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Forum Review

New Insights Regarding the Autoxidation of Polyunsaturated Fatty Acids

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ABSTRACT

Free radical-initiated autoxidation of polyunsaturated fatty acids (PUFAs) has been implicated in numerous human diseases, including atherosclerosis and cancer. This review covers the free radical mechanisms of lipid oxidation and recent developments of analytical techniques to analyze the lipid oxidation products. Autoxidation of PUFAs generates hydroperoxides as primary oxidation products, and further oxidation leads to cyclic peroxides as secondary oxidation products. Characterization of these oxidation products is accomplished by several mass spectrometric techniques. Ag^+ coordination ion spray mass spectrometry has proven to be a powerful tool to analyze the intact lipid peroxides. Monocyclic peroxides, bicyclic endoperoxides, serial cyclic peroxides, and a novel class of endoperoxides (dioxolane-isoprostane peroxides) have been identified from the oxidation of arachidonate. Electron capture atmospheric pressure chemical ionization mass spectrometry has been applied to study lipid oxidation products after derivatization. All eight possible diastereomeric isoprostanes are observed from the oxidation of a single hydroperoxide precursor. 5- and 15-series isoprostanes are more abundant than the 8- and 12-series because the precursors that lead to 8- and 12-series compounds can undergo further oxidation and form dioxolane-isoprostane peroxides. Furthermore, formation of isoprostanes from 15-hydroperoxyeicosatetraenoate occurs from β-fragmentation of the corresponding peroxyl radical to generate a pentadienyl radical rather than a "dioxetane" intermediate, as previously suggested. *Antioxid. Redox Signal.* 7, 170–184.

INTRODUCTION

LIPIDS are essential components of cellular membranes that maintain the biological function of cells. Lipids also play an important role in the control of major cellular activities, such as signal transduction (72). However, lipids with polyunsaturated fatty acids (PUFAs) are primary targets for the attack by reactive oxygen species or free radicals, and the oxidation of these lipids in humans may eventually lead to various diseases, such as atherosclerosis, cancer, diabetes, chronic inflammatory bowel disease, rheumatoid arthritis, and the neurodegenerative disorders including Alzheimer's disease and Parkinson's disease (62). Thus, lipid peroxidation, or reaction of lipid with molecular oxygen, has been an intensive research area for decades (60). Tremendous research efforts have been made to understand the mechanisms of lipid peroxidation and prevent the deleterious effect of lipid autoxidation.

Lipoproteins are major carriers of cholesterol and other lipids in the blood stream. Lipoproteins are divided into several classes according to their density. In order of increasing density (decreasing size), the major lipoproteins in serum are chylomicrons, very-low-density lipoproteins, low-density lipoproteins (LDL), and high-density lipoproteins. The role of LDL is to transport cholesterol to peripheral tissues. The outer layer of an LDL particle contains primarily phospholipids and cholesterol, whereas in the inner neutral core there are two major cholesterol esters, cholesteryl linoleate (18:2)

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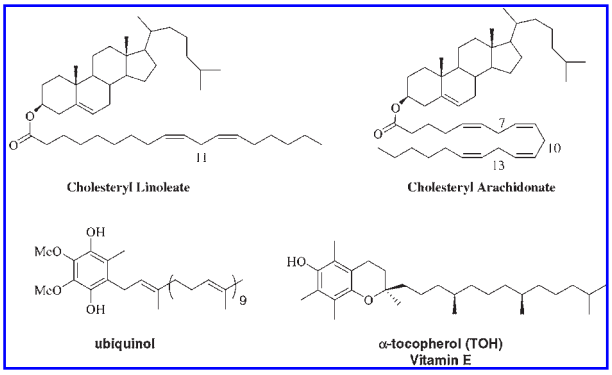
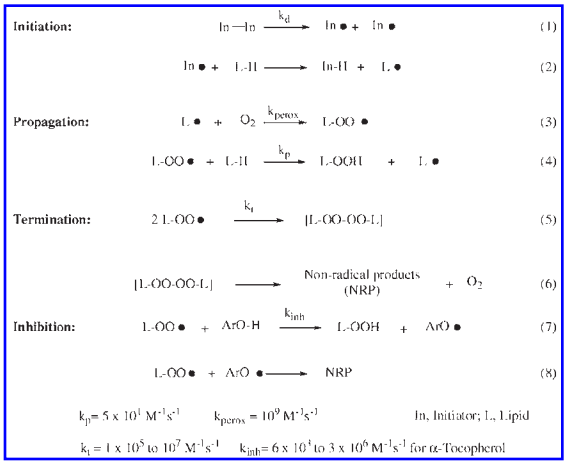


Scheme 1.



Scheme 2.

and cholesteryl arachidonate (20:4) (Scheme 1). In addition to the lipids, there are several lipophilic antioxidants in LDL, such as tocopherols and coenzyme Q-10, which protect the lipids against oxidative stress. Oxidized LDL has been linked to atherosclerosis (11). Mounting evidence supports the oxidative theory: (a) oxidation of LDL accompanies the disease process, and oxidized lipoproteins are present in arterial lesions; (b) a large number of the biological effects of oxidized LDL *in vitro* mimic the events believed to be critical in the generation of atherosclerotic lesions *in vivo*; (c) antioxidants inhibit or delay the formation of lesions in animal models. However, it is still unknown how and where LDL is oxidized *in vivo,* and which of the biological effects demonstrated *in vitro* are relevant to atherosclerosis *in vivo*. It is generally believed that free radical reactions have been involved in the oxidation of PUFAs to generate hydroperoxides and highly oxidized products. The peroxides can also degrade to give highly reactive species, such as malonaldehyde, acrolein, and 4-hydroxynonenal (4-HNE), which can covalently modify protein and DNA (37, 59, 80, 89, 90).

This review will cover free radical mechanisms involved in lipid oxidation and the recent development of analytic techniques that have been applied to analyze lipid oxidation products. A number of mass spectrometric techniques have been used to analyze lipid oxidation products that include Ag^+ coordination ion spray (CIS) mass spectrometry (MS), electron capture (EC) atmospheric pressure chemical ionization (APCI) MS, and gas chromatography (GC) MS. The stereochemistry of isoprostanes will also be summarized, as will the mechanism that generates those compounds from lipid oxidation.

FREE RADICAL MECHANISMS AND LIPID AUTOXIDATION

The mechanism of lipid autoxidation can be understood by a free radical chain process consisting of chain initiation, propagation, and termination steps (Scheme 2) (62).

Initiation

In the initiation step, the key event is the formation of a lipid radical L·. Lipid oxidation can be initiated by a variety of sources, including the use of transition metals such as copper and iron, enzymes, hydroxyl radical, γ-irradiation, and cells that can produce reactive oxygen species and nitrogen species (45, 82). Radicals generated *in vitro* by many methods lead to inconsistent oxidation results. For this reason, *azo* free-radical initiators have proved useful to provide a constant rate of initiating radicals in oxidation reactions (4). LDL oxidation *in vitro* can be initiated either from the aqueous phase or from the interior of the LDL particle by using different initiators. Water-soluble peroxyl radicals must be transferred into the lipid phase to initiate lipid peroxidation in the LDL. On the other hand, the initiation of radical reactions by lipid-soluble initiators proves to be inefficient due to the high cage recombination of radicals produced in the lipid core. Novel amphiphilic *azo* initiators have been tested to overcome the cage return problem. These initiators are designed to produce one lipid-soluble radical in the interior of lipoprotein and one water-soluble radical in the aqueous phase at the same time (14). The hydrophilic/hydrophobic character of the radicals generated from those amphiphilic *azo* initiators is an important factor for separating the two radicals. These radical initiators provide an advantage of free radical production, lipophilic access, and constant radical generation over lipophilic initiators and hydrophilic initiators when they are used to initiate the peroxidation of LDL oxidation for *in vitro* studies (15).

