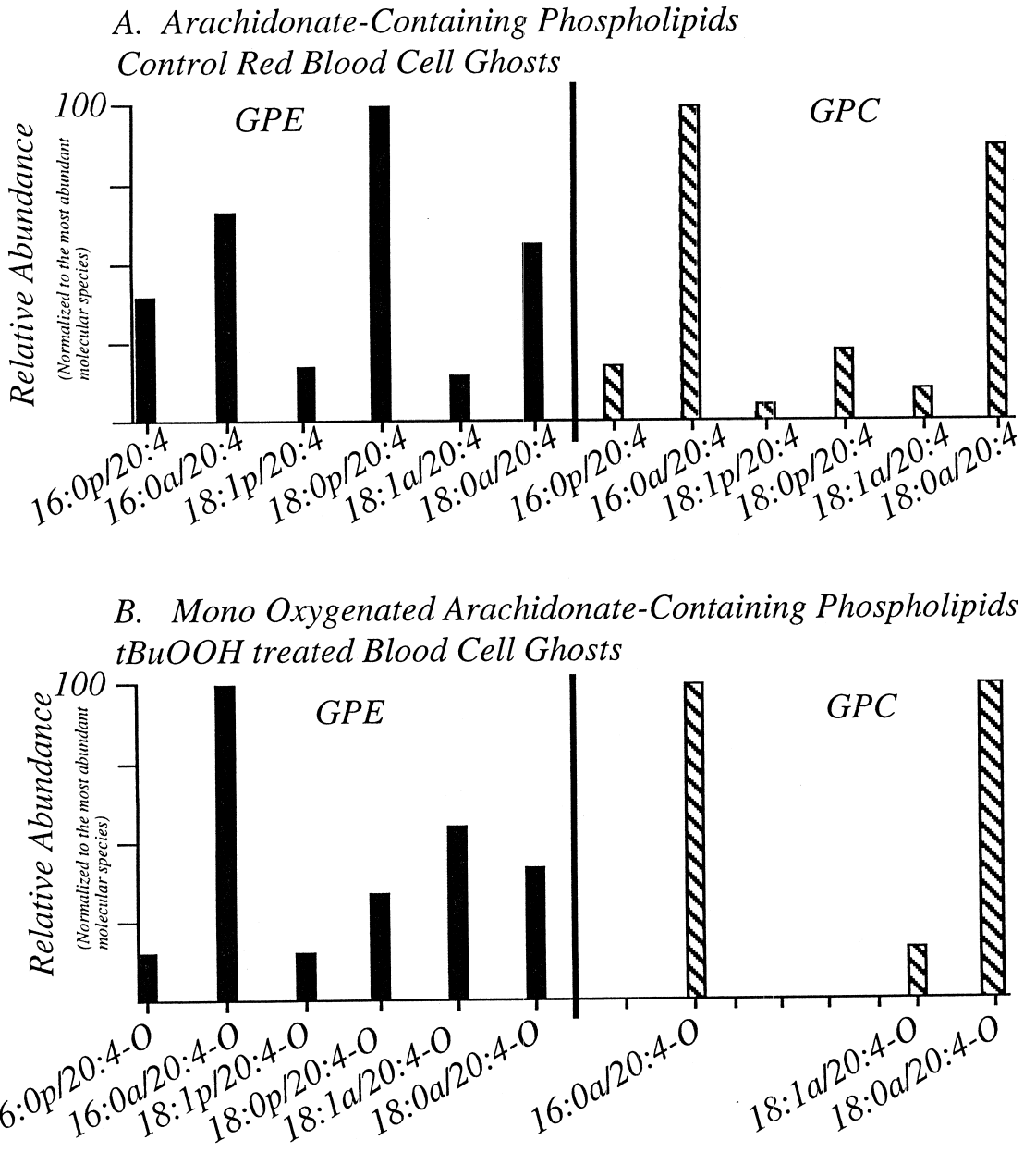

Fig. 4. (A) Major molecular species of GPE and GPC lipids containing esterified arachidonic acid extracted from control red blood cell ghosts. (B) Major molecular species of GPE and GPC phospholipids containing esterified monooxygenated arachidonic acid following treatment of RBC ghosts with tBuOOH (10 m*M*). Molecular species were identified using electrospray ionization tandem mass spectrometry. The abundance of each molecular species was normalized to the most abundant within each class. Abbreviations used to identify individual molecular species have the identity of the radyl group in the *sn*-1 and *sn*-2 positions indicated by the numerical order in which they are written, for example, 16:0p/20:4 GPE corresponds to a 16-carbon radyl group with no double bonds at *sn*-1 and 20:4 corresponds to a 20-carbon fatty acid with four double bonds at *sn*-2. The lower case letter following the *sn*-1 radyl group indicates the type of linkage at *sn*-1, with either 'a' or 'p' representing acyl or vinyl ether, which is commonly referred to as plasmalogen. The most abundant GPE in control RBC ghosts corresponded to plasmalogen molecular species.

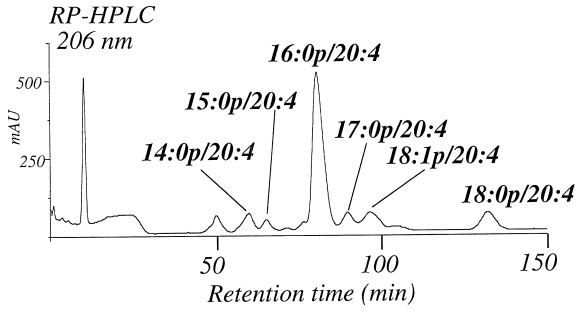

Fig. 5. Reversed-phase HPLC separation of a commercial mixture of semisynthetic glycerophosphocholine lipid molecular species. A 10-μm C_{18} column (250×10 mm) was isocratically eluted using a mobile phase of methanol–acetonitrile–1 m*M* ammonium acetate buffer (90:3:7, v/v/v). The effluent was monitored at 206 nm. Individual molecular species were characterized by electrospray tandem mass spectrometry.

detector, a monitor of UV absorption, is extremely selective for certain molecular species, namely those that contain one or more double bonds in either radyl chain when monitored at 206 nm. The greatest sensitivity is thus obtained for those species containing polyunsaturated fatty acyl substituents. Although reversed-phase HPLC separation may not be complete for all molecular species, on-line mass spectrometric analysis of the HPLC effluent permits unique detection of each of the molecular species present in such a complex mixture of phospholipid classes based on HPLC retention time, molecular ion mass and collision-induced decomposition product ions. Using such techniques as well as precursor ion scanning, it was possible to uniquely define eight major molecular species separated from bovine brain GPE lipids (data not shown), of which, the most abundant corresponded to plasmalogen species containing not only arachidonate and docosahexaenoic acid, but also oleic acid (18:1) esterified at the *sn*-2 position (Fig. 6A).

