Identification and analysis of products formed from phospholipids in the free radical oxidation of human low density lipoproteins

Ginger L. Milne, Jennifer R. Seal, Christine M. Havrilla, Maikel Wijtmans, and Ned A. Porter[1]

Department of Chemistry and the Vanderbilt Institute of Chemical Biology, Vanderbilt University, Nashville, TN 37235

Abstract **Phospholipids reside in the surface layer of LDLs and constitute ~20–25% of the particle by weight. We report a study of the primary products generated from the most abundant molecular species of phosphatidylcholines present in LDL during in vitro free radical oxidations. The 13-hydroperoxides of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC) and 1-stearoyl-2-linoleoyl-*sn*-glycero-phosphocholine (SLPC) and the 15-hydroperoxides of 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (PAPC) and 1-stearoyl-2-arachidonoyl-*sn*-glycero-phosphocholine (SAPC) were found to increase in a time-dependent manner and in significant amounts even in the presence of α-tocopherol. Phospholipid alcohols also formed during the course of the oxidations. Early in the LDL oxidations, while α-tocopherol was still present, the thermodynamically favored *trans,trans* products of PLPC and SLPC were found to form in significantly larger quantities than those formed from cholesteryl linoleate. Additionally, quantities of PAPC 11-hydroperoxide (11-OOH) decreased over time relative to PAPC 15-OOH, even while α-tocopherol was still present in the oxidation, presumably as a result of further oxidation of PAPC 11-OOH to form cyclic peroxide oxidation products. These results suggest that α-tocopherol is more closely associated with the inner cholesteryl ester-rich hydrophobic core of an LDL particle and is not as effective as an antioxidant in the outer phospholipid layer as it is in the lipid core.**—Milne, G. L., J. R. Seal, C. M. Havrilla, M. Wijtmans, and N. A. Porter. **Identification and analysis of products formed from phospholipids in the free radical oxidation of human low density lipoproteins. *J. Lipid Res.* 2005.** 46: **307–319.**

Supplementary key words phospholipid hydroperoxides • phospholipid alcohols • α-tocopherol

Oxidation of LDLs is an important contributor to the pathogenesis of atherosclerosis. In vitro experimental evidence shows that oxidized LDL causes endothelial cells to recruit monocytes into the arterial wall. The monocytes are then transformed into macrophages, which take up the oxidatively modified LDL and eventually become foam cells, early-stage atherosclerotic plaques (1). There is also evidence to support the presence of oxidized LDL in vivo. Oxidized LDL has been extracted from atherosclerotic lesions (2), autoantibodies reactive with oxidized LDL are present in plasma and atherosclerotic plaques of humans and animals (3 and references therein), and small amounts of oxidized LDL have been found in circulating plasma (4, 5). However, despite the vast knowledge of the proatherogenic properties of oxidized LDL, little is known about the mechanism of oxidation in vivo or about which compounds in oxidized LDL are responsible for these properties.

Phospholipids are primary targets of oxidation in an LDL particle. This lipid class resides in the surface layer of LDL particles and makes up ~20–25% of the whole particle (by weight) (6). The structures of a number of bioactive phospholipid oxidation products have been identified in in vitro oxidations of LDL. These products include secondary phospholipid oxidation products, such as isoprostane-containing phosphatidylcholines (PCs), epoxyisoprostane PC (7), and PCs containing an *sn*-2 acyl group with a terminal γ-hydroxy(or oxo)-α,β-unsaturated carbonyl (8, 9), as well as decomposition products of both ester- and ether-containing oxidized glycerophospholipids in

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Abbreviations: AAPH, 2,2′-azobis(amidinopropane) dihydrochloride; BHT, butylated hydroxytoluene; C-0, 2,2′-azobis[2-(2-imidazolin-2-yl) propane] dihydrochloride; CIS, coordination ion-spray; HETE, hydroperoxyeicosatetraenoate; HODE, hydroxy octadecanoate; IPA, isopropanol; LC-MS, HPLC mass spectrometry; MS/MS, tandem mass spectrometry; -OH, alcohol; -OOH, hydroperoxide; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidylcholine; PC, phosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine; PMC, 2,2,5,7,8-pentamethyl-6-chromanol; PPh_3, triphenylphosphine; SAPC, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidylcholine; SLPC, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine; SRM, selected reaction monitoring; UV, ultraviolet.

[1] To whom correspondence should be addressed.
e-mail: n.porter@vanderbilt.edu

which fragmentation of the *sn*-2 fatty acid has occurred (10, 11 and references therein).

Many of these highly oxidized glycerophospholipids have been shown to be involved in biological events associated with atherosclerosis. In vitro studies show that some of these molecules can stimulate the binding of monocytes to endothelial cells (11–13), whereas other molecules cause monocyte activation and other inflammatory responses (10, 14). Also, it has been reported that PCs containing a terminal γ-hydroxy(or oxo)-α,β-unsaturated carbonyl at the *sn*-2 position have a high binding affinity for the macrophage scavenger receptor CD36 (8). This protein is expressed on the surface of endothelial cells and macrophages and has been found to play a key role in the uptake of oxidized LDL and the onset of atherosclerosis.

Not only have these bioactive phospholipids been identified in vitro, but there is also evidence suggesting that these compounds exist in vivo in atherosclerotic lesions and plasma (11, 12, 15–17). Several antibodies that were developed against oxidized LDL have shown specific binding to decomposition products of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-PC (PLPC), a mixture of oxidation products from 1-palmitoyl-2-arachidinoyl-*sn*-glycero-3-PC (PAPC), or phospholipid-apolipoprotein B-100 adducts (16, 18). These antibodies did not show this specific binding to oxidized free fatty acids, oxidized cholesteryl esters, or oxidized apolipoprotein B that was not modified by phospholipids. However, the antibodies did immunohistochemically stain plasma or atherosclerotic lesions from humans, demonstrating that there is accumulation of oxidized phospholipids in vivo, presumably from oxidized LDL. Electrospray ionization tandem mass spectrometry (MS/MS) analysis of phospholipids extracted from atherosclerotic lesions and plasma gives additional proof of the existence of highly oxidized phospholipids in vivo (12).

It seems clear from this discussion that phospholipid oxidation products are of great importance in the pathogenesis of atherosclerosis. However, most of the research reported in the literature has focused on the secondary oxidation products or the decomposition products of phospholipids. There is very little research studying the early-stage oxidation of phospholipids in LDL and the effect of naturally occurring antioxidants, primarily α-tocopherol, on this oxidation. Studying these aspects of LDL oxidation could provide insight into how one might increase the resistance of LDL to oxidation.

Noguchi, Gotoh, and Niki (19) and Stocker and coworkers (20, 21) both have studied the early formation of phospholipid hydroperoxides in oxidations of human lipoproteins. These workers monitored the formation of phospholipid hydroperoxides over time using a method (22) adapted from one first developed by Yamamoto and colleagues (23, 24). This method does not chromatographically separate individual phospholipid oxidation products, however, and no information about the structure of these phospholipid hydroperoxide molecular species was obtained.

Previously, we reported the development of an HPLC coordination ion-spray mass spectrometry (LC-CIS-MS) method for the analysis and identification of phospholipid hydroperoxides, the primary products expected during the early stages of oxidation in LDL (25). In the studies reported here, we used this method to identify and quantify the formation of hydroperoxides from the most abundant molecular species of PC in free radical-initiated in vitro LDL oxidations. Also, we compared the oxidation of the phospholipids in LDL with that of the cholesteryl esters, the most abundant lipid found in the hydrophobic core of LDL, to gain insight into how endogenous antioxidants (e.g., α-tocopherol) influence oxidation in the different regions of LDL.

EXPERIMENTAL PROCEDURES

Materials

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) or from Sigma Chemical Co. (St. Louis, MO) and used without further purification. PLPC was purchased from Sigma as a powder, and PAPC and 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine (SAPC) were purchased from Sigma as chloroform solutions. 1-Stearoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (SLPC) was purchased as a powder from Avanti Polar Lipids. Soybean lipoxygenase (type I-B) and phospholipase D (from *Streptomyces* species; type VII) were purchased as lyophilized powders from Sigma Chemical Co. The free radical initiator 2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (C-0) was generously donated by Wako Chemicals USA, Inc. (Richmond, VA). All chemicals used to make buffers for the LDL experiments were purchased from Sigma Chemical Co. and were of the highest quality (SigmaUltrapure). Solvents were HPLC quality and purchased from either Fisher Chemical (Phillipsburg, NJ) or EM Science (Gibbstown, NJ). Hexanes was purchased from Burdick and Jackson (Muskegon, MI). All other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification.

In general, hydroperoxides and extracts of oxidized lipids from LDL were stored as dilute solutions with 1 mol% butylated hydroxytoluene (BHT) in benzene at −78°C and never exposed to temperatures >40°C.

Instruments

HPLC. Analytical HPLC was conducted using a Waters model 2690 Alliance Separations Model instrument with a Waters 996 photodiode array detector connected to a Waters Millennium^{32} (version 3.20) chromatography station. Semipreparative HPLC was conducted on a Waters model 600E HPLC instrument, with either a Waters model 481 variable wavelength detector or a Waters 2487 dual wavelength absorbance detector and with output to a strip chart recorder.

