J|A|C|S
ARTICLES
Published on Web 04/14/2004

Oxidative Fragmentation of Hydroxy Octadecadienoates Generates Biologically Active γ-Hydroxyalkenals

Mingjiang Sun and Robert G. Salomon*

Contribution from the Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44107-7078

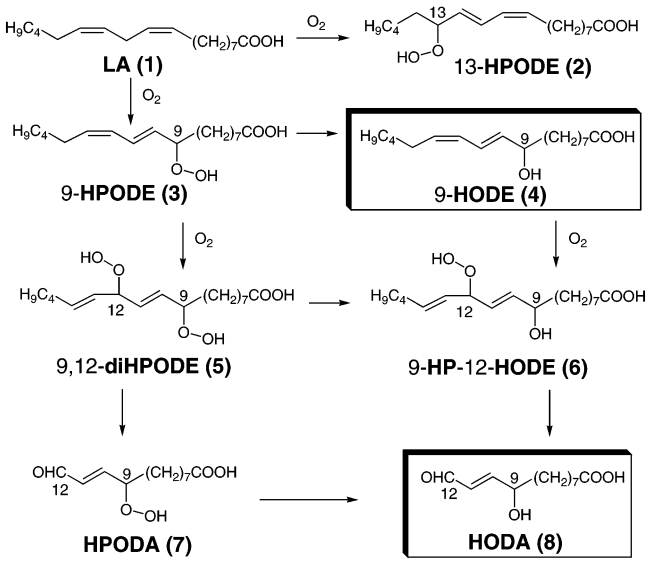
Received September 27, 2003; E-mail: rgs@cwru.edu

Abstract: Oxidative fragmentation of polyunsaturated fatty acids (PUFAs) in vivo generates cytotoxic aldehydes. Among these, 4-hydroxynon-2-enal and analogous γ-hydroxyalkenal phosphatidylcholines (PCs) have attracted attention because these oxidatively truncated lipids are biologically active and have been implicated in diseases. A previous study showed that hydroxydienes, generated by allylic oxygenation of linoleic acid, are unreactive toward oxidative fragmentation. We now show that, in the presence of hydroperoxides, hydroxydienes fragment as readily as the corresponding hydroperoxydienes, generating γ-hydroxyalkenals. In a physiomimetic model study, myeloperoxidase-promoted free radical-induced fragmentation of either hydroperoxy- or hydroxyoctecadienoate esters of 2-*lyso*-PC in small unilamellar vesicles produced the 9-hydroxy-12-oxododec-10-enoic acid (HODA) ester HODA-PC. Therefore, hydroxydienes, that are generally more abundant in vivo than hydroperoxydienes, are plausible intermediates in the production of oxidatively truncated lipids in vivo where a constant flux of radicals and hydroperoxides is present. Our findings also show that the formation of dioxetane intermediates through peroxyradical cyclization is not required to achieve oxidative fragmentation of PUFAs.

Lipid peroxidation generates various cytotoxic aldehydes, among which the γ-hydroxyalkenals are the most studied.[1] Oxidative fragmentation of arachidonate and linoleate phospholipids has pathological significance. We recently showed that it generates biologically active truncated γ-hydroxyalkenal phospholipids that are abundant in atherosclerotic plaques.[2,3] These oxidized phospholipids promote the formation of foam cells, precursors of atherosclerotic plaques, because they are ligands for the macrophage scavenger receptor CD36.[4] They also interfere with the proteolytic degradation of internalized macromolecules by macrophages because, inter alia, they covalently modify a lysosomal protease.[3] In addition, they activate human aortic endothelial cells to bind monocytes, promote the expression of chemokines that are important in monocyte entry into chronic lesions, and inhibit expression of a major adhesion molecule that mediates neutrophil endothelial interactions.[5]

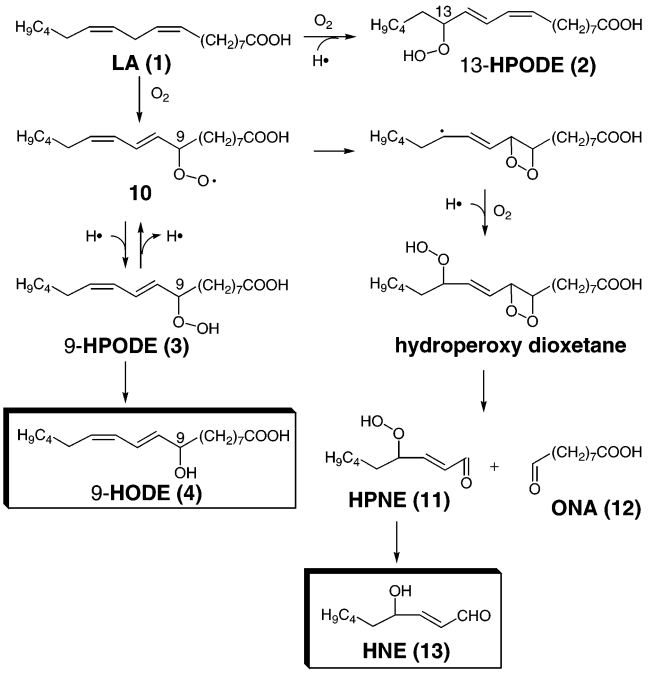
The major primary products of both enzymatic and free radical-induced oxidation of linoleic acid (LA, **1**) are the hydroperoxydienes **2** and **3** (Scheme 1). In vivo, these are efficiently reduced to hydroxydienes by selenium-containing glutathione peroxidases.[6,7] In the aorta of rabbits, the hydroxydiene 9-HODE (**4**) and its regioisomer 13-HODE (**9**) are 5-fold more abundant than corresponding hydroperoxides **3** and **2**.[8] Increased levels of 9-HODE (**4**) are found in human low-density lipoprotein from atherosclerotic patients as compared with individuals who have no cardiovascular disease.[9]

Scheme 1



(1) Carmen Vigo-Pelfrey, E. *Membrane Lipid Oxidation*; CRC Press: Menlo Park, CA, 1990; Vol. I.
(2) Podrez, E. A.; Poliakov, E.; Shen, Z.; Zhang, R.; Deng, Y.; Sun, M.; Finton, P. J.; Shan, L.; Febbraio, M.; Hajjar, D. P.; Silverstein, R. L.; Hoff, H. F.; Salomon, R. G.; Hazen, S. L. *J. Biol. Chem.* **2002**, *277*, 38517−38523.
(3) Hoff, H. F.; O'Neil, J.; Wu, Z.; Hoppe, G.; Salomon, R. L. *Arterioscler., Thromb., Vasc. Biol.* **2003**, *23*, 275−282.
(4) Podrez, E. A.; Poliakov, E.; Shen, Z.; Zhang, R.; Deng, Y.; Sun, M.; Finton, P. J.; Shan, L.; Gugiu, B.; Fox, P. L.; Hoff, H. F.; Salomon, R. G.; Hazen, S. L. *J. Biol. Chem.* **2002**, *277*, 38503−38516.
(5) Subbanagounder, G.; Deng, Y.; Borromeo, C.; Dooley, A. N.; Berliner, J. A.; Salomon, R. G. *Vasc. Pharmacol.* **2002**, *38*, 201−209.
(6) Reinaud, O.; Delaforge, M.; Boucher, J. L.; Rocchiccioli, F.; Mansuy, D. *Biochem. Biophys. Res. Commun.* **1989**, *161*, 883−891.
(7) Kuhn, H. *Prog. Lipid Res.* **1996**, *35*, 203−226.
(8) Upston, J. M.; Witting, P. K.; Brown, A. J.; Stocker, R.; Keaney, J. F., Jr. *Free Radical Biol. Med.* **2001**, *31*, 1245−1253.

