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Critical Insights into Cardiovascular Disease from Basic Research on the Oxidation of Phospholipids: the γ -Hydroxyalkenal Phospholipid Hypothesis

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Abstract

Basic research, exploring the hypothesis that γ -hydroxyalkenal phospholipids are generated *in vivo* through oxidative cleavage of polyunsaturated phospholipids, is delivering a bonanza of molecular mechanistic insights into cardiovascular disease. Rather than targeting a specific pathology, these studies were predicated on the presumption that a fundamental understanding of lipid oxidation is likely to provide critical insights into disease processes. This investigational approach – from the chemistry of biomolecules to disease phenotype – that complements the more common opposite paradigm, is proving remarkably productive.

Keywords

Oxidative injury; oxidized phospholipids; biomolecular chemistry; cardiovascular disease; inflammation; scavenger receptors CD36 and B1; rod photoreceptor cells; foam cells; platelets; endothelial cells; macrophages; hepatocytes

INTRODUCTION

The premise that a fundamental understanding of phospholipid oxidation^{1–11} is likely to provide critical insights into disease processes, e.g., atherogenesis^{12–15}, led us to predict and explore the possibility that γ -hydroxyalkenal phospholipids are generated *in vivo*. The resulting studies have now delivered a bonanza of critical molecular mechanistic insights into the pathogenesis of cardiovascular disease. A key feature of those studies is chemical synthesis of pure samples of putative natural products¹⁶ that facilitated the development of analytical methods for detecting and quantifying their natural occurrence, exploring their biologically important chemistry, and assessing their biological activities. This investigational approach – from biomolecular chemistry to disease phenotype – that complements the more common opposite paradigm, is proving to be remarkably productive. This review focuses on γ -hydroxyalkenal phospholipids, especially their contributions to the pathogenesis of cardiovascular disease. A companion paper describes how the covalent modification of proteins by γ -hydroxyalkenal phospholipids, to generate carboxyalkyl pyrrole derivatives, contributes to age-related macular degeneration, autism, cancer, and wound healing.¹⁷

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γ -HYDROXYALKENAL PHOSPHOLIPIDS: FROM HYPOTHESIS TO DETECTION OF PROTEIN ADDUCTED DERIVATIVES IN VIVO

The γ -hydroxyalkenal phospholipid hypothesis

Oxidative cleavage of bond “a” of 1-palmityl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PA-PC) (Fig. 1) generates 1-palmityl-2-(ω -oxovaleryl)-*sn*-glycero-3-phosphocholine (OV-PC)¹⁸ that is oxidized further to deliver 1-palmityl-2-glutaryl-*sn*-glycero-3-phosphocholine (G-PC). We prepared authentic samples of OV-PC and G-PC by chemical syntheses to facilitate studies demonstrating their biological activities that include promoting adhesion of monocytes to vascular endothelial cells resulting in extravasation into the subendothelial space, an early event in the development of atherosclerosis (*vide infra*).¹⁹ OV-PC and G-PC are components of a complex mixture of oxidized phospholipids present in oxidatively damaged low-density lipoprotein (oxLDL). Another component of the mixture generated by oxidative cleavage of PA-PC exhibited an exact mass m/z = 650. Rather than attempting to isolate and characterize this oxidized phospholipid component of oxLDL, we opted to predict a likely candidate. By analogy with the well known production of the γ -hydroxyalkenal 4-hydroxy-2-nonenal (HNE)²⁰ through free radical-induced oxidative cleavage of bond “c” of PA-PC, we postulated that cleavage of bond “b” would generate a γ -hydroxyalkenal phospholipid, 1-palmityl-2-(5-hydroxy-8-oxooc-6-enoyl)-*sn*-glycero-3-phosphocholine (HOOA-PC) that has an exact mass m/z = 650. Although free HOOA-PC would eventually be detected *in vivo*, we decided to test the γ -hydroxyalkenal phospholipid hypothesis through experiments that used antibodies, raised against their anticipated protein adducts, to secure the first evidence supporting their generation *in vivo*.

Immunological detection of cardiovascular disease-related levels of γ -hydroxyalkenal phospholipid-derived protein adducts *in vivo*

Previously, we found that HNE forms covalent adducts that incorporate the ϵ -amino group of protein lysyl residues in pentylpyrrole protein modifications (PP-protein in Fig. 2). Analogous reactions of γ -hydroxyalkenal phospholipids, followed by lipolysis of intermediate phospholipid adducts, were expected to produce ω -carboxyalkylpyrrole modifications of proteins. Enzyme-linked immunosorbent assays, using antibodies raised against carboxyheptyl pyrrole (CHP) and carboxypropyl pyrrole (CPP) protein modifications (Fig. 2), revealed the presence of CHPs and CPPs in oxLDL²². Since 1-palmityl-2-linoleyl-*sn*-glycero-3-phosphocholine (PL-PC) is the most abundant polyunsaturated phospholipid in LDL, we also measured CHP immunoreactivity in human blood plasma. The mean level (\pm SD) of this protein adduct of HODA in the plasma from patients with end-stage renal disease (209 ± 32 pmol/mL, n = 16) is significantly elevated (P < 0.002) compared to healthy volunteers (169 ± 28 pmol/mL, n = 12). The mean level in the plasma of atherosclerosis patients (209 ± 19 pmol/mL, n = 13) is also significantly elevated (P < 0.003), reminiscent the elevated levels of other lipid-derived oxidative protein modifications associated with atherosclerosis.²³ These observations supported the conclusion that HODA-protein adducts are produced *in vivo* through covalent addition of HODA-PC, a component of the mixture of oxidized lipids derived from PA-PC and referred to collectively as oxPA-PC. The investigation of γ -hydroxyalkenal phospholipids and the ω -carboxyalkylpyrrole derivatives produced from their addition with proteins was not targeted at understanding the molecular basis of a particular disease. Nevertheless, as will be described in this review and a companion paper, it led to major insights into a vast array of pathological and physiological involvements of this biomolecular chemistry.

γ -HYDROXYALKENAL PHOSPHOLIPIDS: FROM SYNTHESIS TO DETECTION IN VIVO AND THE DISCOVERY OF BIOLOGICAL ACTIVITIES

LC-MS/MS characterization of biologically active oxidized lipids in complex mixtures

Free radical-induced oxidation of phospholipids generates a vast array of products. The classical approach for identifying molecular structures of biologically active natural products contained in complex mixtures exploits activity assays²⁴ to guide isolation. An alternative approach is to predict likely candidates by mechanistic speculation or analogy with known products of lipid oxidation and to use authentic samples of the putative natural products, prepared by unambiguous chemical synthesis, to guide their detection *in vivo*. However, the complexity of lipid extracts confounds isolation of analytically pure samples of oxidized lipids. LC-MS/MS analysis provides a workaround often allowing detection of individual species in complex mixtures without the need for isolation. The exquisite complexity of product mixtures generated by free radical-induced oxidation of lipids²⁵ complicates the application of this approach because mass spectra of isobaric compounds not only exhibit identical parent ions, but may also produce nearly identical fragmentation patterns and chromatographic retention times. Consequently LC-MS/MS comparisons, *even when pure authentic samples are available by chemical syntheses*, must include additional chemical tests to confirm the presumed structures of components of product mixtures generated by nonenzymatic oxidation of polyunsaturated fatty acid (PUFA) derivatives. A case in point is provided by two isomeric products generated by oxidative fragmentation of linoleic acid, the ketoaldehyde and an isobaric butenolide shown in Fig. 3. Their mass spectra are nearly identical, and both compounds exhibit identical HPLC retention times with a methanol/water solvent system. Using pure samples available by unambiguous chemical syntheses, we were able to define HPLC conditions that could distinguish them by using an acetonitrile/water solvent system. Furthermore, diagnostic derivatization clearly distinguishes the ketoaldehyde that reacts with methoxylamine (to form a bis methoxime) from the butenolide that does not.

HOOA-PC promotes monocyte entry into chronic lesions

We first executed a chemical synthesis of HOOA-PC (Figs. 1 and 2)²⁶ to facilitate its identification by LC-MS/MS comparison with components of oxPA-PC generated by the nonenzymatic oxidation of PA-PC and to enable biological testing. HOOA-PC exhibited proinflammatory activities²⁷, discovered previously for OV-PC and G-PC¹⁹, which can regulate leukocyte–endothelial interaction resulting in atherogenic extravasation of monocytes into the subendothelial space (Fig. 4a). Thus, HOOA-PC dose-dependently activates human aortic endothelial cells to bind monocytes and increases levels of monocyte chemotactic protein-1 and interleukin-8 (IL-8) – chemokines that are important in monocyte entry into chronic lesions. This suggested that HOOA-PC plays a role in chronic inflammation. In a model of bacterial infection, HOOA-PC also promotes the antiinflammatory inhibition of lipopolysaccharide (LPS)-induced expression of E-Selectin, a major adhesion molecule that mediates neutrophil-endothelial interactions.²⁷ Subsequently, HOOA-PC was also found in lipid extracts from oxLDL and human atheroma (*vide infra*).^{28, 29} Since elevated levels of myeloperoxidase (MPO) are associated with the presence of coronary artery disease³⁰, one likely scenario for atherogenesis involves MPO-initiated free radical-induced lipid oxidation (Fig 4a). The resulting oxPC promote extravasation of monocyte macrophages into the subendothelial space and endocytosis of oxLDL by the macrophages leading to their conversion into foam cells and, ultimately, atheroma formation.

The lipid whisker model for membrane phospholipids

Besides their utility as standards for identification and quantification of oxPC in biological samples, *the availability of pure individual oxPCs through chemical syntheses also enabled a study of their conformations in membranes.* Nuclear Overhauser effect experiments revealed the close proximity of oxidatively truncated sn-2 acyl chains with the choline head group.³¹ Oxidation resulting in the sn-2 acyl chain of phospholipids becoming shorter and richer in polar functional groups increases their hydrophilicity leading to their expulsion from the hydrophobic core of the membrane lipid bilayer (Figure 5). Thus, when cellular membranes are oxidatively damaged, truncated acyl chains sprout from the membrane like whiskers³². Molecular dynamics simulations provide support for this “lipid whisker model” for oxidatively damaged membranes.³³ The conformational change that results in sprouting of lipid whiskers may have several consequences (*vide infra*): (a) facilitating specific binding with receptors (Figure 6), e.g., macrophage or platelet CD36 and hepatocyte SR-BI; (b) inducing higher local positive membrane curvature owing to the presence of one rather than two lipophilic tails inserted into the membrane; (c) allowing interaction and transfer of oxPC, e.g., from oxLDL, to other lipoprotein particles through specific binding interactions, e.g., with small high-density lipoprotein (HDL) particles or peptide fragment analogues of the HDL protein Apo-A1.³⁴

γ -Hydroxyalkenyl phospholipids and their more oxidized derivatives are scavenger receptor CD36 ligands