Propagation

In the propagation step, molecular oxygen is added to the carbon-centered radical L· to generate a peroxyl radical (LOO·) (Scheme 2, Eq. 3). The resultant peroxyl radical abstracts a hydrogen atom from another lipid molecule (L-H) to give another carbon-centered lipid radical (L·) (Scheme 2, Eq. 4), which can carry the chain propagation sequence. The hydrogen abstraction step is the rate-limiting step because oxygen addition to a carbon-centered radical occurs at or near the diffusion controlled rate at oxygen pressure above 100 mm Hg (30). Furthermore, the rate of propagation depends on the C-H bond strength of the lipid that is being oxidized (65). The C-H bonds at the bisallylic positions, such as C11 in

cholesteryl linoleate and C7, 10, and 13 in cholesteryl arachidonate (Scheme 1), are the weakest, and the hydrogen atoms at these positions are preferentially abstracted by a peroxyl radical. The resulting pentadienyl radicals are highly delocalized. The bond dissociation energy (BDE) at these bisallylic positions is ~78–80 kcal/mol and the k_p has been determined to be on the order of 55 M^{-1} s^{-1} (1, 28, 58). In addition to the radical addition and atom transfer (hydrogen atom abstraction), propagation steps may also include fragmentation reactions when the resulting radical is stabilized by delocalization, such as the pentadienyl radical mentioned above (65). Radical cyclization reactions can occur and are responsible for the generation of cyclic peroxides when polyunsaturated lipids, such as arachidonate, are autoxidized (62).

Termination

Nonradical products (NRP) form from the coupling of two radicals in the termination step ($k_t \approx 10^7$ M^{-1} s^{-1}). As hydrogen atom abstraction is the rate-determining step, recombination reactions are primarily between two peroxyl radicals. An unstable tetraoxide intermediate generated from the coupling of two primary or secondary peroxyl radicals decomposes to give an aldehyde, alcohol, and molecular oxygen (Russell Termination) (75).

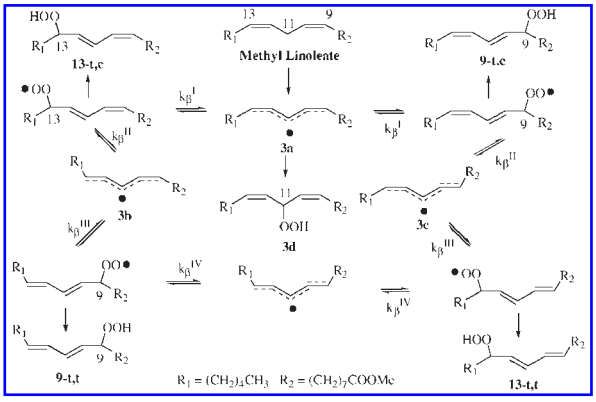
Antioxidants and lipid autoxidation

As lipid peroxidation has been implicated in the pathogenesis of atherosclerosis and other human diseases, a tremendous research effort has been devoted to prevention of lipid peroxidation in LDL by antioxidants. Phenolic antioxidants, such as vitamin E, are among the most active radical chain-breakers in LDL. Vitamin E is a mixture of structurally related phenolic antioxidants known as tocopherols among which α-tocopherol (α-TOH) is the most abundant and potent in LDL. It is generally accepted that α-TOH suppresses lipid peroxidation by trapping lipid peroxyl radicals in homogeneous solutions (Scheme 2). The hydrogen atom-donating ability of α-TOH is excellent. It acts by reducing a lipid peroxyl radical to a hydroperoxide and forming a relatively inert phenoxyl radical in the process (Scheme 2, Eq. 7). The k_{inh} for α-TOH ranges from 6×10^3 M^{-1} s^{-1} in phospholipid bilayers to 3×10^6 M^{-1} s^{-1} in organic solvents (7, 52). k_{inh} of α-TOH in LDL has been recently estimated to be 5.9×10^5 M^{-1} s^{-1} based on cholesteryl linoleate hydroperoxide products formed during autoxidation of intact lipoprotein (16). The α-tocopheroxyl radical generated can rapidly react with another peroxyl radical ($k_t = 2.5 \times 10^6$ to 3×10^8 M^{-1} s^{-1}) to yield NRP. Therefore, each molecule of α-TOH destroys two radicals and terminates two potential radical chains (6). Besides tocopherols, there are other antioxidants present in LDL, such as ubiquinol-10 and ascorbate (vitamin C). Ubiquinol-10 prevents LDL oxidation by reducing α-TO· and generates a water-soluble superoxide radical anion (O_2^{-}·) in the process (4). It is noteworthy that the search for better antioxidants has been an extremely active research topic. Synthesis and study of new antioxidants based on 3-pyridinol and 5-pyrimidinols have been reported recently (64, 87). By incorporation of nitrogen(s) into the phenol ring at either the 3 or the 3 and 5 positions, it is possible to substantially increase the ionization (oxidation) potentials of phenolic compounds without greatly affecting the O-H BDEs. A compound with H-donating ability 90 times better than α-TOH was recently reported (96).

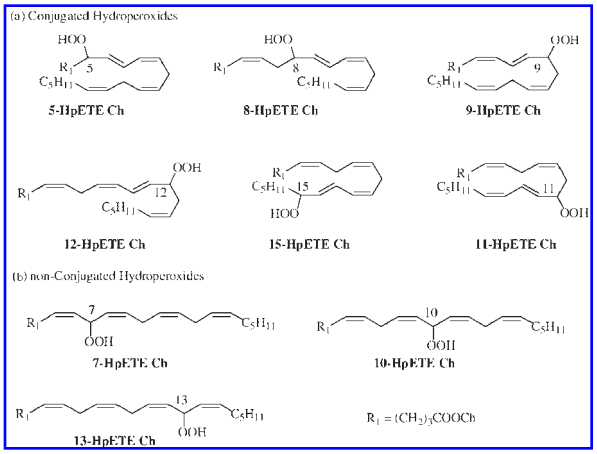
FORMATION OF PRIMARY LIPID OXIDATION PRODUCTS–HYDROPEROXIDES

A tremendous amount of research has been carried out to understand the mechanism of lipid peroxidation since the development of the fundamental concepts of autoxidation in the 1940s. The formation of primary hydroperoxides from the autoxidation process can be understood based on a free radical chain reaction sequence. The autoxidation products of methyl linoleate are shown in Scheme 3. The hydrogen atom abstraction at **C11** of methyl linoleate generates a pentadienyl radical, **3a**. Molecular oxygen addition to either **C9** or **C13** forms the corresponding peroxyl radical. The kinetic products 9-*t*,*c* and 13-*t,c* are obtained by hydrogen atom abstraction if a good hydrogen atom donor is present (t, trans; c, cis). The *t,c* peroxyl radicals at **C9** or **C13** can undergo β-fragmentation to generate the original *c,c* pentadienyl radical (**3a**) or *t,c* pentadienyl radicals (**3b**, **3c**) after single bond rotation. The thermodynamic products, **9** or **13** *t,t* hydroperoxides, can be obtained by oxygen addition to the *t,c* radical followed by hydrogen atom abstraction (62). The rate constants of β-fragmentation vary when pentadienyl radicals with different geometries are generated. The β-fragmentation of pentadienylperoxyl radicals leading to transoid centers occurs at the rate constant of 625 s^{-1} (k_β^{II} and k_β^{IV}), whereas that of generation of cisoid centers occurs with a much slower rate constant of 70 s^{-1} (k_β^{I} and k_β^{III}).

