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Enzymatically oxidized phospholipids assume center stage as essential regulators of innate immunity and cell death

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Enzymatically oxidized phospholipids (eoxPLs) are formed through regulated processes by which eicosanoids or prostaglandins are attached to phospholipids (PLs) in immune cells. These eoxPLs comprise structurally diverse families of biomolecules with potent bioactivities, and they have important immunoregulatory roles in both health and disease. The formation of oxPLs through enzymatic pathways and their signaling capabilities are emerging concepts. This paradigm is changing our understanding of eicosanoid, prostaglandin, and PL biology in health and disease. eoxPLs have roles in cellular events such as ferroptosis, apoptosis, and blood clotting and diseases such as arthritis, diabetes, and cardiovascular disease. They are increasingly recognized as endogenous bioactive mediators and potential targets for drug development. This review will describe recent evidence that places eoxPLs and their biosynthetic pathways center stage in immunoregulation.

Phospholipid (PL) oxidation occurs widely in inflammatory vascular disease but has been long considered to involve uncontrolled chemical mechanisms that are mediated by free radicals. However, emerging evidence indicates that enzymatically oxidized PLs (eoxPLs) generated by lipoxygenases (LOXs) or cyclooxygenases (COXs) are abundant biomolecules formed during acute responses to injury in multiple innate immune cell types. eoxPLs form through the coupling of eicosanoid and prostaglandin pathways with the Lands' cycle enzymes or by direct enzymatic oxidation of PLs. eoxPLs are important players in early innate immunity, particularly in promoting blood clotting and defense against infection. Their discovery and characterization have been enabled by parallel development and application of electrospray ionization (ESI) tandem mass spectrometry (MS/MS) technology. Although this review focuses primarily on eoxPLs, we start by introducing the closely related, nonenzymatically generated oxPLs. This prelude on oxPLs aims to orient readers with the knowledge base that led up to the discovery and characterization of eoxPLs as a distinct but related family of bioactive lipids.

Enzymatic Compared to Nonenzymatic Oxidation of PLs

eoxPLs are considered to be functionally separated from the much larger family of lipids called oxPLs, which have been known since the 1980s. We note that there can be considerable overlap in structures between eoxPL and oxPL families, and thus, determining their specific origin in complex biological samples requires the use of several approaches. These approaches include characterizing their formation in mice lacking eoxPL-generating enzymes, determining enantiomeric composition, and using specific pharmacological inhibitors.

oxPLs are formed by nonenzymatic oxidation of intact PLs in which a hydrogen atom is first abstracted from a bis(allylic) methylene group in a polyunsaturated fatty acid (PUFA) chain, forming a lipid alkyl radical (Fig. 1A). Reactive oxygen species generated during inflammation (such as by cytokine- or agonist-activated immune cells) that can directly oxidize lipids include hydroxyl radical (•OH), hypochlorite (HOCl), peroxynitrite (ONOO–), and nitrogen dioxide ($•NO_2$) (*1*). In contrast, eoxPL formation is enzymatic and results in a more restricted set of products formed through controlled pathways in innate immune cells. Both oxPL and eoxPL generation initially proceed through the same mechanisms involving hydrogen abstraction from PUFAs but differ in several key ways that are outlined below. Although the study of oxPLs has preceded the discovery of eoxPLs, there are many parallels in terms of biological actions and cellular sources, and it is likely that both pathways are functionally linked during chronic inflammatory diseases. Our current view is that eoxPLs are physiological mediators of cell signaling that are generated during innate immunity to limit bleeding and infection. However, it is likely that they are also generated at higher levels in disease, and thus contribute to vascular inflammation. On the other hand, nonenzymatically generated oxPLs are always considered harmful because they contribute to autoimmune and inflammatory diseases and cell death.

Nonenzymatic PL Oxidation: Chemical Mechanisms

The knowledge that lipids can be nonenzymatically oxidized to oxPLs has been appreciated for almost two centuries (*2*). Experiments by Nobel laureate Otto Warburg in the early 1900s found that sea urchin egg lipids were targets of iron-catalyzed oxidation (*3*, *4*). The requirement for PUFA groups was shown around 80 years ago, and the free radical mechanisms were outlined in the 1940s (*5*) [reviewed first in (*6*)].

After the formation of an alkyl radical (a reaction that is called initiation), delocalization and oxygen addition occur at diffusion-limited rates to form a hydroperoxyl radical (•OOH) intermediate (Fig. 1B). This intermediate undergoes various chemical reactions to form various oxPL products, including PL-OOH, by propagation and termination reactions (Fig. 1B and Table 1). Another example of how this form of oxidation can occur is the direct attack by ozone (O_3) on FA groups of PLs that have a single double bond in a non-radical mechanism, leading to the formation of an ozonide (*7*). Both ozonides and PL-OOH undergo many secondary reactions, and multiple oxPLs can be formed including terminal aldehydes and carboxylic acids. Ozonides are formed in the protected environment of

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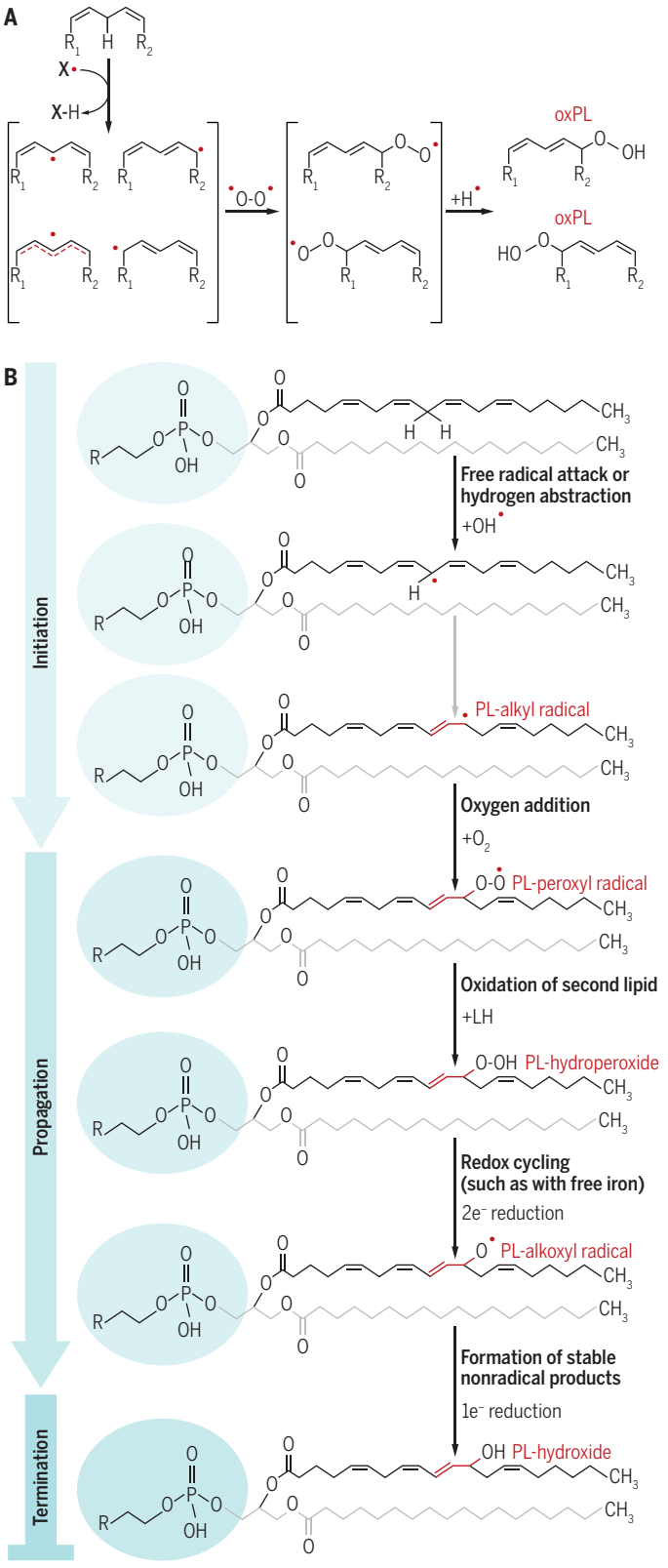