The recognition of oxLDL by the scavenger receptor (SR) CD36 was known to trigger its endocytosis by macrophages. However, unlike the LDL receptor of macrophages that recognizes the protein component apoB100 of LDL, our collaborators at the Cleveland Clinic Foundation discovered that lipid components of oxLDL bind with CD36. To identify oxidized phosphatidylcholines (oxPCs) that bind with CD36, a simpler system, oxPA-PC was separated by HPLC to give three active fractions. Serendipitously, our chemical synthesis of HOOA-PC provided the key to rapid identification of the CD36 ligands in these mixtures. Thus, the identification of HOOA-PC as a component of the complex mixture of oxPCs in one of the CD36 active fractions was confirmed by the identity of its LC-MS/MS retention time and fragmentation pattern with that of a pure sample, prepared by unambiguous chemical synthesis, as well as those of derivatives generated by treatment of the CD36 active fraction with multiple agents (NaBH₄, NaBD₄, and NaCNBH₃) for reducing the aldehyde carbonyl; methylhydroxylamine and dinitrophenyhydrazone for generating a methoxime and hydrazone derivative and subsequent tandem MS analysis in both positive ion and negative ion modes.³⁵ Presuming that the CD36 ligands contained in the other two active fractions from oxPA-PC were derivatives of HOOA-PC, we then postulated that a less polar fraction contained the less polar derivative KOOA-PC (Fig. 7). Unambiguous chemical synthesis of KOOA-PC³⁶ followed by LC/ESI/MS/MS comparison with the less polar fraction and derivatives confirmed the presence of this oxPC in oxPA-PC. We similarly postulated and confirmed by chemical synthesis, derivatizations (including esterification with pentafluorobenzyl bromide) and LC/ESI/MS/MS comparisons that a more polar fraction contained a mixture of HOdiA-PC and KOdiA-PC.³⁶ The phospholipids in oxLA-PC, which are capable of binding with CD36, were similarly identified by chemical syntheses of likely candidates and derivatizations followed by LC/ESI/MS/MS comparisons and shown to be HODA-, KODA-, HDdiA-, and KDdiA-PC (Fig. 7). All of these oxPCs, which contain γ -oxygenated- α , β -unsaturated aldehyde or carboxylic acid functionality, referred to collectively as oxPC_{CD36} (Fig. 7), were confirmed to be especially potent inducers of CD36-mediated endocytosis of oxLDL by macrophage cells.

OxPC_{CD36} accumulate in atherosclerotic lesions and foster foam cell formation

LC/ESI/MS/MS analysis of lipid extracts from rabbit aortas confirmed the presence of each oxPC_{CD36} species *in vivo* and demonstrated 5–7 fold elevated levels of PA-PC-derived oxPC_{CD36} in atherosclerotic versus normal aortas. Identification of each species was based upon the detection of ions with mass to-charge (m/z) ratio identical to that of the parent lipid, which following collision-induced dissociation, subsequently also gave rise to a characteristic daughter ion and retention time determined by analysis of authentic synthetic oxPC species. While their presence in lipid extracts was established by LC-MS/MS comparisons with pure synthetic oxPCs and their characteristic derivatives, analytically pure samples have never been isolated from oxPA-PC, oxPL-PC, oxLDL, or human tissues. Therefore, *chemical syntheses of pure samples of each oxPC_{CD36} were absolutely essential for the unambiguous demonstration of their biological activities.* Addition of pure synthetic oxPC_{CD36} to cholesterol-containing particles promotes CD36-dependent macrophage binding (but not to macrophage cells from CD36 null mice), uptake, and metabolism of cholesterol esters resulting in accumulation of cholesterol and foam cell formation.²⁸ Notably, binding of oxPC_{CD36}-containing particles to CD36 increased with increasing mol % of ligand within the surface of a particle, consistent with enhancement of binding through multivalent, i.e., multiple receptor-ligand interactions. That only a few molecules per LDL particle are needed to confer recognition of oxPC_{CD36}, supports their physiological relevance. Analogously, oxPC_{CD36} promote endocytosis of oxidatively damaged photoreceptor rod cell outer segments (PhROS) by retinal pigmented epithelial (RPE) cells³⁷, a process that replaces the entire stack of photoreceptor disks within these cells every 10 days.

OxLDL is a Trojan horse that delivers toxic electrophilic γ -hydroxyalkenals into macrophage and RPE cells

OxPC_{CD36}-fostered endocytosis of toxic cargo within an oxLDL particle into CD36-expressing macrophages or RPE cells might lead to (1) lipolysis of oxysterol-containing esters that releases toxic oxysterols or (2) covalent adduction of electrophilic oxidized phospholipids or oxysterols to proteins that impairs protein function. Thus, oxPC_{CD36} may indirectly promote biological activities such as oxysterol-induced cytotoxicity³⁸. Covalent adduction of γ -hydroxyalkenal-phospholipids, e.g., HOOA-PC and HODA-PC, with a cysteine thiol in the lysosomal protease cathepsin B, reduces the proteolytic degradation by mouse peritoneal macrophages of macromolecules previously internalized by receptor mediated endocytosis or phagocytosis.²⁹

Processing of the oxidatively damaged protein may also be impaired by HODA-PC through interference with the fusion with lysosomes of endosomes containing oxLDL in macrophage cells. The analogous processing of PhROS by RPE cells is also perturbed by oxLDL.^{39–41} It seemed possible that toxic aldehydes present in oxLDL, such as HODA-PC, might inhibit the fusion of endosomes with lysosomes that is crucial for degradation of oxidatively damaged proteins in endocytosed PhROS. Rab5a is a fusion protein believed to be critical for phagosome and possibly endosome maturation through fusion with lysosomes.⁴² Indeed, HODA-PC blocked the posttranslational modification, i.e., isoprenylation and proteolytic cleavage, of inactive 25 kDa Rab5a within RPE cells into the active 23 kDa form required for phagosome-lysosome fusion in these phagocytes.⁴²

OxPC_{CD36} inhibit HDL binding with hepatocyte SR-B1 impeding delivery of cholesterol to the liver for excretion

The reverse cholesterol transport pathway is the main mechanism whereby HDL protects against the development of atherosclerosis. It results in the transfer of excess cholesterol from peripheral cells, e.g., macrophages in atherosclerotic plaques, to HDL, which

eventually transfers its cholesterol to the liver for excretion. Efflux of excess cholesterol from macrophages to HDL particles (Fig. 8) is followed by conversion of unesterified cholesterol on the particle surface to cholesteryl esters by lecithin:cholesterol acyltransferase (LCAT) and migration to the hydrophobic core. HDL then delivers cholesteryl esters, via the scavenger receptor SR-B1, to the liver where it can be converted into bile acids and excreted. The significant sequence homology and ligands – including HDL, oxLDL, and anionic phospholipids^{43–45} – between SR-CD36 and SR-B1 suggested that oxPC_{CD36} might serve as ligands for SR-B1 and thereby interfere with binding of HDL and reverse cholesterol transport. OxLDL, oxPA-PC, and individual pure oxPC_{CD36} exhibited saturable binding with human SR-B1.⁴⁶ Both HDL and oxPC_{CD36} bound with a purified GST-SR-B1 fusion protein containing the amino acid 144–205 fragment of the extracellular amino-terminal domain of human SR-B1. Importantly, the Kd value of the binding of HDL to this protein was very similar to that determined for small unilamellar vesicles containing oxPC_{CD36}. As expected because of the close proximity of the binding sites for these two ligands on SR-B1, oxLDL and individual pure oxPC_{CD36} inhibited binding of HDL with hepatocytes and almost completely inhibited the major physiological function of SR-B1, the selective uptake of cholesteryl esters from HDL by hepatocytes.⁴⁶ Thus, oxidative stress and accumulation of specific oxidized phospholipids in plasma may promote atherosclerosis not only by inducing uptake of oxLDL by macrophages via CD36 and interfering with lysosomal processing, but can also hamper reverse cholesterol transport by preventing SR-B1-mediated selective uptake of cholesteryl esters into hepatocytes contributing to the development of hypercholesterolemia.

OxPC_{CD36} induce a prothrombotic state through activation of platelet CD36

OxPC_{CD36} also accumulate in plasma of hyperlipidemic mice at concentrations up to 40-fold higher than those found in normolipidemic mice, and they are present in substantial amounts in human plasma at elevated levels in individuals with low HDL.⁴⁷ Through binding with platelet SR-CD36, these levels of oxPC_{CD36} activate platelets, as assessed by activation of platelet fibrinogen receptor integrin αIIβ3 as well as by an increase in P-selectin surface expression.⁴⁷ This primes or sensitizes the platelets for subsequent activation by the classical platelet agonist ADP. A mesenteric thrombosis model revealed that *in vivo* occlusion times are significantly shorter in hyperlipidemic mice than in wild-type mice on a Western diet, and functional deficiency of SR-CD36 protects mice from the hyperlipidemia-related prothrombotic phenotype. These observations suggest a role for oxPC_{CD36} in the pathophysiology of occlusive arterial thrombi associated with myocardial infarction and stroke. They provide a molecular mechanistic link between hyperlipidemia, oxidant stress and a prothrombotic phenotype. It seems reasonable to anticipate that platelet activation through SR-CD36 promotes thrombosis consequent to the rupture of an atherosclerotic plaque owing to the release of a bolus of oxPC_{CD36} into the circulation. In normolipidemic human plasma, oxPC_{CD36} levels are inversely correlated with levels of HDL, but not LDL. They are 2.5 times higher in plasma from subjects in the lowest HDL tertile. This is consistent with the anti-inflammatory and antioxidant effects of HDL⁴⁸, and suggests that it is an interaction between CD36 and oxidative stress, not merely dyslipidemia (or elevated LDL), that is responsible for enhanced platelet reactivity *in vivo*.

Oxidatively truncated ether PCs activate platelets through the PAF receptor

Platelet-activating factor (PAF), 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, is an ether phospholipid with potent, diverse physiological actions, particularly as a mediator of inflammation.⁴⁹ It exerts its effects through a single, highly specific G-protein-coupled receptor. As the most potent phospholipid agonist yet identified, PAF's biosynthesis is closely controlled. In contrast, PAF-like ether phospholipids with oxidatively truncated sn-2 acyl chains (oxPAFs) can be generated nonenzymatically under oxidative stress. We

predicted that oxPAFs shown in Fig 9A would be generated through the autoxidation of PUFA esters of lyso-PAF (LPAF). To facilitate the identification, quantification, and biological testing of these putative oxPAFs, we prepared pure samples by unambiguous chemical syntheses.⁵⁰ The electrospray mass spectrum of lipids extracted from oxLDL exhibited molecular ions corresponding to G-PAF, KOdiA-LPAF, KDdiA-LPAF, KODA-LPAF, and KOOA-LPAF.⁵⁰ The entire family of oxidatively truncated ether phospholipids depicted in Fig 9A is generated through oxidative cleavage of 2-lyso-PAF esters of LA, AA, and DHA in small unilamellar vesicles exposed to the biologically relevant myeloperoxidase (MPO)/H₂O₂/NO₂ system to initiate autoxidation.