Mass spectrometry. CIS-MS was performed using either one of two systems. *1*) A ThermoFinnigan Thermoquest TSQ-7000 (San Jose, CA) triple quadrupole mass spectrometer equipped with a standard API-1 electrospray ionization source outfitted with a 100 mm deactivated fused Si capillary. Data acquisition and spectral analysis were conducted using ICIS software (version 8.3.2) running on a Digital Equipment Alpha Station 200 4/166. Nitrogen gas served as both the sheath gas and the auxiliary gas; argon served as the collision gas. The electrospray needle was maintained at 4.6 kV, and the heated capillary temperature was 250°C. The tube lens and capillary voltages were 90 and 10 V, respectively, and the sheath and auxiliary gases were at 60 and 5–10

pounds per square inch, respectively. For MS/MS experiments, collision gas was typically maintained at a pressure of 2.6–2.9 mTorr, and the offset was 25 eV. For online HPLC sample introduction, a Waters model 2690 Alliance Separations Model instrument was used. *2*) A ThermoFinnigan TSQ Quantum 1.0 SR 1 mass spectrometer coupled with a Surveyor MS Pump 2.0 and a Surveyor Autosampler 1.3 (San Jose, CA). Nitrogen gas served as both the sheath gas and the auxiliary gas; argon served as the collision gas. The electrospray needle was maintained at 5 kV, and the capillary temperature was 310°C. The tube lens voltage was 194 V, and the sheath and auxiliary gases were 49 pounds per square inch and 25 units, respectively. For MS/MS experiments, the collision gas was maintained at 1.5 mTorr, and the offset was 35 eV. Data processing was conducted using Xcaliber software (version 1.3).

Methods

Synthesis of d_9-PLPC, d_9-PAPC, d_9-SLPC, and d_9-SAPC. PLPC, PAPC, SLPC, or SAPC (25 mg, ~0.03 mmol) was dissolved in 1 ml of CH_2Cl_2. d_9-Choline (>98 atom% D, 29 mg, 0.195 mmol) was dissolved in buffer (500 μl) containing 100 mM sodium acetate and 50 mM calcium chloride at pH 6.5 and added to each phospholipid solution. Phospholipase D (50 μl of a 1 U/μl solution in 100 mM sodium acetate) was then added to each reaction. The biphasic reaction mixtures were shaken at 190 rpm for 4 h at 30°C. The reactions were stopped by Folch extraction. The d_9 phospholipids were purified immediately by semipreparative HPLC [Discovery C18 semi-prep column, 21.2 mm × 25 cm; mobile phase: 100% methanol, 10 ml/min; ultraviolet (UV) detection, λ = 210 nm].

Synthesis of d_9-PC hydroperoxides. Soybean lipoxygenase (4 mg for PLPC and SLPC, 26 mg for PAPC and SAPC) was taken up in 5.5 ml of borax buffer that contained 10 mM deoxycholate at pH 9.0. d_9-PLPC, d_9-PAPC, d_9-SLPC, or d_9-SAPC (5 mg, 0.006 mmol) was taken up in 1 ml of the same buffer and added to the enzyme solution (**Fig. 1**). The reaction was stirred at room temperature for 30–60 min. The progress of the reaction was monitored by UV. The reaction was stopped by Folch extraction. To synthesize the alcohol internal standards, the hydroperoxides were made using this procedure and then reduced with triphenylphosphine (PPh_3). The structures of the synthesized standards are shown in **Fig. 2**. The isomeric purity of the d_9-phospholipid hydroperoxides and alcohols were checked by analytical HPLC [Discovery C18 analytical column, 4.6 × 250 mm; mobile phase: methanol-water (95:5), 1 ml/min; UV detection, λ = 234 nm]. The standards were then purified immediately by semipreparative HPLC [Discovery C18 semi-prep column, 21.2 mm × 25 cm; mobile phase: 100% methanol, 10 ml/min; UV detection, λ = 210 nm].

Quantitation and calibration of the d_9-PC hydroperoxides and d_9-PC alcohols. The amount of each pure oxidized internal standard synthesized was quantified by UV using the known ε value for the 13-*cis,trans*-hydroperoxide (13-*cis,trans*-OOH) of linoleates (26) (ε = 27,200) and for arachidonate hydroperoxides (27) (ε = 27,000). Calibration curves were done using LC-CIS-MS/MS. The mass spectrometer was operated in selected reaction monitoring (SRM) mode to monitor the precursor-to-product transition of the PC-OOHs to their corresponding Hock fragments or the loss of the phosphocholine head group from the PC alcohols (PC-OHs). The deuterated phospholipid standards eluted from the HPLC as follows: 13-*cis,trans*-PLPC-OOH, t = 23.40 min; 13-*cis,trans*-PLPC-OH, t = 23.92 min; 13-*cis,trans*-SLPC-OOH, t = 38.83 min; 13-*cis,trans*-SLPC-OH, t = 39.53 min; 15-PAPC-OOH, t = 26.08 min; 15-PAPC-OH, t = 23.38 min; 15-SAPC-OOH, t = 43.39 min; 15-SAPC-OH, t = 42.57 min.

Quantitation and calibration of the unoxidized d_9-PLPC. After synthesis, the amount of d_9-PLPC was determined by weighing. The calibration curve was done using LC-CIS-MS/MS. The mass spectrometer was operated in SRM mode to monitor the loss of the phosphocholine head group from the PLPC parent.

Autoxidation of PAPC. To a solution of PAPC (5 mg, 0.006 mmol) in 1.5 ml of CH_2Cl_2 was added either 0.1 or 0.025 equiva-

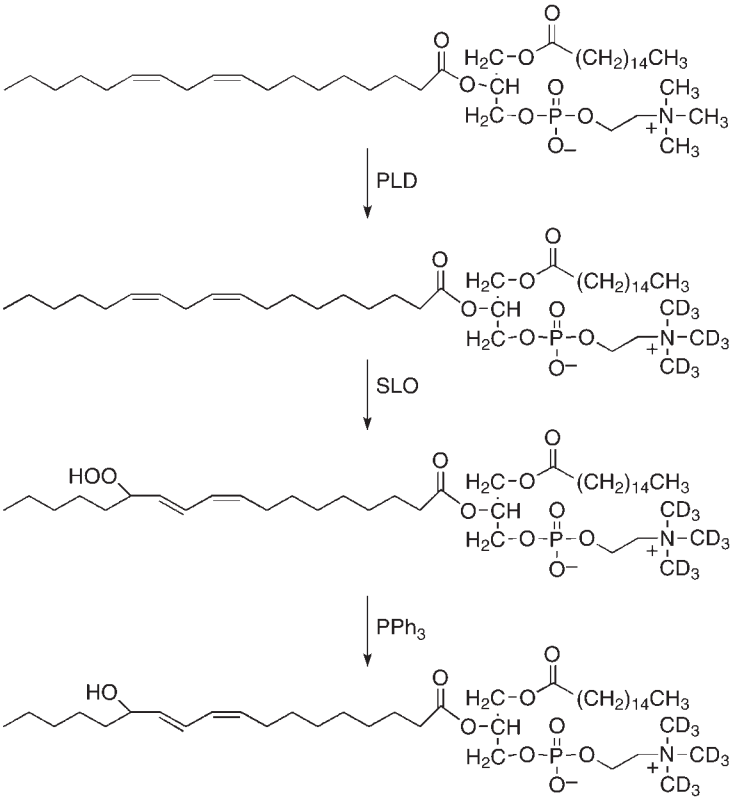


Fig. 1. Synthesis of the d_9-13-*cis,trans*-1-palmitoyl-2-linoleoyl-*sn*-glycero-3-PC hydroperoxide (-OOH) and alcohol (-OH) internal standards. PLD, phospholipase D; PPh_3, triphenylphosphine; SLO, soybean lipoxygenase.

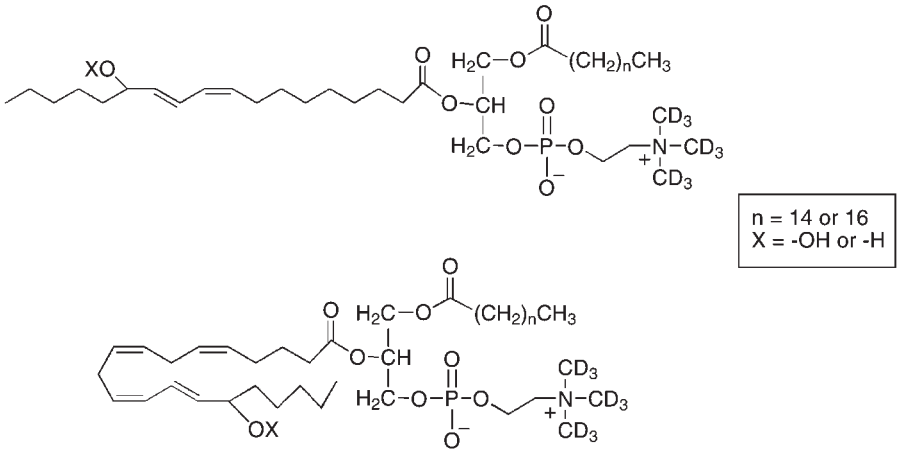


Fig. 2. Structures of the eight internal standards designed to quantify the amount of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-PC (PLPC), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-PC (PAPC), 1-stearoyl-2-linoleoyl-*sn*-glycero-3-PC (SLPC), and 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-PC (SAPC) hydroperoxides and alcohols formed during the autoxidation of LDL.

lent of 2,2,5,7,8-pentamethyl-6-chromanol (PMC; 64 or 16 μl, respectively, of a 10 mM stock solution in CH_2Cl_2). The solution was evaporated to dryness under a stream of argon so that the mixture formed a thin layer on the inside of a 4 ml vial. The vial was then heated to 37°C and exposed to an atmosphere of dry air. After 24 h, the mixture was dissolved in benzene and BHT (~1–2 mg) was added to stop the reaction. The hydroperoxides were analyzed by HPLC.

Isolation and oxidation of LDL. LDL was isolated from the whole blood of fasting, normolipidemic healthy subjects as previously described (28). All blood donations were collected in accordance with guidelines established by the Institutional Review Board at Vanderbilt University (study protocol number 99073), and all donors gave written informed consent. Protein concentration was determined using the modified Lowry (29) assay reported by Morton and Evans (30). SDS-PAGE gels, run on a Ciba-Corning electrophoresis system, and LipoGels, run on a Beckman Instruments Paragon LipoGel system, were used to determine the purity of the LDL.

