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Enzymatically-oxidized phospholipids (eoxPL) assume center-stage as essential regulators of innate immunity and cell death.

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Enzymatically-oxidized phospholipids (eoxPL) form in through regulated highly processes by attaching eicosanoids or prostaglandins to phospholipids (PL) in immune cells. These comprise structurally diverse families of biomolecules with potent bioactivities, and it is becoming increasingly clear that they possess significant immunoregulatory roles in both health and disease. The idea that oxidized PL (oxPL) can even form via enzymatic pathways and signal biologically in their own right has only recently been realized. This new paradigm is changing our understanding of eicosanoid, prostaglandin and PL biology in health and disease. eoxPL display emerging roles in cellular events that include ferroptosis, apoptosis and blood clotting, and diseases such as arthritis, diabetes and cardiovascular disease. They are increasingly being recognized as endogenous bioactive mediators and potential targets for drug development. This review will describe new evidence that is placing eoxPLs and their biosynthetic pathways center stage in immunoregulation.

Introductory paragraph

Phospholipid (PL) oxidation occurs widely in inflammatory vascular disease, but for many years was considered to involve uncontrolled chemical mechanisms that are mediated by free radicals. However, emerging evidence indicates that enzymatically-oxidized PL (eoxPL), generated by lipoxygenases (LOX) or cyclooxygenases (COX), are abundant biomolecules formed during the acute response to injury in multiple innate immune cell types. eoxPL form via the coupling together of well-known eicosanoid/prostaglandin pathways with the Lands' cycle enzymes, or by direct enzymatic oxidation of PL. New studies suggest eoxPL are important players in early innate immunity, particular in promoting blood clotting and defense against infection. Their discovery and characterization was enabled by parallel development and application of electrospray ionization tandem mass spectrometry (ESI/MS/MS) technology. While this review focuses primarily on eoxPL, we start by introducing the closely-related, non-enzymatically generated oxPL. This is to orientate the reader new to this field with the knowledge base that led up to the discovery and characterization of eoxPL, as a distinct but related family of bioactive lipids.

Enzymatic versus non-enzymatic oxidation of PL.

eoxPL are considered to be functionally separate from the much larger family of lipids called oxidized phospholipids (oxPL), that have been known about since the 1980's. We note that there can be considerable overlap in structures between eoxPL and oxPL families, thus determining their specific origin in complex biological samples requires the use of several approaches. This includes characterizing their formation in mice lacking eoxPL generating

enzymes, determination of enantiomeric composition, and careful use of pharmacological inhibitors. These approaches are expanded on below.

oxPL form by **non-enzymatic** oxidation of intact PL where a hydrogen atom is first abstracted from a bis(allylic) methylene group in a polyunsaturated FA (PUFA) chain, forming a lipid alkyl radical (Figure 1 A). Reactive oxygen species (ROS) generated during inflammation (e.g. by cytokine/agonist-activated immune cells) that can directly oxidize lipids include hydroxyl radical ($\bullet\text{OH}$), hypochlorite (HOCl), peroxynitrite (ONOO^-) and nitrogen dioxide ($\bullet\text{NO}_2$) (1). In contrast, eoxPL formation is **enzymatic** and results in a far more restricted set of products formed via highly controlled pathways in innate immune cells. Both oxPL and eoxPL generation proceed initially via the same mechanisms involving hydrogen abstraction from PUFA, but differ in several key ways that are outlined in detail below. The study of oxPL has preceded the discovery of eoxPL, but 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 disease. Our current view is that eoxPL represent physiological mediators of cell signaling that are made during innate immunity to limit bleeding and infection. However it is likely that they are also generated at higher levels and thus contribute to vascular inflammation. On the other hand, oxPL generated non-enzymatically are always considered harmful, contributing to autoimmune and inflammatory disease as well as cell death.

Non-enzymatic PL oxidation: chemical mechanisms

The knowledge that lipids can be non-enzymatically oxidized to oxPL 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 outlined in the 1940s (5). A major review was compiled in the early 1950s (6).