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Fig. 1. Chemical mechanisms of lipid peroxidation. (**A**) The underlying process of lipid peroxidation. The process of hydrogen abstraction and oxygen addition that occurs during lipid oxidation, whether enzymatic or nonenzymatic, is the same. (**B**) A simplified diagram of nonenzymatic phospholipid oxidation.

Table 1. Free radical products that form as oxPLs.

Monosubstituted	Polyoxygenated	Chain-shortened
Hydroperoxide	Dihydroperoxy	ω-Aldehydes
Hydroxy	Dihydroxy	ω-Carboxyl
Keto	Hydroxy, hydroperoxy	ω-Aldehyde-γ-hydroxy
Epoxy	Keto, hydroxy	ω-Carboxy-γ-hydroxy
Nitroalkanes	Hydrohydrins	ω-Carboxy-γ-keto
	Nitro,hydroxy	Furan
	Ozonides	Butanoyl (alkane)
	Isoprostanes	Butenoyl (alkene)
	F2-isoprostanes	
	E2/D2-isoprostanes	
	Epoxy-isoprostanes	
	Isothromboxanes	
	Isoleukotrienes	

the lung where inspired O_3 from pollution encounters PLs in the pulmonary surfactant (*8*). The downstream reactivities and metabolism of PL hydroperoxides (–OOH) and the diversity of products derived from free radical (chemical) auto-oxidation are well characterized (*9*–*11*).

An important feature of nonenzymatic oxidation to form oxPLs is that products form without stereochemical control and are thus racemic and regioisomeric. This feature distinguishes oxPLs from eoxPLs, for which enzymatic mechanisms control oxygen insertion and yield specific enantiomers. Analysis of these products using chiral chromatography can thus help to distinguish oxPLs from eoxPLs.

The Generation of oxPLs in Vascular Disease

Research led by Berliner, Leitinger, Salomon, and colleagues into the vascular formation of oxPL started in the 1980s [reviewed in (*11*)]. This early work showed that oxPLs are present in atherosclerotic plaques, a finding that stimulated the elucidation of vascular oxPL structures and their potential bioactivities (*12*–*15*). The major oxPL class in vascular lesions was reported as phosphatidylcholine (PC), although it is not clear whether this was because other oxPL classes were not assessed or were present in lower amounts. At that time, synthetic oxPCs were generated in vitro through air- and metal-catalyzed oxidation and then applied to cellular or in vivo systems in which pleiotropic bioactivities relevant to inflammation were demonstrated (*11*). This finding led to the idea that oxPLs are formed in excess and promote inflammation, thereby driving atherosclerotic vascular disease. This idea was further supported by Witztum and Hörkko, who found that mice with atherosclerosis generated oxPL-specific immunoglobulin M antibodies from the innate immune system, that the levels of these antibodies were inversely correlated with disease, and that these antibodies provided vascular protection by sequestering oxPLs (*16*–*19*). However, synthetic oxPLs are a complex mixture of hundreds of products. When generated by different laboratories using unstandardized conditions, there was inconsistency in terms of chemical composition, which usually was not defined,

leading to oxPL preparations with opposite biological actions in the same assay systems (*11*). Later, in the 1990s, researchers began to separate the components of synthetic air–oxidized oxPLs. Various forms, including truncated lipids where the PUFA has been oxidatively shortened, were purified and studied in isolation (*20*–*23*) and were also detected in human and murine atheroma lesions (*12*–*15*). These truncated lipids included 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-PC (POVPC), 1-palmitoyl-2-epoxyisoprostane-*sn*-glycero-3-PC (PEIPC), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-PC (PGPC), and others. To date, how these truncated lipids form in vivo is incompletely understood. Although generally assumed to be generated nonenzymatically, we speculate that enzymes such as LOXs or COXs might catalyze the initial oxidation, forming lipid hydroperoxides. If antioxidants are depleted, then these lipid hydroperoxides could be nonenzymatically oxidized to lipid radicals, which would then propagate, leading to racemic downstream oxPL formation. However, the idea that enzymatic initiation of oxidation could lead to downstream oxPL formation is yet to be tested using biological models, such as *Alox*-deficient mice.

Regardless of how oxidation is initiated, the initial product of PL oxidation will always be a PL-OOH, which is chemically reactive and can decompose to products including chain-shortened PL species, such as POVPC. Reduction to the chemically less reactive PL hydroxide (–OH) is catalyzed by a specific PL-OOH reductase, glutathione reductase 4 (GPX4), which limits chemical reactivity of the PL-OOH (*24*). Direct oxidation of cardiolipins containing linoleic acid (LA) also occurs in mitochondria by cytochrome c, leading to the formation of cardiolipin-OOH (*25*).

Formation of eoxPLs Through Enzymatic Oxidation of PLs

In contrast to uncontrolled nonenzymatic oxidation described above, the cellular generation of eoxPLs is a highly controlled process that is mediated by enzymes that catalyze redox chemistry. During oxPL formation, molecular oxygen is inserted into a PUFA group to generate a lipid-OOH; however, in this special case, a more restricted set of structures is formed, including specific regio- and stereoisomers, which are termed eoxPLs (*26*–*29*). The overall mechanism is similar to that of oxPL formation (Fig. 1, A and B), except that the removal of the hydrogen atom is carried out by iron in a metalloenzyme (LOX) or by a tyrosyl radical (COX), and the site of oxygen insertion is controlled because of steric hindrance within the active site. As described below in more detail, several cell types of the innate immune system generate eoxPLs through cell type–specific LOX and COX isoforms (*29*).