Before oxidation, the concentration of the LDL was adjusted to 0.75 mg/ml with PBS. The solution was then magnetically stirred and allowed to equilibrate at 37°C for 5 min. The initiator, either C-0 (50 mM stock solution in PBS) or 2,2′-azobis(amidinopropane) dihydrochloride (AAPH; 50 mM stock solution in methanol) was then added to give a final initiator concentration of either 1 or 0.5 mM, respectively.

For phospholipid analysis, 1.0–1.5 ml aliquots were removed at various time intervals. BHT (100 μl of a 3 mM solution in methanol) was added to all aliquots to stop the oxidation. Each aliquot was immediately extracted with ice-cold CH_2Cl_2 (6 ml), methanol (3 ml), and 0.74% KCl (1.75ml) in sequence. The samples were vortexed after the addition of each solvent and were then centrifuged at 1,700 rpm for 5 min. The organic phase was concentrated under a stream of dry argon, and the resulting residue was stored as a dilute solution in benzene until analysis.

The lipid classes in each aliquot were separated using HPLC equipped with an aminopropyl column (Supelco -NH_2 column; 3.0 × 250 mm). The gradient solvent program used in this separation method employed mixtures of hexanes/tetrahydrofuran (THF) (99:1) and isopropanol-water (85:15) to elute the different lipid classes and acetone/CH_2Cl_2 (2:1) to wash the column. The gradient program is shown in **Table 1**. The cholesteryl esters and other neutral species, including the antioxidants present in LDL, elute between 2 and 5 min, whereas the phospholipids elute between 28 and 30 min.

For cholesteryl ester analysis, LDL was oxidized as described above and 200 μl aliquots were removed at various time points. To each aliquot, BHT (20 μl, 3.0 mM in ethanol) was added. Because many of the cholesteryl linoleate and cholesteryl arachidonate hydroperoxides coelute, the hydroperoxides were converted to the corresponding alcohols by the addition of PPh_3 (20–30 μl, 25 mM in ethanol) to each aliquot. Aliquots were extracted with ice-cold methanol (1.0 ml) and ice-cold hexane (5.0 ml) in sequence, vortexing vigorously after the addition of each solvent (15 s), and then centrifuged at 1,700 rpm for ~1 min. The hexane phase was retained, concentrated under argon, and reconstituted in 200–300 μl of mobile phase for analysis.

Quantification of PC-OOHs and PC-OHs in LDL oxidations. For these experiments, 1 ml aliquots were taken from the LDL oxidation. A known amount of a mixture containing the eight oxidized internal standards was added to aliquots at later times. After work-up and separation of the lipid classes, each phospholipid aliquot was taken up in 2 ml of methanol and subsequently analyzed by LC-CIS-MS/MS [Discovery C-18 microbore column, 1.0 mm × 30 cm, 5-μm (Supelco); mobile phase: 100% methanol, 0.085 ml/min; 16 μl injections] operating in the SRM mode to monitor the precursor-to-product transition of either the PC-OOHs fragmenting to form their corresponding Hock fragments, a common Ag^+ CIS-MS/MS fragmentation pathway for

TABLE 1. HPLC solvent system used to separate lipid classes on a Supelco aminopropyl analytical column

Time	Flow Rate	Solvent A	Solvent B	Solvent C
min	*ml/min*		*% by volume*	
0	1.6	100	0	0
20	1.6	100	0	0
25	1.6	40	0	60
36	1.6	40	0	60
40	2.0	0	100	0
45	2.0	0	100	0
50	1.6	100	0	0
60	1.6	100	0	0

Solvent A, hexanes/tetrahydrofuran (THF) (99:1); solvent B, acetone/CH_2Cl_2 (2:1); solvent C, isopropanol-water (85:15).

hydroperoxides (31, 32), or the PC-OHs losing the phosphocholine head group. Local concentrations were calculated using the oxidation protein concentration, assuming apolipoprotein B-100 is 500 kDa and the total lipid volume in an LDL particle is 3.2×10^{-21} liters (33). A local concentration of 0.001 M is equivalent to approximately two molecules of oxidized lipid per LDL particle.

Determining the extent of oxidation of PLPC and cholesteryl linoleate. To measure the amount of PLPC in LDL, a known amount of the d_9-PLPC standard was added to a t = 0 h aliquot from an LDL oxidation mixture. The sample was worked up in the same manner as the oxidized aliquots. After lipid class separation, the unoxidized phospholipid aliquot was taken up in 2 ml of methanol and subsequently analyzed by LC-CIS-MS/MS [Discovery C-18 microbore column, 1.0 mm × 30 cm, 5-μm (Supelco); mobile phase: 100% methanol, 0.085 ml/min; 8 μl injections] operating in the SRM mode to monitor the precursor-to-product transition of PLPC losing the phosphocholine head group. The amounts of all four PLPC-OOHs and all four PLPC-OHs quantified for each time point were totaled and compared with the starting amount of PLPC to determine the extent of oxidation. Only the 13-*cis,trans*-PLPC-OOH and 13-*trans,trans*-PLPC-OOH were quantified using this method, so the quantified values for each time point were doubled, assuming that the 13-OOHs were formed to the same extent as the 9-OOHs (34), to represent the total amount of PLPC-OOH formed. The local concentration of hydroperoxide was calculated as described above.

For cholesteryl linoleate analysis, a known amount of 13-*trans,trans*-methyl hydroxy octadecanoate (HODE), the internal standard, was added. After extraction, each aliquot was reconstituted in 200 μl of mobile phase and analyzed by HPLC-UV (dual Beckman Si columns; mobile phase: 0.5% isopropanol (IPA) in hexanes, 1.0 ml/min; λ = 234 nm; 50 μl injections). The amounts of all four cholesteryl linoleate alcohols quantified for each time point were totaled and compared with the amount of cholesteryl linoleate in one LDL particle as reported by Esterbauer and coworkers (6) to determine the extent of oxidation.

Measurement of linoleate cis,trans/trans,trans ratios. For these experiments, 1.5 ml aliquots were taken from the LDL oxidation. PPh_3 (100 μl of a 25 mM solution in methanol) was added to each aliquot to reduce the hydroperoxides to the more easily analyzable alcohols. After work-up and separation of the lipid classes, the cholesterol ester oxidation products and the phospholipid oxidation products were converted to the corresponding methyl esters. Each isolated fraction was dissolved in benzene (0.5 ml) and treated with an excess of NaOMe (1 ml of a 0.5 M solution in methanol) for 2 h. The reactions were worked up by the addition of deionized water (5 ml) and acetic acid (100 μl). The aqueous layer was extracted with hexanes (2 × 5 ml). The combined organic layers were dried, concentrated, and analyzed by HPLC [Ultrasphere silica column, 4.6 mm × 250 mm, 5 μm (Beckman); mobile phase: 0.5% 2-propanol, 1 ml/min; λ = 234 nm] and GC [Hewlett-Packard 5890 Series II Gas Chromatograph; HP-5 column, 0.32 mm × 30 m, 0.25 μm (Hewlett-Packard); carrier gas flow rate: 1.5 ml/min; injector/flame-ionization detector: 280°C; temperature program: 100–280°C over 30 min]. The methyl HODEs eluted from the HPLC as follows: 13-*cis,trans*, t = 17.34 min; 13-*trans,trans*, t = 20.07 min; 9-*cis,trans*, t = 24.65 min; 9-*trans,trans*, t = 27.14 min. The methyl HODEs eluted from the GC as follows: 13-*cis,trans*, t = 18.057 min; 9-*cis,trans*, t = 18.390 min; 13-*trans,trans* and 9-*trans,trans*, t = 19.457 min.

Measurement of arachidonate hydroperoxide ratios. For phospholipid analysis, 1.0–1.5 ml aliquots were removed from the oxidation mixture at various intervals from 0 to 5 h. After extraction, the chloroform phase was concentrated using a Thermo Savant SC210A SpeedVac Plus coupled with a RVT400 Refrigerated Vapor Trap (Holbrook, NY). Lipids were reconstituted in 500 μl of hexanes and filtered through a Pall Gelman GHP Acrodisc syringe filter (0.45 μm pore) before lipid class separation. After lipid class separation, half of the phospholipids isolated from each time point were reconstituted in 20 μl of methanol and analyzed by LC-CIS-MS/MS in SRM mode [Discovery C-18 analytical column, 4.6 mm × 25 cm, 5-μm (Supelco); mobile phase: methanol-water (95:5), 1 ml/min with a splitting tee giving a final flow rate into the MS of 240 μl/min]. The PAPC 15-OOH and PAPC 11-OOH SRM peaks were then integrated for analysis.

For analysis of the cholesteryl arachidonate alcohol ratios, the extracted cholesteryl ester oxidation products were reconstituted in 300 μl of HPLC solvent. Each aliquot was analyzed by HPLC-UV (dual Beckman Si columns; mobile phase: 0.5% IPA in hexanes, 1.0 ml/min; λ = 234 nm; 50 μl injections). The cholesteryl-5-hydroperoxyeicosatetraenoate (Ch-5-HETE) and Ch-8-HETE peaks were integrated for analysis.

RESULTS

The oxidation of lipids is a free radical-mediated process. Polyunsaturated fatty acids and esters are especially susceptible to oxidation because they contain bisallylic hydrogen atoms. Bisallylic carbon-hydrogen bonds have a significantly lower dissociation enthalpy than allylic carbon-hydrogen bonds or alkyl carbon-hydrogen bonds, making them especially susceptible to the initial step of oxidation, abstraction of a hydrogen atom. The two most abundant polyunsaturated fatty acids found in humans, and in LDL, are linoleic acid and arachidonic acid. The oxidation of these compounds has been thoroughly investigated, and the primary oxidation products of these fatty acids are shown in **Fig. 3**. Linoleic acid and arachidonic acid are esterified to both phospholipids and cholesteryl esters in LDL. Therefore, our efforts herein focus on the study of the primary oxidation products of these species.