Following formation of an alkyl radical (*initiation*), delocalization and oxygen addition occurs at diffusion-limited rates to form a hydroperoxyl radical ($\bullet\text{OOH}$) intermediate (Figure 1 B). This undergoes various chemical reactions to form a host of oxPL products, including PL-OOH, by *propagation* and *termination* reactions (Table 1, Figure 1 B). A variant of this 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 formation of an ozonide (7). Both ozonides and PL-OOH undergo many secondary reactions and multiple oxPL can be formed including terminal aldehydes and carboxylic acids. Ozonides form in the protected environment of the lung where inspired O_3 from pollution encounters PL in the pulmonary surfactant (8). The downstream reactivities/metabolism of PL hydroperoxides (-OOH) are now well characterised along with the diversity of products derived from free radical (chemical) auto-oxidation, and the reader is guided to relevant references (9-11).

An important feature of non-enzymatic oxidation to form oxPL is that products form without stereochemical control, that are racemic and regioisomeric. This distinguishes oxPL from eoxPL, where enzymatic mechanisms control of oxygen insertion yielding specific enantiomers. Analysis of these products using chiral chromatography can thus help to distinguish oxPL from eoxPL.

The generation of oxPL in vascular disease

Research into vascular formation of oxPL started in the 1980s-90s, led by Berliner, Leitinger, Salomon and colleagues (reviewed in (11)). Early work showed that oxPL are

present in atherosclerotic plaque, and this stimulated 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 looked for, or were present in lower amounts. At that time, synthetic oxPC was generated in vitro via air/metal-catalyzed oxidation and then applied to cellular or in vivo systems, in which pleiotropic bioactivities relevant to inflammation were demonstrated(11). This led to the idea that oxPL is formed in excess and promotes inflammation, driving atherosclerotic vascular disease. This was further supported by findings from Witztum and Hörkko that mice with atherosclerosis generated oxPL-specific IgM antibodies from the innate immune system, whose levels had an inverse relationship with disease, and provided vascular protection through mopping up these lipids(16-19). However, synthetic oxPL is a complex mixture of hundreds of products. When generated by different labs using unstandardized conditions there was inconsistency in terms of chemical composition, which usually was not defined. This led to oxPL preparations showing opposite biological actions in the same assay systems (11). Later, in the 90's, researchers began to separate the components of synthetic air-oxidized oxPL. A number of forms, including truncated lipids where the PUFA has been oxidatively shortened were purified and studied in isolation(20-23). These, including 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 were also detected in human and murine atheroma lesions(12-15). To date there is only an incomplete understanding as to how these form in vivo. While generally assumed to be generated non-enzymatically, we could speculate that enzymes such as LOXs or COXs might catalyze the initial oxidation forming lipid hydroperoxides. If antioxidants are depleted, then these could be non-enzymatically 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 a plethora of 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 LA is also known to take place in the mitochondria by cytochrome c leading to the formation of cardiolipin-OOH(25).

Enzymatic oxidation of PL forms eoxPL.

In contrast to uncontrolled non-enzymatic oxidation described above, we now know that the cellular generation of eoxPL is a highly controlled process, mediated by redox active proteins (enzymes). As for oxPL formation, molecular oxygen is inserted into a PUFA group to generate a lipid-OOH, however, a far more restricted set of structures is formed, including specific regio- and stereoisomers, thus these are termed eoxPL (26-29). The overall mechanism is as in Figure 1, 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 due to steric positioning within the active site. Several cell types of the innate immune system generate eoxPL, with the enzyme isoforms involved being cell-type specific(29).

There are two distinct mechanisms for eoxPL formation, *direct oxidation* of an intact PL, or *indirect oxidation* where a preformed oxidized FA (oxFA) is inserted into a lysoPL via the

enzymes of the Lands remodeling cycle, and these are described below in more detail (Figure 2 A,B).