In contrast with the sensitization of platelets toward activation through binding of oxPC with CD36, oxPAFs are direct platelet agonists that act through the PAF receptor. OxPAFs account for the activation of platelets upon treatment with low concentrations of oxLDL as indicated by surface expression of P-selectin and the production of highly spread aggregates. A large number of these oxPAFs at submicromolar concentrations induce a rapid increase in platelet cytoplasmic Ca²⁺ level (Fig. 9B), some with higher and some lower activity than 2-lysoPAF (LPAF), but the activity of all oxPAFs and oxLDL is completely suppressed by the PAF receptor antagonist WEB 2086.⁵⁰ Furthermore, the action profile is bell shaped, with concentrations of oxLDL from just under 1.5 µg/ml up to 5 µg/mL stimulating intracellular Ca²⁺ flux, but then activation is significantly reduced as the concentration of oxLDL increases above 7 µg/mL, perhaps owing to the presence of antiinflammatory agents in oxLDL.

α,β-Unsaturated carboxylic oxPEs and oxPCs inhibit LPS-induced expression of IL-8

OxLDL inhibits the acute inflammatory response elicited by LPS, a model of bacterial infection, as evidenced by the expression of IL-8 by endothelial cells. Since oxPA-PC and oxPA-PE both mimic this effect, the head group is apparently not important for activity. This presumption and the influence of the structure of the oxidatively truncated *sn*-2 acyl group on biological activity were tested with a variety of analytically pure oxPCs and oxidized phosphatidylethanolamines (oxPEs) available through unambiguous chemical syntheses. Thus, chemical syntheses⁵¹ of the oxPEs depicted in Fig. 10 enabled the subsequent demonstration of their natural occurrence by LC-MS/MS comparisons with lipids extracted from retina.⁵²

While all the oxPEs shown in Fig. 10 have some activity, those containing α,β-unsaturated carboxylic acids (KHdiA, KOdiA, KDdiA, and HDdiA) are much more potent inhibitors of the LPS induction of IL-8 than are those containing short-chain aldehydes or acids, i.e., OV and G.⁵³ In contrast, the latter are among the most active proinflammatory oxPEs, i.e., for fostering monocyte-endothelial interactions. A similar dichotomy is found for oxPCs. Thus oxidation products with different oxidatively truncated *sn*-2 acyl chains – as summarized in Fig. 10 – are most potent in regulating the pro- or anti-inflammatory effects of oxidized phospholipids.

The anti-inflammatory inhibition of LPS induction of IL-8 is apparently mediated, at least in part, by the neutral sphingomyelinase since oxPA-PC treatment activates this enzyme and increases levels of the product of its activity, ceramide. Furthermore, the inhibitory activity of oxPA-PC or KOdiA-PC is abrogated by a neutral sphingomyelinase inhibitor, and cell-permeant C6 ceramide inhibits LPS-induced IL-8 synthesis. These effects may reflect ceramide-induced alterations to lipid rafts and caveolae resulting in deficient assembly of the LPS receptor complex. The noteworthy conclusion is that certain oxPCs can foster modification of cell membrane structure, and thereby alter membrane protein function, e.g. the LPS receptor complex.

γ -HYDROXYALKENAL PHOSPHOLIPIDS: BIOLOGICALLY IMPORTANT CHEMISTRY

Membrane lipid composition profoundly influences the stability of γ -hydroxy- α,β -unsaturated aldehydic PCs

Since phospholipids reside in a membrane environment *in vivo*, membrane composition is likely to influence their biologically important chemistry. The physiologically relevant MPO/H₂O₂/NO₂ system can initiate autoxidation of PUFAS by generating •NO₂ (see Fig. 4) that abstracts doubly allylic hydrogen generating pentadienyl radicals, and these capture oxygen to deliver lipid peroxy (LOO•) and lipid alkoxy (LO•) radicals (Fig. 11). Since PUFA-derived LOO• or LO• can oxidize aldehydes and induce fragmentation of α,β -unsaturated aldehydes while •NO₂ cannot, MPO can only induce these oxidations and fragmentations in membranes that contain PUFAs. We found that PUFAs, e.g., LA-PC in Fig. 11, in a membrane promote fragmentation of the ether phospholipid HOHA-LPAF to OB-PAF and oxidation of HOHA-LPAF to HHdiA-LPAF and KHdiA-LPAF.⁵⁴ On the other hand, these reactions of HOHA-LPAF do not occur readily within membranes composed entirely of saturated diacyl-PCs. Thus, an abundance of PUFAs in a membrane promotes the removal of γ -hydroxy- α,β -unsaturated aldehydic phospholipids, such as HOHA esters, and thereby disfavors their reactions with proteins to produce biologically active adducts, e.g., carboxyalkylpyrroles (see Fig. 2 and the accompanying review¹⁷). However, this chemistry does not abolish recognition by SR-CD36 or SR-B1 because products from oxidation of γ -hydroxy- α,β -unsaturated aldehydic oxidized phospholipids, i.e., γ -oxygenated- α,β -unsaturated carboxylic phospholipids, such as HHdiA-PC and KHdiA-PC, retain high affinity for these receptors.

Spontaneous cyclodehydration of HODA-PCs abolishes recognition by the scavenger receptor CD36

γ -Hydroxyalkenyl phospholipids, i.e., HOHA-, HOOA-, and HODA-PCs, readily undergo another transformation, cyclodehydration, that generates furans, e.g., oxPC-furan(n) where n = the number of methylenes in the alkyl chain (Fig. 12).⁵⁵ This transformation also precludes the reactions of these oxPCs with proteins to produce biologically active adducts, e.g., carboxyalkylpyrroles (see Fig. 2 and the accompanying review), and abolishes recognition by the SR-CD36.

A caveat: lyso-PC is generated by spontaneous nonenzymatic deacylation of oxPCs

As noted above, *chemical syntheses of pure samples of each oxPC_{CD36} have been absolutely essential for the unambiguous demonstration of their biological activities*. Although the LC-MS/MS evidence for their presence in lipid mixtures isolated from biological samples is extensive, *analytically pure samples of these oxidatively truncated phospholipids have never been isolated from oxPA-PC, oxPL-PC, oxLDL, or human tissues*. High purity is crucial when evaluating biological activities because the activities observed could be caused by traces of other components of the original mixture. Furthermore, as noted above, γ -hydroxyalkenals readily undergo free radical-induced spontaneous oxidation to the corresponding γ -ketoalkenals, γ -hydroxyalkenoates, and γ -ketoalkenoates, as well as further oxidative truncation to produce saturated aldehydes, e.g., OB-, OV-, and ON-PC, and carboxylates, e.g., S-, G-, or A-PC, and spontaneous cyclization to oxPC-furans (summarized in Fig. 13).

Lyso-PC, lacking the *sn*-2 acyl group, is generated consequent to oxidation of LDL, accounting for nearly one-third⁵⁶ to one-half⁵⁷ of the PC equivalents in oxLDL. Elevated levels of lysoPC are linked to cardiovascular complications associated with diabetes,

atherosclerosis, and ischemia.^{58, 59} Until recently, it was presumed that lysoPC is produced under physiological conditions by PLA₂-mediated hydrolysis of PC⁶⁰ or from the hydrolysis of oxidized PC by PAF-acetylhydrolase.^{18, 61} However, we recently uncovered yet another process that converts HOHA-PC or HOOA-PC into 2-lyso-PC. Notably, this process involves a spontaneous, nonenzymatic intramolecular transesterification mechanism that generates lactone byproducts (Fig. 13). Under physiological conditions, i.e., 37 °C and pH 7.4 in aqueous solution, the half life for conversion of HOHA-PC into lyso-PC is only 30 min while that for conversion of HOOA-PC into lyso-PC is 2 h.⁶² Furthermore, spontaneous deacylation is not limited to oxPCs that incorporate a hydroxyl group on the γ - or δ -carbon adjacent to the ester functionality in their oxidized acyl moiety. Oxidatively truncated PCs that incorporate an aldehyde or ketone carbonyl on the γ - or δ -carbon adjacent to the ester functionality also spontaneously deacylate. Thus, KOHA-PC, which has a ketone carbonyl on the γ -carbon adjacent to the ester functionality, spontaneously deacylates with a similar $t_{1/2} \sim 40$ min as the hydroxy analogue HOHA-PC. Presumably, the enedione functional array in KOHA-PC is in rapid equilibrium with a hydrate that cyclizes to generate a hemiacetyl with concomitant release of lysoPC (Fig. 14). Therefore, it is absolutely essential to ascertain that biological activities attributed to oxPCs that incorporate a hydroxyl or carbonyl group on the γ or δ -carbon adjacent to the ester are not instead activities of lyso-PC. Aqueous solutions of these pure oxPCs, available by chemical syntheses, are especially valuable for the unambiguous demonstration of biological activities. However, such oxPCs are hydrolytically unstable. Their aqueous solutions must be freshly prepared and kept cold until use.

CONCLUSIONS

One approach to identify oxidized phospholipids formed *in vivo* is to predict likely candidates by mechanistic speculation or analogy with known products of lipid oxidation and to use authentic samples of the putative natural products, prepared by unambiguous chemical synthesis, to guide and confirm LC-MS/MS analyses of biological extracts. Using this approach the formation *in vivo* of families of diacyl oxPEs and oxPCs, as well as *sn*-1-alkyl-*sn*-2-acyl oxPCs containing truncated *sn*-2 acyl groups with γ -hydroxyalkenal or more oxidized functionality were established, and several pathways for their spontaneous decomposition, including the generation of 2-lyso-PC, were discovered. Their involvement as ligands (Fig. 13) for receptor-mediated endocytosis of oxLDL by macrophage cells and of oxidatively damaged rod photoreceptor cells by retinal pigmented endothelial cells, blockage of reverse cholesterol transport to the liver, sensitization or activation of platelets towards thrombosis were demonstrated, as were other involvements in chronic inflammation, and inhibition of normal processing of endocytosed oxLDL, e.g., through covalent protein modification that causes loss of function of proteases and proteins that enable phagosome and possibly endosome maturation through fusion with lysosomes. Covalent γ -hydroxyalkenal-PC-derived protein modifications that result in gain of function are the focus of the accompanying paper.¹⁷

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ABBREVIATIONS

CHP	carboxyheptyl pyrrole
CPP	carboxypropyl pyrrole
G-PC	1-palmityl-2-glutaryl- <i>sn</i> -glycero-3-phosphocholine
HDL	high-density lipoprotein
HNE	γ -hydroxyalkenal 4-hydroxy-2-nonenal
13-HODE-PC	13-hydroxyoctadeca-9,11-dienoyl phosphatidylcholine
HOOA-PC	1-palmityl-2-(5-hydroxy-8-oxo-6-enoyl)- <i>sn</i> -glycero-3-phosphocholine
IL-8	interleukin-8
KOdiA-PC	5-ketoct-6-endioyl phosphatidylcholine
LCAT	lecithin:cholesterol acyltransferase
LDL	low-density lipoprotein
LPAF	lyso-PAF (1-O-hexadecyl-2-lyso- <i>sn</i> -glycero-3-phosphocholine)
LPS	lipopolysaccharide
moxLDL	minimally oxidized low-density lipoprotein
MPO	myeloperoxidase
OV-PC	1-palmityl-2-(ω -oxovaleryl)- <i>sn</i> -glycero-3-phosphocholine
oxLDL	oxidized low-density lipoprotein
oxPC	oxidized phosphatidylcholine
oxPE	oxidized phosphatidylethanolamine
PAF	platelet-activating factor (1-O-hexadecyl-2-acetyl- <i>sn</i> -glycero-3-phosphocholine)
PA-PC	1-palmityl-2-arachidonoyl- <i>sn</i> -glycero-3-phosphocholine
PhROS	photoreceptor rod cell outer segments
PL-PC	1-palmityl-2-linoleyl- <i>sn</i> -glycero-3-phosphocholine
PUFA	polyunsaturated fatty acid
RPE	retinal pigmented epithelial
SR	scavenger receptor

