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Analysis of Epoxyeicosatrienoic and Monohydroxyeicosatetraenoic Acids Esterified to Phospholipids in Human Red Blood Cells by Electrospray Tandem Mass Spectrometry

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Electrospray ionization (ESI) and tandem mass spectrometry (MS/MS) were used to analyze epoxyeicosatrienoic acids (EETs) and monohydroxyeicosatetraenoic acids (HETEs) isolated from human red blood cell membranes following base hydrolysis. ESI results in the formation of an abundant isobaric carboxylate anion at *m/z* 319 for both of these oxidized metabolites of arachidonic acid. The product ion spectra from the collision-induced dissociation of this carboxylate anion could be used to identify each of the isomeric eicosanoids from the unique fragment ions of each eicosanoid. The observed product ion spectra were identical with those previously obtained by fast atom bombardment ionization; however, ESI required less EET and HETE for analysis. Both EET and HETE phospholipids were present in human red blood cells (RBCs) and their abundance could be substantially increased by treatment under conditions that would induce free radical oxidation of membrane phospholipids. Following incubation of human RBCs with *tert*-butyl hydroperoxide (tBuOOH), phospholipids were extracted and purified by normal-phase high-performance liquid chromatography (HPLC) as to glycerophospholipid class containing ethanolamine (GPE), serine (GPS) and choline (GPC) as the polar head group. Each class of phospholipid was hydrolyzed to yield the free carboxylic acid prior to on-line HPLC/ESI-MS/MS analysis. The formation of oxidized arachidonic acid esterified to phospholipids in treated RBCs was found to increase significantly for both esterified EETs in GPE, GPS and GPC which increased 49-, 34- and 59-fold, respectively, and also for esterified HETEs in GPE, GPS and GPC which increased 3-, 4- and 11-fold, respectively, compared with untreated RBCs. These results provide the first characterization of EETs formed non-enzymatically as intact phospholipids in a lipid peroxidation model system. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

Glycerophospholipids are the major components of the lipid bilayer, which is the defining membrane of mammalian cells. These lipid substances are composed of a complex mixture of individual molecular species which can be divided into specific classes depending upon the polar head group esterified to glycerol, that being glycerophosphoethanolamine (GPE), glycerophosphocholine (GPC), glycerophosphoserine (GPS) and glycerophosphoinositol (GPI), which are the most common glycerophospholipid classes found in mammalian cells. The heterogeneity of this mixture is further complicated by the number of individual fatty acyl substituents esterified to two additional hydroxyl groups of glycerol. Glycerophospholipids serve an important structural role as the physical containment of the cell and also serve as a reservoir for arachidonic acid,[1] which is metabolized by various oxidative routes to yield lipid mediators for cellular chemical communication. Arachidonic acid is metabolized by PGH synthase to yield the prostaglandins, thromboxanes and prostacyclin,[2] 5-lipoxygenase to yield the leukotrienes[3] and cytochrome P-450 pathways to yield several biologically active products, including epoxyeicosatrienoic acids.[4] Several other lipoxygenases exist, including 12-lipoxygenase and 15-lipoxygenase which form hydroxyeicosatetraenoic acids (HETEs), 12-HETE and 15-HETE, respectively, which are known to be biologically active, but are not well understood.[5] Epoxyeicosatrienoic acids (EETs) are thought to be generated in a stereospecific manner from free arachidonic acid catalyzed by specific cytochrome P-450 isozymes.[6,7] Several studies have suggested that the epoxyeicosatrienoic acids can subsequently be esterified to glycerophosphocholines forming EET phospholipids in a CoA-dependent process.[8,9] This would then account for the observation of esterified EET phospholipids. Such EET phospholipids could play an important physiological

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role in serving as a reservoir from which EETs can be released following activation of a phospholipase A_2.

Non-enzymatic oxidation of polyunsaturated fatty acids and polyunsaturated acyl groups esterified to glycerophospholipids (lipid peroxidation) is initiated within a biological system by reactive chemical species such as free radicals, generated as a result of normal physiological or pathophysiological conditions. Sufficiently energetic free radical species can react with the homoconjugated diene structural motif common to polyunsaturated fatty acyl groups to form a penta-1,5-diene radical.[10] This radical intermediate can then covalently bind to molecular oxygen to form hydroperoxy radical species. Subsequent reactions of these lipid hydroperoxides result in a large number of products, including malonyldialdehyde,[11] 4-hydroxynonenal[12] and more complex hydroxy and epoxy acids.[13,14] Several lines of evidence now suggest that additional events occurring during lipid peroxidation lead to the formation of compounds isomeric with the enzymatically produced prostaglandins and leukotrienes.[15,16] There is interest in these oxidized phospholipids not only as unique markers of lipid peroxidation, but also from the fact that some products of arachidonic acid free radical oxidation have been found to exert significant biological activity, including isoprostanes[15] and isoleukotrienes.[16]

A large number of model systems have been developed to investigate lipid peroxidation both *in vitro* and *in vivo*.[17] One commonly employed model system involves the use of *tert*-butylhydroperoxide as a lipophilic agent that mimics increased hydroperoxide tone within cellular membranes.[18] In biological systems, lipid hydroperoxides can react to form various oxygen-centered radical species including hydroperoxy and hydroxyl radicals when transition metal ions such Fe(II) are present.[19] Although the exact mechanism responsible for lipid peroxidation products in these model systems is complex, this model system is known to result in the formation of lipid peroxidation in intact cells including red blood cells (RBCs).[20]

Mass spectrometry has played a central role in the structure elucidation of free radical products of arachidonic acid oxidation and in the analysis of complex glycerophospholipids. Fast atom bombardment tandem mass spectrometry was found to generate specific and unique ions for the analysis of EETs present in GPC and GPE phospholipids.[21] In these studies, abundant $[M - CH_3]^-$ and $[M - H]^-$ ions, respectively, could be observed in addition to the collisionally activated formation of product ions that could be used in characterize the structure of the EET-containing phospholipids. In order to analyze the presence of the EET phospholipids in biological extracts, it was necessary to purify such species with sequential normal- and reversed-phase HPLC.