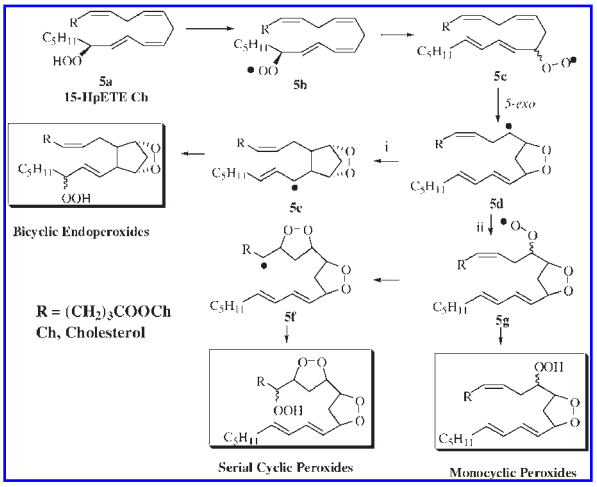
One question that arises in the mechanism of linoleate autoxidation is the formation of bisallylic hydroperoxides from the center carbon of a pentadienyl radical (such as **3a**). Theoretical calculations suggest that the ratio of the spin densities of a pentadienyl radical at the three positions (C9:C11:C13) is 1:1.12:1 (65), whereas electron spin resonance experiments give a ratio of 1:1.2:1 (17, 23). Oxygen addition to the center



Scheme 3.



Scheme 4.



Scheme 5.

carbon may therefore be preferred in comparison with addition to the two terminal positions. However, analysis of methyl linoleate autoxidation products failed to find the bisallylic product until recently, when the 11-hydroperoxide (**3d**) was characterized from autoxidation of methyl linoleate in the presence of α-TOC (5). The difficulty of trapping the peroxyl radicals at the bisallylic position is attributed to the rapid β-fragmentation of this radical. This rate constant was measured to be 1.9×10^6 s^{-1}, which is much faster than k_β from conjugated peroxyl radicals to generate a transoid pentadienyl radical (625 s^{-1}) or cisoid analogue (70 s^{-1}). The difference in rate of β-fragmentation of 9/13-peroxyl radicals and 11-peroxyl radical is best understood in terms of the calculated C-OO BDEs of each of the two peroxyl radicals. The BDE of terminal peroxyl radicals (9/13) leading to the transoid pentadienyl radical is 9.9 kcal/mol, whereas the BDE of the 11-peroxyl radical is only 3.8 kcal/mol (65). This rapid fragmentation also served as a useful radical clock for measuring antioxidant hydrogen atom transfer, k_{inh} (87). The formation of hydroperoxides from other PUFAs can be understood based on free radical mechanisms. Six conjugated and three nonconjugated hydroperoxides are obtained from oxidation of arachidonic acid or its esters, such as cholesteryl arachidonate (Scheme 4).

FORMATION OF CYCLIC PEROXIDES THROUGH LIPID PEROXYL RADICAL CYCLIZATION REACTIONS

Lipid hydroperoxides can be oxidized to give cyclic peroxides under some conditions of oxidation. In addition to the formation of bicyclic endoperoxides, serial cyclic peroxides and monocyclic peroxides were identified from the autoxidation of arachidonic acid or its esters (Scheme 5) (34, 51, 67). The peroxyl radical **5b** is formed under free radical conditions by hydrogen atom abstraction. β-Fragmentation occurs to generate a pentadienyl radical, and O_2 addition to C11 gives the peroxyl radical **5c**. Radical **5c** undergoes *5-exo* cyclization to produce a carbon-centered radical **5d**, which can cyclize again to give another carbon-centered radical **5e** (route **i**) or form a peroxyl radical **5g** by rapid O_2 addition (route **ii**). The bicyclic endoperoxide is formed from **5e** by O_2 addition and hydrogen atom abstraction. Hydrogen atom abstraction of the peroxyl radical **5g** can generate monocyclic peroxides. Alternatively, **5g** can undergo *5-exo* cyclization to give rise to serial cyclic peroxides. Overall, at least three major classes of cyclic peroxides can be generated from the autoxidation of cholesteryl 15-hydroperoxyeicosatetraenoate (15-HpETE Ch), namely, bicyclic endoperoxides, monocyclic peroxides, and serial cyclic peroxides, under conditions in the absence of good hydrogen atom donors. Similar cyclic peroxides can be produced from other regioisomeric hydroperoxides, and a complex mixture will be generated.

ANALYSIS OF LIPID OXIDATION PRODUCTS

Analysis of lipid peroxidation products has been an extremely active area in the research field of lipid oxidation for decades (44). Various methods have been developed to assess lipid peroxidation. Measurement of conjugated dienes with the UV absorption at 234 nm has been used to assess lipid peroxidation. However, the application of this method has been limited due to the complication of other compounds having the same absorption. Light emission during hydroperoxide-induced oxidation of isoluminol has also been used as a chemiluminescence assay for lipid peroxidation (42). This sensitive method is widely used to analyze plasma samples, levels of hydroperoxides from cholesterol esters and phospholipids, when combined with an HPLC separation technique. Iodometric assays are based on the reaction of lipid hydroperoxides with iodide to form iodine or triiodide, which can be measured by a spectrophotometric assay, an anaerobic assay, or a cadmium-based assay. An immunohistochemical technique has been used to raise antibodies against specific

lipid peroxidation products, such as 4-HNE (57). The thiobarbituric acid reactive substance (TBARS) assay is one of the most frequently used methods to assess lipid peroxidation (35). The test is usually performed by heating a lipid peroxidation mixture and thiobarbituric acid in an acidic medium to form a red pigment that shows an absorption maximum at 532 nm (with molecular extinction coefficient of 1.56×10^5) and fluorescence at 532 nm (excitation) and 553 nm (emission). The ferrous oxidation of xylenol (FOX) assay was developed by Wolff and co-workers to analyze the hydroperoxides formed from lipid peroxidation (31, 53). This method is based on the fact that hydroperoxides oxidize ferrous (iron II) to ferric (iron III) and the resulting ferric ion can bind to xylenol orange to produce a colored complex with a strong absorbance at 560 nm ($\epsilon = 4.3 \times 10^4\ M^{-1}\ cm^{-1}$ for hydrogen peroxide and *ter-t*-butyl hydroperoxide). Both the TBARS and FOX assays are nonspecific methods, and highly oxidized peroxides can give a positive response to both even after triphenylphosphine (PPh_3) reduction of the reaction (97).

Ag^+ CIS-MS

Characterization of organic peroxides has been limited by their inherent thermal and chemical instability, and conventional characterization methods have proven difficult when applied to these compounds (98, 99). GC-MS has been used in the analysis of lipid autoxidation products after derivatization [methyl esters or pentafluorobenzyl (PFB) esters and trimethylsilyl (TMS) ethers]. To do this, the peroxides formed during lipid peroxidation must be reduced to alcohols before being derivatized to PFB or methyl esters. An extensive compilation of >700 electron ionization (EI) mass spectra of eicosanoid derivatives derived from oxidation of arachidonic acid has been collected. EI-MS of these derivatives gives specific fragmentation information, such as α-cleavage, which can be used to identify the position of a hydroxyl group in the lipid chain. In addition to EI-MS, the alcohols also can be analyzed under CIS-MS conditions. In this protocol, the alcohols are converted to the PFB esters for analysis under negative ion EC conditions (24).

Direct analysis of lipid peroxidation products without prior derivatization would be of importance because multiple derivatization steps are time-consuming and allow plenty of opportunities for sample loss or artificial lipid oxidation during the sample manipulation. There are a few successful examples in the direct analysis of lipid hydroperoxides. Reverse-phase HPLC coupled with thermospray ionization has been used to identify lipid hydroperoxides and hydroperoxyl fatty acids esterified to phospholipids. Murphy *et al.* applied electrospray ionization (ESI) MS to the direct analysis of lipid hydroperoxides and other oxidation products (25, 39, 49). ESI in the negative ion mode allowed the generation of abundant carboxylate anions from free acid hydroperoxides. By using tandem MS techniques, distinctive fragmentation patterns were obtained, which makes structural elucidation possible.