There are two distinct mechanisms for eoxPL formation: direct oxidation of an intact PL or indirect oxidation in which a preformed oxidized FA (oxFA) is inserted into a lysoPL through the enzymes of the Lands' remodeling cycle. Direct enzymatic oxidation of PLs is catalyzed by 15-LOX (human) or 12/15-LOX (murine) (both encoded by the *ALOX15* gene) in leukocytes (Fig. 2A) (*27*, *30*). 15-LOX is highly expressed in human eosinophils and interleukin-4 (IL-4)/IL-13–treated monocytes and generates free 15-hydroperoxyeicosatetraenoic acid (15-HpETE), which is rapidly reduced by cellular GPXs to the more stable 15-hydroxyeicosatetraenoic acid (15-HETE) (*31*, *32*). High levels of esterified 15-HETE from 15-LOX were first observed by Kühn in human eosinophils stimulated with a Ca^{2+} ionophore (*33*). In human monocytes, 15-HETE was shown to be esterified to PL, specifically phosphatidylethanolamine (PE) (*27*). Similarly, in mice, leukocytes including eosinophils and resident peritoneal macrophages generate 12-HETE-PEs through 12/15-LOX (*30*). Unlike indirect oxidation, eoxPLs generated by 15-LOX or 12/15-LOX are present in unstimulated cells, and their formation does not strictly require agonist activation. These features suggest that some eoxPLs play homeostatic roles in innate immunity. Direct oxidation of PE to form HETE-PE was demonstrated in both mouse and human monocytes and macrophages using a stable isotope dilution MS approach to monitor ^{18}O incorporation after cell activation in ^{18}O-H_2O buffer (*27*, *30*). Lack of incorporation is consistent with direct oxidation because there is no cycle of hydrolysis and re-esterification.

Indirect enzymatic oxidation describes formation of eoxPL through the Lands' pathway (Fig. 2B). The definitive step of this enzymatic reaction involves a coenzyme A (CoA) ester of an oxFA formed by a long-chain fatty acyl-CoA synthetase (ACSL). This product is then esterified onto a lysoPL acceptor by lysoPL acyl transferases (LPATs), also known as membrane-bound O-acyl transferases (MBOATs). The complete cycle of eoxPL formation has been revealed as a rapid and coordinated process that occurs in several immune cell types (*26*, *28*). This process comprises (i) PL hydrolysis by PLA_2 to release FA, (ii) formation of oxFA (typically an eicosanoid or prostaglandin) by FA oxidation catalyzed by COX or LOX, and (iii) re-esterification of the oxylipin to a lysoPL to form eoxPL by fatty acyl Co-A ligase (FACL) and LPAT. This process occurs in platelets and neutrophils, where FA oxidation is primarily catalyzed by 12-LOX (encoded by *ALOX12*) or 5-LOX (encoded by *ALOX5*), respectively (*10*, *28*). In platelets, the most quantitatively abundant eoxPL is 12-HETE attached to PE or PC. This is not surprising because AA is the most abundant long-chain PUFA in immune cell PL membranes, followed by LA and docosahexaenoic acid (DHA). Also, PE and PC are the most abundant PL classes in the plasma membrane and include both acyl and plasmalogen forms. 14-Hydroxydocosahexaenoic acid (HDOHE) PEs are also detected in platelets, which arise from DHA oxidation by 12-LOX (*34*). Similarly, neutrophils stimulated with the bacterial product *f*MLP (*N*-formyl-Met-Leu-Phe) generate primarily 5-HETE-PE or 5-HETE-PC through the action of 5-LOX on AA, followed by esterification of 5-HETE into lysoPL (*26*). A requirement for hydrolysis and reacylation has been confirmed in both platelets and neutrophils using ^{18}O-H_2O stable isotope dilution MS and/or thimerosal to block fatty acyl esterification (*26*, *28*).

Our studies show that 15-LOX and 12/15-LOX mediate direct PL oxidation, whereas 5-LOX and 12-LOX generate HETE-PEs through Lands' cycle remodeling in innate immune cells. These findings are consistent with what is known regarding the substrate preferences of these proteins, based on older in vitro studies using recombinant or purified enzymes (*35*, *36*).

Whether reduction of the HpETE to HETE by GPX occurs before or after esterification is not known, and in both platelets and monocytes, both HpETE-PEs and HETE-PEs have been demonstrated to arise from LOX turnover (*27*, *28*). About a third of all the 12-HETE generated by human platelets is esterified into PC and PE on the same time scale as formation of free 12-HETE (*28*). Thus, eoxPLs form rapidly on cellular activation and are abundant lipids, present at picomole to nanomole levels. This temporal generation suggests that the synthetic enzymes are colocalized and work cooperatively. In support of this notion, exogenously added stable isotope–labeled 12-HETE is not incorporated into PE in thrombin-stimulated platelets on the same short timescale as endogenous 12-HETE-PE formation