Quantification of the primary phospholipid oxidation products in LDL

The major class of phospholipids present in LDL is PC. Many individual molecular species of PC are found in LDL, but the four most abundant species that contain polyunsaturated fatty acids are PLPC, SLPC, PAPC, and SAPC. In these experiments, LDL oxidation was initiated by the addition of a free radical azo initiator, C-0, and the primary hydroperoxides and alcohols formed from the four phosphocholine molecules listed above were quantified. Specifically, the 13-*cis,trans* and 13-*trans,trans* hydroperoxides of both PLPC and SLPC,[2] the four alcohols of PLPC and SLPC,[3] and the 15-OOHs of both PAPC and SAPC[4] were measured using isotope-dilution HPLC-MS/MS

[2] Both 13-hydroperoxides of each linoleate-containing PC were quantified together because they coeluted in HPLC and have the same fragmentation pattern in the mass spectrometer.

[3] All four alcohols of each linoleate-containing PC were quantified together because they coeluted in HPLC and have the same fragmentation pattern in the mass spectrometer. The total value obtained for each time point was divided by 2 to represent just the amount of 13-OHs, assuming that the 9-OHs were formed to the same extent.

[4] The 15-OHs of PAPC and SAPC were observed but were not quantified because they were formed in such low abundance.

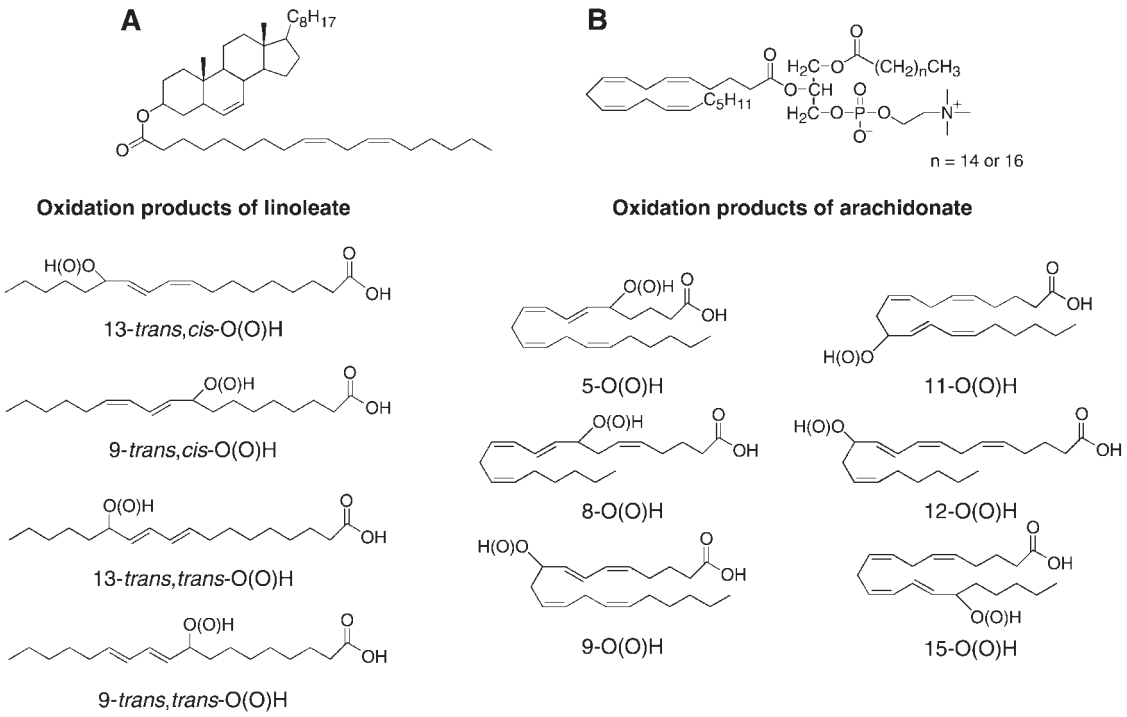


Fig. 3. A: Structure of cholesteryl linoleate. B: Structure of arachidonate containing PCs, PAPC and SAPC. Below are shown the primary oxidation products of linoleic acid and arachidonic acid.

as described in Experimental Procedures. The deuterated standards shown in Fig. 2 were used as internal standards.

As can be seen in **Fig. 4**, all phospholipid hydroperoxides and alcohols measured in these oxidations formed in a time-dependent manner. Significant amounts of the PC-OOHs were formed as early as 0.5 and 1 h, when α-tocopherol, the primary antioxidant in LDL that affects lipid peroxidation, was still present.[5] Interestingly, phospholipid alcohols do not form concomitantly with phospholipid hydroperoxides. At the earliest time points, t = 0.5 h and t = 1 h, little or no PC-OH was detected. However, at t = 2 h, PC-OHs were detected, and the amount of alcohols formed increased slowly during the oxidation. This observation for phospholipid oxidation is in contrast to previous studies of cholesteryl linoleate oxidation (35, 36) in LDL. Cholesteryl ester alcohols formed concomitantly with cholesteryl ester hydroperoxides even at the earliest time points measured for the oxidation. In addition, few of the alcohol oxidation products from cholesteryl linoleate were formed in LDL during oxidation, whereas significant amounts of linoleate phospholipid alcohols were formed.

These quantitation data made it possible to determine the extent to which the phospholipids in LDL were oxidized. However, the amount of each phospholipid molecular species present in LDL particles was not previously known. Esterbauer and coworkers (6) have extensively characterized the lipid components in an LDL particle; the number of molecules of each lipid class in one LDL particle and the number of molecules of each fatty acid per particle as well as the percentage of specific fatty acid molecules esterified in each lipid class were determined. Using these data, the number of molecules of cholesteryl linoleate and cholesteryl arachidonate per particle could be calculated. However, because multiple fatty acids are esterified to a single phospholipid molecule, it was not possible to calculate the number of molecules of specific phospholipid molecular species from these data. Therefore, the number of molecules of relevant phospholipid molecular species in one LDL particle had to be quantified. For these studies, efforts focused on PLPC, an abundant polyunsaturated phospholipid species in LDL, so its percentage oxidation could be compared with that of the most abundant polyunsaturated cholesteryl ester species in LDL, cholesteryl linoleate. Using a synthesized d_9-PLPC standard, it was determined that there are ~140 molecules of PLPC in one LDL particle. To determine the extent of PLPC oxidation, the total amount of PLPC oxidation products, both hydroperoxides and alcohols, quantified for each time point was compared with the starting amount of PLPC. The resulting graph is shown in **Fig. 5**. After 7 h of oxidation, almost 15% of the PLPC was oxidized.

For comparison, the extent to which cholesteryl linoleate is oxidized under these conditions was determined. In a separate, but identical, oxidation, the oxidation products of cholesteryl linoleate that had all been reduced to the alcohols were quantified by HPLC-UV. The

[5] From data collected in our laboratory over the course of many LDL oxidations, we approximate that LDL will contain six to eight molecules of α-tocopherol before oxidation. After 1 h of oxidation, ~70–75% of the α-tocopherol was consumed. Between 3 and 4 h of oxidation, most of the α-tocopherol in the LDL was consumed.

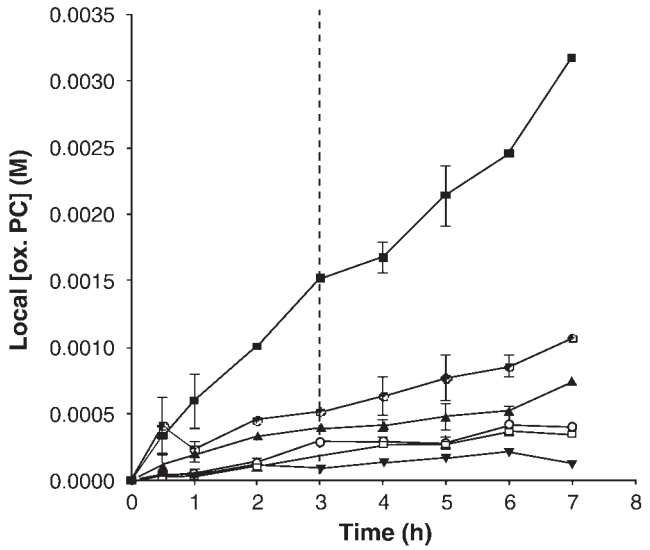


Fig. 4. Free radical autoxidation of LDL (0.75 mg protein/ml) initiated by 1 mM 2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (C-0) at 37°C. At the time points shown, the phospholipid oxidation products were analyzed by HPLC coordination ion-spray tandem mass spectrometry (CIS-MS/MS) as described in the text. Closed squares, 13-*cis,trans*-OOH PLPC and 13-*trans,trans*-OOH PLPC; open squares, 13-*cis,trans*-OH PLPC and 13-*trans,trans*-OH PLPC; closed circles, 13-*cis,trans*-OOH SLPC and 13-*trans,trans*-OOH SLPC; open circles, 13-*cis,trans*-OH SLPC and 13-*trans,trans*-OH SLPC; closed triangles, 15-OOH PAPC; closed inverted triangles, 15-OOH SAPC. Error bars reflect standard deviations where n = 2. The vertical dotted line shows the time in the oxidation at which α-tocopherol can no longer be detected.

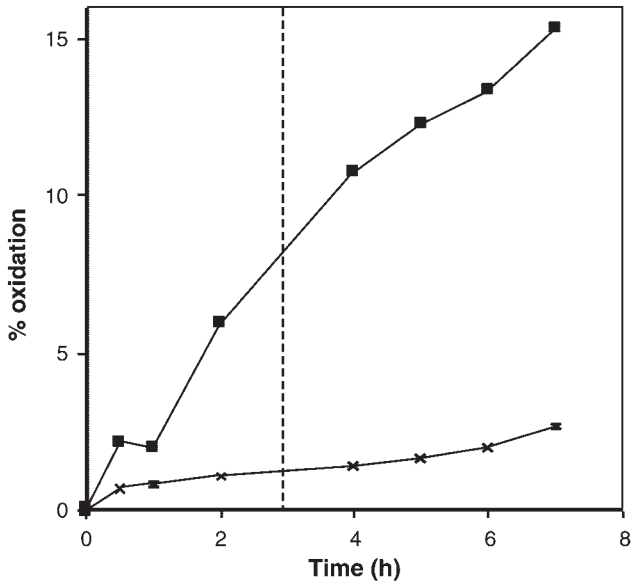


Fig. 5. Percentage of lipids oxidized in a free radical autoxidation of LDL (0.75 mg protein/ml) initiated by 1 mM C-0 at 37°C. Closed squares, PLPC; crosses, cholesteryl linoleate. The vertical dotted line shows the time in the oxidation at which α-tocopherol can no longer be detected.