Oxidation of bovine brain GPE phospholipids led to the rapid formation of multiple products. Solid-phase extraction techniques permitted the separation of lyso-phospholipids from those phospholipids retaining both fatty acyl groups. The lyso-phospholipid fraction was found to be derived from the loss of the *sn*-1 plasmenyl substituent rather than hydrolysis of the *sn*-2 fatty acyl group (Fig. 7). Nevertheless, the products found after oxidation of bovine brain GPE lipids were substantially different from the major products obtained by simple acid hydrolysis of the precursor bovine brain GPE. For example, the three major *sn*-1 lyso products from oxidized bovine brain GPE corresponded to molecular species that had fatty acyl groups esterified to the *sn*-2 positions with octadecanoic acid (18:1), eicosenoic acid (20:1) and docosenoic acid (22:1), with each of these major species containing a single degree of unsaturation in the fatty acyl chain (Fig. 7). In contrast, acid hydrolysis of bovine brain GPE lipids yielded *sn*-1 lyso products with the three most abundant products (Fig. 7) containing *sn*-2 fatty acyl groups corresponding to octadecenoic acid (18:1), arachidonic acid (20:4) and docosahexaenoic acid (22:6).

Analysis of intact phospholipids after oxidation of bovine brain GPE (Fig. 6) revealed that only one minor product retained arachidonic acid and that that product was a diacyl phospholipid containing octadecanoate at *sn*-1 and a monohydroperoxide arachidonate at *sn*-2 (Fig. 8A). The most abundant intact plasmalogen phospholipids remaining in this preparation were found to contain a single double bond present in the *sn*-2 fatty acyl group (Fig. 8B). This rather striking finding that arachidonate- and docosahexaenoate-containing plasmalogen phospholipids were completely destroyed by the Fenton reaction further supported the unique lability of plasmalogen glycerophospholipids containing polyunsaturated fatty acids esterified to the *sn*-2 position of the glycerophospholipid chain.