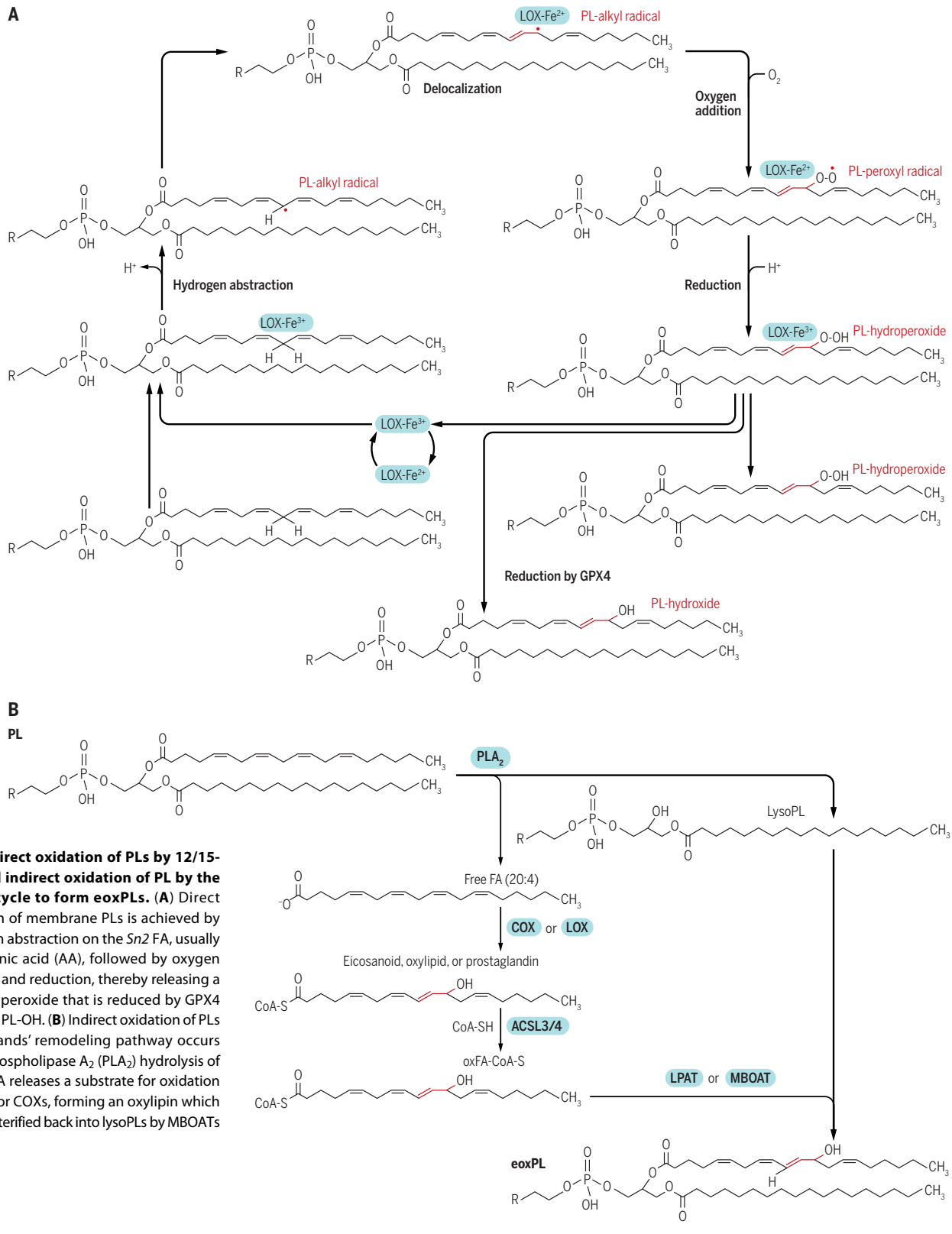

Fig. 2. Direct oxidation of PLs by 12/15-LOX and indirect oxidation of PL by the Lands' cycle to form eoxPLs. (**A**) Direct oxidation of membrane PLs is achieved by hydrogen abstraction on the *Sn2* FA, usually arachidonic acid (AA), followed by oxygen insertion and reduction, thereby releasing a PL-hydroperoxide that is reduced by GPX4 to form a PL-OH. (**B**) Indirect oxidation of PLs by the Lands' remodeling pathway occurs when phospholipase A_2 (PLA_2) hydrolysis of an Sn2 FA releases a substrate for oxidation by LOXs or COXs, forming an oxylipin which is then esterified back into lysoPLs by MBOATs or LPATs.

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(*28*). This result suggests a new paradigm for the purpose of the Lands' cycle, which is to generate immune bioactive eoxPLs in circulating human and murine innate blood cells by remodeling of their biomembranes as an acute response to stimulation.

A second way in which eoxPLs may form through indirect oxidation involves esterification of precursor oxylipins that have been taken up from a different cell type (a process called transcellular uptake). Early studies from Serhan and others in the 1990s focused on this route, adding exogenous oxylipins to cells and measuring their esterification into PLs. For example, exogenous 15-HETE (a LOX product found in monocytes and eosinophils) is incorporated into phosphatidylinositol (PI) in neutrophils and then subsequently released on *f*MLP activation of phospholipases (*37*). However, rates of uptake and esterification of exogenous substrate take place on relatively long time scales (several minutes to hours) and whether this occurs in vivo is unknown. The phenomenon of oxylipin incorporation into lysoPLs has also been observed for epoxyeicosatetraenoic acids (EET) generated by the cytochrome P450 family of enzymes (*38*, *39*). Here, a potential role for EET-PLs in altering membrane microdomain properties or acting as a releasable pool of oxylipins has been proposed (*38*). Esterification of 14,15-EET occurs in mastocytoma cells with half maximal incorporation around 30 min (*40*). Thus, this esterification is on a similar time scale to transcellular oxylipin incorporation described above but is considerably slower than endogenous HETE-PE generation in immune cells. PL incorporation of oxylipins through this transcellular uptake route has been suggested to lead to the formation of a pool of "stored" eicosanoids that can be released later. However, although this is the case, eoxPLs such as HETE-PEs have immunoreactive bioactivities in their own right that require the oxylipin to remain PL esterified.

In addition to 12-LOX, platelets can also generate eoxPL through COX-1, specifically prostaglandin E_2 (PGE_2)-PE and PGD_2-PE (*41*, *42*). However, despite the quantitatively high abundance of thromboxane-B_2, attachment of this eicosanoid to PLs has not been detected. This suggests that esterification pathways may show oxylipin selectivity, in line with findings from Klett *et al.* using purified ACSLs (*43*). Cellular COX-2–mediated generation of eoxPLs could represent a source of esterified prostaglandins during chronic inflammation, although this idea remains to be proven.

Up until 2016, a relatively small number of eoxPL molecular species had been determined in innate immune cells, typically four to six molecular species per cell type. However, we now know that human platelets can rapidly form >100 unique eoxPL structures through 12-LOX and/or COX-1 after thrombin activation (*44*). The most abundant are PEs, although PC forms are also prevalent, and rarer HETE-PI forms have been discovered (*44*, *45*). In addition to AA- or DHA-derived eoxPLs described earlier, –OH forms of 22:4, 22:5, 20:3, and 20:5 are formed but at lower amounts. Rarer multiply oxidized AA species containing two or more oxygens are also generated (*44*). These lower abundance eoxPLs have, so far, not only been measured in platelets but may also be formed during inflammation in other cells expressing high levels of LOXs or COXs. Many eoxPLs have not been fully structurally elucidated, with position of oxidation sites still unassigned. Several MS/MS derived from platelet eoxPLs are available for future characterization (*44*).

Indirect enzymatic oxidation requires agonist activation of innate immune cells, achieved in platelets or neutrophils after stimulation with thrombin, collagen, or *f*MLP. In platelets, thrombin activates protease-activated receptor 1 (PAR1) and PAR4, whereas *f*MLP activates formyl peptide receptor 1 in neutrophils. These stimuli then lead to downstream Ca^{2+} mobilization and phospholipase activation, both of which are required for eoxPL generation (*26*, *28*). The use of pharmacological inhibitors has suggested the involvement of additional signaling pathways in platelets and neutrophils, including protein kinase C, Src tyrosine kinases, phospholipase C, mitogen-activated kinases, $cPLA_2$, $sPLA_2$, and 5-LOX–activating protein (FLAP); although formal studies in genetically deficient mice have not yet been undertaken (*7*, *37*). These studies reinforce the idea that eoxPL generation is a regulated physiological process that is critical for innate immunity.

Approaches to Distinguish eoxPLs from oxPLs in Cellular and Tissue Systems

There is considerable structural overlap between oxPL and eoxPL species, both structurally and functionally. Furthermore, whether oxPLs can be formed through decomposition of eoxPLs (such as through metal-dependent decomposition of LOX-derived HpETE-PEs) is still unclear. Thus, when measuring these lipids, it can be challenging to determine their precise biochemical origin. Furthermore, the biosynthetic pathways for truncated oxPLs detected in plasma are unknown, and there is ongoing debate regarding the origin of lipid oxidation products that drive ferroptosis. Despite these issues, several approaches can help delineate the origin of oxPLs.

The first approach uses tissue or cells lacking *Alox* through genetic deletion or pharmacological inhibition. Mice lacking several *Alox* isoforms are available, and both *Alox12*$^{-/-}$ and *Alox15*$^{-/-}$ mice cannot generate platelet or macrophage HETE-PLs, respectively (*30*, *46*). Pharmacological inhibitors for platelet 12-LOX or leukocyte 15-LOX (12/15-LOX) are not generally specific, and most are either antioxidants or lipid analogs and thus will also inhibit oxPL formation. The exception is MK886, an inhibitor of FLAP, which has been used to implicate 5-LOX in HETE-PL formation in neutrophils (*26*). Pharmacological inhibitors for COXs, including aspirin and indomethacin, or COX isomer–specific inhibitors, such as celecoxib, can also be used to implicate this pathway in esterified prostaglandin formation (*41*).