Analysis of intact cholesteryl ester hydroperoxides derived from the core lipids in a lipoprotein oxidation is difficult to undertake by conventional MS methods. Obviously, GC-MS would not be suitable because these thermally labile compounds cannot survive GC conditions. ESI and APCI are widely used soft ionization modes and may be coupled efficiently to powerful separation techniques such as HPLC for the characterization of complex mixtures. Nonetheless, cholesteryl esters are highly lipophilic and lack ionization sites with sufficient proton affinity that are required for ESI and APCI applications (3, 18, 20). Bayer *et al.* reported the use of a coordinating reagent to ionize highly lipophilic compounds, such as terpenes, sugars, aromatics, and vitamins (2). A variety of coordinating reagents were used to coordinate with the analytes, and the resulting complex can be made suitable for MS analysis through a standard ESI-MS interface. This technique is termed coordination ion spray mass spectrometry (CIS-MS). Silver ion has been used extensively for CIS-MS applications because of its ability to coordinate with double bonds or aromatics. The use of silver-impregnated silica gel is routinely used to improve significantly the separation of unsaturated lipids by chromatography (46). Silver ion adducts show characteristic complexes of $[M + Ag^{107}]^+$ and $[M + Ag^{109}]^+$ doublets in the mass spectrum, because the natural isotopic abundance of silver is 52:48. CIS-MS can also be coupled with on-line liquid chromatography (LC) separations for analysis of complex mixtures of products. Ag^+ CIS-MS has also been used to characterize lipophilic tocopherols and carotenoids (69). This method has been explored to study the lipid peroxidation products by coupling the MS with normal-phase HPLC (26).

EC-APCI-MS

APCI-MS is based on ionization by ion-molecule or EC reactions that are carried out in an ion source operating at atmospheric pressure (10^5 Pa). Although the use of APCI is not as widespread as ESI, the number of reported applications of APCI-MS is burgeoning. APCI-MS has proven to be a very valuable technique for analysis of lipids and other biological compounds. APCI offers the possibility of interfacing with HPLC, which makes it an ideal tool for use in solving some difficult analytical problems (8). APCI-MS can also be operated in the EC negative ionization mode. EC is a sensitive and selective ionization technique for MS. This combination provides an increase in sensitivity of 2 orders of magnitude when compared with the conventional APCI methodology (81). This technique has been widely used to study biomolecules, drugs, and their metabolites in biological fluids owing to its superb sensitivity and the possibility of its coupling with HPLC (21, 22, 29, 38). The extraordinary sensitivity of EC-APCI-MS for a strong electrophore basically derives from the high efficiency of capture of a thermal or near-thermal electron and formation of a dominant anion product. In some cases, zeptomole detection level by GC-EC/MS has been achieved with strong electrophores (93). Pentafluorobenzylation is commonly used because it readily replaces active hydrogen, such as phenol, carboxylic acid, and heterocyclic NH, leading to a product that typically undergoes facile dissociative EC to form an analyte characteristic anion.

IDENTIFICATION OF LIPID OXIDATION PRODUCTS BY LC-AG^+ CIS-MS

In addition to the six conjugated hydroperoxides of cholesteryl arachidonate, nonconjugated hydroperoxides at bisal-

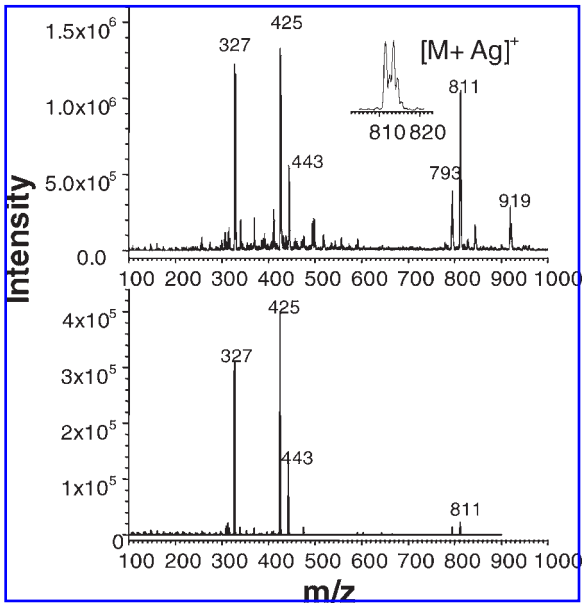
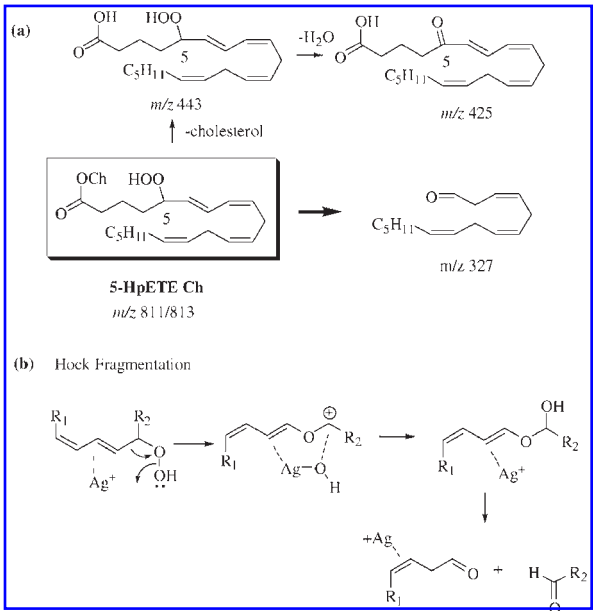


FIG. 1. 5-HpETE Ch analyzed by Ag^+ CIS-MS. (Top) Parent ion scan **(Bottom)** CID of *m/z* 811.



Scheme 6.

lylic positions can be formed when cholesteryl arachidonate is oxidized in the presence of good hydrogen atom donors, such as α-TOH (Scheme 4). Ag^+ CIS-MS has been applied to study the hydroperoxides formed from autoxidation of cholesterol esters, such as linoleate and arachidonate (100).

Characterization of 5-HpETE Ch is taken as an example. The parent ion scan of 5-HpETE Ch and the collision-induced dissociation (CID) spectrum of *m/z* 811 are shown in Fig. 1. The characteristic doublet of *m/z* 811/813 corresponds to the HpETE Ch Ag^+ adduct. The structural information of the parent ion is obtained by a CID experiment that is carried out by selecting *m/z* 811 in the first quadrupole, fragmenting it in the second quadrupole, and scanning all the resulting fragments in the third. The major fragments from the parent ion are summarized in Scheme 6a. Beside the loss of cholesterol and subsequent dehydration, there is a fragment with *m/z* 327 that is consistent with the structure of an aldehyde characteristic of 5-HpETE. The formation of the aldehyde can be understood based on well established Hock fragmentation of hydroperoxide (Scheme 6b). Obviously, the Hock fragmentation can give regioisomeric information about the hydroperoxide functionality of the HpETEs Ch. Other conjugated hydroperoxides of cholesteryl arachidonates can be studied the same way as for 5-HpETE Ch, and they show characteristic Hock fragments. Coupled with HPLC, selective reaction monitoring (SRM) is used to identify the individual hydroperoxide in oxidation mixtures. SRM is carried out in a triquadrupole tandem mass spectrometer by selecting the parent ion (*m/z* 811 for HpETEs Ch) in the first quadrupole, fragmenting it in the second quadrupole, and monitoring a specific fragment (Hock fragments for HpETEs Ch) resulting from the parent ion in the third. The SRM results of the six conjugated hydroperoxides of cholesteryl arachidonate are summarized in Fig. 2. 8-HpETE Ch and 9-HpETE Ch, and 11-HpETE Ch and 12-HpETE Ch, give the same Hock fragments. It is, therefore, impossible to distinguish these isomeric hydroperoxides based on Hock fragmentation alone. There is minor α-cleavage fragmentation in the CID experiment of HpETE Ch, and this fragmentation can be used to distinguish further the regioisomeric HpETEs Ch. The nonconjugated hydroperoxides at bisallylic positions of cholesteryl arachidonate have been detected when cholesteryl arachidonate is oxidized in the presence of good hydrogen atom donors, such as α-TOH. Three possible nonconjugated hydroperoxides from cholesteryl arachidonate, namely, the 7-, 10-, and 13-HpETE Ch, have been detected using Ag^+ CIS-MS. The elution order of the HpETEs Ch is 15-, 13-, 12-, 10-,