Scheme 2



Because the γ-hydroxyalkenals produced through lipid peroxidation in vivo have important biological activities, a fundamental understanding of the mechanism of their formation is of considerable interest. It was suggested that a γ-hydroxyalkenal, 9-hydroxy-12-oxododec-10-enoic acid (HODA, **8**), could be generated through β-scission of a hydroxyalkoxyl radical derived from a hydroxy hydroperoxide intermediate **6** (Scheme 1).[10] It was later found that 9-HODE (**4**) is not readily oxidized under a variety of conditions that cause rapid oxidation of LA (**1**), and it was concluded that hydroxy octadecadienoates are stable end-products that can serve as "markers for lipid peroxidation" in vivo.[11] A possible explanation for the apparent stability of **4** is that oxidative cleavage involves the formation and fragmentation of a hydroperoxydioxetane intermediate that is generated by cyclization of a peroxydiene radical **10** (Scheme 2). No such peroxydiene radical intermediate is accessible from the hydroxydiene **4**. The possible involvement of dioxetane intermediates in the oxidative fragmentation of polyunsaturated fatty acids was suggested previously,[12−14] and the aldehydes **11**−**13** are known products from the autoxidation of linoleic acid (**1**).[15−17]

Another possibility is that HODA (**8**) is only generated by reduction of the known[16] precursor HPODA (**7**) that is produced through the formation and fragmentation of a bishydroperoxide **5** (Scheme 1), but that **8** is not generated through the analogous formation and fragmentation of a hydroxy hydroperoxide **6**. This might be because two hydroperoxy groups are somehow required for the fragmentation process, or because hydroxy hydroperoxide **6** is not readily generated from the hydroxydiene **4**. The latter scenario, which requires that allylic hydrogen atom abstraction from **3** occurs much more readily than from **4**, seemed unlikely. We reasoned that the behavior of the pure hydroxydiene **4** may have little bearing on the biologically relevant chemistry of these lipids. Rather, the chemistry of **4** in vivo would be influenced by the existence of a constant flux of lipid peroxy radicals or hydroperoxides, generated by autoxidation, that seemed likely to promote autoxidation of **4**. We now provide evidence that hydroxy octadecadienoates are not stable end-products, but rather readily undergo oxidative fragmentation that is promoted by lipid hydroperoxides such as 13-hydroperoxyoctadeca-9,11-dienoate (13-HPODE, **2**) (Scheme 1). The physiological relevance of this chemistry is supported by the observation that free radical-induced fragmentation of either hydroxy- (HODE) or hydroperoxyoctecadienoate (HPODE) esters of 2-*lyso*-PC in a model membrane (small unilamellar vesicles) produces the 9-hydroxy-12-oxododec-10-enoic acid (HODA) ester HODA-PC. Our findings also show that the formation of dioxetane intermediates through peroxy-radical cyclization is not required to achieve oxidative fragmentation of polyunsaturated fatty acids.

Results and Discussion

Autoxidation of Both Hydroperoxydienes 2 and 3 Generates Hydroxyalkenal 8. Autoxidations of 13-HPODE (**2**) and 9-HPODE (**3**) were monitored by LC/ESI/MS/MS. This technique allowed simultaneous quantification of specific isomers based upon monitoring unique transitions between the mass-to-charge ratio of the parent ion $[M - H]^-$ and characteristic daughter ions for each species as well as their appropriate retention times. Hydroperoxydiene **2** or **3** was heated as a dry film in an open glass tube at 37 °C, and the amounts of **2** and **3** as well as HODA (**8**) were monitored simultaneously. 13-HPODE (**2**) and 9-HPODE (**3**) behave similarly. After 1 h, only 40−60% of starting compound remains (Figure 1A and B). Furthermore, interconversion of 3-HPODE (**2**) and 9-HPODE (**3**) was observed. This phenomena had been observed previously when the hydroperoxides were stored in organic solvents.[18,19] HODA (**8**) was produced from both hydroperoxides (Figure 1C), in similar yield (5%), and at similar rates.

The isomerization presumably involves reversible generation and rearrangement of peroxy radical intermediates (Scheme 3). It results in loss of regiospecificity and complicates analysis of the fragmentation product distribution. It is difficult to discern which hydroperoxydiene is the immediate precursor of HODA (**8**).

Hydroperoxides Promote Oxidation of Hydroxydienes 4 and 9. Pure 9-HODE (**4**) is not readily oxidized under a variety of conditions that cause rapid oxidation of LA (**1**).[11] These include exposure to air in the presence of Fe^{2+}, or Fe^{3+}, or Fe^{2+} and ascorbate, or Fe^{2+} and H_2O_2. We postulated that alkylperoxyl or alkoxyl radicals, that are generated from alkyl hydro-

(9) Jira, W.; Spiteller, G.; Carson, W.; Schramm, A. *Chem. Phys. Lipids* **1998**, *91*, 1−11.
(10) Loidl-Stahlhofen, A.; Hannemann, K.; Spiteller, G. *Biochim. Biophys. Acta* **1994**, *1213*, 140−148.
(11) Spiteller, P.; Spiteller, G. *Chem. Phys. Lipids* **1997**, *89*, 131−139.
(12) Esterbauer, H.; Zollner, H.; Schaur, R. J. *Membrane Lipid Oxidation*; CRC Press: Boca Raton, FL, 1990; Vol. 1, p 250.
(13) Kaur, K.; Salomon, R. G.; O'Neil, J.; Hoff, H. F. *Chem. Res. Toxicol.* **1997**, *10*, 1387−1396.
(14) Timmins, G. S.; dos Santos, R. E.; Whitwood, A. C.; Catalani, L. H.; Di Mascio, P.; Gilbert, B. C.; Bechara, E. J. H. *Chem. Res. Toxicol.* **1997**, *10*, 1090−1096.
(15) Lee, S. H.; Blair, I. A. *Chem. Res. Toxicol.* **2000**, *13*, 698−702.
(16) Schneider, C.; Tallman, K. A.; Porter, N. A.; Brash, A. R. *J. Biol. Chem.* **2001**, *276*, 20831−20838.
(17) Minamoto, S.; Kanazawa, K.; Ashida, H.; Natake, M. *Biochim. Biophys. Acta* **1988**, *958*, 199−204.
(18) Chan, H. W.; Levett, G.; Matthew, J. A. *Chem. Phys. Lipids* **1979**, *24*, 245−256.
(19) Porter, N. A.; Wujek, J. S. *J. Org. Chem.* **1987**, *52*, 5085−5089.

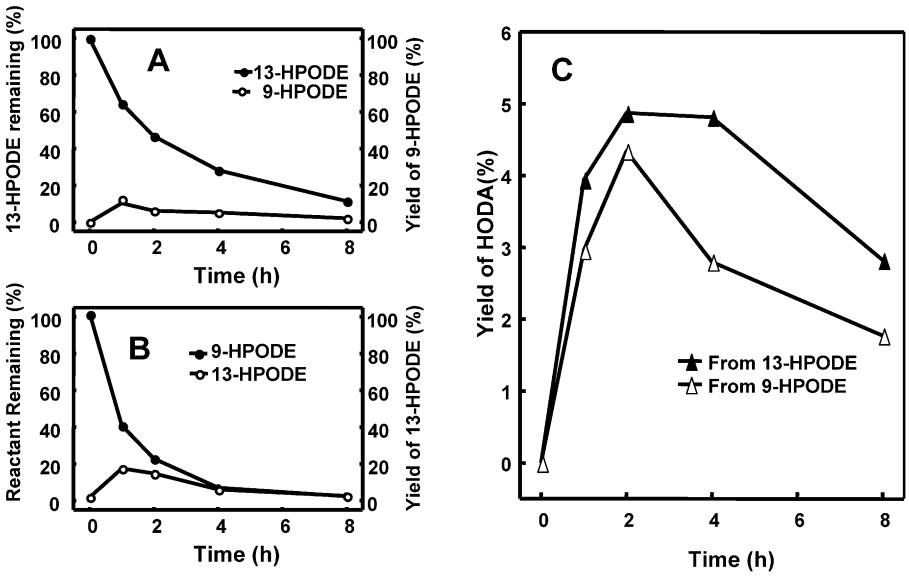
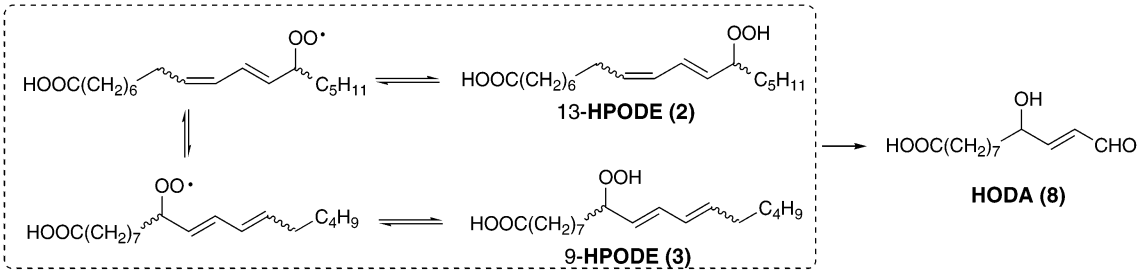


Figure 1. Autoxidation of hydroperoxides from linoleic acid at 37 °C and analyzed by LC-MS. (A) 13-HPODE (**2**), (B) 9-HPODE (**3**), (C) HODA (**8**) generated from hydroperoxide autoxidation.

Scheme 3



peroxides such as hydroperoxyoctadecadienoates (HPODEs) **2** or **3**, are mandatory for the allylic hydrogen atom abstraction required to produce **6** from **4** (Scheme 1). To test this hypothesis, we exposed pure 9-hydroxyoctadeca-10,12-dienoic acid (9-HODE, **4**) or 13-hydroxyoctadeca-9,11-dienoic acid (13-HODE, **9**) to air in the absence or in the presence of 9,10,12,13-tetradeuterated hydroperoxydiene 13-HPODE-d_4 (**2-*d*$_4$**). As expected, the hydroxydienes **4** and **9** were unchanged upon heating in air at 37 °C for 8 h. In contrast, both hydroxydienes decomposed at rates similar to that of the hydroperoxydiene **2** when heated individually in air at 37 °C in the presence of deuterium-labeled **2** (Figure 2).