The second approach relies on reverse phase and chiral chromatography to confirm regio- and enantiomeric specificity. eoxPL formation involves regio- and stereospecific oxygen insertion by enzymes. For example, platelets generate primarily 12(*S*)HETE-containing PL isomers (*28*). Thus, a predominant isomeric composition reflective of enzymatic activity can distinguish oxPLs from eoxPLs. MS/MS fragmentation [to monitor the intact HETE fragment, rather than the internal daughter ions that arise from HETE fragmentation on collision-induced dissociation (CID)] or saponification of purified phospholipids followed by high-performance liquid chromatography–ultraviolet detection of HETE positional isomers can first determine regiospecificity. After this determination, free acid HETEs obtained by saponification can be subjected to chiral chromatography to determine enantiomeric composition. These approaches have been used for platelets, neutrophils, monocytes, and murine macrophages to confirm the involvement of LOX isoforms in eoxPL formation (*26*–*28*, *30*).

A final point relates to the relative amounts and/or importance of eoxPLs compared to oxPLs in specific disease settings and ascribing biological roles in vivo. Currently, little quantitative information exists on this issue, to some extent, due to lack of authentic

standards for measuring their amounts in tissues. Truncated oxPCs are available from Avanti Polar Lipids, and we have synthesized and purified HETE-PE and HETE-PC positional isomers generated by either soybean LOX or air oxidation (*47*). Some of these are now available from Cayman Chemical. Using these standards, isolated platelets and neutrophils were found to generate nanogram amounts of HETE-PEs exclusively through enzymatic mechanisms, whereas the formation of truncated oxPLs is not detected (*26*–*28*, *30*). We found that platelets esterify about 30% of newly synthesized 12-HETE into PL pools and that this process takes place in the same time scale as free 12-HETE synthesis, indicating that it is both fast and controlled and providing an example in which eoxPLs predominate. However, we suspect that during chronic inflammation, in which reactive oxygen and nitrogen species are generated, the situation may change over time. In support of this notion, a comparison of enantiomeric specificity of esterified hydroxyoctadecadienoic acids (HODEs) in human atheroma lesions showed a predominance of the 13(*S*) form in early lesions but showed a racemic mixture at later disease stages (*48*). To test the involvement of lipids in disease, *Alox*-deficient mice are a suitable model; however, these enzymes generate many oxylipins, making it challenging to ascribe functions to specific lipids. To that end, we have administered HETE-PLs to mice, either locally into the tail or systemically through the tail vein and found them to be well tolerated, with acute effects on hemostasis and thrombosis in line with their proposed procoagulant actions (*46*, *49*).

Functions of eoxPL During Innate Immunity

Although LOX isoforms are different gene products, evolutionarily separated with distinct cellular expression profiles, the generation of similar eoxPL classes by different innate immune cells (particularly HETE-PLs) suggests overlapping functions. HETE-PLs are generated by immune cells under conditions relevant to responses to injury or acute trauma, such as following challenge by hemostatic or infectious agents (*29*). These conditions suggest that HETE-PLs have a protective role in these contexts, where prevention of bleeding or infection is a primary goal. Conversely, excessive generation may promote vascular inflammation, particularly arterial thrombosis in atherosclerotic disease or venous thrombosis (*46*, *50*). Accordingly, reports on the bioactions of nonenzymatically generated oxPLs (including structures that we now know are also eoxPLs, such as HETE-PE) demonstrate that they can be either pro- or anti-inflammatory, depending on the context (*11*, *51*). However, in many studies on oxPLs, the lipids were derived by air and/or chemical oxidation, and biological actions cannot be attributed to specific molecules. An exception is POVPC, which is recognized by the scavenger receptor CD36 and thus considered a damage-associated molecular pattern molecule (*52*).

A key difference between eoxPLs and their prostaglandin and eicosanoid precursors is that eoxPLs are not secreted but remain cell associated, residing within membranes, due to their limited solubility in water and lipophilicity. This phenomenon has been shown for HETE-PEs in several cell types (*26*–*28*). Free acid eicosanoids or prostaglandins mediate their potent bioactivities through binding and activating G protein–coupled receptor (GPCR) signaling at subpicomolar concentrations. However, current evidence indicates that HETE-PLs mainly exert their effects through low-affinity interactions with proteins and/or altering membrane electronegativity, leading to changes in how proteins interact with membranes (such as in blood clotting). The lipid whisker model for oxPL signaling, which proposes that more electrophilic oxidized FA moieties protrude into the extracellular space, provides some hints regarding how HETE-PLs might signal (*53*). Introduction of a polar character into the membrane has considerable potential to change the local environment for integral membrane proteins and receptors and may explain many observed effects of the lipids on diverse systems including coagulation. Thus, our current view is that eoxPLs are unlikely to mediate high-affinity receptor-ligand interactions in a manner similar to eicosanoids or prostaglandins.

Innate immune cells undergo substantial changes in membrane structure on agonist activation, including spreading, adhesion, chemotaxis, microvesicle formation, degranulation, and so on. How plasma membranes containing HETE-PLs may influence these phenomena is not currently understood. Hints come from studies on chemical oxidation of membranes. For example, mixtures of oxPLs generated through chemical or air oxidation induce flattening and thinning of artificial membranes and increase water permeabilization, in part, due to the hydrophilic nature of the sn2-oxidized lipid (*54*). Specifically, the introduction of a polar group may cause the acyl chain to partially bend, narrowing the hydrophobic core of the membrane (*54*). In addition, high concentrations (at milligrams per milliliter) of purified 15-LOX cause pore formation in purified organelle membranes through lipid peroxidation, and overexpression of 15-LOX in non-erythroid cells is associated with mitochondrial membrane collapse (*55*). Thus, endogenous LOX in immune cells could mediate profound changes to the plasma membrane on agonist activation (possibly through the generation of HETE-PLs).

Hemostasis and thrombosis

Aminophospholipids (aPLs), specifically native phosphatidylserine (PS) and PE, are essential for effective blood clotting. Normally, PS and PE are retained on the inner leaflet of the plasma membrane; however, when immune cells are activated, they are externalized through scramblase activity (*56*, *57*). Then, negatively charged vitamin K–dependent carboxylation/gamma-carboxyglutamic (Gla) domains of plasma coagulation factors associate with PS and PE on the activated platelet membrane surface through calcium ion binding. Thus, coagulation factors come together on a PL surface, allowing them to effectively interact and facilitating proteolytic cleavage and, ultimately, thrombin generation (*56*, *57*). This assembly of distinct coagulation factors at the platelet surface then promotes fibrin polymerization and clotting, providing a backbone for the stabilization of a platelet-rich thrombus that rapidly forms upon aggregation of these cells in response to vascular injury.