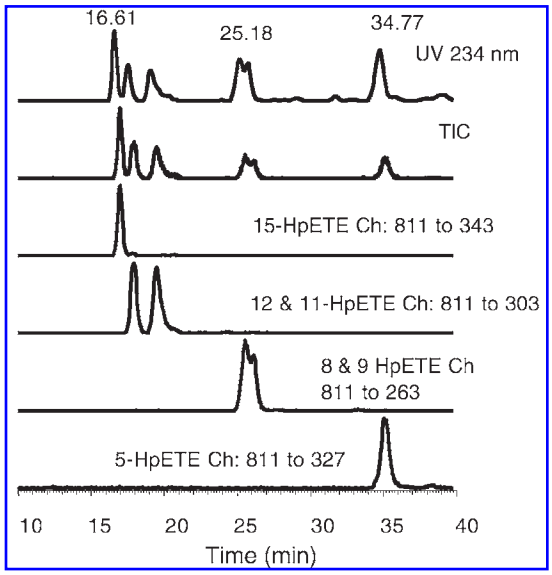


FIG. 2. SRM results of conjugated HpETE Ch analyzed by Ag^+ CIS-MS.

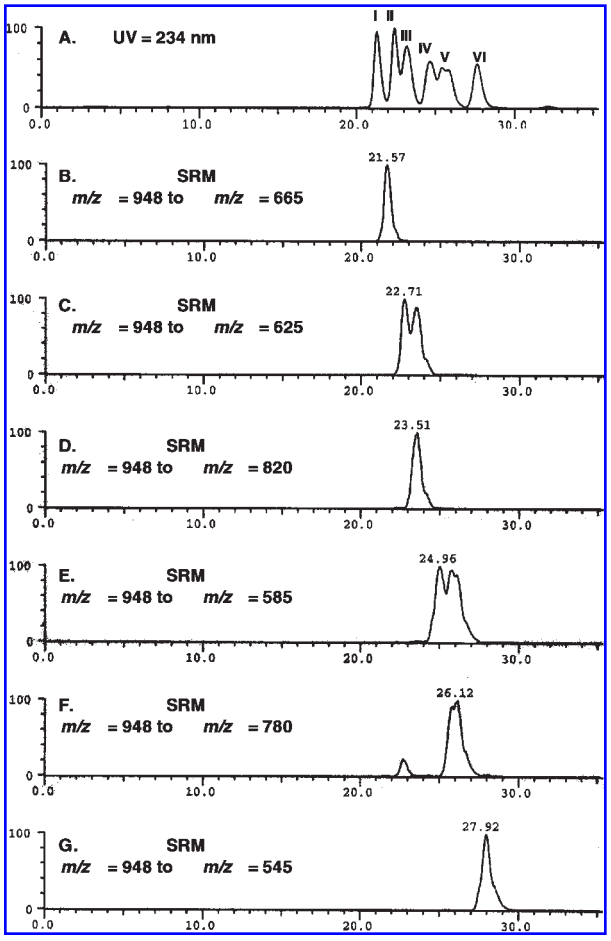


FIG. 3. Chromatograms of the product mixture of SAPC hydroperoxides. (**A**) UV detection at 234 nm: I = 15-HpETE PC, II = 11-HpETE PC, III = 12-HpETE PC, IV = 8-HpETE PC, V = 9-HpETE PC, VI = 5-HpETE PC. (**B–G**) HPLC-CIS-MS/MS in SRM mode.

11-, 9-, 8-, 7-, and 5-HpETE Ch. However, when the hydroperoxides are reduced to the corresponding alcohols by PPh_3, the elution order of these alcohols changes to 12-, 15-, 11-, 13-, 10-, 9-, 8-, 7- and 5-HpETE Ch.

The same technique has been applied to analyze hydroperoxides from phospholipids (41). SRM analysis of the hydroperoxides from 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine (SAPC) is illustrated in Fig. 3. Recently, characterization of 10 hydroperoxides from oxidation of docosahexaenoic acid (DHA) has also been accomplished by the same technique (78).

Oxidation of polyunsaturated lipids can produce many regioisomeric peroxyl radicals, which lead to an extremely complex oxidation mixture (Scheme 5). Biological samples are even more challenging because many types of polyunsaturated lipids are present, increasing the complexity of the lipid autoxidation profile. For the *in vitro* studies, starting from a single isomeric hydroperoxide dramatically reduces the complexity of the autoxidation mixture. After identification of the primary oxidation product hydroperoxides, the individual hydroperoxides can be purified by semipreparative HPLC. 15-HpETE Ch, for example, is used as a model compound for the analysis of the autoxidation products by Ag^+ LC-MS techniques. The possible oxidation products are summarized in Scheme 5 based on a free radical mechanism. Dozens of stereoisomers within each class of peroxides are predicted because several stereogenic centers are formed in these processes (100).

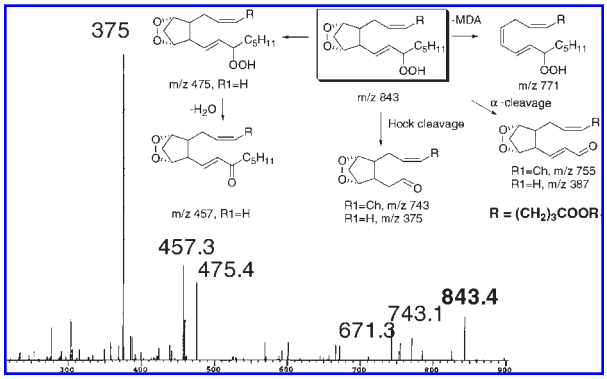
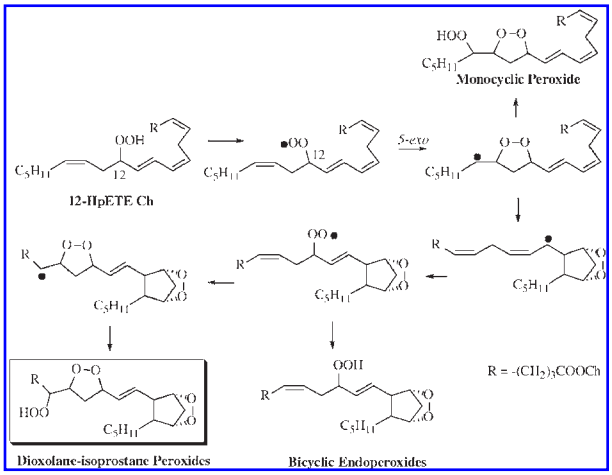


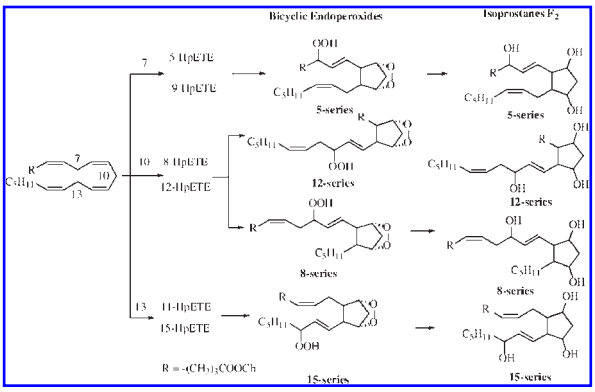
FIG. 4. CID spectrum and fragmentation pathway of bicyclic endoperoxide (*m/z* 843). MDA, malonaldehyde.