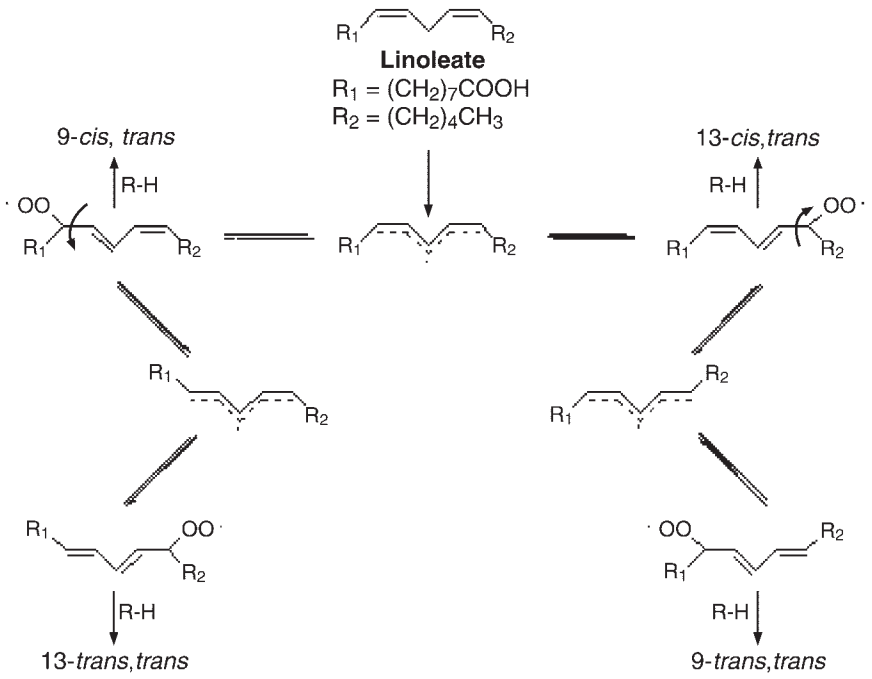
amounts of each product quantified for each time point were totaled and compared with the amount of cholesteryl linoleate reported to be in an LDL particle by Esterbauer and coworkers (6). As can be seen in Fig. 5, cholesteryl linoleate was oxidized to a much lower extent than PLPC. After 7 h of oxidation, only 3% of the cholesteryl linoleate was oxidized under these conditions.

Comparison of phospholipid and cholesteryl ester *cis,trans/trans,trans* ratios

Another goal of these studies was to directly compare the oxidation of the phospholipids with the oxidation of the cholesteryl esters to monitor differences between oxidation on the surface and in the core of LDL and to further understand the role that antioxidants play in each LDL region. One way to compare the oxidation of the two lipid classes is to monitor the linoleate *cis,trans*-to-*trans,trans* product distribution for each lipid class during the course of an LDL oxidation. This method has been used previously to provide information about the efficacy of antioxidants in micelles and lipid bilayers (35–37), and we recently used this product ratio to estimate an inhibition rate constant for the reaction of α-tocopherol with peroxyl radicals derived from cholesteryl linoleate in LDL (38). Monitoring this product distribution is useful to gain insight into the role of antioxidants in the oxidation of lipids in a particular environment because the mechanism by which these linoleate oxidation products form is dependent upon the hydrogen atom-donating capacity of the oxidation medium (**Scheme 1**). The kinetically favored *cis,trans* products will predominantly form in the presence of good hydrogen atom donors, in this case α-tocopherol, whereas the thermodynamically favored *trans,trans* products will form in the absence of hydrogen atom donors, when β-fragmentation is more likely to occur (39, 40).

To obtain these product ratios, the linoleate hydroperoxides and alcohols produced from each of the lipid classes, the cholesteryl esters and the phospholipids,[6] during the oxidation of LDL were converted to the corresponding methyl ester alcohols for analysis by HPLC. Representative chromatograms of the methyl ester alcohols generated from the cholesteryl esters and the phospholipids at 2 h are shown in **Fig. 6**, and the *cis,trans/trans,trans* ratios obtained are shown in **Fig. 7**. The difference in ratios between the two lipid classes is striking. The *cis,trans/trans,trans* ratios for the cholesteryl esters follow the same pattern as previously reported (36). At early time points in the oxidation, such as in Fig. 6, primarily *cis,trans* products are formed (*cis,trans/trans,trans* at 1∼13 h), indicating that α-tocopherol effectively traps these products. As indicated by lower *cis,trans/trans,trans* ratios after 3 h, the *trans,trans* products are formed more abundantly after the depletion of α-tocopherol. The oxidation profile of the linoleate-containing phospholipids is quite different. The *trans,trans* products are formed in significant quantities early in the oxidation (*cis,trans/trans,trans* at 1∼3 h), when α-tocopherol is still present in the LDL particle.

[6] The methyl linoleate alcohols from the phospholipid fraction represented the oxidation products of both PLPC and SLPC.



Scheme 1.

Comparison of PAPC and cholesteryl arachidonate hydroperoxide ratios

The arachidonyl side chain of PAPC contains three bis-allylic hydrogens on carbons 7, 10, and 13. The abstraction of hydrogen from each of these sites results in the formation of three groups of hydroperoxides: the PAPC 5- and 9-OOHs from hydrogen atom abstraction of carbon 7, the PAPC 8- and 12-OOHs from hydrogen atom abstraction of carbon 10, and the PAPC 11- and 15-OOHs from

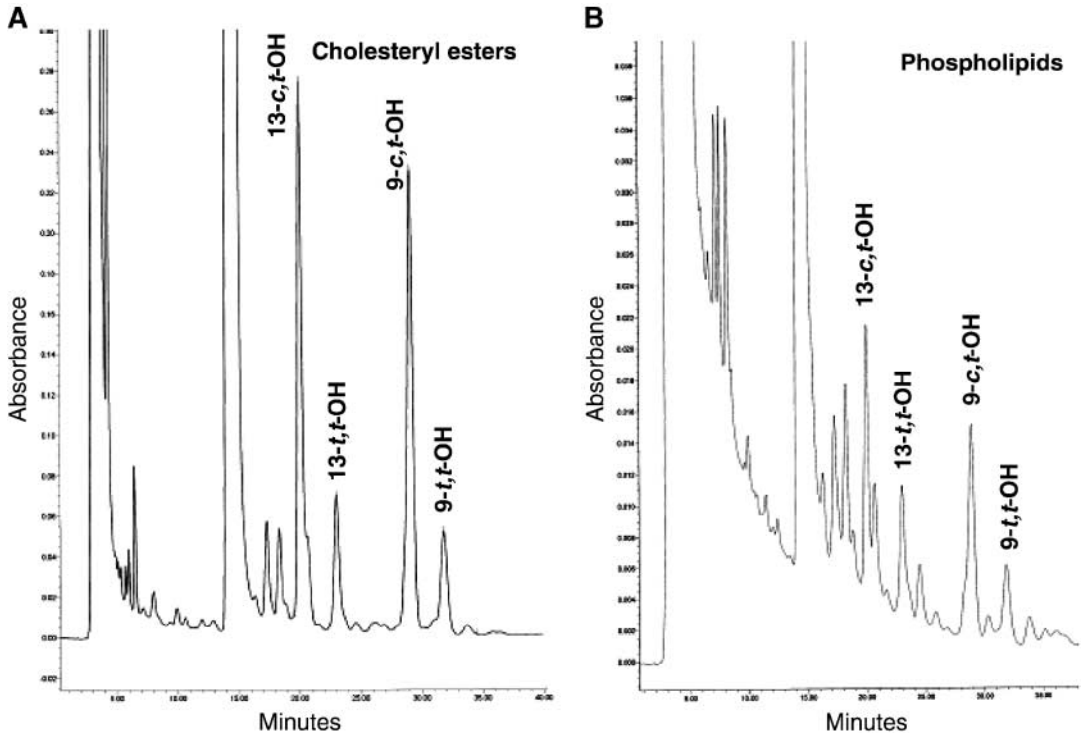


Fig. 6. HPLC of the cholesteryl ester (A) and phospholipid (B) oxidation products at 2 h. The cholesteryl ester and phospholipid hydroperoxides were reduced to the alcohols with triphenylphosphine (PPh_3) and then converted to the corresponding methyl esters for chromatographic analysis [mobile phase, 0.5% isopropanol (IPA) in hexane with ultraviolet detection at 234 nm].