Several studies support an essential role for eoxPLs in promoting PS-dependent coagulation in vitro and in vivo (*46*, *49*, *50*). Specifically, HETE-PE or HETE-PCs enhance the ability of PS to interact with multiple clotting factors, increasing the rates of thrombin generation and ultimately promoting hemostasis (Fig. 3) (*46*, *49*, *50*). Mice genetically lacking HETE-PLs (either through *Alox15* or *Alox12* deficiency) bleed longer when challenged, suggesting that endogenous eoxPL supports hemostasis in vivo (*46*, *50*). Injection of HETE-PEs restores in vivo hemostasis in mice, as evidenced by increased thrombin-antithrombin complexes, and rescues the bleeding phenotype (*46*). Mechanistically, purified HETE-PE or HETE-PCs increase thrombin generation in human plasma, and molecular dynamics simulations show that the –OH group of the HETE is positioned

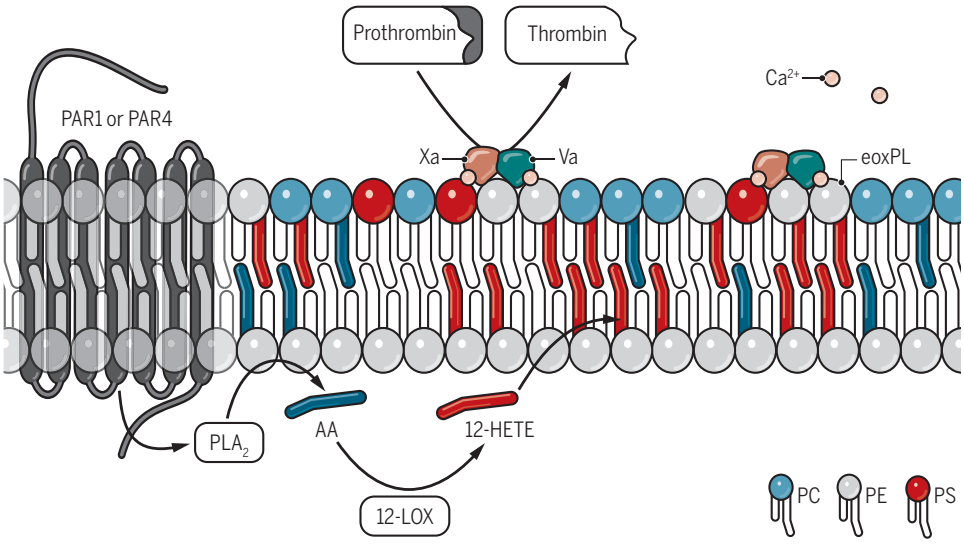

Fig. 3. eoxPLs support the generation of a prothrombotic surface on platelets, facilitating hemostasis. Activated platelets externalize aPLs and eoxPLs to form an electronegative surface that supports Ca^{2+}-dependent binding and activation of coagulation factors, leading to thrombin formation.

close to the polar external face of the membrane, where it interacts with Ca^{2+} and pushes PL headgroups apart (*46*, *49*). HETE-PLs promote Ca^{2+} binding to membrane surfaces, and liposomes containing these lipids are smaller and show greater curvature, also increasing accessibility of charged PS headgroups to coagulation factors (*49*).

12-HETE-PEs form primarily at the inner leaflet of the activated platelet membrane, with a proportion externalizing to the outer face (*28*). 12-HETE-PCs are expected to be found on the outside of the lipid bilayer where PC is primarily located. Externalization of HETE-PE has been detected in platelets and macrophages. Here, exposure of eoxPL parallels that of PS but occurs independently of apoptosis through incompletely defined pathways (*58*). In platelets, the protein TMEM16F contributes to non-apoptotic PS exposure and is likely involved in the exposure of eoxPLs in these and other cells (*58*). In addition to platelets, eosinophils also use HETE-PLs to initiate coagulation. Both human and murine eosinophils generate HETE-PEs, and *ALOX15*-deficient eosinophils poorly support tissue factor–dependent thrombin generation in vitro (*50*). However, unlike platelets, eosinophil HETE-PE levels do not appear to increase on cell activation with adenosine diphosphate (ADP). Instead, their procoagulant activity relies on PS externalization upon ADP stimulation, and the ability of PS to support coagulation is enhanced by *ALOX15*-derived eoxPLs already basally present in the cell membrane (*50*).

HETE-PLs are associated with the pathogenesis of both arterial and venous thrombosis, since they increase in abundance in circulating leukocytes and platelets from patients with antiphospholipid syndrome (also known as human venous thrombosis), where they also stimulate an immune response (*46*). HETE-PLs are likely to be generated in acute vascular events since during stroke and myocardial infarction, 12-LOX–expressing platelets form the major cell type within the initial thrombi and directly attach to sites of vascular injury. In turn, 15-LOX–expressing eosinophils are recruited to the initial platelet-rich thrombus where they contribute to eoxPL and thrombin generation and fibrin polymerization (*50*). Histological analysis of both murine and human thrombi shows that both eosinophils and eosinophil activation markers increase in patients with thrombotic diseases, such as stroke and myocardial infarction (*50*). Similarly, the absence of *ALOX12* or *ALOX15* in eosinophils results in partial protection from venous thrombus formation in mouse models (*46*, *50*).

Conversely, the procoagulant action of HETE-PLs could potentially be harnessed therapeutically in conditions of hemostatic failure, such as traumatic or surgical injury or bleeding disorders. We have found that HETE-PLs can completely prevent bleeding in mice genetically deficient in factor VIII and can improve thrombin generation in human plasma lacking factor VIII, IX, or X (*49*).

Leukocyte antibacterial and inflammation regulatory actions of eoxPLs

Circulating blood leukocytes are the first responders to injury, including neutrophils that engulf bacteria and monocytes, which differentiate into macrophages to participate in resolution of damage or wound healing. Both these cell types generate HETE-PLs that show bioactivities consistent with supporting innate immunity and defense against infection. Neutrophils generate 5-HETE-PLs through 5-LOX upon acute activation by bacteria or bacterial products such as *f*MLP and lipopolysaccharide (LPS) (*26*). These products are found in human bacterial infection in vivo, particularly infections caused by Gram-positive bacteria, and in vitro, they enhance superoxide and IL-8 generation and suppress neutrophil extracellular trap formation (*8*). Macrophages and monocytes generate eoxPLs through 15-LOX and 12/15-LOX, specifically four HETE-PEs and also their reduced carbonyl-containing analogs, ketoeicosatetraenoic acid PEs, which are formed by prostaglandin dehydrogenase reduction of HETE-PEs (*27*, *30*, *59*). We have found that HETE-PEs dampen cytokine generation in monocytes by inhibiting LPS-induced activation of Toll-like receptor 4 (TLR4) (*30*). However, HETE-PEs can also act as TLR4 agonists due to their structural similarity to LPS (*60*). It is likely that their dual action results from either direct weak activation of TLR4 or acting as competitive inhibitors of binding of the far more potent activator, LPS.

Role of eoxPLs and LOXs in Inflammatory and Immune Diseases

LOXs, particularly 15-LOX, are considered to be proinflammatory in murine models, including atherosclerosis, diabetes, hypertension, and arthritis. However, LOXs can generate many oxylipins, including HETEs, HODEs, leukotrienes, hepoxilins, lipoxins, and eoxPLs. In most cases, the specific lipids mediating their inflammatory actions are not clear. Below, we provide a summary of murine and human studies in this area that suggest an involvement for eoxPLs in diverse disease pathologies.

Immune-mediated inflammatory disease and eoxPLs

Multiple forms of oxPL and eoxPL molecular species are detected in both early and late atheroma, although it is still unclear which

of these derive from nonenzymatic oxidation or whether LOXs and/or COXs initiate their formation. In support of a potential role for LOXs, macrophages within plaque express high levels of *ALOX15* (*61*, *62*). Genetic ablation of *Alox15* reduces oxidation of low-density lipoprotein and ameliorates the formation of atherosclerotic lesions in several mouse models (*63*–*65*). Nonenzymatically generated oxPLs such as POVPC and PEIPC trigger the activation of vascular endothelial cells and smooth muscle cells in vitro and in vivo, stimulating the expression of chemokines and adhesion molecules (*66*–*68*). Similarly, these events can also be triggered by artificial overexpression of *ALOX15* in endothelial cells (*69*). In vitro–generated POVPC and PGPC also promote monocyte adhesion and leukocyte transmigration (*15*). Macrophages directly activated by a crude mixture of air-oxidized PC (oxPAPC; which will undoubtedly contain HETE-PCs) substantially change their phenotype, a phenomenon that also occurs in atherosclerotic plaques in vivo (*70*). These studies are consistent with a role of both eoxPLs and oxPLs in driving inflammatory vascular disease in vivo.

