Electrospray Mass Spectrometric Analysis of 5-Hydroperoxy and 5-Hydroxyeicosatetraenoic Acids Generated by Lipid Peroxidation of Red Blood Cell Ghost Phospholipids

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Recent evidence suggests that generation of hydroxyl radicals in the presence of lipid membranes can lead to oxidation of arachidonic acid esterified to glycerophospholipids and the production of compounds isomeric to prostaglandins, thromboxanes, and leukotrienes. Liquid chromatography tandem mass spectrometry and multiple reaction monitoring were employed to quantitate the production of 5-hydroxyeicosatetraenoic acid (5-HETE), 5-hydroperoxyeicosatetraenoic acid (5-HPETE), and 5-oxo-eicosatetraenoic acid (5-oxo-ETE) in red blood cells ghosts treated with *t*-butylhydroperoxide (tBuOOH). Untreated red blood cell ghosts were found to contain low, but measurable quantities of these three 5-oxygenated eicosanoids as phospholipid esters. Following treatment, there was approximately a 53- and 22.5-fold increase in 5-HETE and 5-HPETE, respectively, and an 8.5-fold increase in 5-oxo-ETE. The formation of these compounds was inhibited nearly 90% by the antioxidants butylated hydroxytoluene, ascorbic acid, and resveratrol providing further evidence for free radical mediated oxidation of arachidonic acid. This analytical protocol provided sufficient sensitivity for detection of these compounds in studies in which previous analysis by high-pressure liquid chromatography with UV detection failed to detect their presence. These results reveal that the biologically active eicosanoids 5-HETE, 5-HPETE, and 5-oxo-ETE are formed esterified to phospholipids following exposure of cellular membranes to reactive oxygen species and free radicals in a model system where intracellular antioxidant mechanisms were depleted. (J Am Soc Mass Spectrom 1998, 9, 527–532)

Arachidonic acid is known to be oxidized by several enzymatic pathways including prostaglandin H_2 synthase [1], 5-lipoxygenase [2], and cytochrome *P*-450 dependent epoxygenase reactions [3]. These enzymatic pathways require free arachidonic acid as substrate and result in a host of biologically active lipid mediators including prostaglandins, thromboxanes, leukotrienes, and epoxyeicosatetraenoic acids. These molecules are known to play important roles in both intra and intercellular signaling events within cells [4, 5]. Although considerable attention has been given to the enzymatic production of prostaglandins and leukotrienes, it is now clear that nonenzymatic pathways can lead to the formation of biologically active metabolites of arachidonic acid. These pathways involve the free radical oxidation of arachidonic acid while it is esterified to glycerophospholipids. Compounds, including isoprostanes [6], isothromboxanes [7], and isoleukotrienes [8], have been found as products of both in vivo and in vitro arachidonoyl glycerophospholipid free radical oxidation.

Of particular interest is the fact that free radical oxidation of esterified arachidonic acid can lead to the production of biologically active products including 5-hydroperoxyeicosatetraenoic acid, 5-hydroxyeicosatetraenoic acid, and 5-oxo-ETE. The first two compounds, 5-HPETE and 5-HETE, are racemic at the stereogenic carbon-5 position but still exert significant biological activity [9, 10]. The 5-oxo-ETE product has never been previously reported as a phospholipid ester nor as a free radical product, but is known to exert significant biological activity [11]. Although lipid peroxidation leads to a host of products derived from the major polyunsaturated fatty acids, namely arachidonic and linoleic acids, the most potent biologically active compounds in terms of elevation of intracellular calcium ion in the human neutrophil are those that have been oxygenated at carbon-5 of arachidonate. We previously found that esterified 5-HETE was one of the more abundant products formed following free radical oxidation of intact red blood cells (RBC) by using *t*-butylhydroperoxide (tBuOOH) to increase hydroper-

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1044-0305/98/$19.00
PII S1044-0305(98)00013-0

Received August 23, 1997
Revised November 27, 1997
Accepted December 30, 1997

oxide tone within lipid membranes [12]. These studies suggested that 5-HPETE was initially formed as an arachidonoyl ester that underwent subsequent reactions including reduction to yield the observed 5-HETE. Several pathways for hydroperoxide reduction including glutathione, glutathione-*S*-transferase [13], and phospholipid hydroperoxide glutathione peroxidase [14] exist within cells that limit concentrations of esterified 5-HPETE. The use of the RBC ghosts treated with tBuOOH [15] has been used as a model to investigate initial events of lipid peroxidation as well as a model for cellular events that take place after exhaustion of antioxidant defense mechanisms, which likely occurs as a result of events such as chronic inflammation [16].