Electrospray ionization tandem mass spectrometry (ESI-MS/MS) has been found to be particularly well suited for the analysis of both eicosanoids and phospholipids because of its inherent sensitivity and specificity.[22] Additionally, electrospray ionization is compatible with on-line HPLC separations. Utilizing this methodology, we report here the existence of EET-containing phospholipids by negative-ions ESI-MS/MS. Non-enzymatic generation of epoxyeicosatrienoic acid esterified to glycerophospholipids has not been reported previously. Further, we have found that HETE and EET phospholipids could be generated by free radical oxidation of RBCs as in model of lipid peroxidation injury.

EXPERIMENTAL

Materials

EET and HETE standards were purchased from Cayman Chemical (Ann Arbor, MI, USA). $[^{18}O]_2$-12-HETE was prepared from butyryl cholinesterase and $H_2^{18}O$ as previously described.[23] *tert*-Butyl hydroperoxide (tBuOOH) was purchased from Sigma Chemical (St Louis, MO, USA). Bis(trimethylsilyl) trifluoroacetamide (BSTFA) was purchased from Supelco (Bellefonte, PA, USA). All solvents were of HPLC grade and other reagents were of the highest grade commercially available.

RBC preparation and incubation

Human blood was obtained from healthy volunteers and RBCs were isolated by a two-step centrifugation protocol. The first centrifugation at 120 *g* permitted the removal of the upper layer containing platelet-rich plasma. The lower layer was diluted with five volumes of phosphate-buffered saline (PBS), then centrifuged at 900 *g*. The resulting buffy coat layer containing granulocytes was carefully removed. The RBC layer was washed three times with five volumes of PBS, removing any granulocytes at the upper interface. After the final wash, the packed RBCs were resuspended to a final concentration of 2×10^8 RBC ml^{-1} in PBS. Incubations were carried out with aliquots of this RBC preparation (50 ml), which were incubated with and without (controls) tBuOOH (10 mM final concentration) at 37 °C for 90 min. After incubation, the suspension was centrifuged at 900 *g* for 10 min and the pellet washed three times after resuspension in 10 ml of PBS.

In separate experiments to measure the extent of RBC lysis, the RBC preparation was incubated with tBuOOH (10 mM) for 90 min and then centrifuged at 2000 *g* for 10 min. Hemolysis of the RBCs were determined in this preparation by measuring the absorbance at 410 nm compared with the absorbance obtained from an identical preparation of RBCs treated with tBuOOH, but lysed with distilled water. The extent of RBC hemolysis induced by tBuOOH incubation was found to be less than 1% in two separate experiments.

Phospholipid extraction and purification

Phospholipids were extracted from cell pellets following the method of Rose and Oklander.[24] Briefly, distilled water (1 ml) was added to the RBC pellets and incubated for 15 min, then propan-2-ol (11 ml) was added and vortex mixed thoroughly. The mixture was incubated for 1 h with frequent vortex mixing, which was followed by the addition of chloroform (7 ml). After