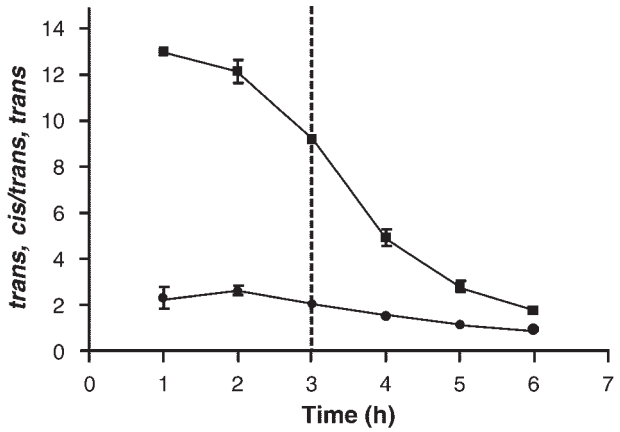
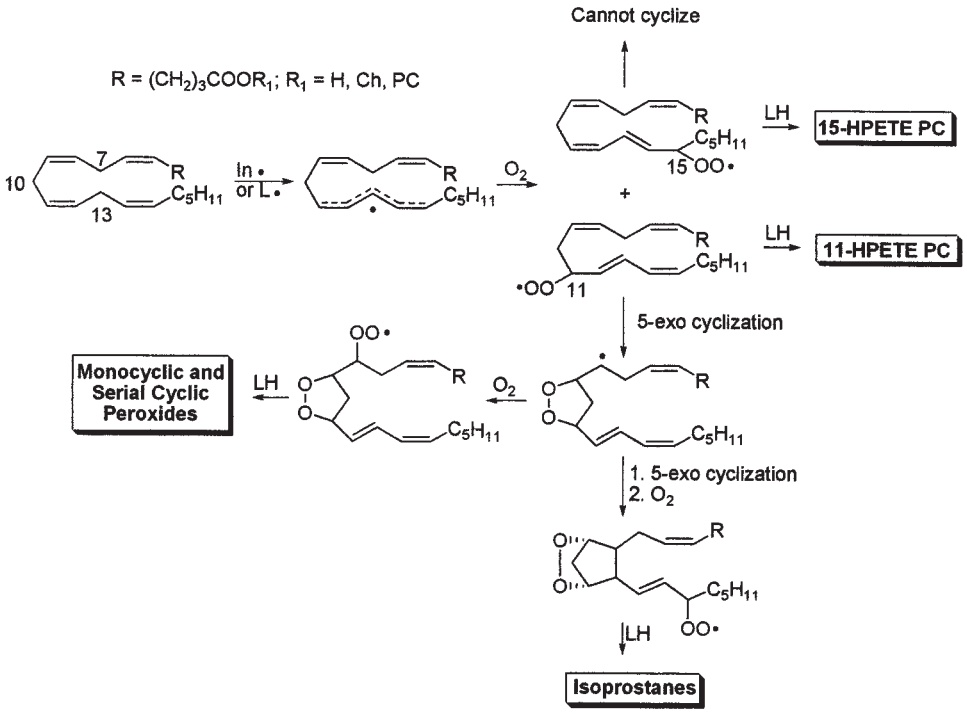


Fig. 7. Ratios of the *cis,trans* and *trans,trans* products formed from cholesteryl linoleate (closed squares) and the linoleate-containing phospholipids PLPC and SLPC (closed circles) in a free radical autoxidation of LDL (0.75 mg protein/ml) initiated by 1 mM C-0 at 37°C. Error bars reflect standard deviations where n = 2 for cholesteryl linoleate and n = 3–4 for the phospholipids. The vertical dotted line shows the time in the oxidation at which α-tocopherol can no longer be detected.

hydrogen atom abstraction of carbon 13 (Fig. 3). In addition to these primary products, the peroxyl radicals generated from the four interior hydroperoxides (C_8, C_9, C_{11}, and C_{12}) have double bonds situated to allow for 5-*exo* radical cyclizations. The result of these cyclizations is the formation of a host of secondary products, such as monocyclic peroxides, serial cyclic peroxides, and isoprostanes (32, 37–40) (**Scheme 2**). In contrast, the exterior peroxyl radicals, C_5 and C_{15}, cannot undergo 5-*exo* radical cyclization and thus cannot form these secondary products. In the presence of a good hydrogen atom donor such as α-tocopherol, the primary hydroperoxides will be trapped. However, in the absence of a good hydrogen atom donor, the interior primary hydroperoxides will undergo further oxidation to form secondary products. This reduces the amount of hydroperoxides with substitution on C_8, C_9, C_{11}, and C_{12} relative to those on C_5 and C_{15}, because the exterior hydroperoxides cannot undergo further oxidation to form secondary products. **Figure 8** shows oxidations of commercial PAPC in the presence of either 0.1 or 0.025 equivalent PMC. As the amount of antioxidant is decreased, the amount of the 8-, 9-, 11-, and 12-hydroperoxides decreases, presumably because of the formation of secondary oxidation products. Similar to the comparison of the *cis,trans* and *trans,trans* products of cholesteryl linoleate, PLPC, and SLPC, the comparison of the exterior hydroperoxides vs. the interior hydroperoxides over the course of an LDL oxidation can provide information on the effect of α-tocopherol in the various regions of an LDL particle.

The 15/11-OOH ratio was monitored over the course of an LDL oxidation initiated with the radical initiator C-0. The phospholipid hydroperoxides generated at various time points during the course of oxidation were measured using LC-CIS-MS/MS with SRM to examine the conversion of each hydroperoxide to its Hock fragment. The signals produced in the mass spectrometer by each hydroperoxide were compared, as shown in **Fig. 9A**. Over the course of the oxidation, the signal of the interior hydroperoxide (C_{11}) decreased relative to the exterior hydroperoxide (C_{15}). As a result, the PAPC 15/11-OOH ratio increased as the oxidation progressed, even at early time points (0–3 h), when α-tocopherol was present. The SAPC



Scheme 2.

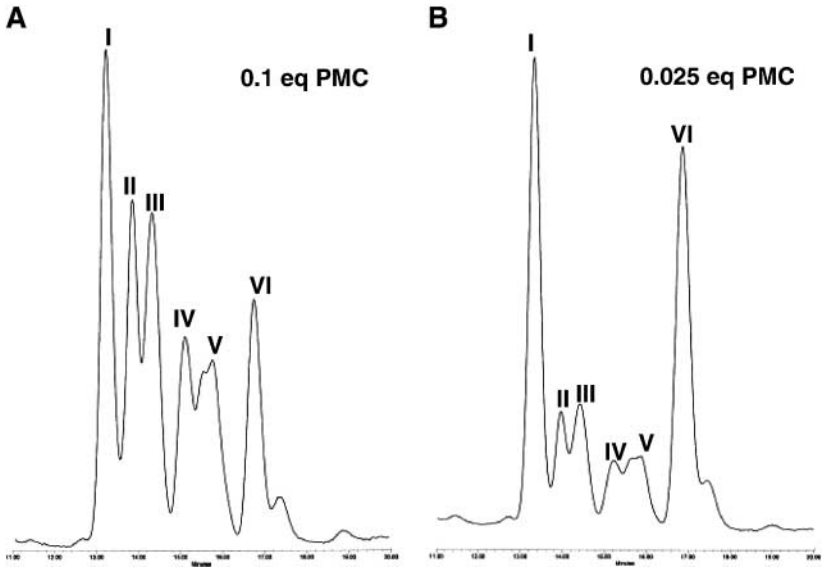


Fig. 8. Standard oxidation of PAPC in the presence of 0.1 equivalent (A) or 0.025 equivalent (B) of 2,2,5,7,8-pentamethyl-6-chromanol (PMC). The PAPC 15-OOH/PAPC 11-OOH ratios are 1.64 ± 0.17 (n = 3 and 4.69, respectively) (mobile phase, 95:5 methanol-water, v/v). HPETE, hydroperoxyeicosatetraenoic acid; PC, phosphocholine. I, 15-HPETE PC; II, 11-HPETE PC; III, 12-HPETE PC; IV, 8-HPETE PC; V, 9-HPETE; VI, 5-HPETE.

15/11-OOH ratio was also analyzed, and the results mirrored those for PAPC (data not shown).

In a separate LDL oxidation initiated with the free radical initiator AAPH, the cholesteryl arachidonate alcohol ratios were compared. Ch-11-HETE overlaps with the 13-*cis,trans*-OH of cholesteryl linoleate. Similarly, Ch-9-HETE overlaps with the 13-*cis,trans*-OH of cholesteryl linoleate. For these reasons, Ch-5-HETE and Ch-8-HETE were compared by HPLC with UV detection at 234 nm. The results are shown in Fig. 9B.[7] In contrast to the results shown for the PAPC-OOHs, the Ch-5-HETE/Ch-8-HETE ratio remained constant at 1.0–1.3 until the α-tocopherol disappeared. After this disappearance, the Ch-5-HETE/Ch-8-HETE ratio increased steadily over the remainder of the oxidation time course.

DISCUSSION

These studies for the first time quantify the formation of individual early-stage phospholipid oxidation products in in vitro free radical-initiated LDL oxidations. The 13-hydroperoxides of PLPC and SLPC as well as the 15-OOHs of PAPC and SAPC were shown to form in a time-dependent manner during the course of oxidation. These hydroperoxides were formed as early as 0.5 and 1 h, when a significant quantity of the antioxidant α-tocopherol was still present. The pathophysiological significance of these phosphocholine hydroperoxides is unknown. However, their formation could be significant because these compounds are highly reactive and unstable. These compounds could modify or adduct with nearby amino acid residues on the backbone of the LDL-associated protein apolipoprotein B-100 (41). In addition, the phospholipid hydroperoxides could themselves undergo either fragmentation or further oxidation to produce known biologically active species such as 4-hydroxynonenal (42) or a class of compounds identified by Podrez and coworkers (8) that contain a terminal γ-hydroxy(or oxo)-α,β-unsaturated carbonyl at the *sn*-2 position and have a high binding affinity for the macrophage scavenger receptor CD36.

Alcohols of PLPC, SLPC, PAPC, and SAPC were also found to form during the course of oxidation, and those from PLPC and SLPC were quantified. The oxidation time course of the phospholipid alcohols was different from that of the corresponding hydroperoxides. At the earliest time points, no alcohols were detected. Indeed, these products were not detected in the oxidation until 2 h, and their levels increased steadily during the remainder of the oxidation. This oxidation time course suggests that the PC-OHs are in all likelihood generated by the reduction of the PC-OOHs during the oxidation.

When Sattler, Christison, and Stocker (35) reported the formation of cholesteryl ester alcohols, they also suggested the presence of a hydroperoxide-reducing activity associated with isolated LDL. Stocker and colleagues (43) have also shown that ebselen, a mimic of the cellular enzyme phospholipid hydroperoxide glutathione peroxidase that is carried by the protein albumin in plasma, can reduce phospholipid and cholesteryl ester hydroperoxides that are associated with LDL. Ebselen uses low-molecular-weight thiols as its reducing agent. Thiols (-SH) are a

[7] To compare the PAPC and cholesteryl arachidonate hydroperoxide analyses on the same time scale, the cholesteryl arachidonate data were corrected based on the rate of decomposition of AAPH (1.8×10^{-6}) relative to C-0 (7.1×10^{-6} s^{-1}).