Measurement of the oxidized metabolites of arachidonic acid including 5-HETE, 5-HPETE, and 5-oxo-ETE has been reported using several strategies. The most widely used protocols are based upon high-pressure liquid chromatography (HPLC) separation followed by UV detection because these molecules have conjugated double bonds with reasonably large extinction coefficients (23,000 5-HETE/5-HPETE at 236 nm and 19,600 at 279 nm for 5-oxo-ETE) [17–19]. Reverse phase HPLC is typically used to separate these eicosanoids and quantitation is possible when 5–10 ng is injected on column [20]. In addition, gas chromatography/mass spectrometry (GC/MS) techniques have been developed to analyze these compounds, but require prior derivatization to render these molecules suitable for gas phase analysis. These GC/MS methods also require the reduction of the conjugated double bond system to reduce the saddle effect observed during GC analysis [21]. Nonetheless, when GC/MS is coupled with electron capture ionization, a sensitive assay for derivatized 5-HETE [14] and 5-oxo-ETE quantitation is possible [15, 22].

Direct analysis of fatty acid hydroperoxides is not possible by GC/MS, which has limited the direct study of 5-HPETE synthesis. With the development of electrospray ionization, LC/MS, and LC/MS/MS techniques, it has become apparent that specific and direct assays can be developed for hydroperoxides [23], which require less sample handling yet retain sufficient sensitivity, thus permitting quantitation of these important products of free radical oxidation of arachidonic acid at levels encountered during in vitro lipid peroxidation. We have used this strategy to develop a direct mass spectrometric assay for 5-HPETE and report here, for the first time, the use of this assay in quantitating production of this biologically active hydroperoxy eicosanoid.

Methods

Materials

Resveratrol, d_8-5(*S*)-HETE, 5(*S*)-HETE, 5(*S*)-HPETE, and 5-oxo-ETE were obtained from Cayman Chemical (Ann Arbor, MI). The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): *t*-butylhydroperoxide (tBuOOH), butylated hydroxytoluene (BHT), and *L*-ascorbic acid. All solvents were HPLC grade (Fisher, Fair Lawn, NJ).

Preparation of RBC Ghosts

Venous blood (30 mL) was taken from normal human volunteers, treated with 3.8% sodium citrate (3.3 mL), and within 60 min blood centrifuged at 300 × g for 20 min to separate serum and buffy coat from RBC. The cells were washed by resuspending them in 50-mL phosphate buffer saline (PBS, 8.0-g NaCl; 0.20-g KCl; 1.51-g Na_2HPO_4; 1.16-g KH_2PO_4 dissolved in 1-L water and adjusted to pH 7.4), then centrifuged at 760 × g for 15 min to pellet the cells. A second wash in 50-mL PBS was employed followed by a final centrifugation at 1700 × g for 15 min to yielded packed RBCs.

RBC lysis was carried out according to the method of Bratton [24] with several modifications. Briefly, aliquots of packed RBC (500 μL) were placed in separate centrifuge tubes after resuspension in 6-mL cold lysis buffer [PBS diluted fivefold, containing 1-mM $MgCl_2$, 0.1-mM egtazic acid (EGTA)] for 1 h on ice. Following lysis, the suspension was spun at 26,000 × g for 15 min, supernatant discarded, and pellet resuspended in 6-mL water. This suspension was then passed several times through a 22-gauge syringe needle to insure lysis was complete. The process of centrifugation and resuspension in distilled water was repeated 4–5 times to obtain the substantially white RBC ghosts; however, traces of hemoglobin were still present. The ghosts were resuspended in 1.7-fold concentrated PBS (4 mL) and sealed by placing in a 37°C water bath for 30 min, then stored at 4°C overnight. The ghosts were centrifuged at 1700 × g for 15 min, lysed with distilled water on ice, resealed, and pelleted by centrifugation (1700 × g, 15 min) immediately before incubation. Protein was measured by the bicinchoninic acid protein assay [25].