While the studies of the intact phospholipids by tandem mass spectrometry permitted a description of the number of oxygen atoms added to arachidonate, it was not sufficiently powerful to uniquely analyze the various regioisomers that make up the monooxygenated arachidonate species. In order to obtain unambiguous data concerning the chemical nature of the oxidized arachidonate moiety, it was found necessary to carry out a hydrolysis of the fatty acyl group prior to reversed-phase LC–MS and LC–MS–MS analysis. The strategy of this approach can be illustrated by the analysis of the free radical-derived monooxygenated arachidonate species isolated from intact RBCs treated with tBuOOH [9]. Saponification of each phospholipid class purified by normal-phase HPLC (vide supra) followed by reversed-phase LC–MS–MS revealed the unique elution of ten monooxy-

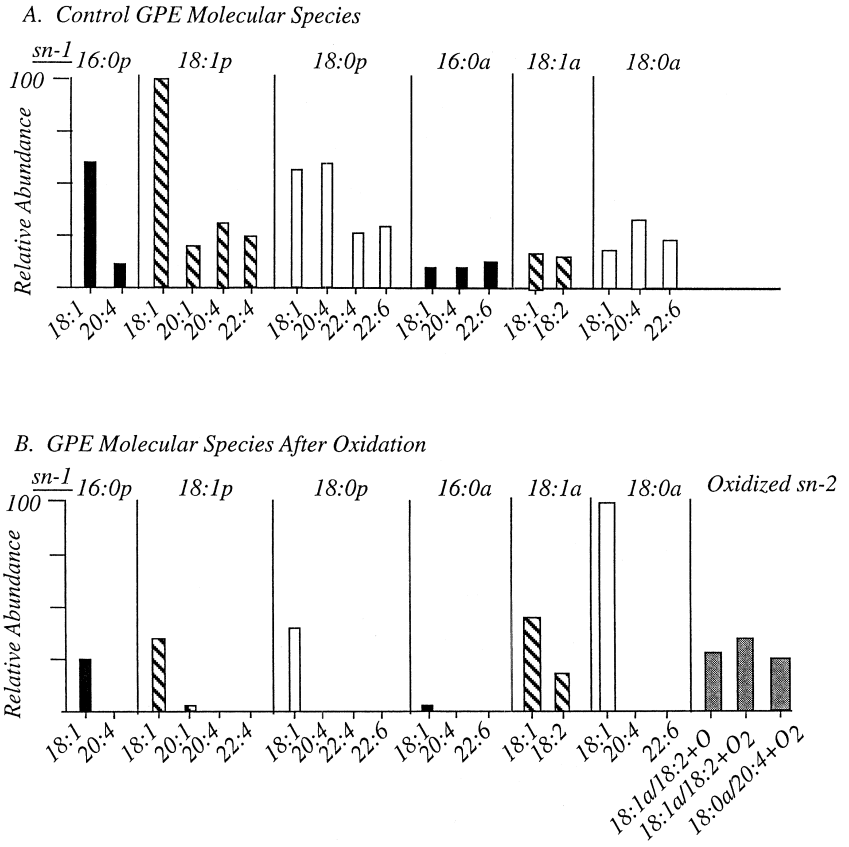

Fig. 6. (A) Major molecular species of GPE phospholipids from bovine brain extracts grouped by the *sn*-1 substituent with a fatty acyl moiety at the *sn*-2 position on the ordinant. (B) Major molecular species of GPE phospholipids that remain following treatment with H_2O_2 (70 mmol) and $CuCl_2$ (100 μmol). The molecular species were identified using electrospray ionization tandem mass spectrometry. The abundance of each of the molecular species is presented, corresponding to the abundance of the molecular ions (negative ions) normalized to the most abundant molecular species (18:1p/18:1-GPE for control and 18:0a/18:1-GPE for oxidized GPE). Abbreviations used to identify the molecular species are identical to those used for Fig. 2.