Direct enzymatic oxidation of PL is catalyzed by the leukocyte 15-lipoxygenase (LOX) (human) or 12/15-LOX (murine) (both from the *ALOX15* gene) (Figure 2 A) (27,30). This is highly expressed in human eosinophils and 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 following stimulation by calcium ionophore(33). Later, human monocytes were shown to have 15-HETE esterified to PL, specifically phosphatidylethanolamine (PE)(27). Similarly, in mice, leukocytes including eosinophils and resident peritoneal macrophages generate 12-HETE-PEs via *Alox15*(30). Unlike indirect oxidation (described below), eoxPL from *ALOX15* are present in resting cells, and their formation doesn't strictly require agonist activation. This suggests they play homeostatic roles in innate immunity. Direct oxidation of PE to form HETE-PE was demonstrated in both mouse and human monocyte/macrophages using a stable isotope dilution MS approach, monitoring for ¹⁸O incorporation following cell activation in ¹⁸O-H₂O buffer (27,30). Lack of incorporation is consistent with direct oxidation, since there is no cycle of hydrolysis and re-esterification.

Indirect enzymatic oxidation describes formation of eoxPL via the Lands' pathway (Figure 2 B). The definitive step of this enzymatic reaction involves a CoA ester of an oxFA formed by a long-chain fatty acyl CoA synthetase (ACSL). This is then esterified onto a lysoPL acceptor, by lysoPL acyl transferases (LPAT), which have also been termed membrane bound O-acyl transferases (MBOAT). Recently, the complete cycle of eoxPL formation was

revealed as a rapid and co-ordinated process that occurs in several immune cell types(26,28). This comprises (i) PL hydrolysis by PLA₂ releasing FA, (ii) formation of oxFA (typically an eicosanoid or prostaglandin) by FA oxidation catalyzed by COX or LOX, then (iii) re-esterification of the oxylipin to a lysoPL to form eoxPL by FACL+LPAT. This occurs in platelets and neutrophils, where FA oxidation is primarily catalyzed by 12-LOX (*ALOX12*) or 5-LOX (*ALOX5*) respectively(10,28). In platelets, the most quantitatively abundant eoxPL comprise 12-HETE attached to PE or PC. This is not surprising since AA represents the most abundant long-chain PUFA in immune cell PL membranes, followed by linoleic acid (LA) and docosahexanoic acid (DHA). Also, PE and PC are the most abundant PL classes in the plasma membrane, and include both acyl and plasmalogen forms. 14-hydroxydocosahexanoic (HDOHE) acid-PEs are also detected in platelets, arising from DHA oxidation by 12-LOX(34). Similarly, fMLP-stimulated neutrophils generate primarily 5-HETE-PE/PCs via the action of 5-LOX on AA, followed by esterification of 5-HETE into lysoPL(26). A requirement for hydrolysis and re-acylation was confirmed in both platelets and neutrophils using ¹⁸O-H₂O stable isotope dilution MS, and/or thimerosal to block fatty acyl esterification (26,28).

Our studies show that *ALOX15* mediates direct PL oxidation, while *ALOX5* and *ALOX12* generate HETE-PEs via Land's cycle remodeling in innate immune cells. This is fully in line with what has been known regarding the substrate preferences of these proteins, based on older *in vitro* studies using recombinant or purified enzymes for many years. Unlike other LOX isoforms, the purified 15-LOX1/12/15-LOX was well known to be capable of direct oxidation of PL substrates(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- and HETE-PEs have been detected, arising from LOX turnover(27,28). Approximately a third of all the 12-HETE generated by human platelets is esterified into PC and PE, on the same timescale as formation of free 12-HETE(28). Thus, eoxPL form rapidly on cellular activation and are abundant lipids, present at picomole to nanomole levels. This temporal generation suggests that the synthetic enzymes are co-located and working co-operatively. In support, exogenously added stable-isotope labeled 12-HETE is not incorporated into PE in thrombin-stimulated platelets during endogenous 12-HETE-PE formation(28). This indicates a new paradigm for the Lands' cycle, generating immune bioactive eoxPL lipids in circulating human and murine innate blood cells via remodeling of their biomembranes, as an acute response to stimulation.