Incubation with t-Butylhydroperoxide

Ghosts were incubated at a final concentration of 0.2-mg/mL total protein and 10-mM tBuOOH for 90 min at 37°C. Identical aliquots of the ghost preparation were incubated for 90 min at 37°C without addition of tBuOOH as the control experiment for endogenous lipid peroxidation products in the RBC ghost preparation. In separate experiments, RBC ghost preparations were incubated with 1-mM BHT, 5-mM ascorbic acid, or 0.44-mM resveratrol for 1 min prior to the addition of tBuOOH followed by a 90-min incubation at 37°C. Each reaction was terminated by the addition of 5-mg BHT dissolved in 1-mL methanol. This also inhibited any further oxidation during the lipid extraction (see below).

Lipid Extraction and Saponification

Phospholipids were extracted from RBC ghosts essentially by the method of Bligh and Dyer [26] substituting methylene chloride for chloroform. The methylene chloride layer was removed, taken to dryness, then resuspended in 1.5-mL 85% methanol. Hydrolysis of the fatty acyl groups was carried out at room temperature for 1 h by the addition of 500-μL NaOH(1N). The reaction mixture was acidified by the addition of 50-μL 88% formic acid and the sample vacuum concentrated to approximately 200 μL. The sample was then diluted to 1 mL with a final concentration of 30% B (see below). The stable isotope labeled internal standard, d_8-5(S)-HETE (25 ng) was added to each sample at this point.

Reverse Phase HPLC/Mass Spectrometry (RP-HPLC/MS)

An Ultremex 5-μm 4.6-mm $\times$ 250-mm column (Phenomenex, Torrance, CA) with an ODS Guard-pak precolumn (Waters, Marlborough, MA) was used for separation of saponified fatty acyl groups. The solvent system was as follows: Solvent A: 8.3-mM acetic acid adjusted to pH 5.7 with ammonium hydroxide; solvent B: methanol:acetonitrile (35:65). The flow rate was 1 mL/min with the following stepwise gradient: 30–55% B over the first 10 min, 55–75% B over the next 15 min, 75–100% B over 5 min, then hold at 100% B. The effluent was split postcolumn with 30% diverted to the mass spectrometer and 1 mL of sample injected.

Electrospray Ionization Multiple Reaction Monitoring (MRM) Mass Spectrometry

Mass spectrometry analysis was performed on a Sciex API-III$^+$ (PE-Sciex, Toronto, Canada) operated in the negative ion mode with an orifice voltage of -55 V. Curtain gas flow was 1.2 L/min, nebulizer pressure 40 lb/in.^{2}, and ion spray voltage was -3500 V (nitrogen as nebulizing gas). Product ion spectra were obtained using a collision energy of 15 eV and collision gas thickness (argon) of 211 $\times$ 10^{13} molecules/cm^2.

Results

Electrospray ionization of 5-HETE, 5-HPETE, and 5-oxo-ETE results in the formation of an abundant negative ion corresponding to the carboxylate anion from each of these eicosanoids. Collision induced decomposition of these carboxylate anions yields a large number of decomposition ions, several of which carry unique structural information as has been previously investigated [23, 27]. For example, collision induced decomposition of 5-HPETE (m/z 335) yields abundant ions at m/z 203, 129, and 59. Collision induced decomposition of the isobaric compound leukotriene B_4 also produces the same ions; however, they are substantially less abundant compared to the major product ion at