genated arachidonate species (Fig. 9). The characterization of each isobaric molecule required the combined separation power of reversed-phase HPLC as well as tandem mass spectrometry. Such a separation is illustrated in Fig. 10 for each of these isobaric monooxygenated arachidonate species by LC–MS–MS. Reversed-phase HPLC resulted in separation of most, but not all, of the components from each other and, since these molecules were isobaric, it was insufficient to measure the abundance of the molecular anion (m/z 319) in order to uniquely quantitate each component. For example, 5,6-EET and 8,9-EET did not fully separate by the HPLC system employed. Furthermore, 8-HETE and 12-HETE could not be separated from each other. However, collision-induced decomposition of the carboxylate anion for each of these species (m/z 319) was found to yield a unique set of decomposition product ions [9,18] that could be used to unambiguously characterize the elution of each of these isobaric substances from the HPLC (Fig. 10). For example, one could monitor the ion transition m/z 319 → m/z 155 to detect the elution of 8,9-EET, separate from the elution of 5,6-EET, which was characterized by the mass transition m/z 319 → m/z 191. Some ion transitions were identical for two different isobaric species and, in particular, for EET regioisomers, which were similar in structure to the

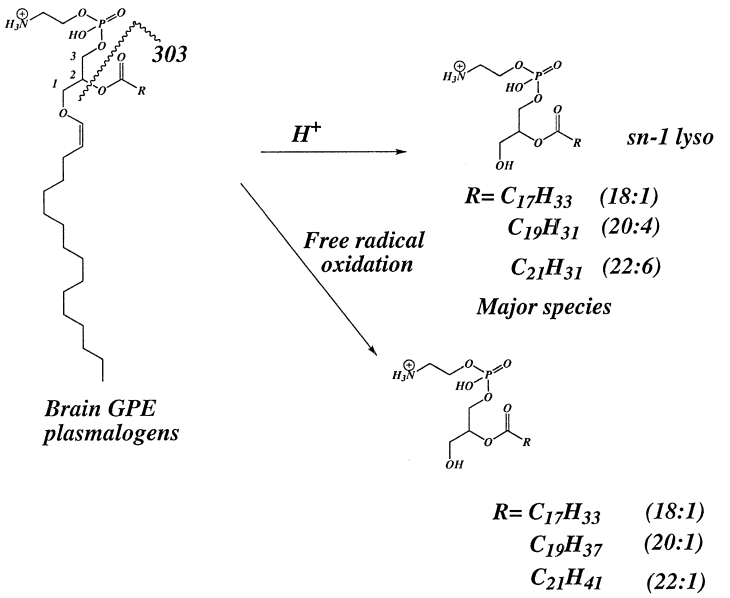

Fig. 7. Treatment of bovine brain GPE lipids with either acid or free radical oxidative conditions resulted in structurally diverse lyso (*sn*-1) products.

corresponding HETE isomer. For example, m/z 319 $\rightarrow$ m/z 155 was an abundant ion transition not only for 8-HETE, but also for 8,9-EET. Therefore, the separation of these two isomers by reversed-phase HPLC was essential for identifying the formation of both of these individual monooxygenated arachidonate species in RBCs treated with tBuOOH. The use of a stable-isotope-labeled internal standard, added to

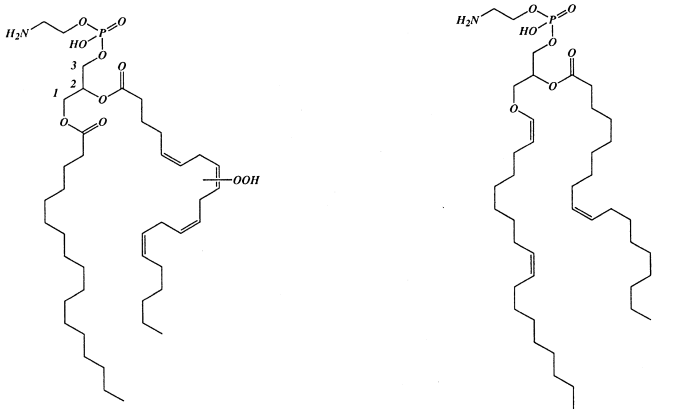

Fig. 8. Major (A) arachidonate-containing GPE lipid and (B) plasmalogen molecular species remaining as intact glycerophospholipids after exposure to free radical oxidative conditions.