A second way in which eoxPL may form via *indirect oxidation* involves transcellular uptake of precursor oxylipins (generated in a different cell type), followed by their esterification. Early studies from Serhan and others in the '90s focused on this route, adding exogenous oxylipins to cells and measuring their esterification into PLs. For example, exogenous 15-HETE (a monocyte/eosinophil LOX product) is incorporated into PI in neutrophils, and then subsequently released on fMLP activation of phospholipases(37). However, rates of uptake and esterification of exogenous substrate take place on relatively long timescales (several minutes - hrs) and whether this occurs *in vivo* is unknown. The phenomenon of oxylipin incorporation into lysoPLs has also been observed for epoxyeicosatetraenoic acid (EETs) generated by cytochrome P450 enzymes (38,39). In that example, a potential role for EET-PLs in altering membrane microdomain properties or acting as a releasable pool of oxylipins was proposed (38). Esterification of 14,15-EET occurs in mastocytoma cells with half

maximal incorporation around 30 min (40). Thus it is on a similar timescale to transcellular oxylipin incorporation described above, but considerably slower than endogenous HETE-PE generation in immune cells. It has been suggested that PL incorporation of oxylipins through this transcellular uptake route leads to formation of a pool of “stored” eicosanoids that can be released later. However, even if this is the case, eoxPL such as HETE-PEs possess immunoreactive bioactivities in their own right that require the oxylipin to remain PL esterified. These are described in detail later.

In addition to *ALOX12*, platelets can also generate eoxPL via COX-1, specifically PGE₂-PE and PGD₂-PE (41,42). However despite the quantitative abundance of thromboxane-B₂ (TXB₂), attachment of this eicosanoid to PLs has never been detected. This suggests that esterification pathways may show oxylipin selectivity, in line with recent studies from Klett using purified ACSLs (43). To date, studies examining cellular COX-2 generation of eoxPL have not been undertaken, but this could represent a source of esterified prostaglandins during chronic inflammation.

Up to 2016, a relatively small number of eoxPL molecular species had been determined in innate immune cells, typically 4-6 molecular species per cell type. However, human platelets were recently shown to rapidly form > 100 unique eoxPL structures, from *ALOX12* and/or COX-1 on thrombin activation(44). The most abundant are PEs, however PC forms are also prevalent, and rarer HETE-PI forms were recently discovered(44,45). As well as AA or DHA-derived eoxPL 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 either >2 - 3 oxygens are also generated(44). These lower abundance eoxPLs have so far only been measured in platelets but may also be formed in other cells expressing high levels of LOXs

or COXs during inflammation. Many eoxPL are not fully structurally elucidated yet, with position of oxidation sites still unassigned. Several tandem mass spectra are available, derived from platelet eoxPL, for future characterization(44).

Indirect enzymatic oxidation requires agonist activation of innate immune cells, achieved in platelets or neutrophils by thrombin, collagen or fMLP. In platelets, thrombin activates protease activated receptors (PAR)1 and PAR4, while fMLP activates FPR1 in neutrophils. This then leads to downstream calcium mobilization and phospholipase activation, both required for eoxPL generation(26,28). Pharmacological inhibitors have suggested involvement of additional signaling pathways in platelets and neutrophils, including protein kinase C, src tyrosine kinases, phospholipase C, MAP Kinases, cPLA₂, sPLA₂ 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 of importance to innate immunity.