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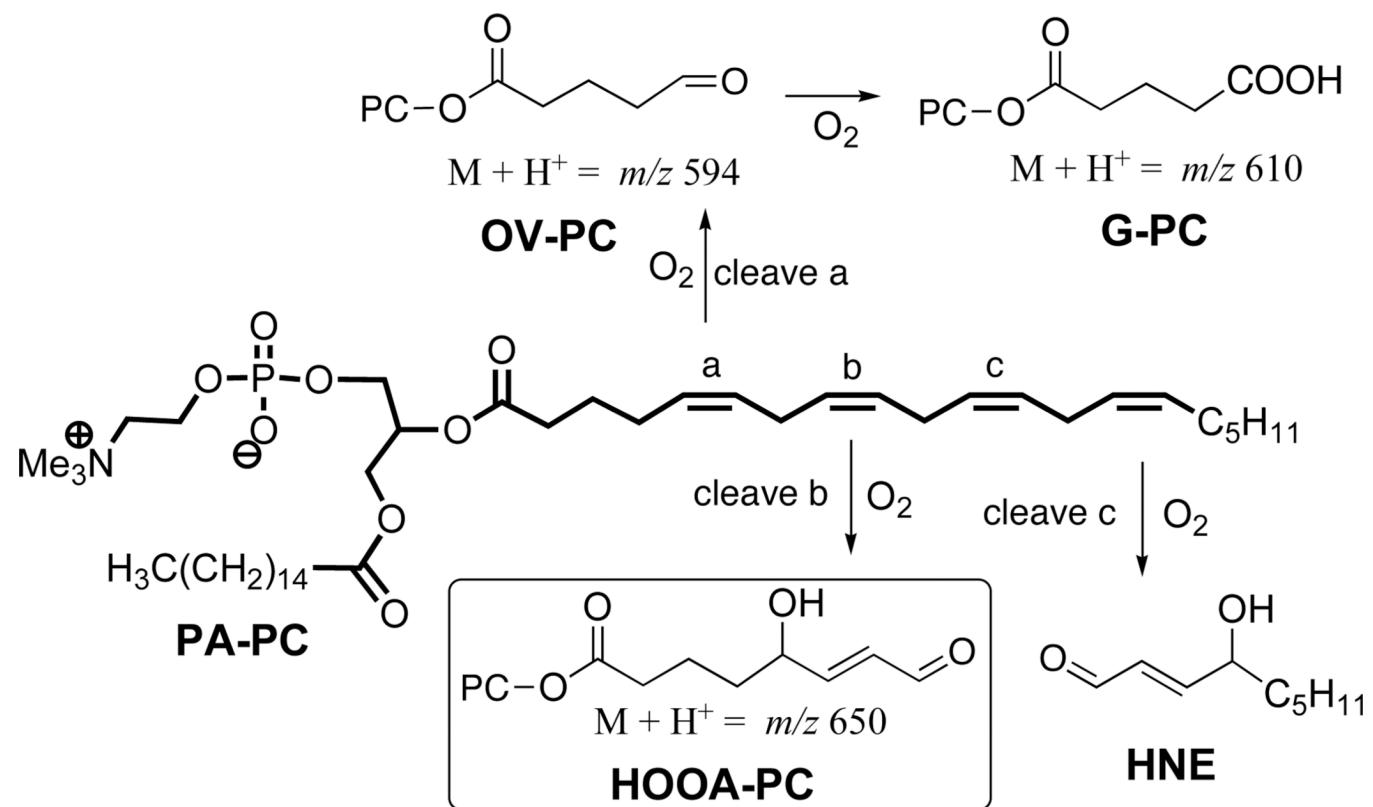
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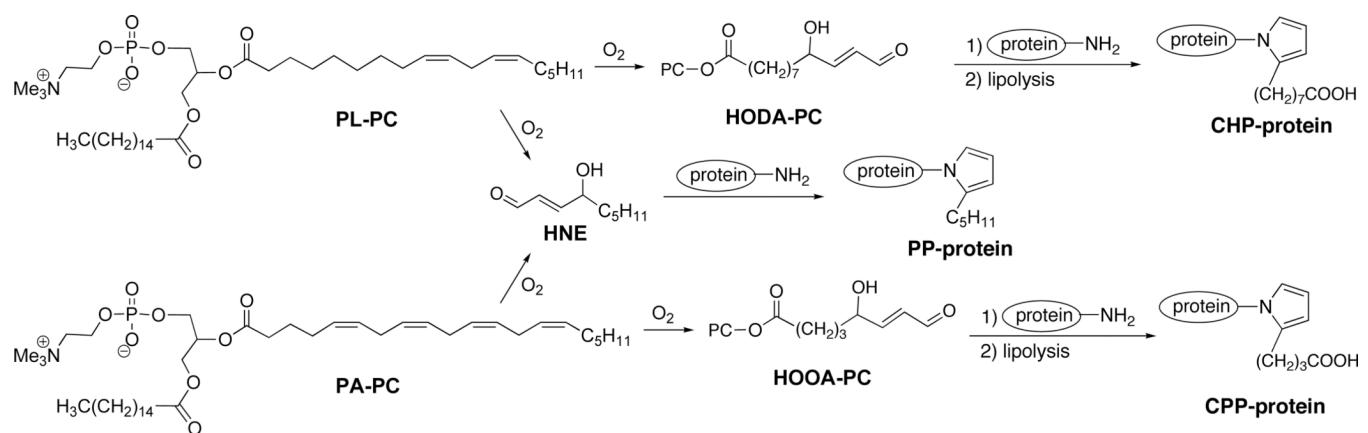
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**Figure 1.**

Free radical-induced oxidative cleavage of PA-PC to generate HOOA-PC, a γ -hydroxyalkenal phospholipid analogue of HNE.

**Figure 2.**

Covalent addition of γ -hydroxyaldehydes with proteins generates alkyl and ω -carboxyalkyl pyrrole modifications.

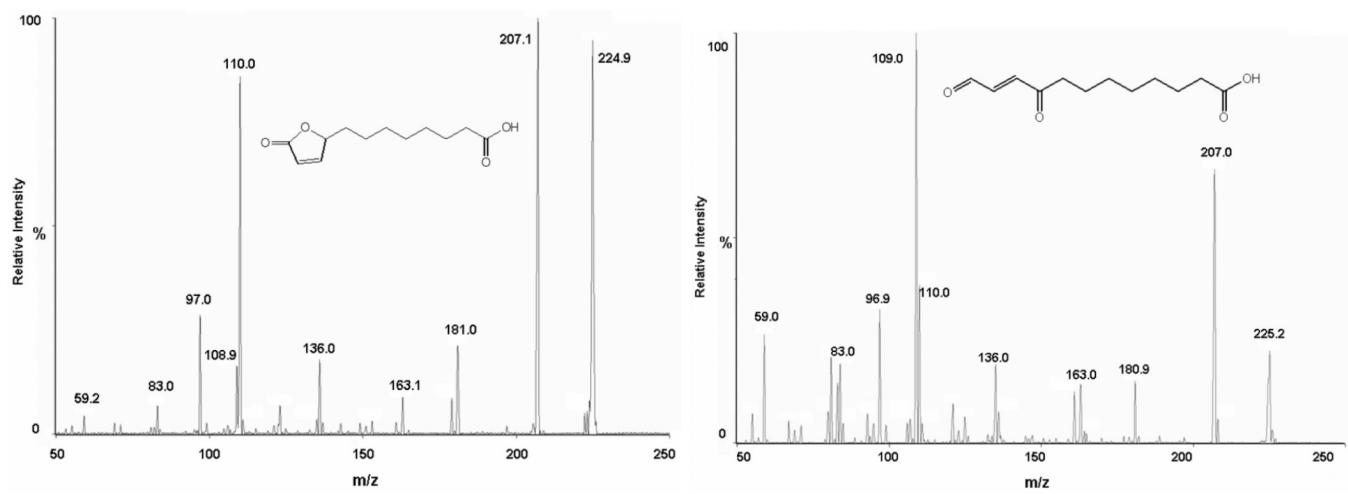
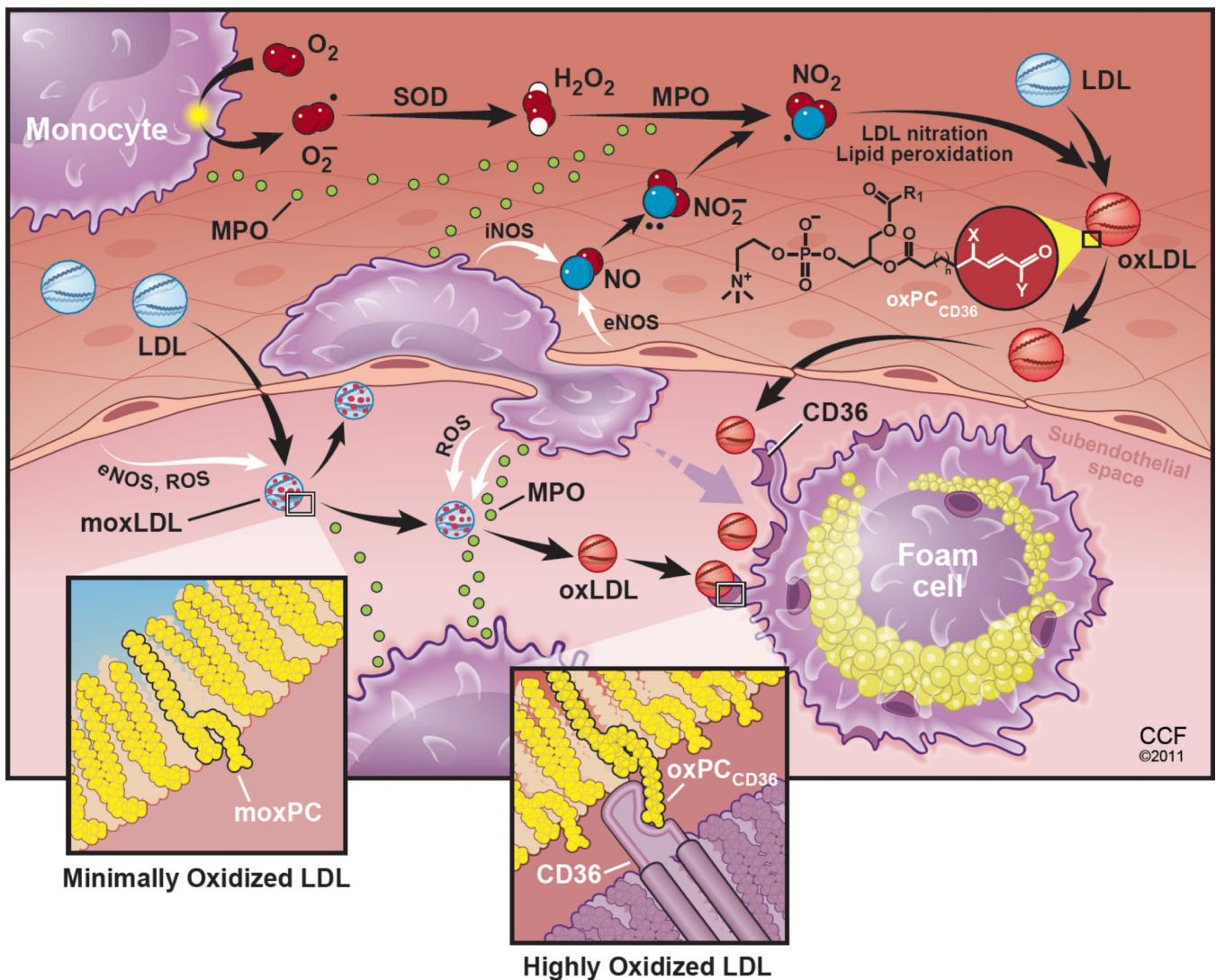


Figure 3.
Negative ion ESI-MS/MS of isomeric products from oxidative fragmentation of linoleate.