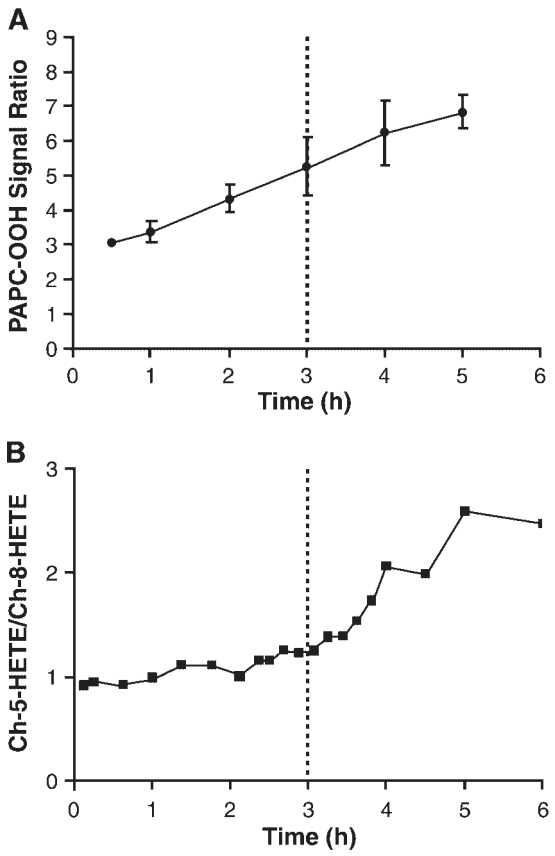


Fig. 9. Comparison of PAPC and cholesteryl arachidonate product ratios. A: Ratios of PAPC 15-OOH and 11-OOH products in a free radical autoxidation of LDL (0.75 mg protein/ml) initiated by 1 mM C-0 at 37°C. Hydroperoxides were analyzed by silver CIS-MS/MS operating in the selected reaction monitoring mode. The conversion of each hydroperoxide to its corresponding Hock fragment was monitored. Each peak was integrated, and the hydroperoxide signal ratios were plotted. Error bars reflect standard deviations where n = 4–5. The vertical dotted line shows the time in the oxidation at which α-tocopherol disappears from the oxidation. B: Ratio of cholesteryl-5-HETE and cholesteryl-8-HETE products formed in a free radical autoxidation of LDL (0.75 mg protein/ml) initiated by 0.5 mM 2,2′-azobis(amidinopropane) dihydrochloride at 37°C. The vertical dotted line shows the time in the oxidation at which α-tocopherol can no longer be detected.

common chemical entity found in vivo that are known to reduce lipid hydroperoxides to alcohols. Garner and coworkers (44) reported that methionine residues of apolipoproteins A-I and A-II play a significant role in the reduction of lipid hydroperoxides in HDL. Furthermore, Burlet and coworkers (45) reported that cysteine and methionine residues in apolipoprotein B-100 can be oxidized during LDL oxidations. Interestingly, most of the cysteine residues in the LDL protein are located in the phospholipid region of the particles (46 and references therein). Taken together, these results suggest that cysteine and methionine residues in apolipoprotein B-100 could be involved in the generation of the phospholipid alcohols detected in the experiments reported here.

The quantitation studies also revealed differences in oxidation in the phospholipid region of LDL and the cholesteryl ester region of LDL. The most striking difference was the extent to which these two lipid classes are oxidized. PLPC was ~9% oxidized under our conditions, whereas cholesteryl linoleate was only ~3% oxidized. This suggests that α-tocopherol, the primary antioxidant in LDL that affects lipid peroxidation, is not as effective at preventing oxidation on the LDL surface as it is in the LDL hydrophobic core.

To gain further insight into how α-tocopherol affects LDL oxidation, the formation of the *cis,trans* and *trans,trans* oxidation products of the linoleate-containing lipids in each class was measured and the oxidation of the arachidonate-containing lipids from each class was compared.

The difference in the oxidation of the linoleate-containing PCs, PLPC and SLPC, and cholesteryl linoleate was striking (Fig. 7). At time points before 3 h, when α-tocopherol was still present in the LDL oxidation, the major oxidation products observed for cholesteryl linoleate were the kinetically favored *cis,trans* alcohols. As expected, at later time points in the oxidation, when α-tocopherol was depleted, the *cis,trans/trans,trans* ratio decreased, indicating the formation of the thermodynamically favored *trans,trans* products. These results are consistent with previously reported studies (34) and suggest that when present, α-tocopherol effectively traps peroxyl radicals formed in the LDL core. In contrast, α-tocopherol does not seem to trap peroxyl radicals formed in the phospholipids on the surface layer of LDL as effectively. During the oxidation of linoleate-containing PCs, β-fragmentation reactions seem competitive to tocopherol trapping, because even at time points as early as 1 h, significant amounts of *trans,trans* products were formed in large amounts compared with the *cis,trans* products.

The oxidation profile obtained from arachidonate PCs (PAPC in particular) in LDL also suggests that α-tocopherol is not as effective an antioxidant in the phospholipid layer of LDL as it is in the cholesteryl ester-rich LDL core. The ratios of the primary oxidation products of PAPC and those of cholesteryl arachidonate were compared over the course of an LDL oxidation. It was found that the ratio of PAPC 15-OOH/PAPC 11-OOH increased as a function of time (Fig. 9A). The increase in this ratio results from a depletion of the 11-OHH of PAPC relative to the 15-OHH, presumably because the 11-OHH undergoes further oxidation to form the various cyclic peroxides described above. Interestingly, even at 30 min, the PAPC 15-OOH/PAPC 11-OOH ratio was 3.07 ± 0.10 (n = 5), which is nearly twice the ratio for a standard oxidation of PAPC in the presence of a good hydrogen atom donor (1.64 ± 0.17; n = 3). More importantly, this ratio increased even at early time points, while α-tocopherol was present, suggesting that α-tocopherol is not an effective antioxidant in the phospholipid layer. In contrast, for the oxidation products from cholesteryl arachidonate, the Ch-5-HETE/Ch-8-HETE ratio did not increase substantially until after α-tocopherol disappeared at 12 h (Fig. 9B). This is further evidence that α-tocopherol behaves as an antioxidant in the cholesteryl ester core but is not as effective an antioxidant on the surface layer of LDL.

Together, the findings reported here suggest that in LDL particles, α-tocopherol is a better antioxidant for the cholesteryl esters than it is for the phospholipids. These findings may have relevance to questions in biology and medicine. Since the oxidative theory of atherosclerosis was introduced more than a decade ago (1), α-tocopherol (or vitamin E) has been the focus of much research and many clinical trials because it is a good antioxidant in in vitro lipid environments and it is the most abundant antioxidant in LDL. For this reason, it was thought that supplementing the diet with high levels of vitamin E would prevent or reduce the oxidation of LDL in vivo and thus attenuate atherogenic events caused by LDL oxidation. However, many clinical trials have shown that supplementation with vitamin E has little effect on the progression of cardiovascular disease or cardiovascular event risk. The results of this study suggest that vitamin E may not have an effect on atherosclerosis because it does not effectively inhibit surface lipid peroxidation of the LDL particle. It is of some interest that dietary supplementation with both vitamin E and a water-soluble antioxidant, such as vitamin C, has shown promise in the attenuation of atherogenic events (47).