15-LOX–derived HETE-PLs increase in abundance in monocytes incubated with type 2 cytokines such as IL-4 and IL-14 (which induce this enzyme) and are detected in type 2 inflammation, including mouse models of lung allergy (*30*). In addition, *ALOX15* expression and HETE-PE abundance increased in bronchial epithelial cells and were observed in infiltrating alternatively activated macrophages (AAMs) and eosinophils (*27*, *50*). During asthma, 15-LOX has been implicated in mucus hypersecretion through HETE-PE generation and can contribute to airway epithelial injury (*71*).

Role of eoxPLs in immune tolerance

15-LOX and HETE-PEs have essential roles in controlling self-tolerance and the resolution of inflammation (*72*–*76*). Both enzyme expression and levels of HETE-PLs peak during resolution, coinciding with the appearance of eosinophils and AAMs and the reemergence of tissue-resident macrophages (*30*, *76*). During resolution, 15-LOX modulates the cytokine response of infiltrating immune cells and contributes to the nonimmunogenic removal of apoptotic cells by *Alox15*-expressing tissue-resident macrophages (*75*, *77*). 12-HETE-PEs are expressed on the surface of resident macrophages where they facilitate clearance of apoptotic cells by this macrophage subset (*75*). However, they appear to conversely interfere with the uptake of apoptotic cells by immunocompetent monocyte-derived macrophages and dendritic cells during inflammation (*75*). Generation of HETE-PEs thus facilitates a "silent waste disposal" that is executed by tissue-resident macrophages, ensuring a nonimmunogenic clearance of apoptotic cell-derived autoantigens, and enables maintenance of self-tolerance during inflammation (Fig. 4) (*75*). Generation of 15-LOX–derived HETE-PC and HpETE-PC by dendritic cells, in turn, seems to increase their activation threshold and helps these cells maintain an immature phenotype under basal conditions (*72*, *78*), an effect that partially involves activation of the redox-sensitive transcription factor Nrf2 (*72*, *79*). The absence of *Alox15* or inhibition of 15-LOX results in spontaneous activation and maturation of dendritic cells (*72*). Immune regulation by 15-LOX–derived HETE-PLs may explain why *Alox15*-deficient animals show spontaneous autoimmunity, including the presence of nuclear antibodies and exacerbated disease in models of systemic lupus erythematodes, multiple sclerosis, rheumatoid arthritis, and systemic sclerosis (*72*, *75*, *80*–*82*).

The context dependence of the proinflammatory and anti-inflammatory actions of eoxPLs

It must be emphasized that mechanisms underlying the proinflammatory, as well as the anti-inflammatory, and immune modulatory properties of oxPLs and eoxPLs are still incompletely understood and likely depend on the cell type, the site of generation, and the type and amount of the individual phospholipid species, including the repertoire of FA side chains. Studies addressing the biological properties of nonenzymatically formed oxPLs, primarily oxPC derivatives, show that these lipids share common immunomodulatory features of eoxPLs, such as HETE-PLs. Low concentrations of oxPCs and eoxPLs are anti-inflammatory and block LPS activation and maturation of dendritic cells, macrophages, and endothelial cells (*30*, *72*, *78*, *79*, *83*, *84*). Proposed anti-inflammatory mechanisms involve blockade of the cell surface and soluble pattern recognition receptors (PRRs) such as TLR4, CD14, and LPS-binding protein, as well as activation of Nrf2 (*72*, *79*, *83*, *84*). On the other hand, high amounts of oxPCs or increased or overwhelming production of eoxPLs can result in the inflammatory activation of these same cell types. Here, a shift from a blockade to an activation of TLRs, coupled with binding to additional PRRs such as CD36, suggests partial agonistic properties on TLR and PRR signaling (*60*, *85*, *86*). In addition, binding of POVPC to GPCRs such as the platelet activating factor (PAF) receptor has been described, although given the requirement for PAF receptor activating lipids to be oxidatively truncated, a role for cell-generated HETE-PLs is unlikely in this case (*87*). High concentrations of oxPAPC mixtures trigger the intracellular inflammasome pathway, leading to the activation of caspase 11 and release of IL-1β (*88*). These findings suggest that biological properties of

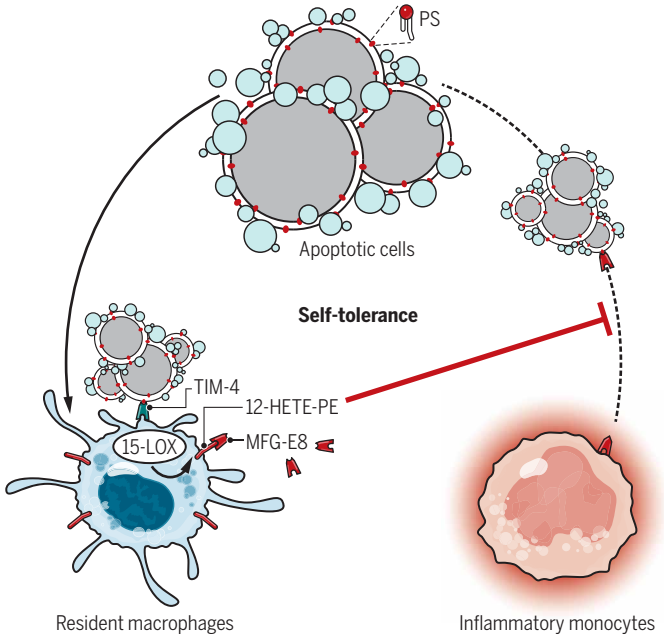

Fig. 4. eoxPLs on the surface of resident macrophages facilitate clearance of apoptotic cells. In this mechanism, MFG-E8 binds to PS on apoptotic cells and macrophages, acting as a bridge to facilitate recognition and uptake by the macrophages themselves. Conversely, this mechanism prevents uptake of apoptotic cells by inflammatory monocytes that do not generate eoxPLs. Resident macrophages use the PS receptors TIM-4 and MFG-E8 to clear apoptotic cells, whereas inflammatory monocytes recognize PS with MFG-E8.

both eoxPLs and nonenzymatically generated oxPLs are dose- and context-dependent and that the generation of eoxPLs needs to be tightly balanced to maintain homeostasis. We note that oxPAPC that contains hundreds to thousands of products is still often used for biological studies, including some cited above. Unfortunately, the use of these mixtures makes it unclear which specific lipid mediates the biological effect and difficult to delineate the effective concentrations and biological relevance of active lipids.