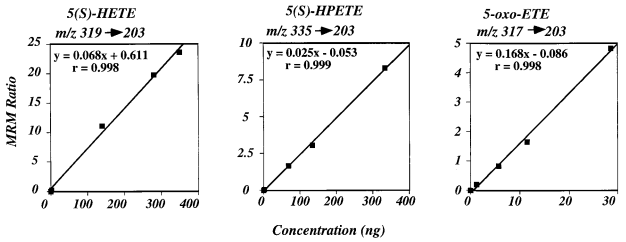

Figure 1. MRM standard curves. Solutions were prepared containing authentic standards of 5(S)-HETE, 5(S)-HPETE, and 5-oxo-ETE and analyzed by MRM LC/MS/MS as described in Methods. Amounts ranging from 1.4 to 350 ng were used for 5(S)-HETE, 1.3–334 ng for 5(S)-HPETE, and 1.1–29 ng for 5-oxo-ETE, as well as samples with no target eicosanoid added, with the following ion transitions monitored: m/z 319 $\rightarrow$ 203, m/z 335 $\rightarrow$ 203, and m/z 317 $\rightarrow$ 203. The d_8-5(S)-HETE internal standard (25 ng) was monitored as the ion transition m/z 327 $\rightarrow$ 210. The MRM ratio was calculated as the ratio of the abundance of target eicosanoid ion transition to that of the abundance of the internal standard ion transition.

m/z 195 [28]. Collision induced decomposition of the 5-oxo-ETE carboxylate anion (m/z 317) yields the same ions observed for decomposition of the molecular anion of 5-HPETE (m/z 335) because of a facile dehydration reaction [23]. The orifice potential was reduced to -55 V to reduce the extent of this reaction prior to the first quadrupole mass filter, but even at this voltage, the dehydration ion was quite abundant. Collision induced decomposition of 5-HETE (m/z 319) yields relatively few decomposition ions, but they are quite abundant and specific for this eicosanoid (m/z 203 and 115). It is not unexpected that similar product ions are observed for these compounds as they are members of a structurally related family of molecules. Therefore, analysis of these eicosanoids required separation prior to MS/MS analysis and fortunately, all of these eicosanoids were readily separated by RP-HPLC.

Stable isotope labeled 5-HETE [25-ng, d_8-5(S)-HETE] was employed as internal standard for the assay with the following specific ion transitions monitored: m/z 327 $\rightarrow$ m/z 210 for d_8-5(S)-HETE; m/z 319 $\rightarrow$ m/z 203 for 5-HETE; m/z 335 $\rightarrow$ m/z 203 for 5-HPETE; m/z 317 $\rightarrow$ m/z 203 for 5-oxo-ETE (Figure 1). The d_8-5(S)-HETE also served as a marker for HPLC retention time. Typical calibration curves (Figure 1) for all compounds studied showed excellent linearity in the range of 1–350 ng of oxidized arachidonic acid using 25 ng of d_8-5(S)-HETE as internal standard. The detection limits for this protocol were assessed in separate experiments and a signal-to-noise ratio greater than 3 was observed when 1 ng was injected onto the column for 5-HETE and 5-HPETE and 300 pg injected onto the column for 5-oxo-ETE.

The quantitative assay for these 5-oxygenated eicosanoids was used to investigate whether these compounds were present in the normal plasma membrane preparation from red blood cells and if an agent that increased membrane peroxide tone [15] would induce

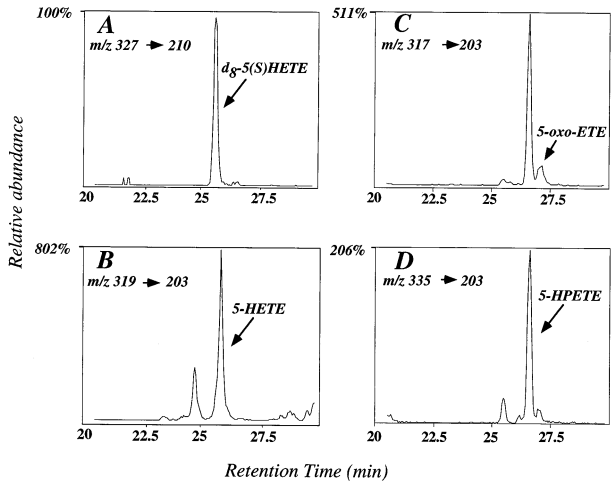

Figure 2. MRM LC/MS/MS chromatogram from tBuOOH treated RBC ghosts. Representative chromatogram of target eicosanoids produced after tBuOOH (10 mM) treatment of RBC ghosts (0.2-mg protein). (**A**) The ion transition m/z 327 → 210 [d_8-5(S)-HETE] was used to normalize the MRM signals for target eicosanoids in (**B**), (**C**), and (**D**). (**B**) Ion transition m/z 319 → 203 used for MRM analysis of 5-HETE. (**C**) Ion transition m/z 317 → 203 used for MRM analysis of 5-oxo-ETE. (**D**) Ion transition m/z 335 → 203 used for MRM analysis of 5-HPETE.