Oxidative Fragmentation of Hydroxydiene 4 but Not 9 Generates γ-Hydroxyalkenal 8. These observations supported the possibility that the hydroxydienes **4** and **9** can serve as intermediates in the free radical-induced oxidative fragmentation of linoleic acid. Detection of the fragmentation product **8** generated in the oxidative decomposition of the hydroxydiene **4** confirmed this presumption. By using tetradeuterated hydroperoxydiene **2-*d*$_4$** in these experiments, LC-MS analysis allowed a distinction between hydroxyalkenal **8-*d*$_3$** generated from the hydroperoxide and hydroxyalkenal **8-*d*$_0$** produced from the hydroxydiene. The oxidative fragmentation product **8** was generated upon air oxidation of both hydroxydiene **4** (in >5% yield) and hydroperoxydiene **2-*d*$_4$** but not from oxidation of hydroxydiene **9** (Figure 3).

To further verify that HODA (**8**) was produced from 9-HODE (**4**) in the presence of hydroperoxydiene 2-d_4, both standard

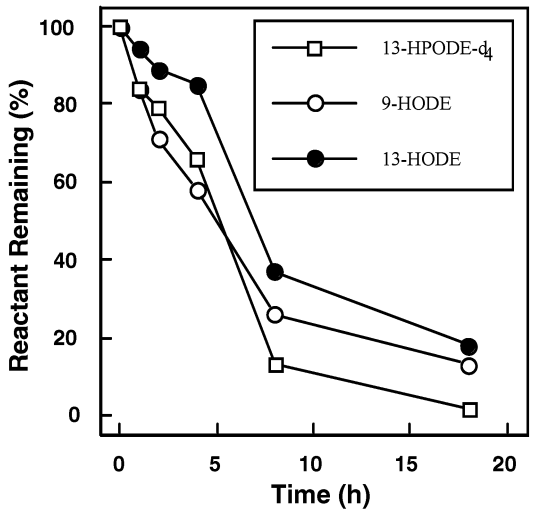


Figure 2. Decomposition of hydroxydiene 9-HODE (**4**) or 13-HODE (**9**) in the presence of the hydroperoxyoctadecadienoate 13-HPODE-d_4 (**2-*d*$_4$**).

HODA (**8**) and the oxidation reaction product mixture were derivatized with methoxylamine hydrochloride in pyridine. HODA-methoxime ($[M - H]^-$, *m*/*z* 256) produces a dominant daughter ion *m*/*z* 197. The HPLC chromatogram of the HODA methoxime derivative was monitored by ESI/MS/MS with monitoring of the *m*/*z* 256 to *m*/*z* 197 mass transition. After derivatization, the peak corresponding to HODA (**8**) in the autoxidation reaction product mixture disappeared, and a new

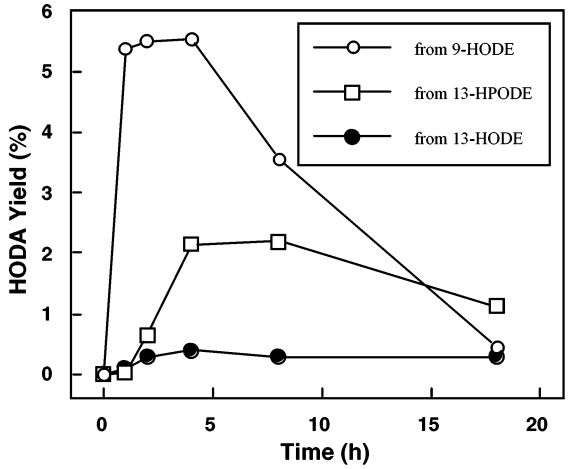


Figure 3. Yield of HODA (**8**) from oxidative fragmentation of 9-HODE (**4**) or 13-HODE (**9**) in the presence of 13-HPODE-d_4 (**2-*d*$_4$**).

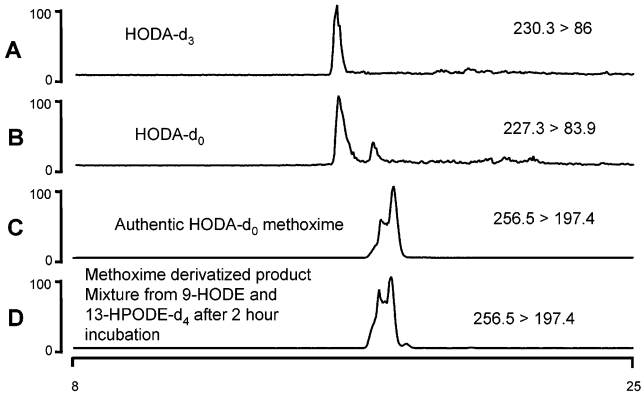


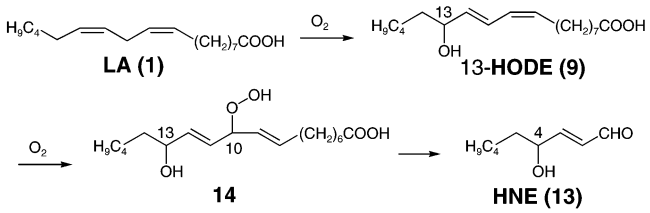
Figure 4. HPLC chromatogram of HODA and HODA methoxime derivative. All chromatograms were monitored by LC-MS in the negative ion mode with MRM of appropriate mass transitions as noted. (A) HODA-d_3 produced from autoxidation (2 h) of 13-HPODE-d_4 in the presence of 9-HODE. (B) HODA-d_0 produced from autoxidation (2 h) of 9-HODE in the presence of 13-HPODE-d_4. (C) Methoxime derivative of authentic HODA-d_0. (D) Methoxime derivative of HODA-d_0 produced from autoxidation (2 h) of 9-HODE in the presence of 13-HPODE-d_4.

peak at the position of the HODA methoxime derivative appeared (Figure 4).

The contrasting behavior of the hydroperoxydiene 13-HPODE (**2**) and the hydroxydiene 13-HODE (**9**) is noteworthy. Autoxidation of **2** generates HODA (**8**), but autoxidation of **9** does not. Furthermore, the yield of **8** from the hydroperoxydiene **2** is only about one-half the yield of **8** from the regioisomeric hydroxydiene 9-HODE (**4**). This is understandable if it is presumed that: (i) the generation of **8** from the 13-hydroperoxydiene requires prior isomerization to the 9-hydroperoxydiene 9-HPODE (**3**), (ii) the formation of **8** through isomerization to **3** competes with alternative fragmentation reactions that are only accessible to the regioisomeric hydroperoxydiene 13-HPODE (**2**) or hydroxydiene 13-HODE (**9**), and (iii) while the regioisomeric hydroperoxydienes **2** and **3** readily interconvert, the corresponding regioisomeric hydroxydienes **4** and **9** do not.

Oxidative Fragmentation of Hydroxydiene 9 Generates γ-Hydroxynonenal (13). The failure of the oxidation of pure 13-HODE (**9**) to produce the γ-hydroxyalkenal **8** is understandable if C−C bond cleavage is presumed to require the involvement of a hydroperoxy group or an alkoxyl radical on one terminus of the scissile C−C bond. Oxidative fragmentation

Scheme 4



of the hydroxy hydroperoxide **14** derived from **9** is expected to produce a different γ-hydroxyalkenal, 4-hydroxynon-2-enal (HNE, **13** in Scheme 4). This cytotoxic product of lipid oxidation in vivo[20,21] forms protein adducts, causing the inhibition of enzymes.[22] Elevated levels of HNE and HNE-derived protein modification have been implicated in diseases.[21,23−25]