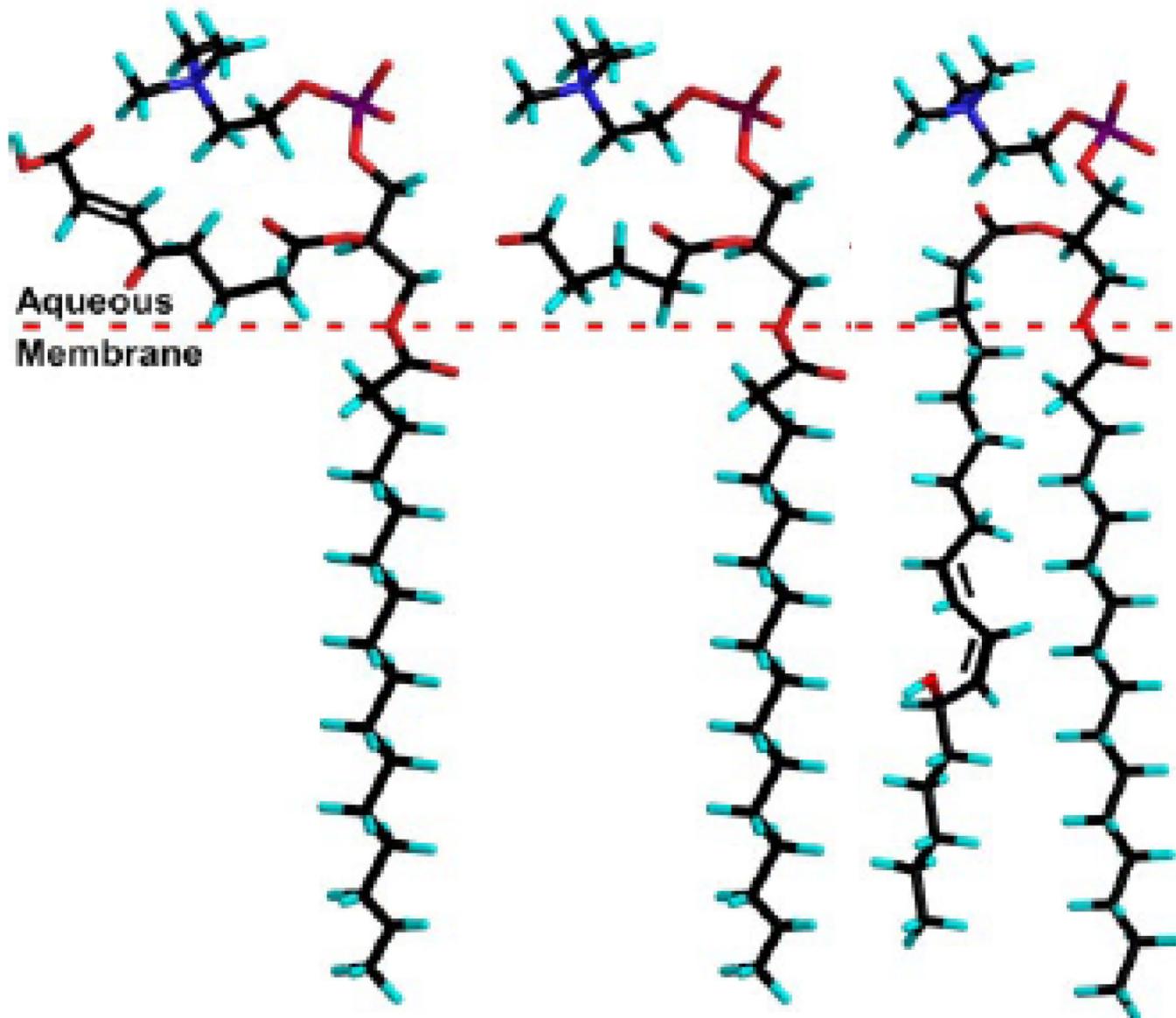
**Figure 4.**

Monocytes and endothelial cells contribute to one pathway leading to oxidation of low-density lipoprotein (LDL) by iNOS- or eNOS-promoted generation of NO that is converted into the reactive nitrogen radical $\bullet\text{NO}_2$ by myeloperoxidase (MPO)-catalyzed reaction with H_2O_2 . Low levels of oxidatively truncated phospholipids, present in minimally oxidized (mox)LDL, are sufficient to promote adhesion of monocytes to the vascular endothelium that facilitates extravasation into the subendothelial space where they become macrophages. There a family of γ -oxygenated- α,β -unsaturated aldehydic and carboxylic oxidatively truncated phospholipids ($\text{oxPC}_{\text{CD}36}$) – that protrude like whiskers from the outer shell of oxLDL – bind strongly with the scavenger receptor CD36, promoting endocytosis of oxLDL by the macrophages and leading to foam cell formation and atherosclerosis. Illustration by David Schumick, BS, CMI. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2011. All Rights Reserved.