The autoxidation mixture from reactions of 15-HpETE Ch was first analyzed by a direct liquid infusion (DLI) experiment, which was carried out by continuous infusion of a mixture of a $AgBF_4$ solution and the autoxidation mixture into a triple quadrupole tandem mass spectrometer operating in Q_1 (parent scanning) mode. The DLI experiment clearly showed that all three classes of cyclic peroxides (bicyclic, monocyclic, and serial cyclic) are present in the autoxidation mixture of 15-HpETE Ch, in addition to the starting hydroperoxide. CID experiments were carried out by coupling of the MS with HPLC to characterize further these cyclic peroxides. A CID spectrum of *m/z* 843 corresponding to a bicyclic endoperoxide and the possible fragmentation pathways are shown in Fig. 4. All the fragmentation reactions observed have well established precedence in solution. The CID experiment unambiguously supports the presence of bicyclic endoperoxide structures in the product mixture. Monocyclic



Scheme 7.

peroxides and serial cyclic peroxides have also been identified by analogous CID experiments. SRM chromatograms of the oxidation products of 15-HpETE Ch revealed a number of diastereoismers within each class of cyclic peroxides. The oxidation mixtures from 11-, 5-, and 9-HpETE Ch have also been studied by the same technique, and similar cyclic peroxides are identified. In addition, a novel class of cyclic peroxides has been identified from an oxidation mixture of 12- and 8-HpETE (102). These compounds are termed dioxolane-isoprostane peroxides because a dioxolane functional group and a bicyclic peroxide moiety are present in the same molecule (Scheme 7). The novel structure of these compounds is further confirmed by other MS methods.



Scheme 8.

STEREOCHEMISTRY OF ISOPROSTANES

Eicosanoids are biologically active metabolites derived from arachidonic acid. Following its release from membrane phospholipids by phospholipases in response to a variety of nonspecific stimuli, arachidonic acid can be converted to prostaglandin (PG) G_2 and PGH_2 by prostaglandin synthase (PGH_2 synthase)-1 or -2, also referred to as cyclooxygenase COX (COX)-1 or COX-2. COX isozymes catalyze a two-step reaction, first cyclizing arachidonic acid to PGG_2, and then reducing the 15-hydroperoxyl group to form PGH_2. Cell-specific prostaglandin synthases catalyze the conversion of PGH_2 to other biologically active end products, such as PGE_2, PGD_2, PGF_2, PGI_2, and thromboxane (TxA_2), known collectively as prostanoids (91). These compounds are highly bioactive and have been implicated in inflammation, thrombosis, gastroprotection, and immune response (10, 63, 73, 74). Clearly, the formation of these potent compounds through controlled enzymatic pathways is essential for proper physiological function (36).

On the other hand, arachidonic acid can also undergo free radical-initiated peroxidation to produce a mixture of hydroperoxides and cyclic peroxides. These peroxides are always racemic mixtures, contrary to the products from the enzymatic pathways. It has been demonstrated that arachidonic acid, in both the esterified form and free acid, can form a wide array of oxidation products. The bicyclic endoperoxides, for example, differ in stereochemistry from enzymatically produced prostaglandins PPG_2, and these compounds can generate other isomers, called isoprostanes, upon reduction. In 1990, Morrow *et al.* reported the discovery of the formation of PGF_2-like compounds *in vivo* in humans by nonenzymatic free radical-induced peroxidation of arachidonic acid (47, 48). The initial discovery of F_2-isoprostanes was made possible by the use of stable isotope dilution GC negative ion chemical ionization (NICI) MS (71). Although this MS methodology is expensive and time-consuming, it is highly specific and sensitive. The accuracy of this assay is 96% and the precision is ±5%. Measurement of F_2-isoprostanes by this method has become one of the most reliable biomarkers for oxidative stress *in vivo*. There are several favorable attributes that make it a reliable indicator of oxidative stress: (a) F_2-isoprostanes are specific products of lipid peroxidation; (b) they are stable compounds; (c) their levels are present in detectable quantities in all normal biological fluids and tissues, allowing definition of a normal range; (d) their formation increases dramatically *in vivo* in a number of animal models of oxidant injury; (e) their formation is modulated by antioxidant status; and (f) their levels are not affected by dietary lipid content.

Bicyclic endoperoxides from cholesteryl arachidonate have been identified by Ag^+ CIS-MS techniques coupled with HPLC (100), and these compounds can be reduced to give a mixture of stereoisomeric isoprostanes (Scheme 8). It is conceivable that isoprostanes and their endoperoxide precursors with different stereochemistry from the natural prostaglandins (diastereochemistry and regiochemistry) may contribute to human diseases. Therefore, the study of the stereochemistry of isoprostanes may shed light into the biological activity of these compounds. Basically, four types of bicyclic endoperoxide can be formed from autoxidation of arachidonates, depending on the sites of the initial hydrogen atom abstraction. Moreover, eight racemic diastereomers can be generated from a single regioisomeric peroxyl radical. Thus, a total of 64 stereoisomers of isoprostanes can be generated from the oxidation of arachidonate. It is noteworthy that the stereochemistry of these endoperoxides generated from free radical autoxidation is quite different from the enzymatic oxidations by COX enzymes (see below). Therefore, the stereochemistry of the resulting isoprostanes is an important factor to distinguish the sources of these biologically potent compounds.

The stereochemistry of isoprostanes has been of interest for decades. Porter and Funk originally provided experimental evidence for the formation of bicyclic endoperoxides from PUFAs (61). Mihelich and O'Connor *et al.* furthered the original work of Porter by the isolation and extensive characterization of monocyclic peroxide and bicyclic endoperoxides derived from methyl linolenate hydroperoxides (40, 55, 56). The major isomers of the bicyclic endoperoxides were those with a *cis*-substituted dioxolane ring. Free radical generation of bicyclic products having the natural *trans* prostaglandin ring stereochemistry was highly disfavored. In addition, all these compounds are racemic, whereas those formed from enzymatic oxidation are optically pure.

As shown in Scheme 8, the 15-series bicyclic endoperoxides can be formed from 15- or 11-HpETE Ch. During the formation of these endoperoxides, eight diastereomers are anticipated. Fortunately, most of the stereoisomers of this class

of isoprostanes have been synthesized (83–86). Therefore, it is possible to determine the stereochemistry of the resulting isoprostanes in an autoxidation mixture of cholesteryl arachidonate hydroperoxides by comparison with the known synthetic standards.

A derivatization scheme for bicyclic endoperoxides of cholesteryl arachidonate has been reported (100). In this protocol, the oxidation mixture of 15-HpETE Ch containing the cholesteryl ester of bicyclic endoperoxides was first reduced to the trihydroxyl isoprostane by reaction with excess PPh_3. After basic hydrolysis, the electrophoric derivatization was accomplished by making the PFB ester. TMS derivatization facilitates the volatility of the resulting derivatives for GC-MS analysis. A selective ion monitoring (SIM) experiment was then carried out to monitor the negative carboxylate ion of isoprostanes with *m/z* 569, which is generated by the loss of PFB radical. The GC-MS chromatograms of SIM monitoring *m/z* 569 are shown in Fig. 5. Four pairs of peaks with similar patterns were observed. These peaks were assigned by spiking with known synthetic standards. The stereoisomers with *cis* dioxolane structures (**c** and **d**, **g** and **h**) are major products in comparison with those with *trans* configuration (**a** and **b**, **e** and **f**), which is consistent with the previous reports by Mihlich (40) and O'Connor *et al.* (55, 56).