their synthesis. Treatment of RBC ghosts for 90 min at 37°C with tBuOOH (10 mM) resulted in the formation of abundant oxidized arachidonic acid and specifically 5-HPETE, 5-HETE, and to a lesser extent 5-oxo-ETE as revealed by a representative LC/MS/MS trace for these components (Figure 2). The specific formation of 5-HETE was observed at 25.8 min from the transition m/z 319 → 203, although other monohydroxyeicosatetraenoic acids were also formed in this reaction as revealed by the components eluting at 23.6 and 24.7 min which yielded m/z 203 as a minor product ion from decomposition of m/z 319. Six different monohydroxyeicosatetraenoic acid isomers were possible in the free radical oxidation of arachidonic acid, but their formation was not further investigated.

The second most abundant 5-oxygenated eicosanoid corresponded to 5-HPETE, which was detected by the specific transition m/z 335 → m/z 203 at 26.6 min. Other monohydroperoxy eicosanoids were also likely present (isobaric at m/z 335), however, only minor product ions at m/z 203 were formed by these products, which also elute at a different retention time. This chemically reactive hydroperoxide would be the immediate precursor of 5-HETE and was likely an initial oxidized product of the reaction of molecular oxygen with arachidonate esterified to glycerophospholipids in the RBC ghosts. The least abundant 5-oxygenated eicosanoid corresponded to 5-oxo-ETE, which eluted at approximately 26.9 min. The elution of this component was also characterized by significant absorption at 280 nm characteristic for a conjugated dienone in 5-oxo-ETE (data not shown). The transition m/z 317 → m/z 203 also indicated the elution of 5-HPETE at 26.6 min, as

Table 1. Quantitation of 5-oxygenated arachidonate esterified to phospholipids in the plasma membrane of red blood cells (RBC ghosts) before and after treatment with t-butylhydroperoxide

	5-HETE	5-HPETE	5-Oxo-ETE
Control RBC	4.7 ± 2.3	8.5 ± 6.0	1.8 ± 1.8
tBuOOH (10 mM)	264 ± 140	191 ± 116	17.1 ± 1.8

m/z 317 is a facile dehydration product ion of 5-HPETE formed during the electrospray ionization process [23].

Untreated RBC ghosts were found to contain both 5-HPETE and 5-HETE and a substantially lower quantity of 5-oxo-ETE. Following the treatment of RBC ghosts, a striking increase in 5-HETE, 5-HPETE, and to a lesser extent 5-oxo-ETE was observed (Table 1). Several antioxidants were employed to address whether or not 5-HETE production could be inhibited in this lipid peroxidation model. Butylated hydroxytoluene (1 mM), ascorbic acid (5 mM), and resveratrol (0.44 mM), a natural product isolated from grapes [29] were added to the isolated RBC ghosts immediately prior to the addition of the tBuOOH. The 5-oxygenated eicosanoids were then analyzed using the LC/MS/MS protocol (Figure 3). The production of all of these oxygenated eicosanoids was found to decrease dramatically. The quantity of oxidized metabolites found in the resveratrol treated RBC ghosts was indistinguishable from levels of 5-HETE and 5-HPETE initially present in untreated RBC ghosts.

Discussion

Electrospray ionization mass spectrometry has enabled development of a sensitive and specific quantitative assay for the eicosanoid, 5-HPETE. This assay was used to investigate whether or not 5-HPETE was a major product of lipid peroxidation involving red blood cell

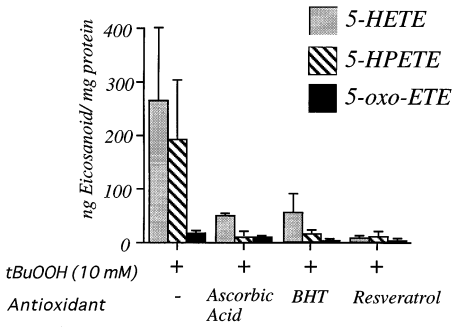

Figure 3. MRM quantitation. Amount of 5-HETE (filled square), 5-HPETE (open square), and 5-oxo-ETE (lined square) quantitated by MRM analysis after treatment of RBC ghosts for 90 min at 37°C with tBuOOH (10 mM) alone or with tBuOOH (10 mM) in addition to the following antioxidants: ascorbic acid (5 mM), resveratrol (0.44 mM), and BHT (1 mM). Control RBC ghosts were incubated under identical conditions for 90 min at 37°C with no addition of tBuOOH or antioxidants. Results are averages of 3 samples from different donors ± SEM and have been normalized by the amount of total protein (0.2 mg) in the incubation.