Approaches to distinguish eoxPL from oxPL in cellular and tissue systems

There is considerable structural overlap between oxPL and eoxPL species. Furthermore, whether oxPL can be formed via decomposition of eoxPL (e.g. 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 oxPL detected in plasma are unknown, and there is ongoing debate regarding the origin of lipid oxidation products that drive ferroptosis. Despite this, several approaches can help delineate the origin of oxidized PL as follows:

1. *Tissue or cells lacking Alox through genetic deletion or pharmacological inhibition.*

Mice lacking several *Alox* isoforms are available, and both *Alox12^{-/-}* and *Alox15^{-/-}*

mice have been shown to be unable to 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 to this is MK886, an inhibitor of 5-LOX activating protein (FLAP) which as used to implicate 5-LOX in neutrophil HETE-PL formation (26). Pharmacological inhibitors for COX including aspirin, indomethacin or COX isomer specific inhibitors such as celecoxib can also be used to implicate this pathway in esterified PG formation(41).

2. *Reverse phase and chiral chromatography to confirm regio- and enantiomeric specificity.* EoxPL formation involves regio- and stereo-specific oxygen insertion by enzymes. For example, platelets generate primarily 12SHETE-PL isoforms(28). Thus, a predominant isomeric composition reflective of enzymatic activity can distinguish oxPL from eoxPL. MS/MS fragmentation (monitoring the intact HETE fragment, versus internal daughter ions that arise from HETE fragmentation on collision-induced-dissociation), or alternatively saponification of purified phospholipids followed by HPLC-UV detection of HETE positional isomers can first determine regio-specificity. Following this, free acid HETEs obtained by saponification can be subject 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/importance of eoxPL vs oxPL 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 -PC positional isomers generated by either soybean LOX or air oxidation (47). Using these, isolated platelets and neutrophils were found to generate ng amounts of HETE-PEs exclusively via enzymatic mechanisms, while truncated oxPL were not detected to form (26-28,30). Indeed, platelets esterify approximately 30% of newly synthesized 12-HETE into PL pools during the same timescale as free 12-HETE synthesis. This provides an example where eoxPL predominate, however we suspect that in chronic inflammation, where reactive oxygen and nitrogen species are generated, the situation may change over time. In support, a previous study comparing enantiomeric specificity of esterified HODEs in human atheroma lesions showed a predominance of the 13S form in early lesions, but a racemic mixture in more progressed disease (48). For testing involvement of lipids in disease, *Alox*-deficient mice are a suitable model, however these enzymes make many oxylipins making ascribing function to specific lipids challenging. To that end we recently administered HETE-PLs to mice, either locally into the tail, or systemically via tail vein and found them to be well tolerated, with acute effects on hemostasis and thrombosis being seen in line with their proposed pro-coagulant actions (46,49).

Functions of eoxPL during innate immunity.

Although LOX isoforms are different gene products, separated in evolution 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 “response to injury” or acute trauma, such as following challenge by haemostatic or infectious agents(29). This suggests a protective role in this context, where prevention of bleeding or infection is a primary goal.

Conversely, their generation in excess may promote vascular inflammation, in particular arterial thrombosis in atherosclerotic disease, or venous thrombosis(46,50). Of relevance to this, a large literature on the bioactions of non-enzymatically-generated oxPL (including structures that we now know are also eoxPL, such as HETE-PE) exists showing they can be either pro- or anti-inflammatory depending on the context(11,51). However, in many studies on oxPL, the lipids were derived by air/chemical oxidation and biological actions cannot be attributed to any specific molecules. An exception to this is POVPC, which is recognized by the CD36 scavenger receptor, and thus considered a damage-associated molecular pattern (DAMP) molecule(52).

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

high affinity receptor-ligand interactions, in a manner similar to eicosanoids or prostaglandins.

Innate immune cells undergo significant changes to their membrane structure on agonist activation, including spreading, adhesion, chemotaxis, microvesicle formation, degranulation, etc. How plasma membranes containing HETE-PLs may influence these phenomena is currently not understood. Hints come from studies on chemical oxidation of membranes. For example, mixtures of oxPL 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). Also, high concentrations (mg/ml) of purified 15-LOX cause pore formation in purified organelle membranes, through lipid peroxidation, and its overexpression 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 via generation of HETE-PLs).

Alternatively eoxPL can act as low-affinity ligands for nuclear receptors, such as PPAR γ or regulate signaling by Toll like receptors via their structural similarity with bacterial lipids such as lipopolysaccharide (LPS)(30,56). These actions will be summarized below.