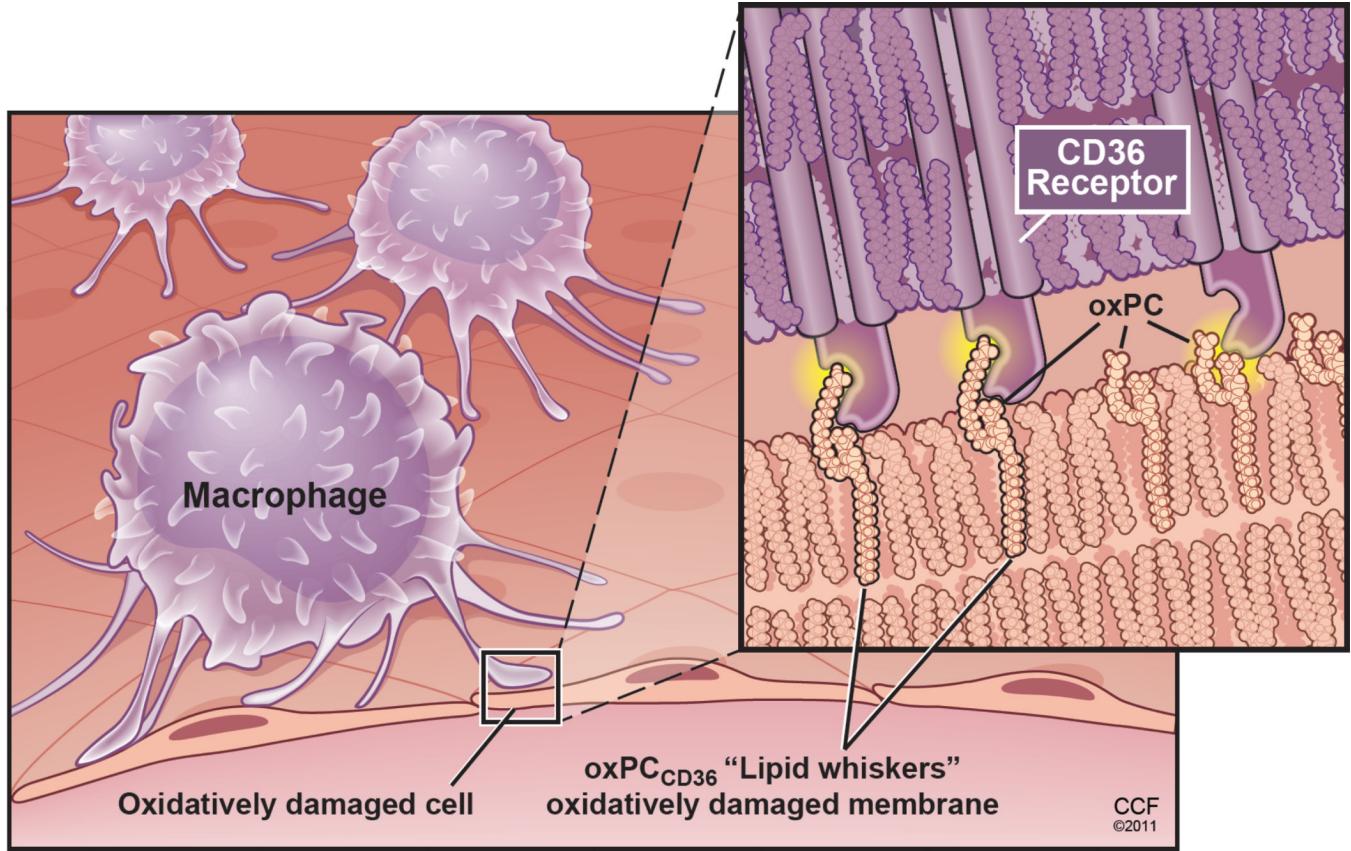
KOdiA-PC

OV-PC

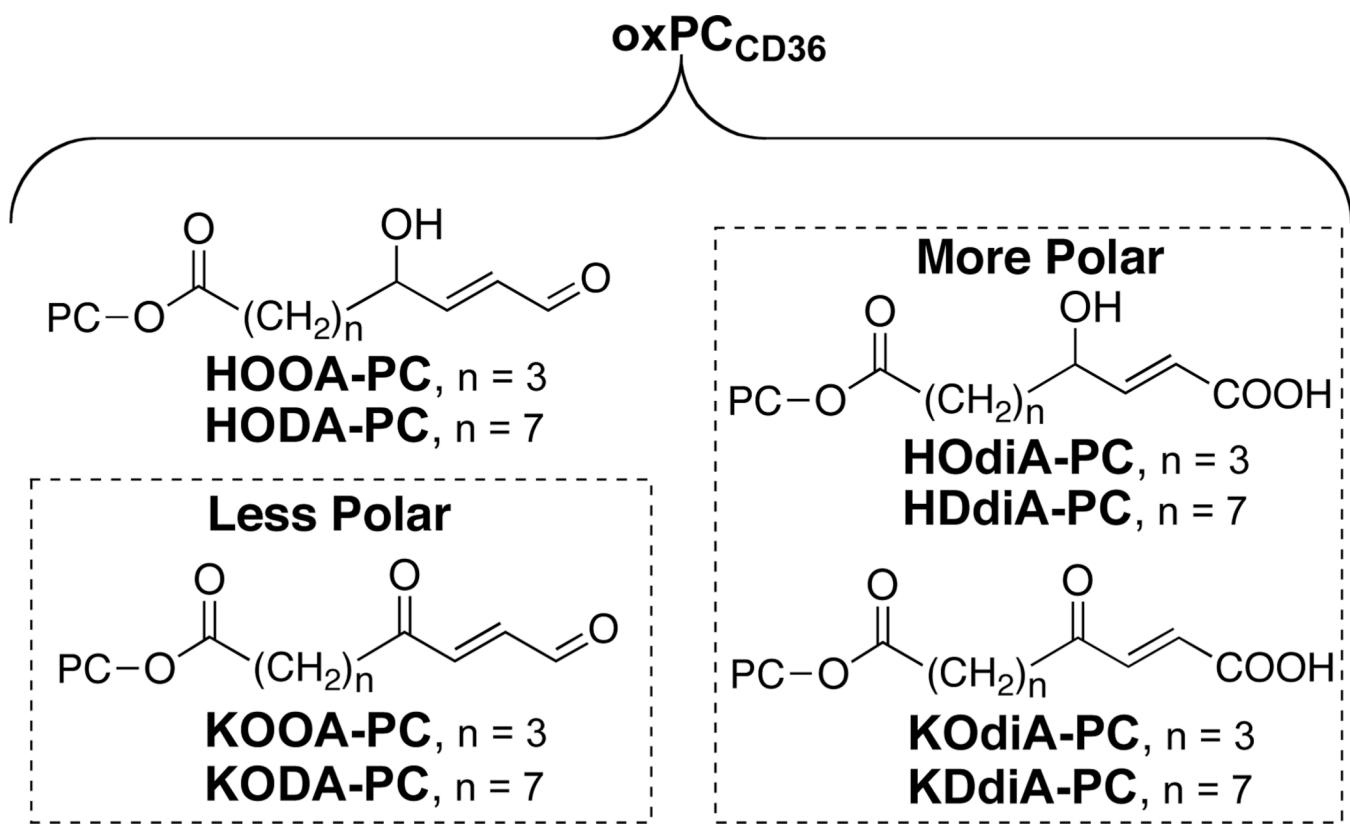
13-HODE-PC

**Figure 5.**

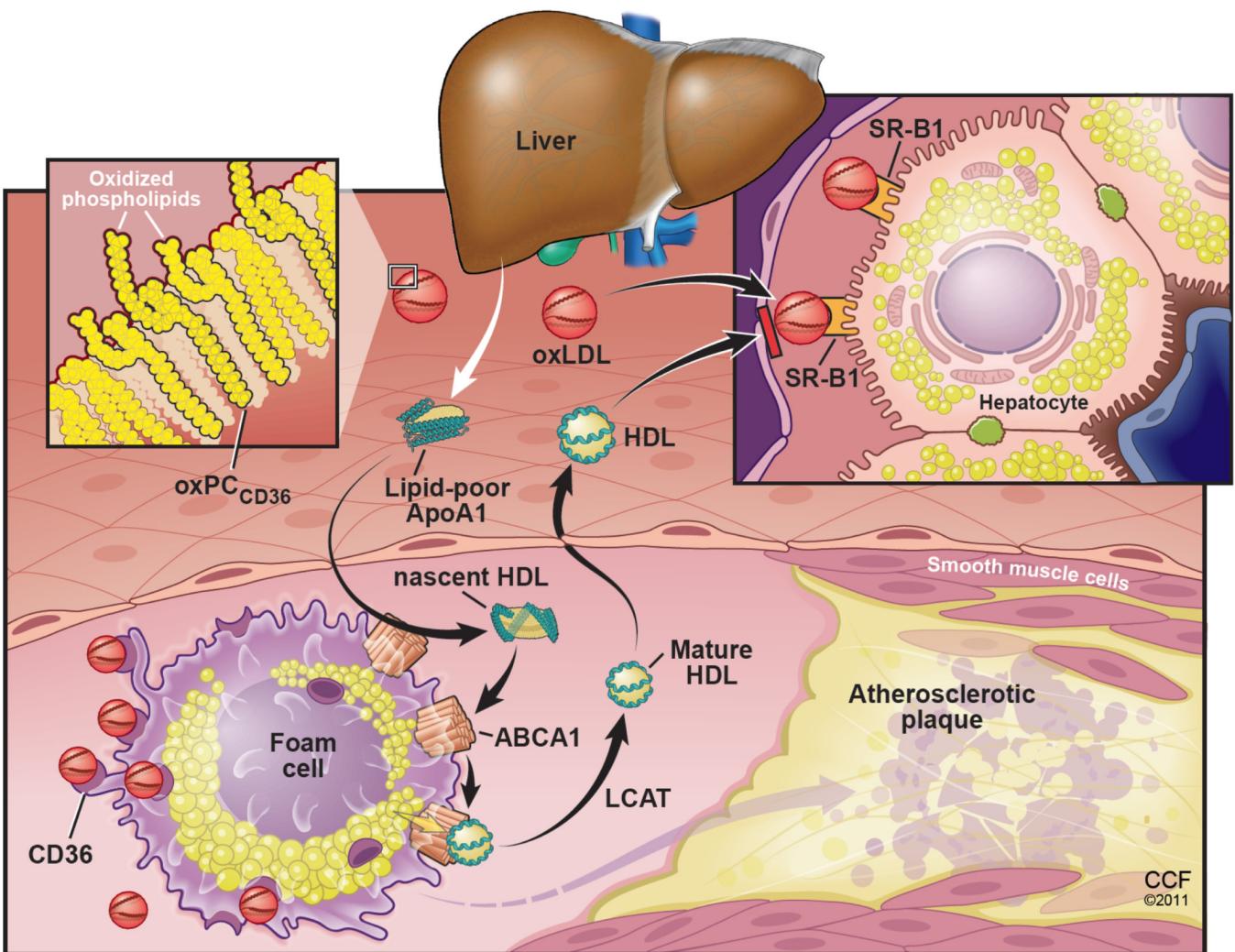
The lipid whisker model of oxidatively damaged membranes. The more hydrophilic ω -oxovaleryl and 5-ketoct-6-endioyl-PC (KOdiA-PC), but not the more lipophilic 13-hydroxyoctadeca-9,11-dienoyl-PC (13-HODE-PC) side chains protrude from the membrane where they are in close proximity with the choline head group and well positioned for interaction with receptors, e.g., on macrophages, platelets, or retinal pigmented epithelial (RPE) cells.

**Figure 6.**

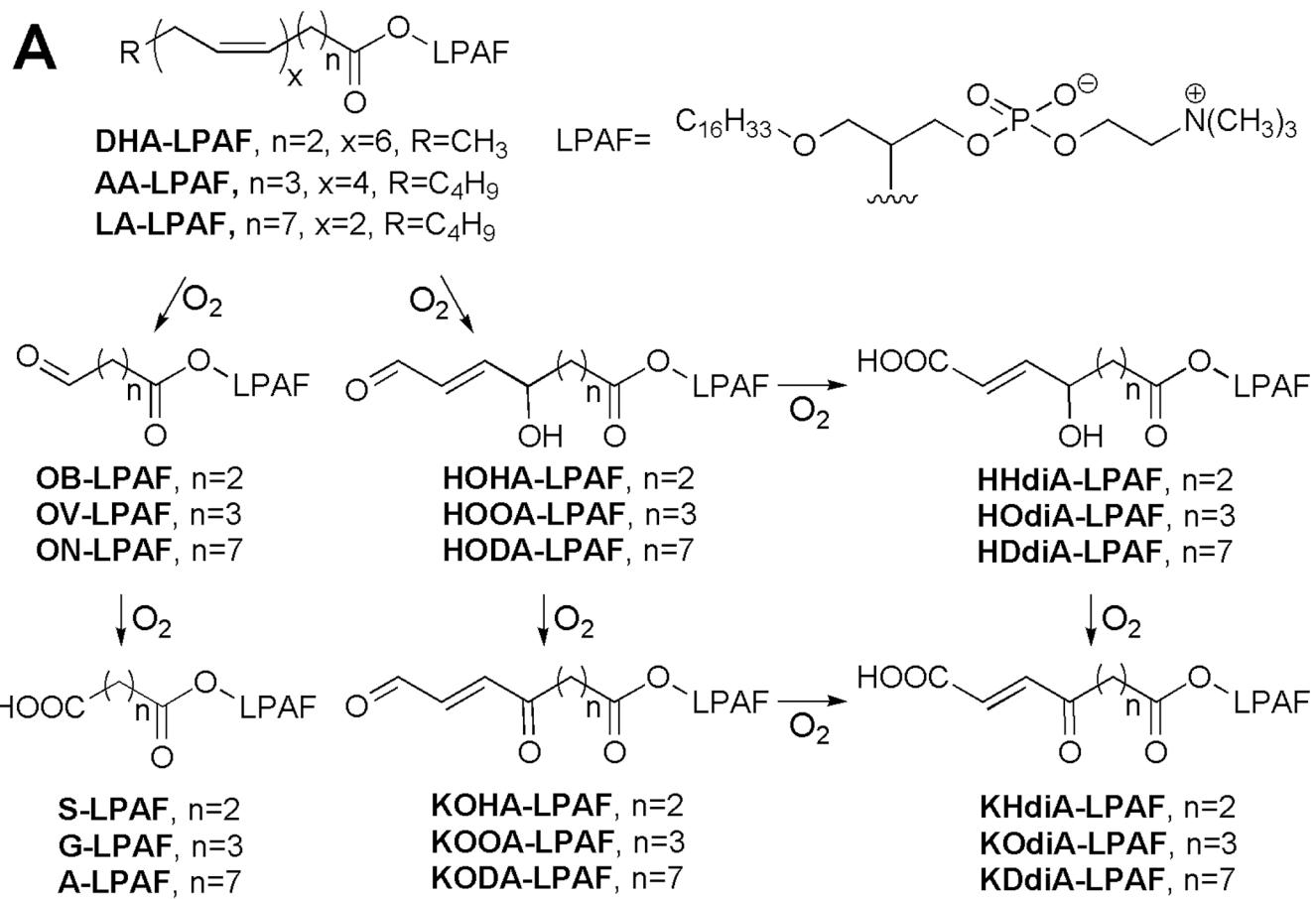
Macrophage scavenger receptor-CD36 detects “lipid whiskers”, oxidized truncated acyl chains that protrude from an oxidatively damaged cell membrane. Binding is especially strong with oxPC_{CD36}, i.e., γ -hydroxyalkenal phospholipids and their more oxidized derivatives, that incorporate a terminal γ -oxygenated α,β -unsaturated carbonyl. Illustration by David Schumick, BS, CMI. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2011. All Rights Reserved.