ghosts. This model of lipid peroxidation of cellular plasma membranes focuses attention on peroxidative events that take place in cells with little antioxidant defense mechanisms [15]. Such antioxidant mechanisms are responsible for the chemical reduction of this chemically reactive, yet biologically active hydroperoxide. This mass spectrometric approach was also used to quantitate 5-HETE and 5-oxo-ETE, in addition to the 5-HPETE, by RP-HPLC/tandem mass spectrometry operating in the MRM mode. This was an attractive alternative to other methods of analysis of these eicosanoids such as RP-HPLC with ultraviolet detection and GC/MS. Although GC/MS based protocols can be used to quantitate the biologically active 5-HETE and 5-oxo-ETE, it is not compatible with the analysis of 5-HPETE.

The LC/MS/MS method described here had detection limits in the picogram to low nanogram level (injected quantity) permitting investigations of in vitro production of this reactive eicosanoid in phospholipid precursors. Significantly enhanced sensitivity would be expected if smaller diameter HPLC columns were employed with corresponding lower flow rates that would increase analyte concentrations in the effluent. The assay described here utilized only 30% of the effluent for MS/MS analysis permitting collection of the remaining effluent. Nevertheless, the sensitivity of this LC/MS/MS assay permitted accurate determination of each of these free radical derived metabolites from RBC ghosts when formation of these metabolites was inhibited greater than 90%. By using the RP-HPLC assay with a UV monitor, it was not possible to detect the occurrence of 5-HETE, 5-HPETE, or 5-oxo-ETE present in unstimulated RBC ghosts. Sufficient sensitivity was available in this electrospray mass spectrometric assay to permit detection of these components present in unstimulated RBC ghosts. Of further advantage was the minimal sample handling required prior to the mass spectrometric assay when compared to a GC/MS based assay. In large part, this was made possible because of formation of abundant carboxylate anions during electrospray ionization of the nonderivatized carboxylic acid. A reduced orifice potential was employed in this assay to lessen the dehydration ions $[M–H_2O]^-$ and correspondingly increase the sensitivity of this assay.

The esterification of 5-HETE into glycerophospholipids has been previously examined and found to be a facile process for 5-lipoxygenase generated 5(*S*)-HETE [30]. However, studies reported here show that esterified 5-HETE, and in particular esterified 5-HPETE, can be found in relatively high levels as a result of free radical oxidation of arachidonate in situ. There have been no previous reports of esterified 5-HPETE in phospholipids. The use of the RBC ghost model eliminated the possibility that the observed esterified 5-HETE and 5-HPETE were derived from a reacylation mechanism from the corresponding free acids. First, 5-lipoxygenase is not present in the intact RBC or RBC ghost. Furthermore, the RBC ghost is depleted of the cofactors necessary for the formation of CoA ester of 5-HETE or 5-HPETE required for the reacylation mechanism. Thus, the observed 5-HETE and 5-HPETE glycerophospholipids were a result of direct lipid peroxidation and not an indirect effect of enzymatic formation of these molecules in this model system.

In the present study, oxidized arachidonic acid was found to be present at fairly low levels as evidenced by the abundance of 5-HETE, 5-HPETE, and 5-oxo-ETE in normal RBC ghosts. These three lipid peroxidation products increased significantly following treatment with tBuOOH. Due to the highly reactive nature of the free radical species involved during lipid peroxidation, it would be expected that variation in the observed quantity of these oxidized products may result as was observed (Table 1). We have previously found the formation of 5-HETE in tBuOOH treated intact RBC along with formation of the isobaric epoxyeicosatrienoic acids (EET) [12]. Specific analysis of EET esters was not investigated in this model system. Although the intermediacy of esterified 5-HPETE was suggested in these previous studies, the presence of numerous antioxidant mechanisms and high intracellular concentration of glutathione in the intact RBC led to a rapid reduction of phospholipid hydroperoxides to phospholipid alcohols. The RBC ghost has been used to model those situations in vivo when such antioxidant mechanisms are absent or substantially reduced [15].