(i) Haemostasis and thrombosis

Aminophospholipids (aPL), 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 via

scramblase activity(57,58). Then, negatively-charged Gla domains of plasma coagulation factors associate with PS and PE on the activated platelet membrane surface *via* calcium ion binding. Thus, coagulation factors come together on a PL surface, allowing them to effectively interact, facilitating proteolytic cleavage and ultimately thrombin generation(57,58). 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 recent studies support an essential role for eoxPL 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 rates of thrombin generation and ultimately promoting haemostasis (Figure 3) (46,49,50). Mice genetically lacking HETE-PLs (either *Alox15* or *Alox12* deficient) bleed longer on challenge, suggesting that endogenous eoxPL support haemostasis *in vivo*(46,50). Injection of HETE-PEs restores *in vivo* hemostasis in mice, evidenced by elevated thrombin-anti-thrombin (TAT) complexes, and rescues the bleeding phenotype(46). The mechanisms have been extensively investigated. Purified HETE-PE or HETE-PCs increase thrombin generation in human plasma, and molecular dynamics simulations show that the –OH group of the HETE resides close to the polar external face of the membrane, interacting with calcium and pushing PL headgroups apart(46,49). HETE-PLs promote calcium 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 should be found on the outside of the lipid bilayer, since this is where PC primarily is located. Externalization of HETE-PE has been detected in platelets and macrophages. Here, exposure of eoxPL parallels that of PS, but occurs independent of apoptosis via incompletely defined pathways(59). In platelets, the protein TMEM16F contributes to non-apoptotic PS exposure and is likely involved in the exposure of eoxPL in these and other cells(59). Notably, platelets are not the only cell type that utilizes HETE-PLs to initiate coagulation. Eosinophils also play a key role in this process, with recent studies showing that both human and murine eosinophils generate HETE-PEs, and that *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 ADP). Instead, their procoagulant activity relies on PS externalization being stimulated by ADP, with the ability of PS to support coagulation being significantly enhanced by *ALOX15*-derived eoxPL already basally present in the cell membrane(50).

Recent evidence indicates that HETE-PLs are involved in the pathogenesis of both arterial and venous thrombosis. For example, they are found elevated in circulating leukocytes and platelets from patients with antiphospholipid syndrome (human venous thrombosis), where they also stimulate an immune response(46). During stroke and myocardial infarction, *ALOX12*-expressing platelets form the major cell type within the initial thrombi and directly attach to sites of vascular injury. *ALOX15*-expressing eosinophils, in turn, are recruited to the initial platelet-rich thrombus where they seem to essentially contribute to eoxPL generation as well as to thrombin generation and fibrin polymerization(50). Histological analysis of both murine and human thrombi shows both eosinophils and eosinophil activation markers are

elevated in patients with thrombotic diseases such as stroke and myocardial infarction(50). Similarly, absence of *ALOX12*, *ALOX15* or of eosinophils results in partial protection from venous thrombus formation in mouse models(46,50).

Conversely, the pro-coagulant action of HETE-PLs could potentially be harnessed therapeutically in conditions of hemostatic failure, such as trauma/surgical injury or bleeding disorders. We found that HETE-PLs can completely prevent bleeding in mice genetically deficient in Factor VIII (FVIII), and can improve thrombin generation in human plasma lacking either FVIII, IX or X(49).