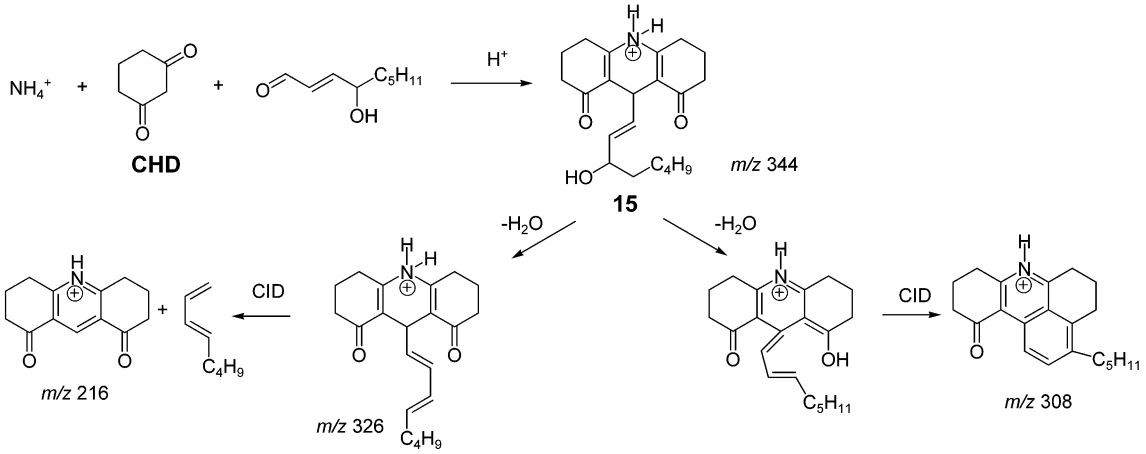
Generation of HNE (**13**) upon oxidation of 13-HODE (**9**) in the presence of 13-HPODE-d_4 (**2-*d*$_4$**) was confirmed. To increase the sensitivity of HNE (**13**) detection by ESI-MS, **13** was derivatized with cyclohexanedione (CHD) and ammonium acetate in aqueous acetic acid (Scheme 5).[26] The ESI-MS spectrum of the HNE CHD derivative **15** showed a predominant pseudo parent $[M - H_2O + H]^+$ *m*/*z* 326 ion in addition to the parent $[M + H]^+$ *m*/*z* 344 ion. Tandem MS/MS analysis of the dehydrated ion revealed *m*/*z* 308, in addition to *m*/*z* 216 which was assigned previously.[27] We propose that the HNE derivative **15** can be dehydrated at two different positions; one of them leads to the *m*/*z* 216 ion, and the other leads to the *m*/*z* 308 ion by loss of another molecule of water through electrocyclization followed by dehydration.

An LC-ESI-MS/MS method[27] was employed to quantify HNE (**13**) after derivatization based on monitoring unique transitions between the mass-to-charge ratio of the pseudo parent ion $[M + H - 18]^+$ *m*/*z* 326 and the characteristic daughter ion *m*/*z* 216 and its appropriate retention time. Before derivatization, benzaldehyde was added as internal standard, and the amount of HNE was calculated from a calibration curve (see Experimental Procedures). As predicted, HNE was produced from 13-HODE, but not from 9-HODE, upon autoxidation in the presence of 13-HPODE-d_4 (Figure 5). The yield of HNE (**13**) peaked at about 4 h and is comparable with the yield of HODA (**8**) from the autoxidation of 9-HODE (**4**) in the presence of hydroperoxydiene-d_4 (**2-*d*$_4$**). This observation further confirmed that **4** and **9** can serve as intermediates for production of HODA (**8**) or HNE (**13**).

Hydroxy- and Hydroperoxydienes Are Similarly Reactive Precursors for γ-Hydroxyalkenals. The present study demonstrates that hydroxydienes **4** and **9** are not stable end-products

(20) Benedetti, A.; Comporti, M.; Esterbauer, H. *Biochim. Biophys. Acta* **1980**, *620*, 281−296.
(21) Esterbauer, H.; Schaur, R. J.; Zollner, H. *Free Radical Biol. Med.* **1991**, *11*, 81−128.
(22) Benedetti, A.; Casini, A. F.; Ferrali, M.; Comporti, M. *Biochem. Pharmacol.* **1979**, *28*, 2909−2918.
(23) Hoff, H. F.; O'Neil, J.; Chisolm, G. M. d.; Cole, T. B.; Quehenberger, O.; Esterbauer, H.; Jurgens, G. *Arteriosclerosis* **1989**, *9*, 538−549.
(24) Requena, J. R.; Fu, M. X.; Ahmed, M. U.; Jenkins, A. J.; Lyons, T. J.; Baynes, J. W.; Thorpe, S. R. *Biochem. J.* **1997**, *322*, 317−325.
(25) Palinski, W.; Yla-Herttuala, S.; Rosenfeld, M. E.; Butler, S. W.; Socher, S. A.; Parthasarathy, S.; Curtiss, L. K.; Witztum, J. L. *Arteriosclerosis* **1990**, *10*, 325−335.
(26) Matsuoka, M.; Imado, N.; Maki, T.; Banno, K.; Sato, T. *Chromatographia* **1996**, *43*, 501−506.
(27) O'Brien-Coker, I. C.; Perkins, G.; Mallet, A. I. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 920−928.

Scheme 5



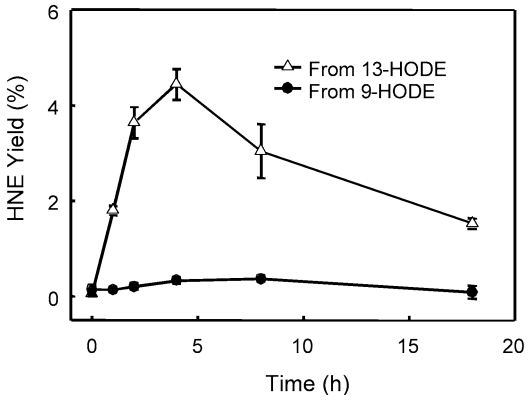
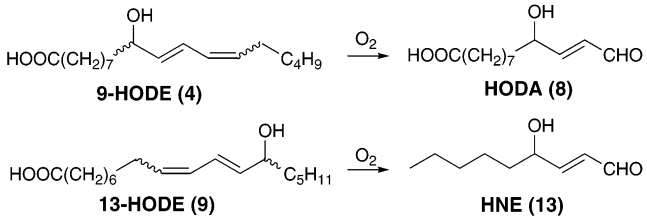


Figure 5. HNE production from autoxidation of HODEs and 13-HPODE-d_4 in air at 37 °C.

of lipid oxidation. In fact, under identical reaction conditions, that is, in the presence of hydroperoxides that can initiate autoxidation, the hydroxydienes and hydroperoxydienes are consumed (Figure 2) and generate fragmentation products (Figure 3) at similar rates. Thus, rather than being stable[11] markers for lipid peroxidation, the hydroxydienes **4** and **9** are reactive precursors to biologically active γ-hydroxyalkenals HODA (**8**) and HNE (**13**). The increased levels of 9-HODE (**4**) found in human low-density lipoprotein from atherosclerotic patients as compared with individuals who have no cardiovascular disease[9] may have pathological significance because it can serve as a precursor of HODA (**8**). The ester of HODA (**8**) with 2-*lyso*-phosphatidylcholine is a strong ligand for the scavenger receptor CD-36 of macrophage cells.[4] It promotes recognition of oxidatively damaged low-density liproprotein, leading to unregulated endocytosis.[2] This avid uptake contributes to the production of foam cells and atherosclerotic plaques. Because hydroperoxydienes are rapidly reduced in vivo,[6,7] hydroxydienes are generally more abundant[8] and, consequently, their oxidative cleavage could be the primary route to γ-hydroxyalkenals. The production of HNE from 13-HODE (**9**) may also be a pathologically important process because the elevated levels of HNE (**13**), produced through lipid oxidation in vivo, contribute to various disease processes through covalent adduction with proteins and consequent inhibition of enzymes.[20−25]

Their similar reactivities toward autoxidation also suggest that oxidative fragmentations of both the hydroxydienes and the hydroperoxydienes proceed by identical mechanisms. Autoxidation of the hydroperoxydienes 13-HPODE (**2**) and 9-HPODE (**3**) both afford HODA (**8**). However, this complexity is undoubtedly the result of the interconversion of **2** and **3** under the autoxidation reaction conditions. In contrast, only one hydroxydiene, 9-HODE (**4**) and not 13-HODE (**9**), affords **8** upon autoxidation. Furthermore, only autoxidation of one regioisomeric hydroxydiene, 13-HODE (**9**) and not 9-HODE (**4**), affords HNE (**13**). Thus, in contrast with the chemistry of the regioisomeric hydroperoxydienes, the immediate precursor of the fragmentation products HODA (**8**) and HNE (**13**) is unambiguously defined in the autoxidation reactions of the regioisomeric hydroxydienes.



Oxidative Fragmentation of HODE-PC in Unilamellar Vesicles. In vivo, hydroperoxy- and hydroxydienes are present mainly esterified, for example, in phospholipids. Therefore, we compared free radical-induced oxidative fragmentation of the hydroperoxy- and hydroxyoctadecadienoate esters of 2-*lyso*-phosphatidylcholine (PC), HPODE-PC (**16**) and HODE-PC (**17**), respectively. Regioisomeric 13- and 9-hydroperoxyoctadecadienoate esters **16a** and **16b** (4:1) are produced from 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (LA-PC) by lipoxygenase-promoted autoxidation. Reduction with triphenylphosphine provides the corresponding regioisomeric hydroxydienes **17a** and **17b**. To evaluate the oxidative fragmentation of hydroperoxy and hydroxydienes in a model membrane under physiologically relevant conditions, we compared the free radical-induced oxidation of unilamellar vesicles comprised of HPODE-PC (**16**) or HODE-PC (**17**) esters in pH 7.4 aqueous buffer at 37 °C. Myeloperoxidase (MPO) serves as an enzymatic catalyst for initiation of lipid peroxidation in vivo,[28] and we recently showed that free radical-induced phospholipid peroxidation promoted by the MPO/H_2O_2 system of leukocytes serves as one

(28) Zhang, R.; Brennan, M. L.; Shen, Z.; MacPherson, J. C.; Schmitt, D.; Molenda, C. E.; Hazen, S. L. *J. Biol. Chem.* **2002**, *277*, 46116−46122.