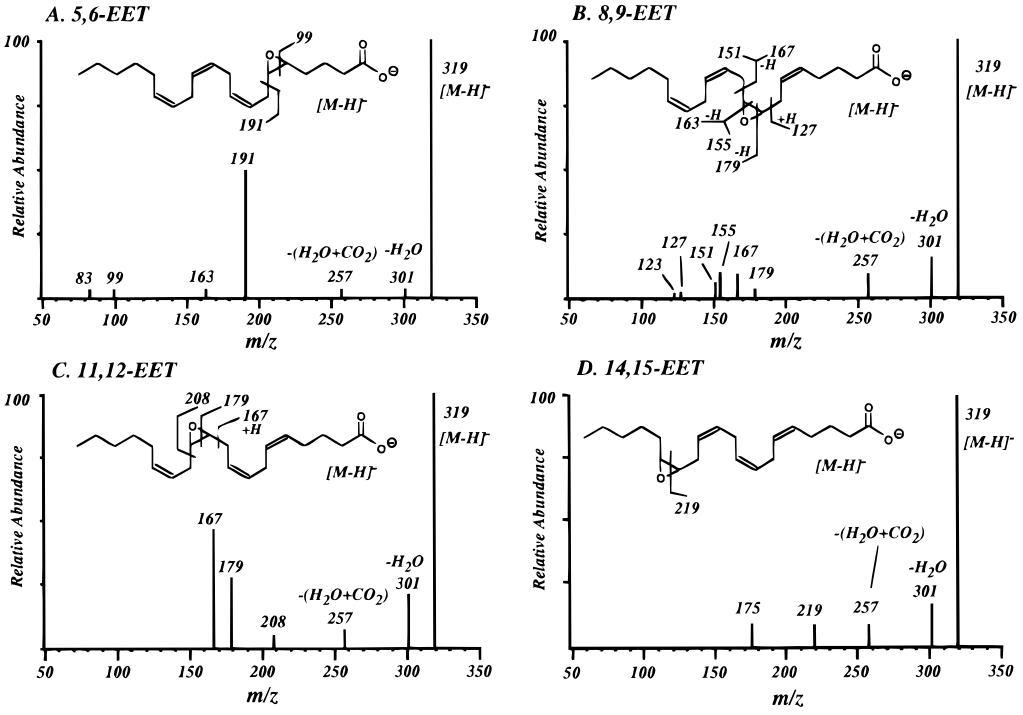

Figure 1. Negative ion product ion spectra from carboxylate anions generated by electrospray ionization of (A) 5,6-EET, (B) 8,9-EET, (C) 11,12-EET and (D) 14,15-EET. The collision energy was 11 eV with argon as a collision gas at a thickness of 240×10^{12} molecules cm^{-2}. The orifice potential was −55 V.

vortex mixing, the mixture was allowed to stand for 1 h, then centrifuged (500 *g*) for 30 min and the supernatant evaporated to dryness under nitrogen. Phospholipids were purified and separated into their GPE, GPS and GPC classes by normal-phase HPLC essentially following a previously published protocol.[25] GPI is a minor phospholipid class in RBC and was not collected for study. Briefly, normal-phase HPLC was performed using a 5 μm silica (Licrosorb; Phenomenex, Torrance, CA, USA) analytical column (250 × 4.6 mm i.d.) with a gradient of 62.5% solvent A (propan-2-ol–hexane–260 mM ammonium acetate, pH 7.0 (58:40:2) programmed to 100% solvent B (propan-2-ol–hexane–260 mM ammonium acetate, pH 7.0 (50:40:10) over a 20 min period. The solvent flow rate was 1 ml min^{-1} and fractions were collected.

Incorporation of 5-HETE into RBC membranes

RBCs (5×10^8) were suspended in 25 ml of PBS and incubated with [^{3}H]-5-HETE (0.3 ng, 50 000 dpm; New England Nuclear, Boston, MA, USA) for 90 min at 37 °C in the presence and absence of 10 mM tBuOOH. Following incubation, the cell suspension was centrifuged at 900 *g* and the phospholipids were extracted from the cell pellets as described above. The recovery of [^{3}H]-5-HETE in the phospholipid extract was 6% in untreated RBCs and 3% of added [^{3}H]-5-HETE in tBuOOH-treated RBCs. Normal-phase HPLC was performed as described above and the effluent monitored with an HPLC radioactive monitor (Flow 1Beta, Radiomatic, Tampa, FL, USA). No radiolabeled phospholipids could be identified in these extracts and the only radioactive component eluted at the retention time of 5-HETE.

Phospholipid hydrolysis

Normal-phase HPLC fractions were hydrolyzed with 1 M sodium hydroxide (2 ml) at room temperature for 90 min. The fractions were then acidified (pH 3) with 2 M hydrochloric acid and [^{18}O]$_2$-12-HETE (10 ng) was added as an internal standard. Lipids were extracted twice with hexane (5 ml). Immediately before analysis, the hexane extract was blown to dryness with a stream of dry nitrogen and the residue dissolved in 100 μl of solvent C [methanol–acetonitrile–6.5 mM ammonium acetate, pH 5.7 (24.5:45.5:30)] in preparation for analysis by reversed-phase HPLC/MS.

Mass spectrometry

EET standards were evaporated to dryness under nitrogen and the residues dissolved in solvent system C at a final concentration of 1–3 μM. Samples were introduced into a Sciex API-III$^+$ triple-quadrupole mass spectrometer (Perkin-Elmer Sciex, Toronto, Canada) by continuous flow injection at a flow rate of 10 μl min^{-1}. Spectra were acquired at 3 s per scan with a spray voltage of −2800 V, an orifice voltage of −55 V and a collisional offset potential of 11 eV. Collision-induced dissociation (CID) spectra were obtained using argon in the second quadrupole equivalent to 240×10^{12} molecules cm^{-2}. The nitrogen curtain gas flow rate was 1.2 l min^{-1} and nebulizer gas flow rate was held at 0.6 l min^{-1}.

Analysis of hydrolyzed phospholipids by HPLC was performed using a 5 μm ODS 100A (Prodogy; Phenomenex) analytical column (250 × 1.0 mm i.d.). The mobile phase consisted of gradient from 20% 6.5 mM ammonium acetate (pH 5.7) programmed to 100%