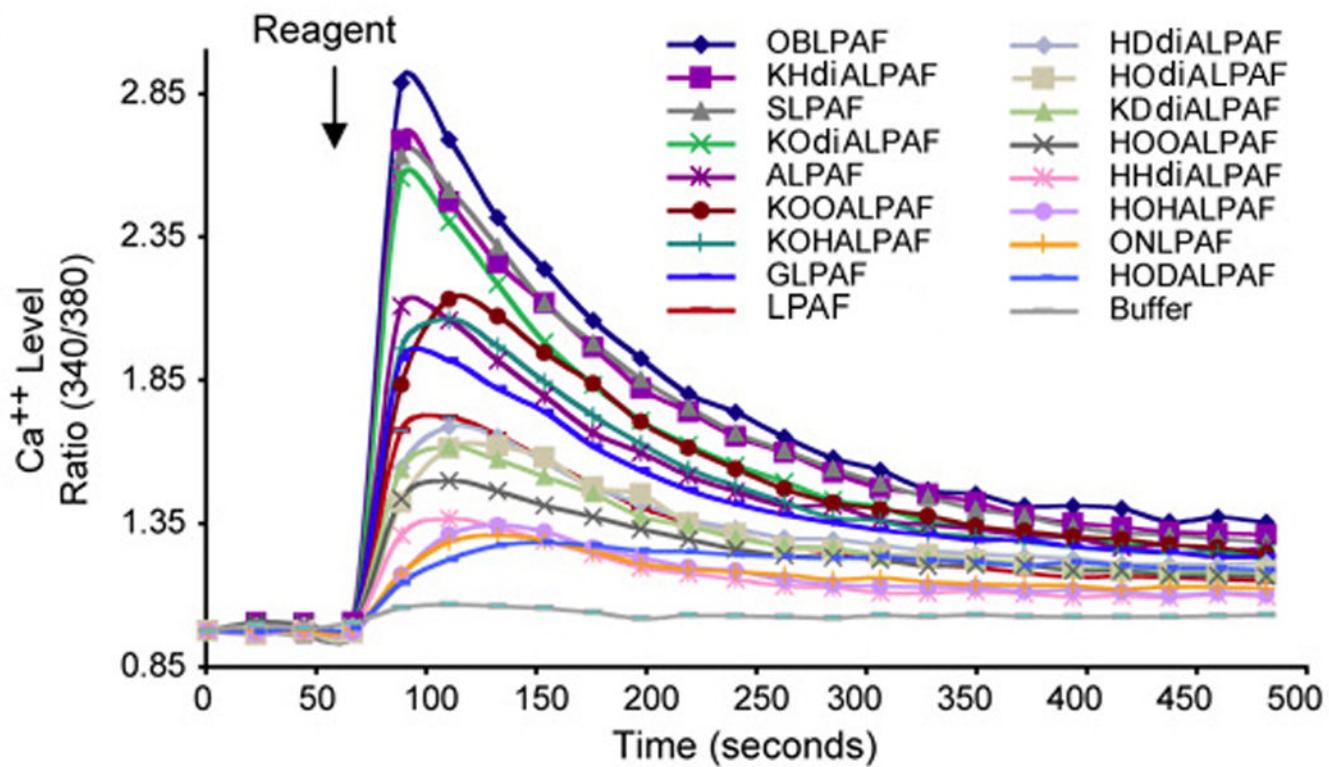
**Figure 7.**

Oxidatively truncated phospholipids with a terminal γ -oxygenated α,β -unsaturated carbonyl, referred to collectively as oxPC_{CD36}, bind avidly with the scavenger receptor CD36.

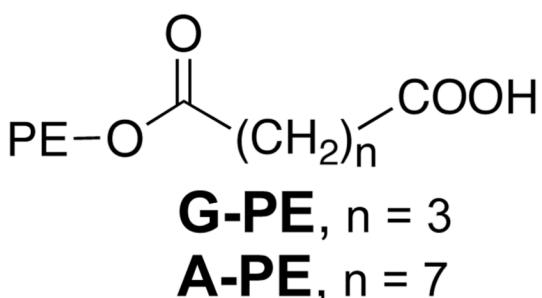
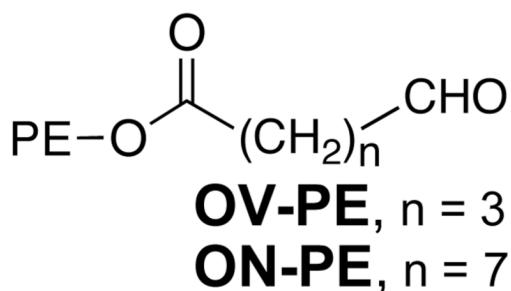
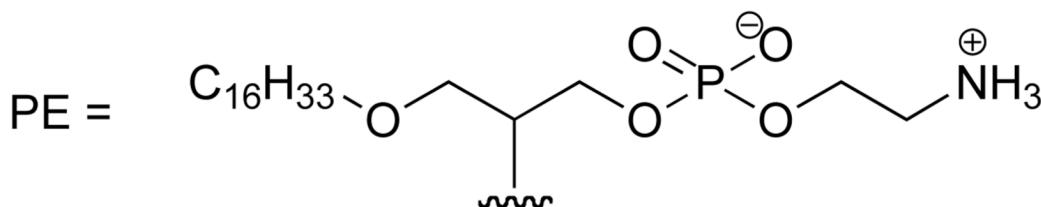
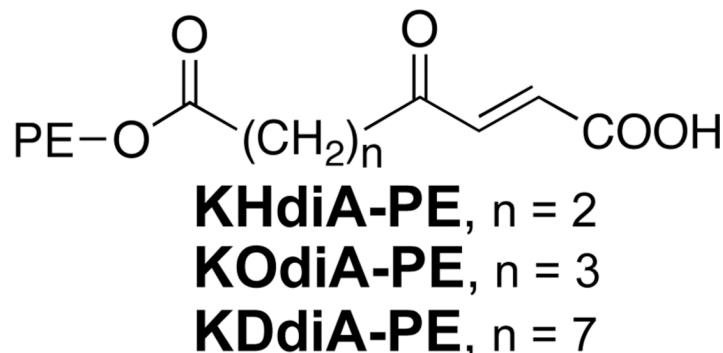
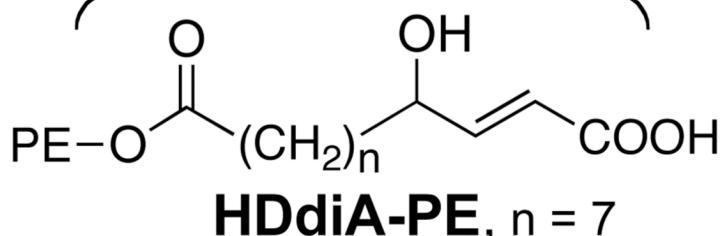
**Figure 8.**

Binding of HDL to hepatocyte SR-B1, that promotes reverse cholesterol transport, e.g., from foam cells to the liver, is impeded by binding with SR-B1 of oxPC_{CD36} lipid whiskers protruding from the outer phospholipid shell of oxLDL (see Figs. 4 and 5). The consequent accumulation of foam cells in the subendothelial space leads to atherosclerotic plaque formation. Illustration by David Schumick, BS, CMI. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2011. All Rights Reserved.

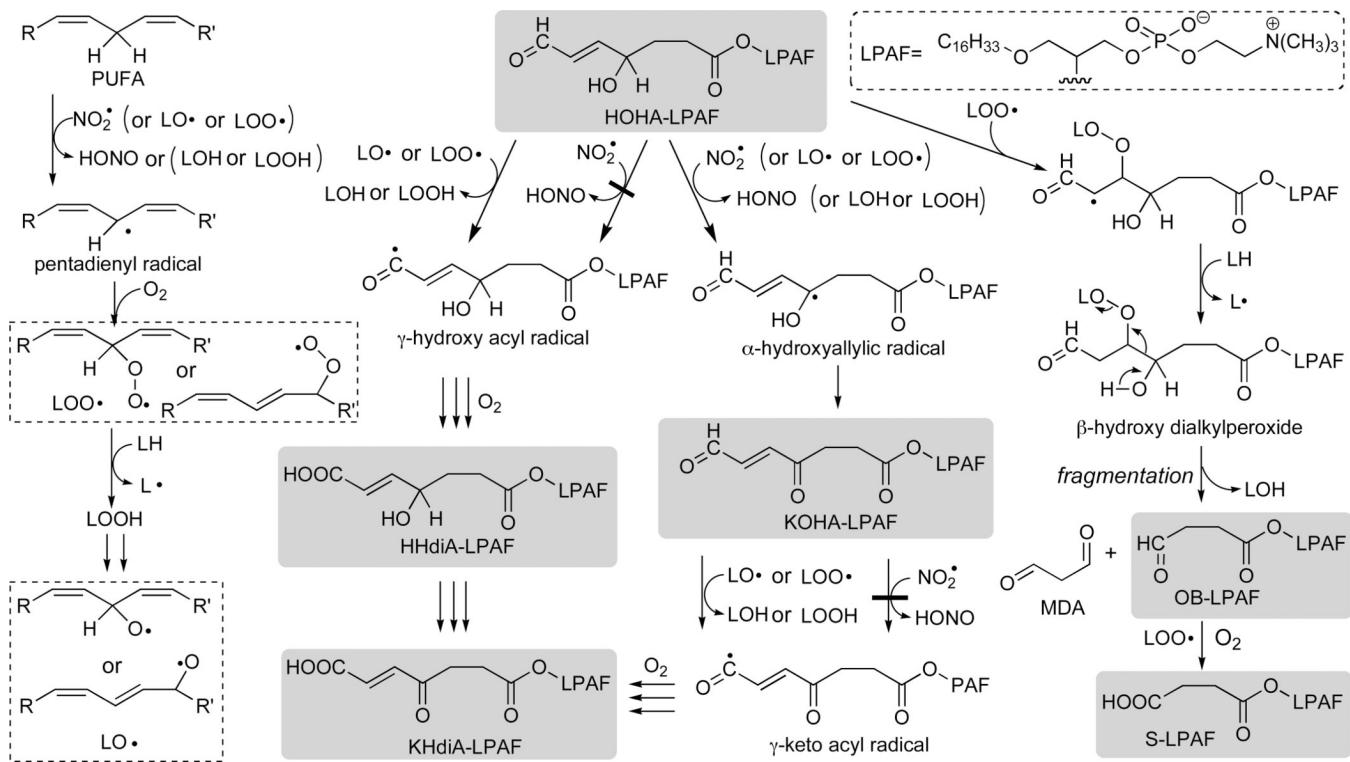


**Figure 9.**

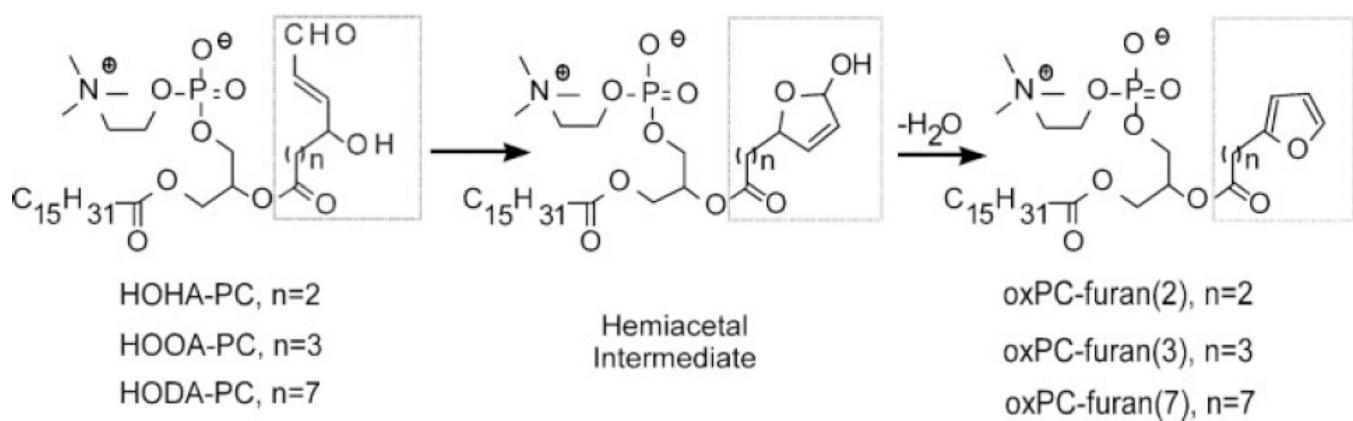
Oxidatively truncated ether phospholipids, referred to collectively as oxPAFs: (A) generation of oxPAFs by oxidative cleavage of PUFA esters of lyso-PAF (LPAF); (B) platelet stimulation by oxPAFs as measured by intracellular Ca^{2+} as a function of time in platelets treated with 0.5 mmol/L of each phospholipid. This research was originally published in Chen, R., Chen, X., Salomon, R. G. and McIntyre, T. M. (2009) Platelet activation by low concentrations of intact oxidized LDL particles involves the PAF receptor. *Arterioscler Thromb Vasc Biol* 29, 363–371. © American Heart Association.