The formation of the other types of bicyclic endoperoxides from the isomeric HpETEs Ch was confirmed by the Ag^+ CIS-MS. The corresponding isoprostanes are derived from the bicyclic endoperoxides (Scheme 9) (74). The autoxidation mixtures of 12-, 8- and 9-, 5-HpETE Ch can be derivatized in the same way as that described above for 15-HpETE Ch, and our GC-MS results show that the formation of 8- and 12-series of isoprostanes from 8- and 12-HpETE Ch occurs to a lesser extent than the formation of 5- and 15-series isoprostanes. This suggestion is consistent with the results from autoxidation of arachidonic acid and *in vivo* studies of the rat (94, 95). Murphy *et al.* proposed that a random distribution of

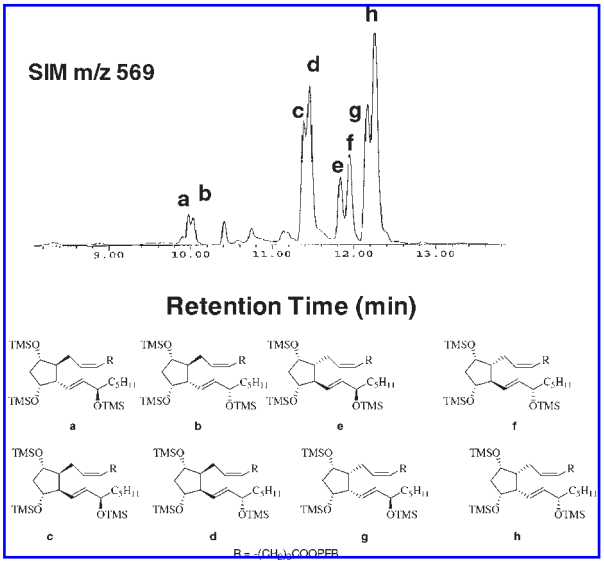
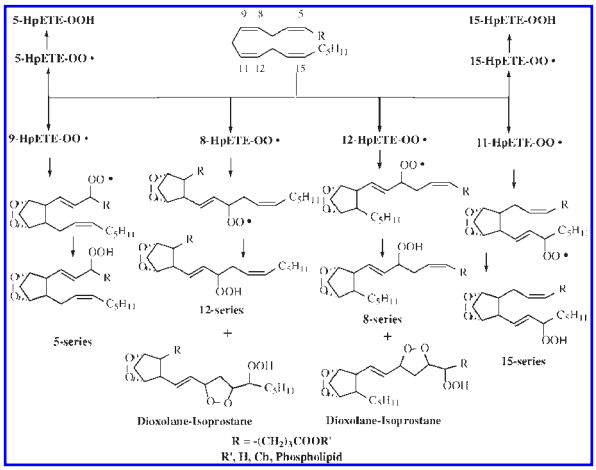


FIG. 5. GC-MS results (SIM *m/z* 569) of diastereoisomers of 15-series isoprostanes formed from 15-HpETE Ch.

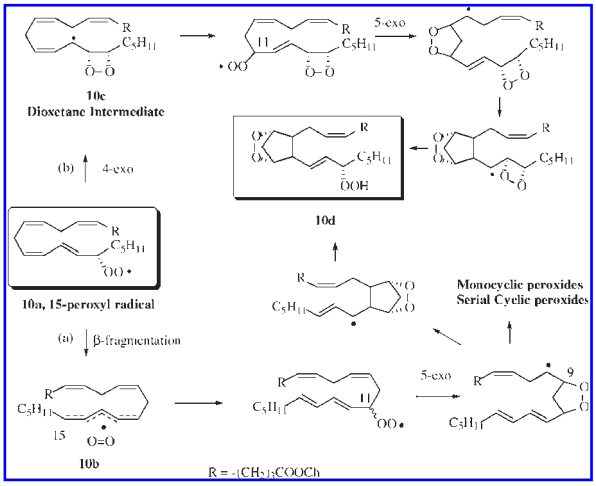


Scheme 9.

these four regioisomers of isoprostanes 15:8:12:5 should be 2:1:1:2 because both the 8- and 12-series of isoprostanes resulted from hydrogen abstraction at C10, whereas the 5- and 15-series formed from hydrogen abstraction at C7 and C13, respectively (95). Although there is merit to this proposal, we note that the 15- and 5-HpETEs are usually found in excess of the other hydroperoxides in oxidation mixtures. The peroxyl radicals that lead to the 5- and 15-hydroperoxides cannot cyclize to give isoprostanes, but they do have other pathways available to them. Another explanation for the preferential formation of the 15- and 5-series isoprostanes compared with the 8- and 12-series is the generation of the dioxolane-isoprostane peroxides from 8- or 12-peroxyl radicals (Scheme 9) (102). The 8- and 12-series isoprostanes are formed in less abundance than 5- and 15-series because only the peroxyl radicals that lead to 8- and 12-series can be oxidized further to dioxolane-isoprostane peroxides.

FORMATION OF BICYCLIC ENDOPEROXIDES FROM 15-HPETE: "DIOXETANE" MECHANISM OR β-FRAGMENTATION OF THE PEROXYL RADICAL?

The enzymatic oxidation of arachidonic acid by COX enzymes generates optically pure compounds, whereas the uncontrolled free radical oxidation process produces racemic mixtures. Furthermore, arachidonic acid is the substrate for enzymatic reactions, whereas the free acid as well as arachidonate esterified to cholesterol or phospholipids can be oxidized by nonenzymatic free radical reactions. A variety of other highly oxidized products are detected from the nonenzymatic reactions, such as monocyclic peroxides, bicyclic endoperoxides, serial cyclic peroxides, and dioxolane-isoprostanes. A unified free radical mechanism based on β-fragmentation of the 15-peroxyl radical (**10a**) was proposed to explain the experimental facts (Scheme 10a) (26, 62). The key step in these transformations is the β-fragmentation of the



Scheme 10.

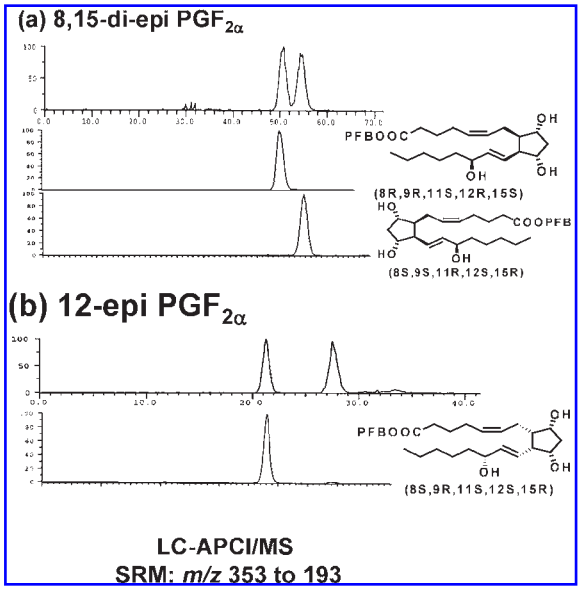


FIG. 6. LC-APCI/MS results of isolated iosprostanes analyzed by chiral HPLC (SRM *m/z* 353 to 193).

15-peroxyl radical to generate a pentadienyl radical (**10b**) followed by O_2 readdition to **C11** to give rise to the 11-peroxyl radical, which then undergoes subsequent cyclization to generate the bicyclic endoperoxides (**10d**). This mechanism can also explain the formation of monocyclic peroxide and serial cyclic peroxides. Another interesting mechanism was proposed by Corey *et al.* based on the formation of a "dioxetane" intermediate (Scheme 10b) (13). In this scheme, the 15-peroxyl radical undergoes *4-exo* cyclization to give a "dioxetane" intermediate (**10c**) with a carbon-centered radical that could add O_2 to form 11-peroxyl radical. Sequential *5-exo* cyclization generates the bicyclic endoperoxides. This mechanism has been recognized as an alternative to explain the formation of other regioisomeric bicyclic endoperoxides from other arachidonate-containing lipid in the literature (12, 74). Dioxetane structures have also been proposed active intermediates in some other chemical transformations. The existence of this unstable intermediate is extremely difficult to prove experimentally, especially for the generation of dioxetane from a peroxyl radical by *4-exo* cyclization. The formation of 4-HNE from hydroperoxides of linoleic acid was first proposed via a dioxetane intermediate by Esterbauer *et al.* (19). Several alternative mechanisms of HNE formation have also been invoked (66, 77). Little experimental evidence supports the dioxetane mechanism of HNE formation (33, 88).