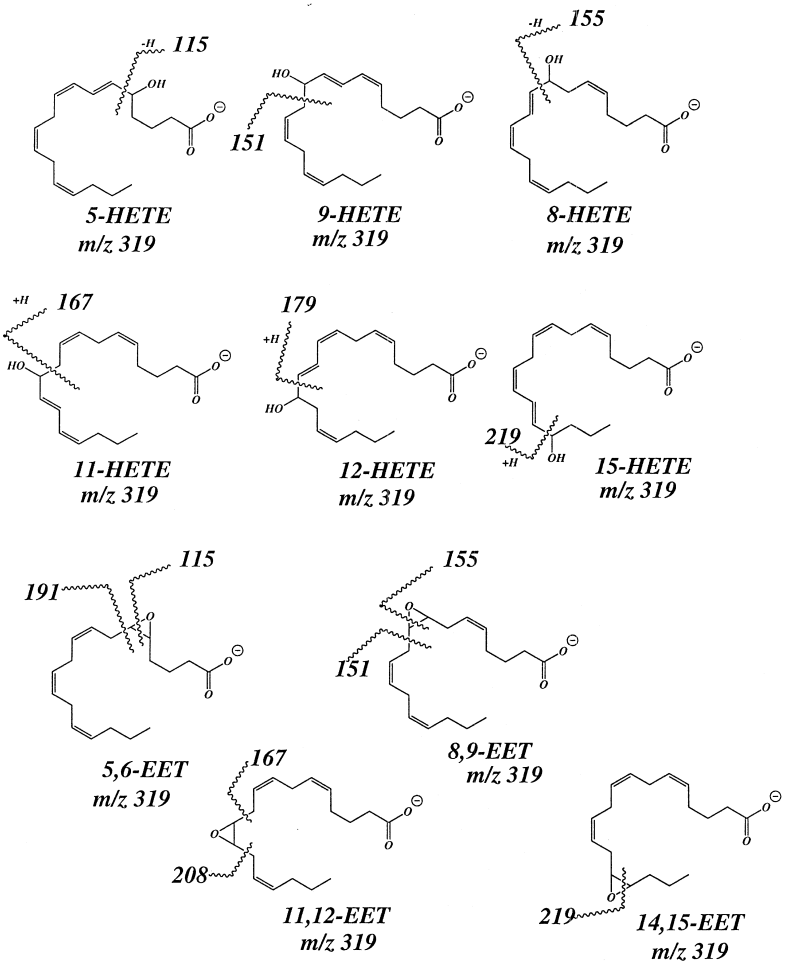

Fig. 9. Ten eicosanoids derived from the monooxygenation of arachidonic acid. Negative ion electrospray ionization yields the same isobaric ion, m/z 319, from each eicosanoid. Specific ions formed by collisional activation of each species yields the product ions indicated.

each of these saponified mixtures, permitted a quantitative analysis of each of these isomeric species by measuring the abundance of each ion transition relative to the internal standard ion transition. It was also possible to quantitate the monooxygenated arachidonate species present in RBC ghosts using an alternative internal standard, namely [$^{18}O_2$]12-HETE [19].

4. Discussion

HPLC and tandem mass spectrometry have proven to be a powerful combination in the analysis of complex mixtures of closely related molecules, such as those found as naturally occurring glycerophospholipid molecular species in cellular membranes as well as the products formed by lipid peroxidation mechanisms acting on cellular membranes. Several strategies have emerged as useful approaches for the unique detection of specific lipid molecules. The use of the elevated orifice voltage and single stage mass spectrometry permits one to uniquely identify the emergence of phospholipids containing a specific fatty acyl group. This strategy involves the collision-induced decomposition of phospholipids in the electrospray interface. Since the focus of many of the studies in this laboratory have involved an under-