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REFERENCES

1. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
2. Yla-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. Butler, J. L. Witztum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified LDL in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84:** 1086–1095.
3. Palinski, W., S. Horkko, E. Miller, U. P. Steinbrecher, H. C. Powell, L. K. Curtiss, and J. L. Witztum. 1996. Cloning of monoclonal antibodies to epitopes of oxidized lipoproteins from apo-E-deficient mice. Demonstration of epitopes of oxidized LDL in human plasma. *J. Clin. Invest.* **98:** 800–814.
4. Itabe, H., H. Yamamoto, T. Imanaka, K. Shimamura, H. Uchiyama, J. Kimura, T. Sanaka, Y. Hata, and T. Takano. 1996. Sensitive detection of oxidatively modified LDL using a monoclonal antibody. *J. Lipid Res.* **37:** 45–53.
5. Holvoet, P., G. Theilmeier, B. Shivalkar, W. Flameng, and D. Collen. 1998. LDL hypercholesterolemia is associated with accumulation of oxidized LDL, atherosclerotic plaque growth, and compensatory vessel enlargement in coronary arteries of minature pigs. *Arterioscler. Thromb. Vasc. Biol.* **18:** 415–422.
6. Esterbauer, H., M. Dieber-Rotheneder, G. Waeg, G. Striegl, and G. Jurgens. 1990. Biochemical, structural, and functional properties of oxidized low-density lipoprotein. *Chem. Res. Toxicol.* **3:** 77–92.
7. Watson, A. D., G. Subbanagounder, D. S. Welsbie, K. F. Faull, M. Navab, M. E. Jung, A. M. Fogelman, and J. A. Berliner. 1999. Structural identification of a novel pro-inflammatory epoxyisoprostane phospholipid in mildly oxidized LDL. *J. Biol. Chem.* **274:** 24787–24798.
8. Podrez, E. A., E. Poliakov, Z. Shen, R. Zhang, Y. Deng, M. Sun, P. J. Finton, L. Shan, B. Gugiu, P. L. Fox, H. F. Hoff, R. G. Salomon, and S. L. Hazen. 2002. Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. *J. Biol. Chem.* **277:** 38503–38516.
9. Podrez, E. A., E. Poliakov, Z. Shen, R. Zhang, Y. Deng, M. Sun, P. J. Finton, L. Shan, M. Febbraio, D. P. Hajjar, R. L. Silverstein, H. F. Hoff, R. G. Salomon, and S. L. Hazen. 2002. A novel family of atherogenic oxidized phospholipids promotes macrophage foam cell formation via the scavenger receptor CD36 and is enriched in atherosclerotic lesions. *J. Biol. Chem.* **277:** 38517–38523.
10. Marathe, G. K., S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 2001. Oxidized LDL contains inflammatory PAF-like phospholipids. *Trends Cardiovasc. Med.* **11:** 139–142.
11. Berliner, J. A., G. Subbanagounder, N. Leitinger, A. D. Watson, and D. Vora. 2001. Evidence for a role of phospholipid oxidation products in atherogenesis. *Trends Cardiovasc. Med.* **11:** 142–147.
12. Watson, A. D., N. Leitinger, M. Navab, K. F. Faull, S. Horkko, J. L. Witztum, W. Palinski, D. Schwenke, R. G. Salomon, W. Sha, G. Subbanagounder, A. M. Fogelman, and J. A. Berliner. 1997. Structural identification by MS of oxidized phospholipids in minimally oxidized LDL that induce monocyte/endothelial interactions and evidence for their presence in vivo. *J. Biol. Chem.* **272:** 13597–13607.
13. Leitinger, N., T. R. Tyner, L. Oslund, C. Rizza, G. Subbanagounder, H. Lee, P. T. Shih, N. Mackman, G. Tigyi, M. C. Territo, J. A. Berliner, and D. Vora. 1999. Structurally similar oxidized phospholipids differentially regulate endothelial binding of monocytes and neutrophils. *Proc. Natl. Acad. Sci. USA.* **96:** 12010–12015.
14. Marathe, G. K., S. S. Davies, K. A. Harrison, A. R. Silva, R. C. Murphy, H. Casro-Faria-Neto, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1999. Inflammatory PAF-like phospholipids in oxidized LDL are fragmented alkyl phosphatidylcholines. *J. Biol. Chem.* **274:** 28395–28404.
15. Schlame, M., R. Haupt, I. Wiswedel, W. J. Kox, and B. Rustow. 1996. Identification of short-chain oxidized phosphatidylcholine in human plasma. *J. Lipid Res.* **37:** 2608–2615.
16. Itabe, H. 1998. Oxidized phospholipids as a new landmark in atherosclerosis. *Prog. Lipid Res.* **37:** 181–207.
17. Morrow, J. D., J. A. Awad, H. J. Boss, I. A. Blair, and I. L. J. Roberts. 1992. Non-cyclooxygenase-derived prostanoids (F_2-isoprostanes) are formed in situ on phospholipids. *Proc. Natl. Acad. Sci. USA.* **89:** 10721–10725.
18. Horkko, S., D. A. Bird, E. Miller, H. Itabe, N. Leitinger, G. Subbanagounder, J. A. Berliner, P. Friedman, E. A. Dennis, L. K. Curtiss, W. Palinski, and J. L. Witztum. 1999. Monoclonal antibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized LDL. *J. Clin. Invest.* **103:** 117–128.
19. Noguchi, N., N. Gotoh, and E. Niki. 1993. Dynamics of the oxidation of low density lipoprotein induced by free radicals. *Biochim. Biophys. Acta.* **1168:** 348–357.
20. Stocker, R., V. W. Bowry, and B. Frei. 1991. Ubiquinol-10 protects human LDL more efficiently against lipid peroxidation than does alpha-tocopherol. *Proc. Natl. Acad. Sci. USA.* **88:** 1646–1650.
21. Mohr, D., and R. Stocker. 1994. Radical-mediated oxidation of isolated human VLDL. *Arterioscler. Thromb.* **14:** 1186–1192.
22. Sattler, W., D. Mohr, and R. Stocker. 1994. Rapid isolation of lipoproteins and assessment of their peroxidation by HPLC postcolumn chemiluminescence. *Methods Enzymol.* **233:** 469–489.
23. Yamamoto, Y., and B. N. Ames. 1987. Detection of lipid hydroperoxides and hydrogen peroxide at picomole levels by an HPLC and isoluminol chemiluminescence assay. *Free Radic. Biol. Med.* **3:** 359–361.
24. Yamamoto, Y., M. H. Brodsky, J. C. Baker, and B. N. Ames. 1987. Detection and characterization of lipid hydroperoxides at picomole levels by HPLC. *Anal. Biochem.* **160:** 7–13.
25. Milne, G. L., and N. A. Porter. 2001. Separation and identification of phospholipid peroxidation products. *Lipids.* **36:** 1265–1275.
26. Chan, H. W-S., and G. Levett. 1977. Autoxidation of methyl linoleate. Separation and analysis of isomeric mixtures of methyl linoleate hydroperoxides and methyl hydroxylinoleates. *Lipids.* **12:** 99–104.
27. Porter, N. A., J. Logan, and V. Kontoyiannidou. 1979. The preparation and purification of arachidonic acid hydroperoxides of biological importance. *J. Org. Chem.* **44:** 3177–3181.
28. Culbertson, S. M., F. Antunes, C. M. Havrilla, G. L. Milne, and N. A. Porter. 2002. Determination of the alpha-tocopherol inhibition rate constant for peroxidation in low-density lipoprotein. *Chem. Res. Toxicol.* **15:** 870–876.

29. Lowry, T. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
30. Morton, R. E., and T. A. Evans. 1992. Modification of the bicinchoninic acid protein assay to eliminate lipid interference in determining lipoprotein protein content. *Anal. Biochem.* **204:** 332–334.
31. Hock, H., and O. Schrader. 1936. Der Mechanismus der autoxydation einfacher Kohlenwasserstoffe als beitrag zur Kentnis der Autoxydation von brennstoffen. *Angew. Chem.* **49:** 595–596.
32. Havrilla, C. M., D. L. Hachey, and N. A. Porter. 2000. Coordination (Ag^+) ion spray-mass spectrometry of peroxidation products of cholesteryl linoleate and cholesteryl arachidonate: HPLC-MS analysis of peroxide products from polyunsaturated lipid autoxidation. *J. Am. Chem. Soc.* **122:** 8042–8055.
33. Bowry, V. W., and R. Stocker. 1993. Tocopherol-mediated peroxidation. The prooxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. *J. Am. Chem. Soc.* **113:** 6029–6044.
34. O'Connor, D. E., E. D. Mihelich, and M. C. Coleman. 1984. Stereochemical course of the autoxidative cyclization of lipid hydroperoxides to prostaglandin-like bicyclo endoperoxides. *J. Am. Chem. Soc.* **106:** 3577–3584.
35. Sattler, W., J. Christison, and R. Stocker. 1995. Cholesteryl ester hydroperoxide reducing activity associated with isolated HDL and LDL. *Free Radic. Biol. Med.* **18:** 421–429.
36. Kenar, J. A., C. M. Havrilla, N. A. Porter, J. R. Guyton, S. A. Brown, K. F. Klemp, and E. Selinger. 1996. Identification and quantification of the regioisomeric cholesteryl linoleate hydroperoxides in oxidized human LDL and HDL. *Chem. Res. Toxicol.* **9:** 737–744.
37. Porter, N. A., M. O. Funk, D. Gilmore, R. Isaac, and J. Nixon. 1976. The formation of cyclic peroxides from unsaturated hydroperoxides: models for prostaglandin biosynthesis. *J. Am. Chem. Soc.* **98:** 6000–6005.
38. Porter, N. A. 1986. Mechanisms for the autoxidation of polyunsaturated lipids. *Acc. Chem. Res.* **19:** 262–268.
39. Porter, N. A., S. E. Caldwell, and K. A. Mills. 1995. Mechanisms of free radical oxidation of unsaturated lipids. *Lipids.* **30:** 277–290.
40. Yin, H., C. M. Havrilla, J. D. Morrow, and N. A. Porter. 2002. Formation of isoprostane bicyclic endoperoxides from the autoxidation of cholesteryl arachidonate. *J. Am. Chem. Soc.* **124:** 7745–7754.
41. Kawai, Y., H. Fujii, Y. Kato, M. Kodama, M. Naito, K. Uchida, and T. Osawa. 2004. Esterified lipid hydroperoxide-derived modification of protein: formation of a carboxyalkylamide-type lysine adduct in human atherosclerotic lesions. *Biochem. Biophys. Res. Commun.* **313:** 271–276.
42. Schneider, C., K. A. Tallman, N. A. Porter, and A. R. Brash. 2001. Two distinct pathways of formation of 4-hydroxynonenal. Mechanisms of nonenzymatic transformation of the 9- and 13-hydroperoxides of linoleic acid to 4-hydroxyalkenals. *J. Biol. Chem.* **276:** 20831–20838. [Erratum. 2001. *J. Biol. Chem.* **276:** 32392.]
43. Sattler, W., M. Maiorino, and R. Stocker. 1994. Reduction of HDL- and LDL-associated cholesteryl ester and phospholipid hydroperoxides by phospholipid hydroperoxide glutathione peroxidase and ebselen (PZ 51). *Arch. Biochem. Biophys.* **309:** 214–221.
44. Garner, B., R. Waldeck, P. K. Witting, K-A. Rye, and R. Stocker. 1998. Oxidation of HDL. II. Evidence for direct reduction of lipid hydroperoxides by methionine residues of apolipoproteins AI and AII. *J. Biol. Chem.* **273:** 6088–6095.
45. Burlet, O., C. Y. Yang, J. R. Guyton, and S. J. Gaskell. 1995. Tandem mass spectrometric characterization of a specific cysteic acid residue in oxidized human apoprotein B-100. *J. Am. Soc. Mass Spectrom.* **6:** 242–247.
46. Yang, C. Y., and Z. W. Gu. 1994. Primary structure of apo B-100. *Chem. Phys. Lipids.* **67–68:** 99–104.
47. Upston, J. M., L. Kritharides, and R. Stocker. 2003. The role of vitamin E in atherosclerosis. *Prog. Lipid Res.* **42:** 405–422.