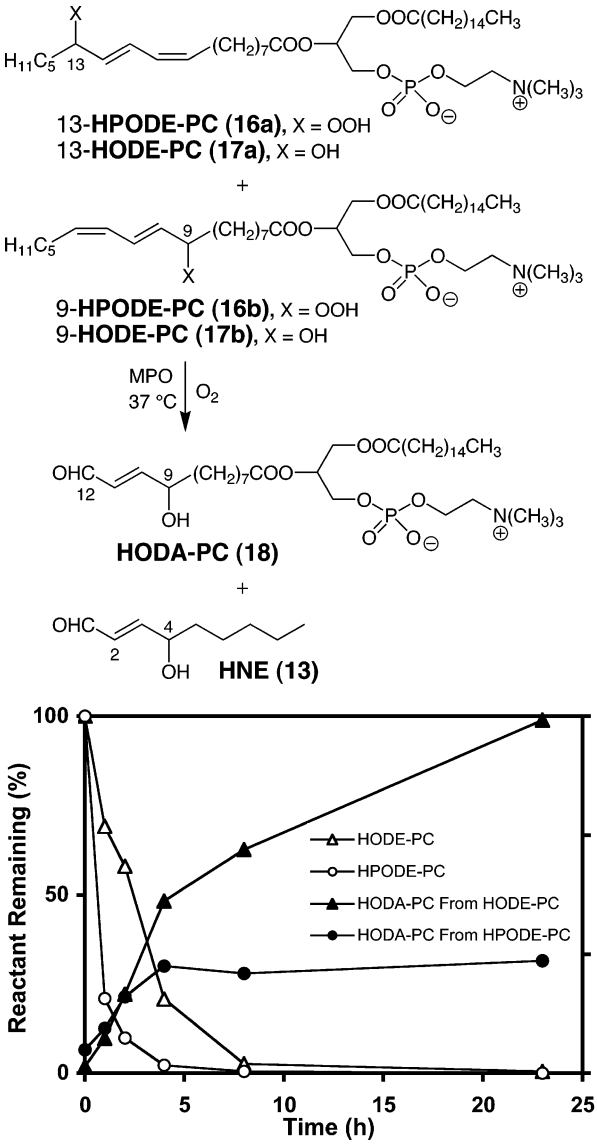
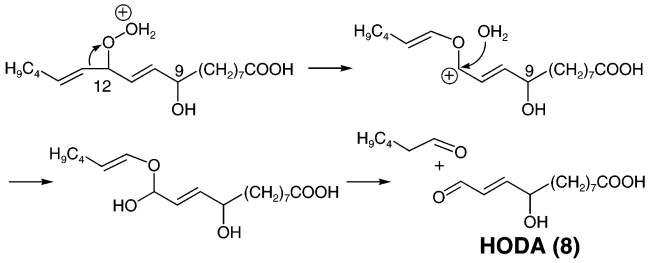


Figure 6. MPO-promoted autoxidation of HPODE-PC or HODE-PC in unilamellar liposomes.

mechanism for the generation of isolevuglandins in vivo.[29] To provide free radicals that can initiate autoxidation, we used the MPO−H_2O_2−NO_2^- system and generated a constant flux of the H_2O_2 through the glucose oxidase-promoted oxidation of glucose. The consumption of starting dienes and production of the γ-hydroxyalkenal phospholipid HODA-PC (**18**) through oxidative fragmentation of HPODE-PC (**16**) or HODE-PC (**17**) were monitored by LC-ESI-MS/MS (Figure 6).

Both HPODE-PC (**16**) and HODE-PC (**17**) were readily consumed under these physiomimetic autoxidation reaction conditions. Oxidative fragmentation of both **16** and **17** produced the biologically active truncated phospholipid HODA-PC (**18**). The yield of γ-hydroxyalkenal **18** was 3-fold higher from the hydroxydiene **17** than from the hydroperoxydiene **16**. HNE (**13**) is also produced in the MPO-promoted autoxidation of **16** and **17**. Yields reached a maximum within 5 h and were about 5% from the hydroxydiene **17** and about 3% from the hydroperoxydiene **16**. Therefore, hydroxydienes, for example, **17**, that are generally more abundant in vivo than hydroperoxydienes,

Scheme 6



for example, **16**, are plausible intermediates in the production of oxidatively truncated lipids, HNE (**13**) and HODA-PC (**18**), in vivo where a constant flux of radicals and hydroperoxides is present.

The Oxidative Fragmentations Do Not Require Peroxycyclization to Dioxetane Intermediates. The mechanisms that generate HODA (**8**) from **4** and HNE (**13**) from **9** remain to be determined. However, the peroxyradical cyclization pathway outlined in Scheme 2 is not feasible for oxidative fragmentation of hydroxydienes. The presumption of identical mechanisms for fragmentations of both the hydroxydienes and the hydroperoxydienes leads to the conclusion that the process involving cleavage of a dioxetane intermediate, formed through peroxyradical cyclization, is not a significant pathway for oxidative fragmentations of hydroperoxydienes. A recent study discounted the intermediacy of a similar dioxetane in the generation of isoprostanes from cholesteryl-15-hydroperoxyeicosatetraenoate.[30]

The contrasting chemistry of 9-HODE (**4**) and 13-HODE (**9**) summarized in Figures 3 and 5 supports our presumption that C−C bond cleavage requires the involvement of a hydroperoxy group or an alkoxyl radical on one terminus of the scissile C−C bond involved in oxidative fragmentation. As suggested previously, the formation of **8** upon oxidation of LA (**1**) may involve allylic oxygenation of **4** followed by β-scission of a hydroxy alkoxyl radical derived from an intermediate hydroxy hydroperoxide **6** (Scheme 1).[10] As expected for free radical oxidations, the presence of 10 wt % of vitamin E, a lipophilic antioxidant, inhibited the fragmentations of both hydroperoxydiene **2** and hydroxydiene **4**. Fragmentation of both **2** and **4** occurred simultaneously, albeit more gradually, after a delay period during which the antioxidant was consumed. However, these observations do not rule out the possibility that formation of γ-hydroxyalkenal **8** from **6** involves a polar Hock-cleavage (Scheme 6) analogous to that proposed recently to explain the formation of HNE (**13**) through fragmentation of a dihydroperoxide corresponding to the hydroxy hydroperoxide **14** in Scheme 4.[16] Because the hydroxydienes **4** and **9** do not interconvert, in contrast with the corresponding hydroperoxydienes 13-HPODE (**2**) and 9-HPODE (**3**), the reaction product mixtures generated from autoxidation of the hydroxydienes are less complex than those generated from the HPODEs. Consequently, studies of the 9-HODE (**4**) to HODA (**8**) and the 13-HODE (**9**) to HNE (**13**) conversions are especially attractive for further studies focusing on this fragmentation process.

Experimental Procedures

General. High performance liquid chromatography (HPLC) purification was performed with HPLC grade solvents using a Waters M600A solvent delivery system and a Waters U6K injector or a Waters 717

(29) Poliakov, E.; Brennan, M. L.; Macpherson, J.; Zhang, R.; Sha, W.; Narine, L.; Salomon, R. G.; Hazen, S. L. *FASEB J.* **2003**, *17*, 2209−2220.

(30) Yin, H.; Havrilla, C. M.; Gao, L.; Morrow, J. D.; Porter, N. A. *J. Biol. Chem.* **2003**, *278*, 16720−16725.

Table 1. Optimized Parameters for the Mass Spectrometer

	acids	CHD derivs	PFB derivs
ion mode	negative	positive	positive
capillary (kV)	3	3	4
cone (V)	30	30	30
hex 1 (V)	0	10	10
aperture (V)	0	0	0
hex 2 (V)	0.5	0.5	0.5
LM 1 resolution	10	19	15
HM 1 resolution	10	19	15
ion energy 1	1	1	1
LM 2 resolution	15	19	15
HM 2 resolution	15	19	15
ion energy 2	2	2	2

autosampler. The eluants were monitored using an ISCO V^4 UV−vis detector or a Waters 2996 photodiode array detector. Linoleic acid-d_4 (9,10,12,13-tetradeutero linoleic acid), 9(*S*)-hydroxyoctadeca-10*Z*,12*E*-dienoic acid (9-HODE), 9(*S*)-hydroperoxyoctadeca-10*Z*,12*E*-dienoic acid (9-HPODE), 13(*S*)-hydroxyoctadeca-9*Z*,11*E*-dienoic acid (13-HODE), and 13(*S*)-hydroperoxyoctadeca-9*Z*,11*E*-dienoic acid (13-HPODE) were from Cayman Chemical Co. (Ann Arbor, MI), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (LA-PC) was from Avanti Polar Lipids (Alabaster, AL), catalase (bovine liver, thymol-free) and glucose oxidase (grade II) were from Boehringer Mannheim (Indianapolis, IN), and myeloperoxidase (human leukocytes) was from Sigma (St. Louis, MO).