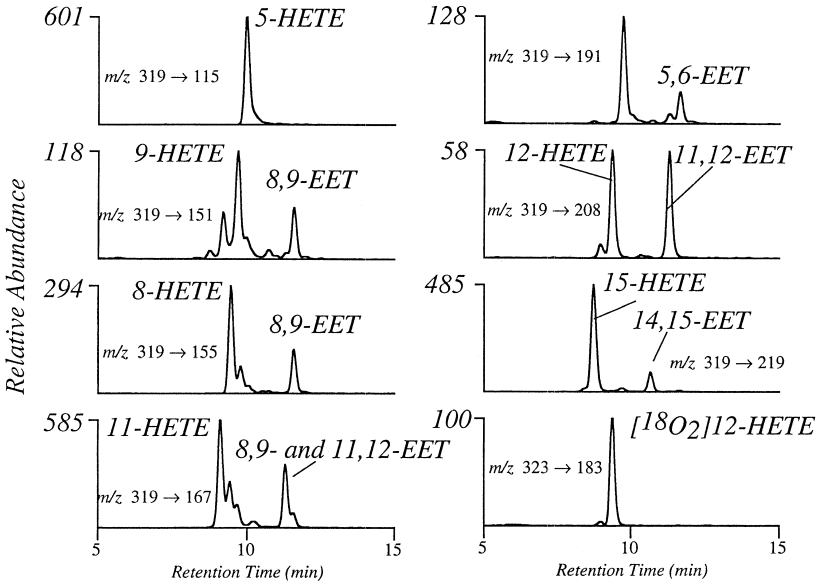

Fig. 10. LC–MS–MS analysis of individual monohydroxyeicosatetraenoic acid (HETE) and epoxyeicosatrienoic acid (EET) isomers esterified to GPC lipids in red blood cell membranes treated with tBuOOH (10 mmol). Extracted phospholipids were saponified to release the free carboxylic acid prior to tandem mass spectrometric analysis. Each ion transition chromatogram used for eicosanoid identification is presented individually with absolute abundance normalized to the added internal standard [$^{18}O_2$]12-HETE (m/z 323→183) ion transition.

standing of the role of arachidonic acid and mechanisms relevant to remodeling of this polyunsaturated fatty acid into different phospholipid classes, this is a particularly useful technique for detecting arachidonate-containing glycerophospholipids eluting from the normal-phase HPLC separation of phospholipid classes (Fig. 2). The elevated orifice potential approach is made possible by the unique gas phase ion chemistry inherent in glycerophospholipid negative ions that render them particularly susceptible to the formation of abundant carboxylate anions as a result of a charge-driven reaction mechanism and concomitant formation of a neutral cyclic phosphate diester [15]. Such experiments can be carried out in either single stage electrospray mass spectrometers, tandem quadrupole mass spectrometers or ion-trap mass spectrometers.

A more complete structural characterization of phospholipid molecular species containing arachidonic acid can be achieved if one carries out true tandem mass spectrometry. For example, the precursor ion scanning strategy can be used to uniquely identify molecular species containing arachidonic acid through determination of precursor molecular mass. When dealing with a purified phospholipid class, such as GPE phospholipids, the molecular mass is often a sufficient parameter to define whether or not a specific molecular species is a 1,2-diacyl, 1-*O*-alkyl ether or a plasmalogen glycerophospholipid [20]. While the partial separation of molecular species can be achieved by RP-HPLC separation of a pure lipid class, e.g., GPE-lipids (Fig. 5), the mass spectrometer serves as a critical detector to follow the elution of all species, independent of UV-absorption characteristics.

The detailed chromatographic and mass spectrometric analysis of oxidized RBC ghost membranes suggested that plasmalogen phospholipids containing polyunsaturated fatty acid, specifically arachidonic acid, were uniquely susceptible to oxidation. Detailed analysis of GPE phospholipids subjected to radical oxidant conditions supported this observation. Arachidonate-containing plasmalogen molecular species are probably the initially oxidized components of plasma membranes and, in this way, they possibly serve as antioxidant molecules [21] that protect the cellular constituents from reactive oxygen species. The chemical structures of the products that

result from the oxidation of arachidonate are under further investigation.

The oxidation of intact RBCs by tBuOOH revealed the formation of ten different isomers of monooxygenated arachidonic acid. The quantitation of each of these isomers required the combination of both chromatographic separations as well as tandem mass spectrometric separations (Fig. 10). Unique collision-induced decomposition product ions could be found to characterize closely related molecules. The tBuOOH treatment of RBCs led not only to abundant formation of monooxygenated arachidonate (HETE), but also to the formation of epoxyeicosatrienoic acids (EETs). This latter class of eicosanoids most likely resulted from the direct addition of an oxygen atom to one of the four double bonds of arachidonic acid by an enzymatic process within the intact RBC. Each of the four regioisomers of arachidonic acid were observed following oxidation of intact RBC ghosts. We have suggested that hemoglobin may play an important catalytic role in this oxidative step [9], largely because EETs were not abundant products in the oxidation of RBC ghost membranes.

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