inflammatory**antiinflammatory****Figure 10.**

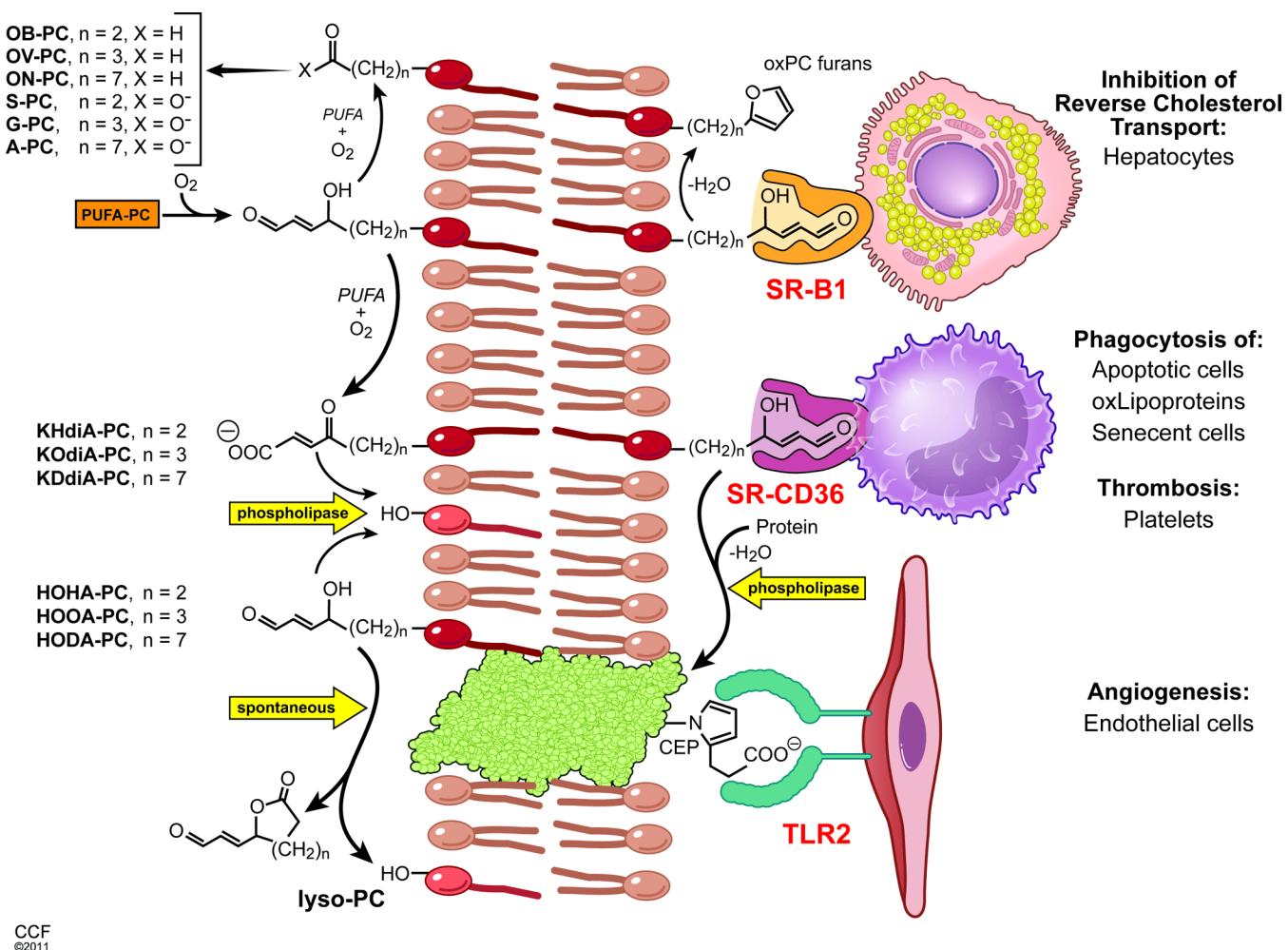
OxPEs exhibit the same action profile as the corresponding oxPCs. Thus, the *sn*-2 acyl chain, and not the polar head group, is the primary determinant of the biological activities tested.

**Figure 11.**

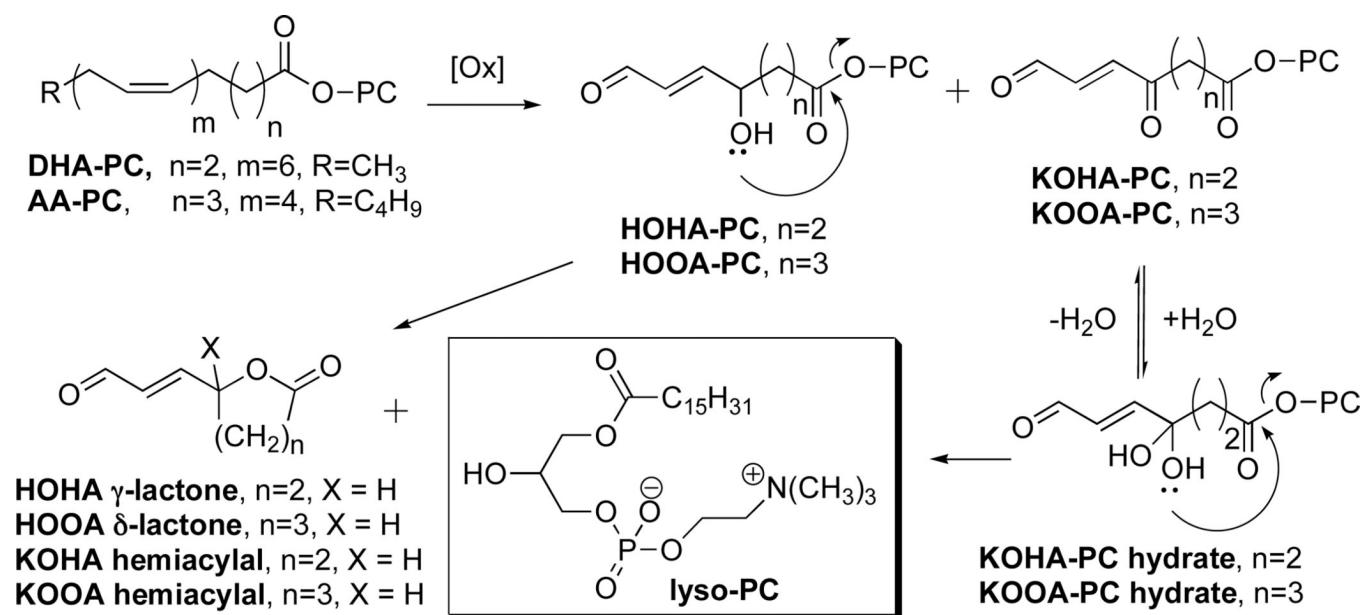
Proposed mechanisms for the fragmentation of HOHA-LPAF and the oxidative conversion of HOHA-LPAF into HHdiA-LPAF and KHdiA-LPAF, which are promoted by PUFAs (LH). LO[•] and LOO[•] are alkoxy and alkylperoxy radicals derived by hydrogen abstraction from LH. LOH, hydroxylipid; MDA, malondialdehyde. Isolable products of these reactions are highlighted. This research was originally published in *The Journal of Lipid Research*. Chen, X., Zhang, W., Laird, J., Hazen, S. L. and Salomon, R. G. (2008) Polyunsaturated phospholipids promote the oxidation and fragmentation of gamma-hydroxyalkenals: formation and reactions of oxidatively truncated ether phospholipids. *J Lipid Res* 49, 832–846. © the American Society for Biochemistry and Molecular Biology.

**Figure 12.**

Generation of oxPC-furans by cyclodehydration of γ -hydroxyalkenals phospholipids. This research was originally published in The Journal of Biological Chemistry. Gao, S., Zhang, R., Greenberg, M. E., Sun, M., Chen, X., Levison, B. S., Salomon, R. G. and Hazen, S. L. (2006) Phospholipid hydroxyalkenals, a subset of recently discovered endogenous CD36 ligands, spontaneously generate novel furan-containing phospholipids lacking CD36 binding activity in vivo. *J Biol Chem* 281, 31298–31308. © American Society for Biochemistry and Molecular Biology.

**Figure 13.**

Summary of the formation and receptor binding of oxPC_{CD36} and the derived CEP-protein modifications, and their biological sequelae. Binding of the oxidatively truncated γ -hydroxyalkenal PCs, e.g., HOOA-PC, and their more oxidized derivatives, e.g., KOdiA-PC (referred to collectively as oxPC_{CD36}) to the scavenger receptor CD36 activates phagocytic cells or platelets, promoting endocytosis of oxLDL or thrombosis, respectively, while binding with SR-B1 inhibits interaction with HDL and, thus, interferes with reverse cholesterol transport. The oxidatively truncated γ -hydroxyalkenal PCs, e.g., HOOA-PC, and their more oxidized derivatives, e.g., KOdiA-PC, readily undergo enzyme-catalyzed phospholipolysis to release lyso-PC. Spontaneous intramolecular transacylation also generates lyso-PC and 5- or 6-membered lactone by-products. Biologically active shorter chain oxidized PCs can be generated by further fragmentation, e.g., HOOA-PC \rightarrow OV-PC, while cyclodehydration to oxPC-furans abolishes recognition by CD36. As described in the companion review, covalent adduction of HOHA-PC with proteins in conjunction with phospholipolysis generates a carboxyethylpyrrole (CEP) that activates endothelial cell toll-like receptor (TLR) 2 resulting in migration, proliferation, and tube formation leading to angiogenesis. Illustration by David Schumick, BS, CMI. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2011. All Rights Reserved.

**Figure 14.**

Generation of lyso-PC from γ - and δ -hydroxy or -keto esters by a nonenzymatic spontaneous transesterification mechanism.