Liquid Chromatography/Mass Spectrometry. LC/ESI/MS/MS analysis of autoxidation reaction product mixtures was performed on a Quattro Ultima (Micromass, Wythenshawe, UK) connected to a Waters 2790 solvent delivery system with an auto-injector. The source temperature was maintained at 100 °C, and the desolvation temperature was kept at 200 °C. The drying gas (N_2) was maintained at ca. 450 L/h, and the cone flow gas was kept at ca. 50 L/h. The multiplier was set at an absolute value of 500. Optimized parameters for acids, cyclohexanedione (CHD) derivatives of HNE, and pentafluorobenzyl (PFB) derivatives of acids are presented in Table 1. MS scans at *m*/*z* 20−400 were obtained for standard compounds.

Argon was used as collision gas at a pressure of 5 psi for MS/MS analysis. For MS/MS analysis, the collision energy was optimized for each compound. For multiple reaction monitoring (MRM) experiments, the optimum collision energy (giving the strongest signal) was determined for each *m*/*z* ion pair. For acidic compounds, the mass spectrometer was operated in the negative ion mode. For CHD-derivatized HNE and PFB derivatives, it was operated in the positive ion mode.

Online chromatographic separation was achieved using a 150 × 2.0 mm i.d. Prodigy ODS-2, 5 μ column (Phenomenex, UK), with a binary solvent (water and methanol) gradient. The solvents were supplemented with 0.2% formic acid, whenever the mass spectrometer was operated in positive mode. For acidic compounds, the gradient started with 100% water and rose to 100% methanol linearly in 15 min, and elution was continued for 5 min with 100% methanol. The gradient was then reversed to 100% water in 0.5 min, and then held for 9.5 min at 100% water. For CHD-derivatized HNE, the gradient started with 70% methanol and rose to 100% methanol linearly in 10 min, and was then held for 5 min with 100% methanol. The gradient was then reversed to 70% methanol in 0.2 min, and then held for 4.8 min at 70% methanol. For dipentafluorobenzyl ester compounds, the gradient started with 85% methanol and rose to 88% methanol linearly in 12 min, and then rose to 100% methanol linearly over 3 min, which was held for 12 min. The gradient was then reversed to 85% methanol in 4 min, and held for 10 min. The solvents were delivered at 200 μL/min.

13-Hydroperoxyoctadeca-9,11-dienoic Acid (2, 13-HPODE, Cayman). ESI-MS/MS analysis yielded the following diagnostic fragments: *m*/*z* 311 [M − H]$^-$; *m*/*z* 293; *m*/*z* 223; *m*/*z* 113 (see Supporting Information Figure S1A).

Table 2. HPLC Gradients for the Purification of Hydroperoxidiene

time (min)	flow (mL/min)	methanol (%)	water (%)
0	3	70	30
20	3	100	0
25	3	100	0
30	3	70	30

9-Hydroperoxyoctadeca-10,12-dienoic Acid (3, 9-HPODE, Cayman). ESI-MS/MS analysis yielded the following diagnostic fragments: *m*/*z* 311 [M − H]$^-$; *m*/*z* 293; *m*/*z* 197; *m*/*z* 185; *m*/*z* 125 (see Supporting Information Figure S1B).

13-Hydroxyoctadeca-9,11-dienoic acid (9, 13-HODE, Cayman) was purified by the same HPLC system as 13-HPODE-d_4. ESI-MS/MS analysis yielded the following diagnostic fragments: *m*/*z* 295 [M − H]$^-$; *m*/*z* 278; *m*/*z* 195; *m*/*z* 112 (see Supporting Information Figure S2A).

9-Hydroxyoctadeca-10,12-dienoic acid (4, 9-HODE, Cayman) was purified by the same HPLC system as 13-HPODE-d_4. ESI-MS/MS analysis yielded the following diagnostic fragments: *m*/*z* 295 [M − H]$^-$; *m*/*z* 278; *m*/*z* 171; *m*/*z* 125 (see Supporting Information Figure S2B).

13-Hydroperoxy-9,10,12,13-tetradeuterio-octadeca-9,11-dienoic acid (13-HPODE-d_4, 2-d_4) was prepared by incubating 9*Z*,12*Z*-octadecadienoic-9,10,12,13-d_4 acid (**1-d_4**, Cayman) with lipoxygenase (Sigma, type V)[31] and purified with a RP-HPLC column (Phenomenex LUNA, 10.0 × 250 mm) using methanol/water linear gradients (Table 2). Products were monitored at 234 nm. The retention time of the product is about 12 min. ESI-MS/MS analysis yielded the following diagnostic fragments: *m*/*z* 315 [M − H]$^-$; *m*/*z* 297; *m*/*z* 227; *m*/*z* 198; *m*/*z* 114 (see Supporting Information Figure S3A).

9-(2-Oxanyloxy)-11-(3,3-dimethyl-2,4-dioxolanyl)undec-10-enoic acid was used as internal standard and was prepared as described previously.[32] ESI-MS/MS analysis yielded the following diagnostic fragments: *m*/*z* 384 [M − H]$^-$; *m*/*z* 281; *m*/*z* 223; *m*/*z* 101 (see Supporting Information Figure S3B).

9-Hydroxy-12-oxo-10-dodecenoic acid (**HODA, 8**) was synthesized according to the published protocol.[32] ESI-MS/MS analysis yielded the following diagnostic fragments: *m*/*z* 227 [M − H]$^-$; *m*/*z* 209; *m*/*z* 163; *m*/*z* 84 (see Supporting Information Figure S4A).

9-Hydroxy-12-oxo-9,10,12-trideuterio-10-dodecenoic acid (**HODA-d_3, 8-d_3**) was prepared by the vitamin C-catalyzed oxidation of 13-HPODE-d_4. 13-HPODE-d_4 (500 μg) was dissolved in 50 mM pH 7.4 phosphate buffer with vitamin C (2 mM).[33] The mixture was heated at 37 °C under air for 2 h. Catalyst was added to the mixture followed by BHT. The mixture was then acidified to pH 3 with 0.2 M HCl. The products were extracted and then fractionated on a RP-HPLC column (Phenomenex LUNA, 1.0 × 25 cm) with a methanol/water gradient at 0.8 mL/min. The fractions containing HODA-d_3, that is, with retention time similar to HODA-d_0, were combined and concentrated by rotary evaporation. ESI-MS/MS analysis yielded the following diagnostic fragments: *m*/*z* 230 [M − H]; *m*/*z* 212; *m*/*z* 166, *m*/*z* 86 (see Supporting Information Figure S4B).

9-Hydroxy-12-methoxyimino-dodec-10-enoic Acid (HODA) Methoxime. Synthetic HODA (100 μg) in chloroform was transferred to a 3.7-mL vial, and the solvent was evaporated with a stream of dry N_2. The freshly distilled pyridine (200 μL) containing 2 wt % of methoxime hydrochloride was added.[34] The mixture was sealed under nitrogen and kept at room temperature overnight with stirring. Pyridine was then evaporated with a stream of nitrogen, and the residue was dissolved in 0.5 mL of water (pH 3). The solution was extracted with ethyl ether (3 × 0.5 mL). The combined organic layer was washed with water (pH

(31) Funk, M. O.; Isaac, R.; Porter, N. A. *Lipids* **1975**, *11*, 113−117.
(32) Deng, Y. H.; Salomon, R. G. *J. Org. Chem.* **1998**, *63*, 7789−7794.
(33) Lee, S. H.; Oe, T.; Blair, I. A. *Science* **2001**, *292*, 2083−2086.
(34) Gardner, H. W. In *Advances in Lipid Methodology-Four*; Christie, W. W., Ed.; Oily Press: Dundee, Scotland, 1997; pp 1−43.

3). Solvent was evaporated with a stream of nitrogen, and the residue was analyzed with ESI-MS/MS, producing the following fragments: *m*/*z* 256 [M − H]$^-$; *m*/*z* 197 (see Supporting Information Figure S5).