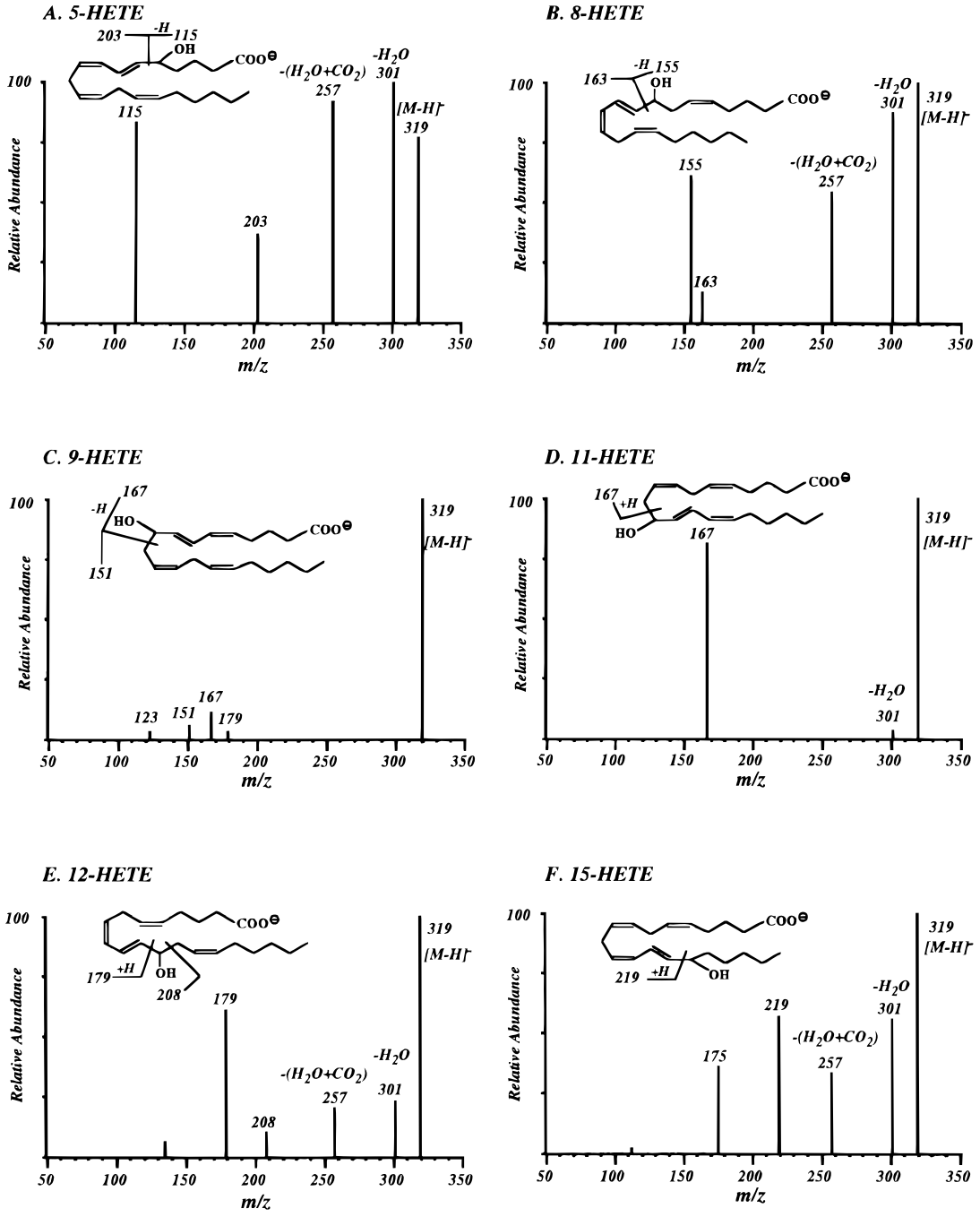

Figure 2. Negative ion product ion spectra from carboxylate anions generated by electrospray ionization of (A) 5-HETE, (B) 8-HETE, (C) 9-HETE, (D) 11-HETE, (E) 12-HETE and (F) 15-HETE. Conditions as in Fig. 1.

solvent D (methanol–acetonitrile (35:65 over a 15 min period with a mobile phase flow rate of 50 μl min^{-1}. For these experiments, a 20 μl sample aliquot was injected.

RESULTS

The ESI mass spectrum of regioisomeric EETs consisted of a single carboxylate anion $[M - H]^-$ at *m/z* 319. The abundance of this anion was greatest at an orifice voltage of 55 V and at this setting there was no further fragmentation of the EETs (data not shown). Collisional activation of the carboxylate anion resulted in product ions characteristic of each of the regioisomers of the EETs and also common product ions formed by loss of water (*m/z* 301) and the loss of water and CO_2 from the carboxylate group (*m/z* 257). The product ions obtained in ESI-MS/MS were strikingly similar to those observed in the collisional activation of the carboxylate anion of the regioisomer of EETs obtained by fast atom bombardment (FAB) MS.[21] The only significant difference in the observed product ion masses of the EET negative ions analyzed by FAB compared with ESI was fewer fragment ions for the latter ionization process, suggesting that during ESI these ions arose from a much less energetic ionization process. Since the observed ions were identical, the

mechanisms previously suggested for the formation of these ions were probably identical.[21]

HETEs are reduced products of the reaction of molecular oxygen autoxidation with arachidonic acid or reduced metabolites formed by lipoxygenase-catalyzed arachidonate oxidation and are isobaric with the EET regioisomers. The CID of the carboxylate anions of monohydroxyeicosatetraenoic acid obtained by FAB[26] and ESI[27] have been published previously. The ESI product ion spectra presented in Fig. 2 were obtained under CID conditions identical with those used to decompose the carboxylate anions of the EET regioisomers (Fig. 1).