Emerging Roles for eoxPL in Ferroptosis and Pathogen Biology

Ferroptosis is a regulated form of necrosis that is implicated in diverse processes including cell death during tissue turnover, cancer cell death, and aggravation of tissue injury (*89*). Ferroptosis involves the rapid and massive generation of eoxPLs (mostly PE, including oxidized adrenic acid–containing forms) by PL peroxidation in an iron-dependent manner (*90*–*92*). The presence of PLs with long polyunsaturated ω6 FAs within cellular membranes is a prerequisite for eoxPL formation during ferroptosis and, in turn, depends on enzymes involved in PL synthesis, such as ACSL4 (*90*). Onset of lipid peroxidation during ferroptosis involves insufficiency and shutdown of antioxidative enzymes such as GPX4 and LOX-mediated peroxidation that at least partially involves 15-LOX (Fig. 5). Insights into the physiological and pathological implications of this type of cell death are currently emerging.

Although most of our knowledge on eoxPL biology comes from their generation in murine or human systems, pathogens such as *Pseudomonas aeruginosa* and *Toxoplasma gondii* express enzymes with 15-LOX activity. The genes encoding these enzymes have been most likely acquired by horizontal gene transfer by these organisms because they display a high sequence homology to mammalian *ALOX15* (*93*, *94*). These LOXs can generate HETE-PLs and other eoxPLs and might contribute to the pathogenicity of these pathogens, for example, by enabling immune evasion or the induction of hemolysis or even ferroptosis (*95*, *96*).

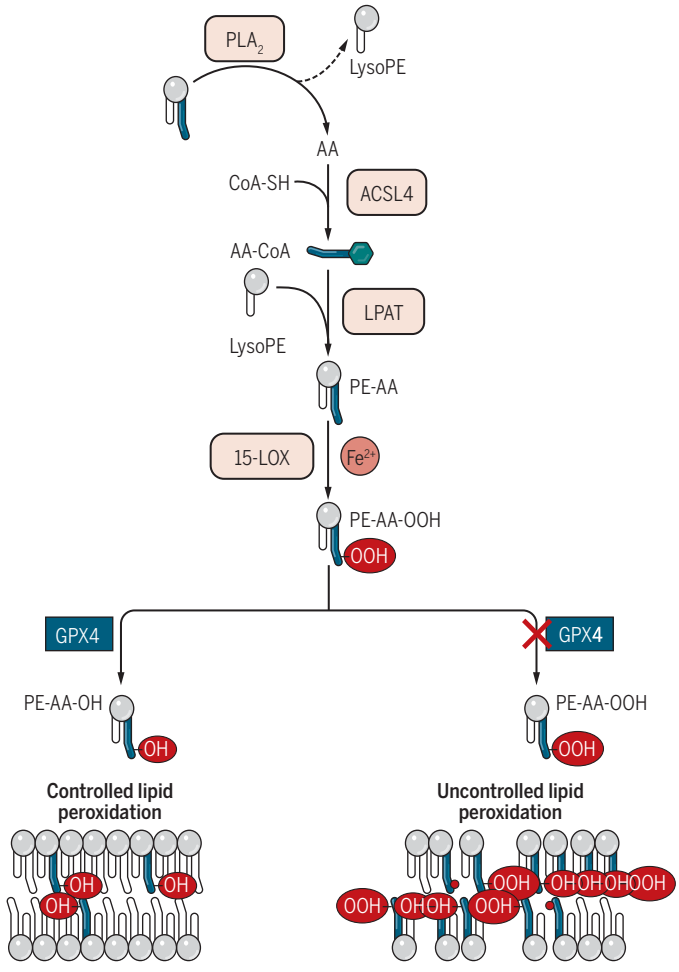

Fig. 5. eoxPL formation is required for ferroptosis. The following mechanism of how eoxPLs support ferroptosis has been proposed, although the detailed enzymology is not yet fully clear. First, the formation of PLs with long PUFA chains is facilitated by ACSL4. Then, oxidation of PE to form hydroperoxides is mediated by 15-LOX. Last, iron-dependent lipid peroxidation during ferroptosis involves insufficiency and shutdown of antioxidative enzymes such as GPX4 and LOX-mediated peroxidation that at least partially involves 15-LOX.

The Historical Context of the Analysis of oxPLs with MS

The ability to analyze oxPLs and eoxPLs as chemical entities emerged with the advent of modern biological MS. The first report of molecular ions generated from intact PC used field desorption ionization (*97*). However, it was not until fast atom bombardment (FAB) ionization was introduced by Barber in 1981 that intact PC could be widely studied (*98*, *99*). FAB was developed in parallel with MS/MS and is the most widely used method of PL analysis today. In this method, collisional activation (through multiple collisions with a neutral gas) is used to initiate carbon-carbon bond cleavage, forming product or daughter ions that can be separately analyzed (*100*). This method is also called collision-induced dissociation (CID). The terminal sector of tandem instruments then enabled determination of the mass/charge ratio (*m*/*z*), providing the molecular mass of the PL. Another important advance was the development of high-performance MS/MS instruments that enabled measurement of precursor and product ions with a high mass accuracy, enabling the calculation of the exact elemental composition (high-resolution MS). Understanding the gas phase ion chemistry that occurs as a result of CID permitted one to propose structures consistent with the elemental composition of FA, PL, and oxPL ions. A detailed knowledge of PL and eicosanoid and prostaglandin gas phase chemistry has developed over the past few decades due to studying the MS behavior of known lipids (*101*, *102*). Thus, it is now possible to characterize not only the structures but also the mechanisms of lipid oxidation by either enzymatic or nonenzymatic pathways in cell and tissue samples using MS analysis (*103*).

The ionization methods most often used to analyze FAs, eicosanoids, prostaglandins, PLs, oxPLs, or eoxPLs are electrospray ionization (ESI), discovered by Nobel laureate John Fenn, and matrix-assisted laser desorption ionization (MALDI) (*104*) and were developed in the mid-1980s. Ions generated by MALDI or ESI undergo similar CID decomposition mechanisms. Thus, understanding the ion chemistry of oxPLs is relevant to observations made by either method. ESI is widely used because of its ease of implementation and the breadth of molecules that can be analyzed including nonvolatile molecules such as PLs and their various oxidation products. In addition, with ESI, it is easy to interface the mass spectrometer to the effluent of a liquid chromatographic column, thus presenting purified material for either MS or MS/MS. Chromatography is

important because it enables the separation of closely related PLs including isobaric species (with the same *m/z* values) and isomers so that their MS behavior can be determined separately. For readers wishing to learn more about MS/MS of these oxidized lipid species and for libraries of MS/MS spectra, we direct the reader to reviews on this topic and to the LIPID MAPS (www.lipidmaps.org) database and resource (*101*–*103*). In addition, LIPID MAPS have introduced new tools to aid in the analysis of these lipids, including dedicated structure drawing tools and a computationally generated database of oxPL chain composition masses.

Summary

Here, we have summarized the state of the art relevant to the chemistry, biochemistry, and cellular and clinical biology of oxPL forms that are generated by physiological enzymatic pathways. Individual cell types can generate large numbers of eoxPLs, with the most abundant being HETE-PLs, and the full cellular complement of eoxPLs in health and disease remains to be characterized in detail. The first discovered were mainly PE lipids, and additional analogs are being found in human cells that include other PL classes such as PIs. Much work remains to be done to fully understand the diversity of eoxPLs and their detailed biological functions in innate and adaptive immunity. The possibility of targeting the lipids and their biosynthetic enzymes to prevent inflammatory, immune, or thrombotic diseases is now emerging.

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