1-Palmitoyl-2-(13-hydroperoxyoctadeca-9*Z*,10*E*-dienoyl)-*sn*-glycero-3-phosphatidylcholine (16a) and 1-Palmitoyl-2-(9-hydroperoxyoctadeca-10*Z*,12*E*-dienoyl)-*sn*-glycero-3-phosphatidylcholine (16b). An 81:19 regioisomeric mixture of hydroperoxydienes **16a** and **16b** was prepared by enzyme-catalyzed oxidation of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (LA-PC) by a modification of the method reported previously.[35] LA-PC (20 mg, 0.026 mmol, Avanti Polar Lipids, AL) was suspended in sodium borate buffer (26 mL, pH 9.0, 0.1 M, pretreated with Chelex 100) and was emulsified with vigorous magnetic stir for 5 min at room temperature. Sodium cholate (112 mg) was then added. After the suspension became transparent, 26 mg of soybean lipoxygenase (Fluka) was added. The reaction was terminated after 1.5 h by the addition of 2:1 $CHCl_3$/MeOH (25 mL) and centrifugation. The organic layer was collected, and the aqueous layer was further extracted with chloroform (3 × 25 mL). The combined organic extracts were concentrated, and the product was purified on a silica gel column ($CHCl_3$/MeOH/H_2O: 65/24/4, TLC: R_f = 0.4) to give the title hydroperoxydienoyl phospholipids (15 mg, 73%). The hydroperoxy functional group was detected on TLC plates with ammonium thiocyanate-ferrous sulfate solution spray. The ratio of regioisomeric hydroperoxydienes was determined after reduction to the corresponding hydroxydienes (vide infra). ^{1}H NMR ($CDCl_3$): δ 6.54 (dd, J = 14.7, 11 Hz, 1H), 6.03 (dd, J = 10.8, 10.8, 1H), 5.61 (dd, J = 17.4, 7.8 Hz, 1H), 5.46 (dd, J = 17.4, 8.4 Hz, 1H), 5.24 (m, 1H), 4.3−4.4 (4H), 4.17 (dd, J =12.2, 7.4 Hz, 1H), 3.9−4.1 (2H), 3.77 (bm, 2H), 3.35 (bs, 9H), 2.0−2.4 (6H), 1.5−1.7 (4H), 1.1−1.45 (42H), 0.85−0.95 (6H). HRMS (ESI-MS): *m*/*z* 791.0 (MH^+) calcd for $C_{42}H_{81}NO_{10}P$ found 791.0; *m*/*z* 813.0 calcd for $C_{42}H_{80}NO_{10}PNa^+$ found 813.0 (see Supporting Information Figure S6A).

1-Palmitoyl-2-(13-hydroxyoctadeca-9*Z*,10*E*-dienoyl)-*sn*-glycero-3-phosphatidylcholine (17a) and 1-Palmitoyl-2-(9-hydroxyoctadeca-10*E*,12*Z*-dienoyl)-*sn*-glycero-3-phosphatidylcholine (17b). The corresponding hydroperoxides (5 mg, 0.0068 mmol) in $CHCl_3$ (5 mL) were reduced with Ph_3P (16 mg, 0.06 mmol). The mixture was stirred for 10 min and then concentrated. The residue was purified on a silica gel column (65/24/4 $CHCl_3$/MeOH/H_2O, TLC R_f = 0.4) to give the title hydroxydienoyl phospholipids (4 mg, 82%). ^{1}H NMR ($CDCl_3$): δ 6.41 (dd, J = 15, 11.4 Hz, 1H), 5.9 (dd, J = 10.8, 10.8 Hz, 1H), 5.60 (dd, J = 15, 6.6 Hz, 1H), 5.34 (dd, J = 18, 7.8 Hz, 1H), 5.14 (m, 1H), 4.3−4.4 (3H), 4.0−4.1 (4H), 3.94 (2H), 3.89 (bm, 2H), 3.33 (bs, 9H), 2.1−2.3 (4H), 2.05−2.15 (2H), 1.5−1.7 (4H), 1.1−1.45 (42H), 0.85−0.95 (6H). HRMS (ESI-MS): *m*/*z* 775.0 (MH^+) calcd for $C_{42}H_{81}NO_9P$ found 775.0; *m*/*z* 797.0 calcd for $C_{42}H_{80}NO_9PNa^+$ found 797.0 (see Supporting Information Figure S6B). To determine the ratio of the 9-hydroxy to 13-hydroxy regioisomers, the mixture (20 μg) was hydrolyzed with 0.2 N NaOH (1 mL) at 45 °C for 1 h. The resulting solution was acidified to pH 4 and extracted with chloroform (4 × 1 mL). The ratio of 13-hydroxyoctadeca-9*Z*,10*E*-dienoic acid to 9-hydroxyoctadeca-10*E*,12*Z*-dienoic acid was determined to be 81:19, respectively, by quantifying both isomers using LC-MS (Figure 7).

Autoxidation of 13-HPODE and 9-HPODE. Five 1 mL glass vials containing 10 μg of 13-HPODE or 9-HPODE in each were heated at 37 °C in an incubator. At 0, 1, 2, 4, and 8 h, a vial was removed from the oven and stored at −80 °C. Before analysis, 100 μL of internal standard solution (0.2 μg/mL) in methanol was added, and 50 μL of the mixture was injected into the LC-MS/MS system. HPLC conditions and MS conditions are the same as those in other experiments for other acidic compounds. The amounts of 13-HPODE, 9-HPODE, and HODA in the autoxidation reaction mixture were monitored simultaneously.

Preparation of Cyclohexanedione (CHD) Reagent. Cyclohexanedione (CHD) was freshly recrystallized from ethyl acetate. First, 125 mg of CHD, 5 g of ammonium acetate, and 2.5 mL of acetic acid were dissolved in deionized water and diluted to 50 mL. The pale yellow solution was heated at 60 °C for 1 h, cooled with an ice bath, and purified by passing through a C18 SPE cartridge (1 mL), which was preconditioned with 5 mL of water and 5 mL of methanol. The elute was collected and stored at 4 °C.[27]

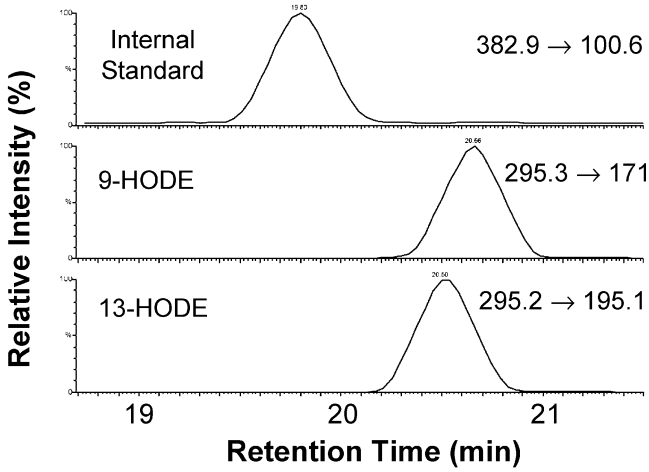


Figure 7. LC/ESI/MS/MS analysis of 9-HODE and 13-HODE. For calibration curves and experimental details, see Quantification of Acids section.

Derivatization of 4-Hydroxy-2-nonenal and Benzaldehyde with CHD/NH_4OAc. Stock solutions of HNE and benzaldehyde (100 ng/μL) were prepared. First, 10 μL of benzaldehyde solution was mixed with 10 μL, 50 μL, 100 μL, 500 μL, and 1000 μL of HNE solution. The solvent was removed with a steam of nitrogen, and the residue was redissolved in 100 μL of methanol. CHD reagent (1 mL) was added, and the mixture was incubated at 60 °C for 1 h. The resulting solution was cooled, and then passed through a C18 SPE cartridge, which had been washed with 5 mL of methanol and 5 mL of water sequentially. The cartridge was washed with 5% acetonitrile in water (9 mL), and the product was washed off with acetonitrile containing 5% water (9 mL). The solvent was removed with a stream of nitrogen. Before LC-MS/MS analysis, the residue was dissolved in methanol containing 25% water, and diluted to 0.01 ng/μL benzaldehyde, of which 0.25 ng was injected into the LC-MS for quantification. ESI-MS/MS analysis of the HNE-CHD derivative produced characteristic fragments: *m*/*z* 326 ([MH − 18]$^+$); *m*/*z* 308 ([MH − 18 − 18]$^+$); *m*/*z* 216. ESI-MS/MS analysis of the benzaldehyde-CHD derivative produced characteristic fragments: *m*/*z* 294 ([MH]$^+$); *m*/*z* 216 (see Supporting Information Figure S7).

Autoxidation of HODEs with and without the Presence of the Hydroperoxide 13-HPODE-d_4. Into five 1-mL glass vials was placed 100 μL of 9-HODE or 13-HODE (50 μg/mL, in methanol). Solvent was evaporated with a stream of nitrogen. The vials were incubated at 37 °C for 1, 2, 4, and 8 h and were stored at −80 °C immediately after incubation. Into six 1-mL glass vials were placed 100 μL of 9-HODE or 13-HODE (50 μg/mL, in methanol) and 100 μL of 13-HPODE-d_4 (100 μg/mL, in methanol). Solvent was evaporated with nitrogen. The vials were incubated at 37 °C for 1, 2, 4, 8, and 18 h and were stored at −80 °C immediately after incubation. To each vial was added 100 μL of methanol with 20 ng internal standard before analysis. The solution (50 μL) was injected onto a RP-HPLC column (Phenomenex Prodigy, 2.0 × 150 mm) using methanol/water gradients at 200 μL/min. The eluant was monitored by an ESI-MS/MS (Quattro Ultima) in the negative ion mode. To confirm the presence of HODA in the oxidation product mixture, a product mixture obtained from the mixture of 10 μg of 9-HODE and 20 μg of 13-HPODE-d_4 after 2 h autoxidation was derivatized with methoxylamine hydrochloride and then analyzed following the same procedure as used for characterizing authentic HODA-methoxime (Figure S5). To quantify HNE, to each vial

(35) Brash, A. R.; Ingram, C. D.; Harris, T. M. *Biochemistry* **1987**, *26*, 5465−5471.