A comparison of the mass spectra revealed striking similarities in the product ions, e.g., the CID mass spectra for 14,15-EET displayed the same ions as the CID of 15-HETE. As the distance between the carboxylate anion and the hydroxy or epoxide group became shorter, differences in the mass spectra became more evident. The mass spectrum of 11,12-EET [Fig. 1(C)] is the composite of the product ions obtained by CID of 12-HETE [Fig. 2(E)] and 11-HETE [Fig. 2(D)]. This included the interesting odd-electron ion observed at *m/z* 208 for 12-HETE and 11,12-EET. Aside from the common ions due to loss of water and loss of water and CO_2, product ions obtained from collisional activation of 5-HETE (*m/z* 115 and 203) were completely absent as product ions following CID of 5,6-EET. The abundant ion at *m/z* 191 in the latter compound was probably a result of the cleavage of the carbon–oxygen bond at C-5, driven by the carboxylate anion charge site, ultimately leading to a neutral 6-oxo neutral acid and the hydrocarbon anion at *m/z* 191 as suggested previously.[21] Such a mechanism was not possible for 5-HETE and there is no 6-HETE product of autoxidation or lipoxygenase action. The identity of these two classes of molecules in terms of product ion spectra, except as noted, makes the unique characterization of these two isomeric classes of molecules difficult when employing only MS/MS of their common carboxylate anion at *m/z* 319.

Reversed-phase HPLC separation of the regioisomers of EETs and HETE isomers was readily achieved. A mixture of EETs (5,6-, 8,9-, 11,12- and 14,15-EET) and HETEs (5-, 8-, 9-, 11-, 12- and 15-HETE) were injected on to a reversed-phase HPLC column on-line with the mass spectrometer. ESI provided abundant signals for the carboxylate anions at *m/z* 319 for each EET and HETE (Fig. 3). Incomplete separation of the EETs was evident by co-elution of 5,6- and 8,9-EET at 11.9 min using *m/z* 319 to detect both isomers. Co-elution of 12- and 8-HETE and incomplete separation from 9-HETE were also observed with this column. Nonetheless, specific detection of each of the components could be achieved using MS/MS and monitoring specific transitions for each EET as indicated in Fig. 3(B). For example, the presence of both 8,9- and 5,6-EET in HPLC at 11.9 min was revealed by the unique transitions for 8,9-EET (*m/z* 319 → 151) and *m/z* 319 → 191 for 5,6-EET. In fact, a slight separation of 5,6-EET eluting at 11.9 min from 8,9-EET eluting at 11.8 min could be detected when these specific ion transitions were used to detect the EETs. Using such multiple reaction monitoring modes, it was possible to identify each

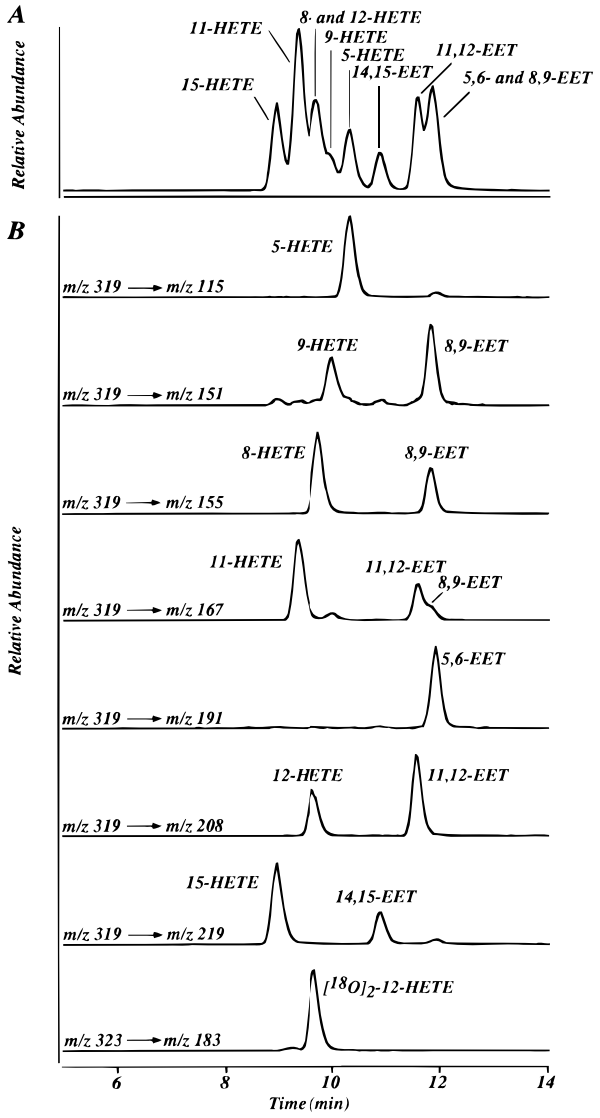

Figure 3. HPLC/MS/MS analysis of EET, HETE (*m/z* 319) and [$^{18}O_2$]-12-HETE (*m/z* 323) standards via reversed-phase HPLC. A mixture of each standard (2 ng per component) was injected and then eluted from a 250 × 1.0 mm i.d. ODS column under gradient conditions. The mass spectrometer was operated in the multiple reaction monitoring mode. (A) Summation of all ion current transitions of *m/z* 319 to *m/z* 115, 151, 155, 167, 191, 208 and 219 and *m/z* 323 to *m/z* 183. (B) Transitions specific for each EET and HETE isomer are indicated above each trace and individually normalized.

of the HETE isomers eluting from the HPLC column and also the four regioisomeric EETs, even though the same ion transitions were used for certain identifications. The chromatographic separation of 8-HETE from 8,9-EET permitted the use of the transition *m/z* 319 → 155 to detect both compounds.