Experiments have been carried out to distinguish the two mechanisms of the formation of PGG_2–like compounds from 15-HpETE, namely, β-fragmentation of a 15-peroxyl radical and the dioxetane mechanism. An optically pure compound (**10d**) would be generated from an optically pure starting compound **10a** if the transformation does go through a dioxetane intermediate. On the other hand, β-fragmentation would generate a racemic mixture of **10d** even if it started from an optically pure **10a**. Therefore, these two mechanisms can be distinguished by measuring the optical purity of the resulting isoprostanes.

The optically pure 15(*S*)-HpETE was synthesized by soybean lipoxygenase (26). HPLC determination using a Chiralpak AD column showed that the enzymatic product 15(*S*)-HpETE has enantio excess of 98.9% (76). The 15(*S*)-HpETE Ch was incubated for 24 h at 37°C with oxygen under free radical conditions. The autoxidation mixture of 15(*S*)-HpETE Ch was analyzed by LC-Ag^+ CIS/MS technique. In addition to the bicyclic endoperoxides, monocyclic peroxide and serial cyclic peroxides were also observed in this mixture. An aliquot of the autoxidation mixture was derivatized by reduction of PPh_3, followed by basic hydrolysis and PFB esterification. The PFB esters were separated by normal-phase HPLC using tandem analytical silica gel columns. An aliquot of each fraction was treated with *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) to make TMS derivatives for GC-EC-NICI-MS analysis. The GC-MS results showed single SIM peaks for each separated fraction. The rest of each PFB derivative was analyzed by LC-APCI/MS using a chiral HPLC column. SRM experiments were carried out by monitoring the characteristic fragmentation pathways of 15-series isoprostanes from *m/z* 353 to 193. Two major fractions from the autoxidation mixture of 15(*S*)-HpETE Ch were analyzed by the LC-APCI-EC-NICI-MS technique using a Chiralpak HPLC column (Fig. 6). The PFB derivatives from 8,15-di-epi PGF_{2a} and 12-epi PGF_{2a} showed them to be racemic mixtures. It is concluded that the conversion of 15(*S*)-HpETE Ch to isoprostanes results in the complete loss of optical purity of the starting hydroperoxide in the product isoprostanes. These results are consistent with the β-fragmentation pathway rather than the dioxetane intermediate.

CONCLUSIONS AND FUTURE DIRECTIONS

This review covers the recent progress on the understanding of the free radical mechanisms of lipid peroxidation and recent developments of the analytical techniques to analyze

the lipid oxidation products. Autoxidation of PUFAs in the presence of good hydrogen donors generates hydroperoxides as primary oxidation products. Secondary oxidation products are obtained from further oxidation. Autoxidation of regioisomeric hydroperoxides (such as HpETEs) of cholesteryl arachidonate, one of the two major lipids in the neutral core of LDL, gives rise to oxidation products derived from specific hydroperoxyl radicals. This strategy significantly simplifies the autoxidation pattern. Autoxidation of 5-, 9-, 11-, and 15-HpETE generate monocyclic peroxides, bicyclic endoperoxides, and serial cyclic peroxides, whereas 8- and 12-HpETEs give monocyclic peroxides, bicyclic endoperoxides, and a novel class of endoperoxides (termed dioxolane-isoprostane peroxides). Characterization of these oxidation products is accomplished by a variety of MS techniques coupled with separation methods such as HPLC and GC. Ag^+ CIS-MS has proven to be a powerful tool to analyze intact lipid peroxides. EC-APCI-MS is applied to study lipid oxidation products after derivatization. For the first time, eight possible diastereomer isoprostanes are observed from the oxidation of 15-HpETE by using GC-MS with NICI. Diasteoromers with *cis* side chains are formed preferentially over those with *trans* configurations. The 5- and 15-series of isoprostanes are more abundant than 8- and 12-series because the precursors that lead to 8- and 12-series isoprostanes can undergo further oxidation and give rise to dioxolane-isoprostane peroxides. Furthermore, formation of isoprostanes from 15-HpETE by β-fragmentation of the peroxyl radical seems to generate a pentadienyl radical rather than the "dioxetane" intermediate.

There are two major directions in which the research field of lipid peroxidation can proceed. These are (a) further understanding the chemistry that is involved in the process and (b) the development of new analytical techniques. The knowledge of autoxidation of cholesteryl arachidonate may aid in the understanding of the peroxidation of other PUFAs, such as DHA (C22:6) and eicosapentaenoic acid (C20:5). These two PUFAs are rich in fish oil, and extensive research has been carried out to understand the beneficial effects of fish oil (9, 27, 50, 54, 68, 79, 92). DHA is also present in abundant quantities in the brain, and its oxidation products have been implicated in the pathogenesis of Alzheimer's disease (70,71). The formation of F-ring isoprostane-like compounds with four double bonds during free radical-induced peroxidation of DHA *in vitro* and *in vivo* was recently reported (43). These isoprostane-like compounds were termed F_4-neuroprostanes and the levels of F_4-neuroprostanes are elevated in cerebrospinal fluid from patients with Alzheimer's disease. There are six bisallylic positions in DHA, and 10 conjugated regioisomers of DHA hydroperoxide can be formed. The secondary oxidation products of DHA are more complicated than those of arachidonate. However, with the knowledge of oxidation mechanisms of arachidonate, the oxidation products from DHA can be predicted based on the same free radical chemistry. Thus, the study of basic peroxidation pathways of DHA may shed light on the pathogenesis of Alzheimer's disease and other disorders in which this lipid is relevant.

Development of analytical techniques is another important aspect that will aid in understanding lipid oxidation. With advances in analytical instrumentation, such as MS, numerous new applications are anticipated in the near future. Selection, specificity, and sensitivity in the analysis of lipid oxidation products *in vitro* and *in vivo* will be likely much improved. Application of proteomic approaches to study the protein adducts that are formed in lipid oxidation is another important approach that has emerged in recent years (32). Thus, new analytical methodologies will yield important information about the pathogenesis of atherosclerosis and other diseases that are linked to lipid oxidation.

ACKNOWLEDGMENTS

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ABBREVIATIONS

APCI, atmospheric pressure chemical ionization; BDE, bond dissociation energy; CID, collision-induced dissociation; CIS, coordination ion spray; COX, cyclooxygenase; DHA, docosahexaenoic acid; DLI, direct liquid infusion; EC, electron capture; EI, electron ionization; ESI, electrospray ionization; FOX, ferrous oxidation of xylenol; GC, gas chromatography; 4-HNE, 4-hydroxynonenal; HpETE Ch, cholesteryl hydroperoxyeicosatetraenoate; LC, liquid chromatography; LDL, low-density lipoprotein; MS, mass spectrometry; NICI, negative ion chemical ionization; NRP, nonradical products; PC, phosphatidylcholine; PFB, pentafluorobenzyl; PG, prostaglandin; PPh_3, triphenylphosphine; PUFA, polyunsaturated fatty acid; SAPC, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidylcholine; SIM, selective ion monitoring; SRM, selective reaction monitoring; TBARS, thiobarbituric acid reactive substance; TMS, Trimethylsilyl; α-TOH, α-tocopherol.

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