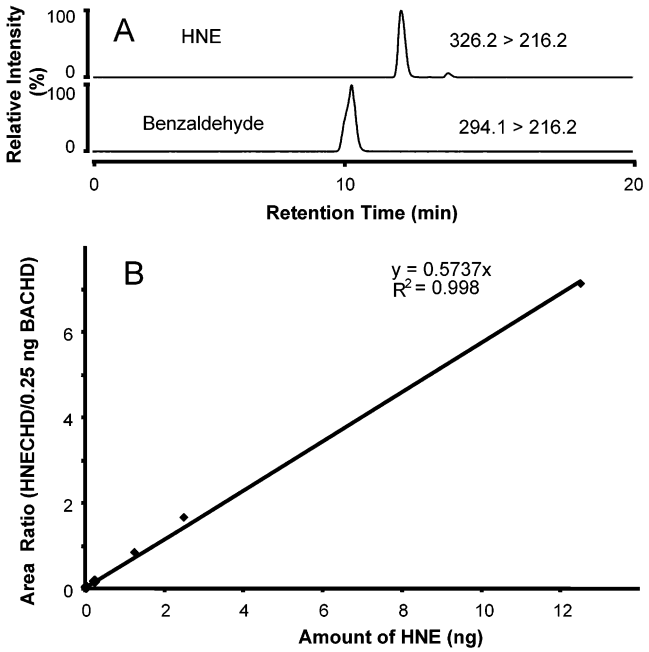


Figure 8. (A) Chromatograms of HNE and benzaldehyde after derivatization by CHD reagent and (B) calibration curve for HNE.

containing 5 μg of 9-HODE (or 13-HODE) and 10 μg of 13-HPODE-d_4 was added 10 ng benzaldehyde in 100 μL of methanol, and the resulting mixture was derivatized with CHD reagent as described for HNE derivatization (see above).

Quantification of HNE as the CHD Derivative 15. The CHD derivative **15** of HNE and the analogous derivative of benzaldehyde were identified individually by reverse phase HPLC coupled to tandem mass spectrometry in the positive ion mode using multiple reaction monitoring (MRM). The mass transition *m*/*z* 396 to 216 was monitored for the HNE-CHD derivative, and *m*/*z* 294 to 216 was monitored for the benzaldehyde-CHD derivative (Figure 8A). The amount of benzaldehyde injected into LC-MS was 0.25 ng. A standard curve (Figure 8B) was generated by preparing samples containing a fixed amount of internal standard (benzaldehyde) and various amounts of HNE, and plotting peak area ratio versus amount of HNE. Each point in the figure is the mean of triplicate determinations.

Quantification of Acids. 13-HPODE-d_4, 13-HPODE, 13-HODE, 9-HODE, HODA, and HODA-d_3 were quantified by LC-ESI-MS/MS with MRM. For 13-HPODE-d_4, the mass transition *m*/*z* 315.4 to 114.6 was monitored. For 13-HPODE, the mass transition *m*/*z* 311.4 to 112.6 was monitored. For 13-HODE, the mass transition *m*/*z* 295 to 195 was monitored. For 9-HODE, the mass transition *m*/*z* 295 to 171 was monitored. For HODA, the mass transition *m*/*z* 227.3 to 84 was monitored. For HODA-d_3, the mass transition *m*/*z* 230.3 to 86 was monitored. Addition of 9-(2-oxanyloxy)-11-(3,3-dimethyl-2,4-dioxolanyl)undec-10-enoic acid as internal standard to the sample prior to analysis permitted for adjustment for ionization variations for each injection. For internal standard, the mass transition *m*/*z* 382.9 to 100.6 was monitored. Calibration curves were built by injecting various amounts of 13-HPODE, 13-HODE, 9-HODE, HODA, and 10 ng internal standard into the LC/MS/MS (Figure 9).

Myeloperoxidase-Promoted Oxidation of HPODE-PC and HODE-PC. Vesicles comprised of the 13-hydroperoxy regioisomer **16a** (81%) and the 9-hydroperoxy regioisomer **16b** (19%) of HPODE-PC or the corresponding regioisomers **17a** and **17b** of HODE-PC were prepared by extrusion as described previously.[4] Autoxidation promoted by myeloperoxidase (MPO) was performed as described previously.[36]

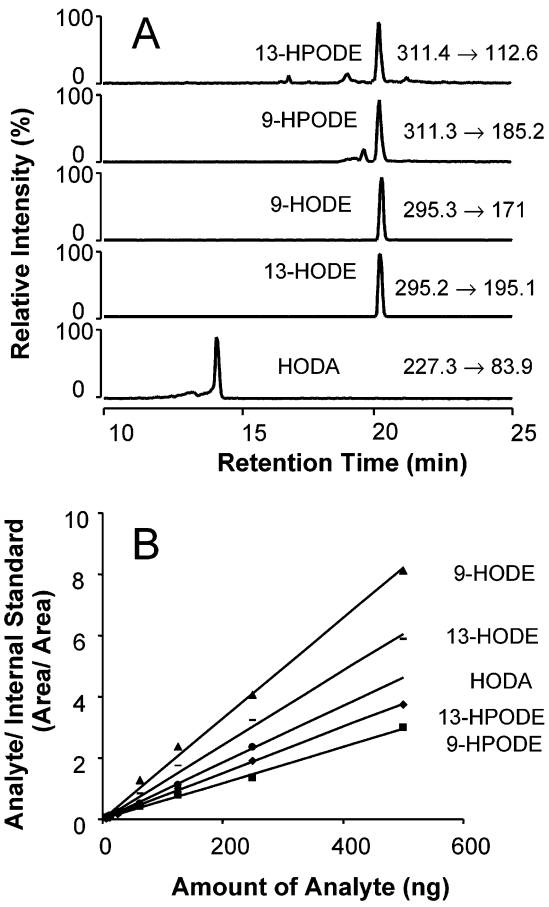


Figure 9. LC/ESI/MS/MS analysis of lipid oxidation products. (A) Each compound was identified individually by reverse phase HPLC coupled to tandem mass spectrometry in the negative ion mode. Multiple reaction monitoring analysis for individual compounds was performed by monitoring the characteristic parent to daughter ion transitions noted. (B) Calibration curves generated by incorporating a fixed amount of internal standard and varying levels of each analyte, and plotting peak area ratio versus nanograms for each analyte.

Briefly, vesicles of HPODE-PC or HODE-PC (0.13 μmol/mL), MPO (60 nM), sodium nitrite (50 μM), glucose (100 μg/mL), and glucose oxidase (20 ng/mL) were incubated in sodium phosphate buffer (50 mM, pH 7.4) supplemented with DTPA (200 μM) at 37 °C. For analysis of the reaction mixture, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC, 7.4 nmol/mL) was added as an internal standard, and lipids were extracted immediately by the method of Bligh and Dyer.[37] The extracts were dried over nitrogen and stored at −80 °C for less than 24 h before analysis. The extracts were analyzed by LC-ESI-MS/MS. HODA-PC, HPODE-PC, and HODE-PC were quantified by monitoring the mass transition from the parent ion (MH^+) to a common fragment (*m*/*z* 184) as described previously.[4] To determine the production of HNE, an aliquot (60 μg) in 100 μL of methanol was treated with benzaldehyde (10 ng) in methanol (20 μL) followed by addition of CHD reagent (1 mL) as described above.

Acknowledgment. We thank the National Institutes of Health for support of this research by grants GM21249 and HL53315. We thank Lian Shan for guiding our LC-ESI-MS/MS analyses. All mass spectrometry experiments were performed in the Cleveland Clinic Foundation Mass Spectrometry Core Facility.

Supporting Information Available: Negative ion ESI-MS/MS spectra of 13-HPODE (**2**), 13-HPODE-d_4 (**2-d_4**), 9-HPODE

(36) Gu, X.; Sun, M.; Gugiu, B.; Hazen, S.; Crabb, J. W.; Salomon, R. G. *J. Org. Chem.* **2003**, *68*, 3749−3761.

(37) Bligh, E. G.; Dyer, W. J. *Can. J. Biochem. Physiol.* **1959**, *37*, 911−917.

(**3**), 9-HODE (**4**), HODA-d_0 (**8**), HODA-d_3 (**8-d_3**), 13-HODE (**9**), and HODA methoxime. Positive ion ESI-MS/MS spectra of HPODE-PC (**16**), HODE-PC (**17**), and CHD derivatives of HNE (**13**) and benzaldehyde. ^{1}H NMR spectra of HPODE-PC (**16**) and HODE-PC (**17**). This material is available free of charge via the Internet at http://pubs.acs.org.

JA038756W