As can be seen in Fig. 3, the stable isotope labeled [$^{18}O_2$]-12-HETE was identified by the ion transition *m/z* 323 → 183. A quantitative assay for both EETs and HETEs present in the same sample was established using [$^{18}O_2$]-12-HETE as an internal standard and multiple reaction monitoring for the specific ions indicated in Fig. 3(B) and chromatographic separation permitted the quantitation of each specific eicosanoid. The calibration graphs for all compounds studied showed excellent correlation in the range 0.5–50 ng of EETs

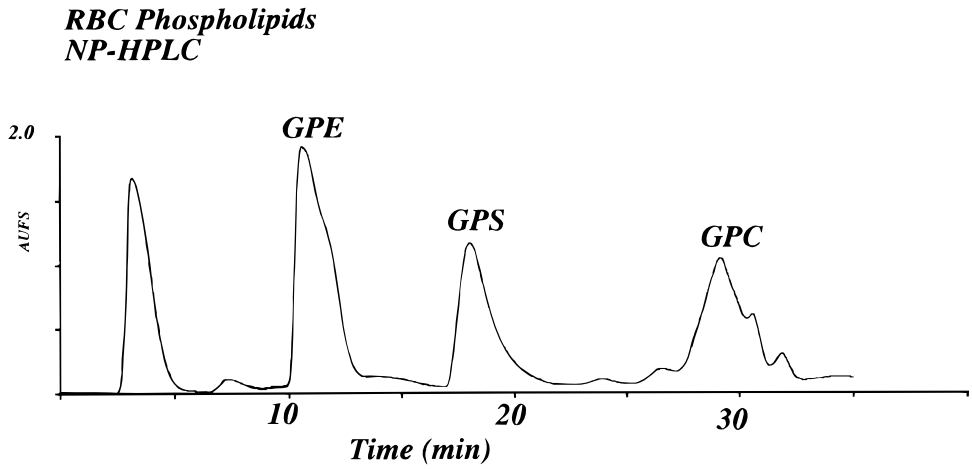

Figure 4. Normal-phase HPLC separation of phospholipids extracted from human RBCs incubated with 10 mM tBuOOH for 90 min. The chromatogram was monitored with UV absorption at 205 nm.

and HETEs using 10 ng of [$^{18}O_2$]-12-HETE as an internal standard.

Analysis of EETs and HETE phospholipids in human red blood cells

Previous investigations have established the presence of phospholipids containing EETs in human plasma[28] and in human platelets, with the most abundant EETs appearing in GPI phospholipid molecular species.[29] Investigations were carried out to determine whether such EET phospholipid molecular species were present in the membranes of human RBCs. Furthermore, human RBCs were incubated with tBuOOH and then analyzed for EET- and HETE-containing phospholipids. Phospholipid extracts from human RBCs were separated into their phospholipid classes of GPE, GPS and GPC by normal-phase HPLC as indicated in Fig. 4. The effluent from the normal-phase separation was monitored at 205 nm to detect non-specifically the elution of those phospholipid species containing polyunsaturated fatty acyl groups. Normal-phase HPLC achieved good resolution of each phospholipid class and each fraction with retention times corresponding to GPE (10–13 min), GPS (17–22 min) and GPC (25–33

Table 1. Formation of HETE isomers esterified to phospholipids in human red blood cells following 90 min of incubation with 10 mM *tert*-butyl hydroperoxide

	ng HETE/10^{10} RBC[a]					
	GPE		GPS		GPC	
Isomer	Untreated	Treated	Untreated	Treated	Untreated	Treated
5-HETE	10.7 ± 5.6	54.6 ± 14.8	6.4 ± 2.8	44.2 ± 17.3	2.4 ± 0.7	37.1 ± 4.0
8-HETE	9.0 ± 4.6	21.3 ± 3.5	6.9 ± 1.2	18.2 ± 5.7	1.8 ± 0.9	16.3 ± 2.3
9-HETE	12.6 ± 2.7	44.8 ± 10.0	13.4 ± 1.6	36.7 ± 10.6	2.0 ± 0.8	26.3 ± 1.3
11-HETE	7.4 ± 3.6	11.8 ± 0.3	3.7 ± 0.6	8.0 ± 2.0	1.2 ± 0.3	8.1 ± 0.1
12-HETE	8.3 ± 2.3	20.6 ± 5.6	6.0 ± 1.1	18.3 ± 5.4	2.3 ± 0.5	15.8 ± 2.2
15-HETE	8.5 ± 2.8	31.6 ± 9.1	4.6 ± 0.7	20.9 ± 6.1	1.5 ± 0.4	20.3 ± 2.6
Total	56.5 ± 21.6	184.5 ± 42.5	41.1 ± 6.9	146.3 ± 46.9	11.1 ± 3.5	123.8 ± 11.2

[a] Mean of incubations of RBC from three separate human subjects, ±SEM.
[b] Esterified arachidonate content of phospholipid classes of untreated RBC is 58% in GPE, 26% in GPS and 16% in GPC according to the measured total phosphorus content of each phospholipid class[31] and the mol.% arachidonate content of each phospholipid class.[30]

Table 2. Formation of EET isomers esterified to phospholipids in human red blood cells following 90 min of incubation with 10 mM *tert*-butyl hydroperoxide