The nonenzymatic nature of lipid peroxidation induced by tBuOOH was supported by the observed inhibition of product formation using the antioxidant compounds BHT, ascorbic acid, and resveratrol. Resveratrol, 3,5,4′-trihydroxy-trans-stilbene, is a natural product isolated from grapes, which has been found to possess chemopreventive activity in each of the stages of carcinogenesis initiation, promotion, and propagation [29]. Furthermore, resveratrol has been shown to possess antioxidant properties as demonstrated by the inhibition of TPA induced free radical formation in human promyelocytic leukemia cells [29]. Resveratrol, as well as ascorbic acid and BHT, were capable of inhibiting the formation of 5-HETE, 5-HPETE, and 5-oxo-ETE, further demonstrating potential of resveratrol as a lipid antioxidant. The MRM assay had sufficient sensitivity and precision to determine inhibition of the formation of these oxidized arachidonic acid isomers at levels greater than 75% and to levels observed of esterified 5-oxygenated eicosanoids in untreated RBC ghosts.

Formation of these phospholipid products of arachidonate free radical oxidation is of interest from several points of view. Such molecules can serve as markers of free radical oxidation of biological membranes since monohydroperoxides are likely the initially formed products of the reaction between phospholipid radical species with molecular oxygen [31]. However, these compound can be derived from 5-lipoxygenase catalyzed mechanisms, which limits their value as unique markers. Nevertheless, detection of 5-HPETE suggests limited or diminished antioxidant defense mechanisms. More interestingly, the targets of

this study, 5-oxo-ETE, 5-HPETE, and 5-HETE are known to exert potent biological activities on different tissues. Natural killer cell mediated cytotoxicity has been found to be enhanced by 5-HPETE [32] as well as eosinophil and neutrophil chemotaxis [33]. Racemic 5-HPETE has also been found to be a potent agonist of neutrophil degranulation [34]. Pharmacologic studies have shown both 5(*S*)-HETE and 5-oxo-ETE to increase intracellular calcium ion in the human polymorphonuclear leukocytes in a dose dependent manner [35, 36]. The 5(*R*)-HETE isomer was found to be substantially less potent in this biological assay. The most potent activities for these class of molecules has been reported for 5-oxo-ETE. The degranulation of the human neutrophil was also found to be stimulated by 5-oxo-ETE in conjunction with GM-CSF or G-CSF in substantially lower concentrations than that required for other monooxygenated products of arachidonic acid [37]. 5-Oxo-ETE has also been shown to increase O_2^- anion production in neutrophils [37] and a considerable amount of interest has focused on the activities of 5-oxo-ETE because of its potency as a chemotactic agent for the human eosinophil [38].

Further reactions of these free radical products are also possible; for example, 5-HPETE is readily degraded enzymatically by peroxidases [39] or nonenzymatically through reaction with heavy metals [31], to 5-HETE. Dehydration of 5-HPETE to 5-oxo-ETE can take place by nonenzymatic mechanisms. Oxidation of 5-HETE to 5-oxo-ETE is known to be catalyzed by the enzyme 5-hydroxyeicosanoid dehydrogenase [22]. Interestingly, 5-HPETE can be converted into the leukotriene precursor LTA_4 by 15-lipoxygenase [40].

Because the biological activities of 5-HETE and 5-HPETE, as well as isoprostanes and isoleukotrienes, were studied as the corresponding free acids, mechanisms responsible for liberation of the oxidized arachidonoyl ester from the phospholipid have become of interest. New evidence has emerged for specific phospholipases that can rapidly hydrolyze oxidized fatty acyl esters from phospholipids. These enzymes include calcium-dependent phospholipase A_2 [41] and platelet activating factor acetyl hydrolase [42]. The assay reported here can be used to independently assess hydrolysis of esterified 5-HETE and 5-HPETE.

Acknowledgment

This work was supported in part by a grant from the National Institutes of Health (HL34303).

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