	EET (ng/10^{10} cells)[a]					
	GPE		GPS		GPC	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
5,6-EET	1.5 ± 0.3	109.9 ± 20.9	1.3 ± 0.3	71.3 ± 38.8	0.5 ± 0.2	63.3 ± 42.6
8,9-EET	1.1 ± 0.2	86.9 ± 7.3	1.8 ± 0.1	53.1 ± 11.2	0.7 ± 0.2	38.3 ± 10.7
11,12-EET	2.3 ± 0.7	49.7 ± 3.4	1.6 ± 0.6	42.2 ± 2.5	0.7 ± 0.3	24.4 ± 5.5
14,15-EET	1.0 ± 0.5	43.1 ± 5.4	1.2 ± 0.6	33.5 ± 3.0	0.6 ± 0.2	24.2 ± 6.9
Total	6.0 ± 1.0	289.5 ± 19.4	5.9 ± 1.4	200.2 ± 31.6	2.5 ± 0.8	150.2 ± 61.7

[a] Mean of three separate incubations, three different subjects, ± SEM.

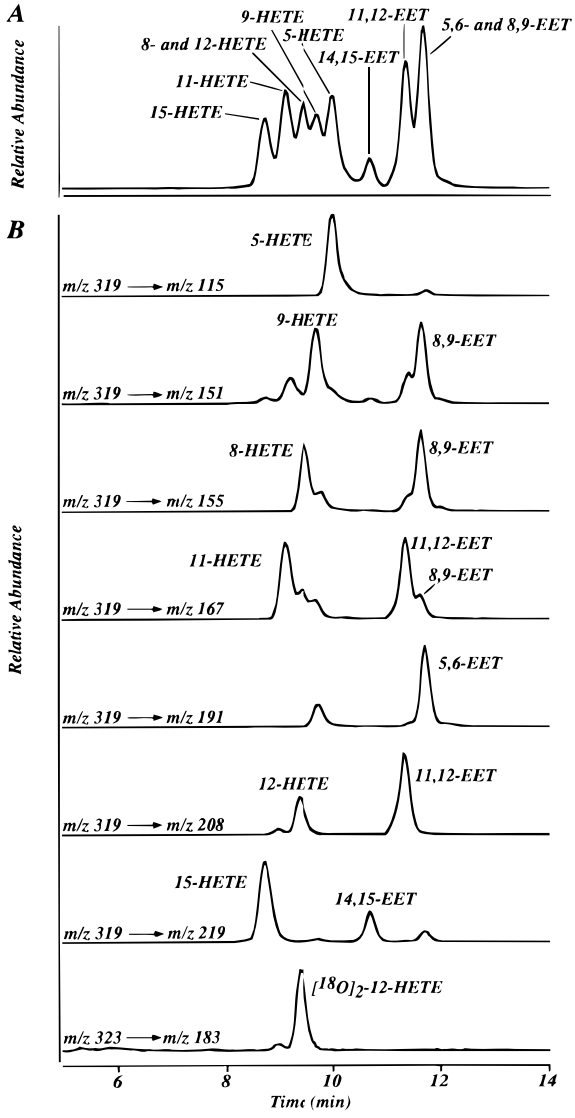

Figure 5. LC/MS/MS analysis of EET and HETE isomers formed in human RBCs following incubation with 10 mM tBuOOH. Lipids extracted from RBCs were separated into GPE, GPS and GPC phospholipid classes by normal-phase HPLC. The GPE fraction was hydrolyzed with 1 M NaOH, acidified, then analyzed by multiple reaction monitoring HPLC/MS/MS by reversed-phase HPLC. The mass spectrometer was operated as described in Fig. 1. (A) Summation of all ion current transitions of *m/z* 319 to *m/z* 115, 151, 155, 167, 191, 208 and 219 and *m/z* 323 to *m/z* 183. (B) Transitions specific for each EET and HETE isomer are indicated above each trace and individually normalized.

min) were collected individually. These phospholipid classes are known to contain the highest quantities of esterified arachidonic acid in normal human RBCs.[30,31]

Each phospholipid class was saponified and the fatty acid components were analyzed by HPLC/MS/MS using the multiple reaction monitoring specific for EETs and HETEs. The results from the analysis of untreated and treated RBCs are summarized in Tables 1 and 2. In general, the highest concentrations of HETEs were found in GPE followed by GPS and GPC phospholipids (Table 1). All six HETE isomers possible from autoxidation of arachidonic acid were observed in approximately the same relative concentration. The rank order of abundance of the total amount of HETE was similar to the rank order of arachidonic acid content present in RBCs with most arachidonate being in GPE and the least in GPC.[30] In 5–10-fold lower abundance were the EET regioisomers (Table 2), again in the approximate rank order of concentration to that of arachidonic acid in each phospholipid class. All of the four EET regioisomers could be identified as esterified phospholipids in normal RBCs.

Initiation of lipid peroxidation by tBuOOH in the intact RBCs resulted in a substantial increase in the production of both HETEs and EETs esterified to glycerophospholipids. A representative analysis of these eicosanoids esterified to GPE phospholipids after tBuOOH treatment is shown in Fig. 5. HETEs increased 3–11-fold and all six HETE isomers were observed consistent with a direct oxidation of arachidonic acid present in the phospholipid bilayers of RBCs (Table 1). The formation of the four regioisomeric EETs were strikingly enhanced by treatment with tBuOOH. All four regioisomers were formed in approximately equal amounts except for a somewhat larger abundance of 5,6-EET present in GPI, GPS and GPC phospholipids. The formation of GPE, GPS and GPC EET phospholipids in treated RBCs was found to be 49-, 34- and 59-fold higher, respectively, than in untreated RBCs. In separate experiments, [^{3}H]-5-HETE was incubated with RBC in the presence and absence of tBuOOH (10 mM) and phospholipids were extracted. No incorporation of 5-HETE into phospholipids could be found by normal-phase HPLC analysis and the small amount of radiolabel present in the phospholipid extract eluted with a retention time of 5-HETE free acid.

Previously published methods to detect and measure EET isomers employed gas chromatography/mass spectrometry and negative ion chemical ionization after derivatization to the pentafluorobenzyl ester.[32,33] This GC/MS technique was used to confirm the substantial increase in EET and HETE isomers in RBCs as reported in Tables 1 and 2 following treatment with tBuOOH (data not shown).

DISCUSSION

Electrospray mass spectrometry has made a significant impact on the analysis of a large number of biologically relevant molecules, including metabolites of arachidonic acid.[22,27] This ionization process was found to generate abundant carboxylate anions from the four regioisomeric epoxyeicosatrienoic acids (EETs) which are cytochrome P-450 products of arachidonic acid metabolism. Collisional activation of these carboxylate anions led to a population of product ions which were unique for each of the EET regioisomers. However, the CID mass spectra for the EETs were similar to, if not identical with, certain isobaric HETEs. In order to use ESI and MS/MS for the characterization of EETs present in biological extracts, it was found necessary to separate HETEs from EETs. This could be readily achieved using reversed-phase HPLC and each of the specific oxidized forms of arachidonic acid could then be analyzed by multiple reaction monitoring in the HPLC/

MS/MS format. Using such an approach with [$^{18}O_2$]-12-HETE as an internal standard, a quantitative assay for ten different isomeric molecules of the EET and HETE family was established.

The specific assay for EETs and HETEs was used to study the appearance of these corresponding eicosanoids esterified to individual phospholipid classes of human RBCs. The results of these studies revealed the appearance of HETE and EET fatty acyl groups esterified to phospholipids including glycerophosphoserine phospholipids. The production of HETE isomers esterified to all of RBC glycerophospholipid classes which contained arachidonic acid markedly increased following treatment with tBuOOH. The quantity of EET regioisomers present in RBC glycerophospholipids, GPE, GPS and GPC in normal RBCs increased even greater following tBuOOH treatment, suggesting that the EET phospholipids were formed as a result of non-enzymatic lipid peroxidation.

Previous studies have revealed the presence of EETs esterified to phospholipids, including *cis*-14,15-epoxyeicosatrienoic acid in GPI isolated from human platelets.[29] More recently, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acid esters of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol were isolated from the phospholipids extracted from rat liver.[8] In this study, 5,6-EET estrified phospholipid was not detected. The formation of these epoxyeicosatrienoyl phospholipids was found to be a result of incorporation of the endogenous EET free acids into the phospholipid pools in a CoA-dependent process.[8,9] The formation of the 14,15-EET GPI in human platelet was suggested to be a result of the direct epoxidation of arachidonic acid esterified to GPI in a unique enzymatic process.[29]

The present study reveals the striking accumulation of EET-containing phospholipids following initiation of lipid peroxidation. The EETs analyzed were present as intact phospholipids and were probably formed *in situ* rather than as a result of reacylation of lysophospholipid precursors based on studies of [^{3}H]-5-HETE incorporation into RBC phospholipids. The mechanism of phospholipid epoxidation is currently unknown. The epoxidation of olefins by hemoglobin has been reported in a free radical system.[34] However, susceptible olefins do not include phospholipids.[35] Hematin can catalyze the oxidation of linoleic acid into epoxyhydroxy products[36] when linoleic acid is non-esterified.

Lipid peroxidation initiated by tBuOOH resulted in the abundant production of the HETEs (Table 1) and an even more striking formation of EET-containing phospholipids (Table 2). The non-enzymatic nature of this epoxidation was supported by the observed formation of six isomeric HETEs in addition to the four individual regioisomeric EETs. Previous investigations with tBuOOH treatment of RBC does support the hypothesis of a free radical-based peroxidation of phospholipids.[20] Esterified EETs have been reported to be present in rat plasma esterified to phospholipids of circulating lipoproteins, but chiral analysis of these EETs revealed the formation of a single enantiomer, which suggested they were enzymatically derived from cytochrome P-450 epoxygenase.[28]

Certain EETs have been shown to have profound biological activities, including stimulation of glucagon and insulin release from isolated rat pancreatic islet cells,[37] relaxation of coronary arteries as an endothelium-derived hyperpolarization factor[38] and serving as modulators of epidermal growth factor-induced increases in intracellular calcium and mitogenesis.[39] The studies of the biological actions of the regioisomeric EETs have centered around cytochrome P-450 as the mechanism of EET generation, but the results of this study suggest an alternative pathway for EET formation, namely as a result of the direct oxidation of arachidonic acid esterified to glycerophospholipids. These non-enzymatically derived EET-containing phospholipids represent another class of biologically active eicosanoids derived from a direct chemical oxidation mechanism including the isoprostanes and isoleukotrienes. The mechanism of formation of the EETs is currently under investigation.

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