(ii) Leukocyte antibacterial and inflammation regulatory actions of eoxPL

Circulating blood leukocytes are the first responders to injury, including neutrophils which engulf bacteria, and monocytes that differentiate into macrophages to participate in resolution of damage/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 via *ALOX5*, on acute activation by bacteria or their products such as fMLP and LPS(26). These are found in human bacterial infection in vivo, particularly Gram^{+ve}, and in vitro they enhance superoxide and IL-8 generation, while suppressing neutrophil-extracellular trap formation(8). Macrophages/monocytes generate eoxPL via *ALOX15*, specifically four HETE-PEs and also their reduced carbonyl-containing analogs, ketoeicosatetraenoic acid (KETE)-PEs formed by prostaglandin dehydrogenase (PGDH) reduction of HETE-PEs(27,30,56). We found that HETE-PEs dampen cytokine generation in monocytes by inhibiting LPS-activation of TLR4(30). However, HETE-PEs can also act as TLR4 agonists due to their structural similarity with LPS(60). It is likely that

their dual action results from either (i) direct weak activation of TLR4, or (ii) acting as competitive inhibitors of binding of the far more potent activator, LPS.

Role of eoxPL/LOXs in inflammatory and immune disease.

Multiple studies show that LOXs, in particular *ALOX15* are pro-inflammatory in murine models, including atherosclerosis, diabetes, hypertension and arthritis. However LOXs can generate many oxylipins, including HETEs, HODEs, leukotrienes, hepoxilins, lipoxins, etc, as well as eoxPL and 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 strongly suggest an involvement for eoxPL in diverse disease pathologies.

(i) Immune mediated inflammatory disease and eoxPL

Multiple forms of oxPL/eoxPL molecular species are detected in both early and late atheroma, however, as described earlier, it is still unclear which of these derive from non-enzymatic oxidation or whether LOXs/COXs initiate their formation. In support of a potential role for LOXs, macrophages within plaque express high levels of *ALOX15* (61,62). Genetic absence of *ALOX15* reduces oxidization of LDL and ameliorates formation of atherosclerotic lesions in several mouse models(63-65). Non-enzymatically generated oxPLs such as POVPC and PEIPC trigger activation of vascular endothelial cells and smooth muscle cells in vitro and in vivo, stimulating expression of chemokines and adhesion molecules(66-68). Similarly, these events can be also 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 (which will undoubtedly contain HETE-PCs) substantially change

their phenotype, a phenomenon that is also seen in atherosclerotic plaques in vivo (70). These studies are consistent with a role of both eoxPL and oxPL in driving inflammatory vascular disease in vivo.

ALOX15-derived HETE-PLs are upregulated in monocytes by incubation with type 2 cytokines, IL4/IL14, and are detected in type 2 inflammation, including mouse models of lung allergy (30) Also *ALOX15* expression and HETE-PE are elevated in bronchial epithelial cells and observed in infiltrating alternatively-activated macrophages (AAM) and eosinophils(27,50). During asthma *ALOX15* has been implicated in mucus hypersecretion via HETE-PE generation and can contribute to airway epithelial injury (71).

(ii) Role of eoxPL in immune tolerance

Recent studies uncovered an essential role for *ALOX15* and HETE-PEs during controlling self-tolerance and resolution of inflammation(72-76). Both enzyme expression and levels of HETE-PLs peak during resolution coinciding with appearance of eosinophils and AAMs and the re-emergence of tissue resident macrophages (30,76). During resolution, *ALOX15* modulates the cytokine response of infiltrating immune cells and contributes to the non-immunogenic removal of apoptotic cells by *ALOX15*-expressing tissue-resident macrophages (75,77). In this, 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 immune-competent 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 non-immunogenic clearance of apoptotic cell-derived autoantigens, and enabling maintenance of self-tolerance during

inflammation (Figure 4) (75). Generation of *ALOX15*-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 during steady state (72,78). This partially involves activation of the redox-sensitive transcription factor Nrf2(72,79). Absence or inhibition of *Alox15* results in spontaneous activation and maturation of dendritic cells(72). Immune-regulatory features of *ALOX15*-derived HETE-PLs may explain why *Alox15*-deficient animals show spontaneous autoimmunity including the presence of anti-nuclear antibodies, and exacerbated disease in models of systemic lupus erythematoses, multiple sclerosis, rheumatoid arthritis and systemic sclerosis(72,75,80-82).

(iii) The pro- vs anti-inflammatory actions of eoxPL are context dependent.

It has to be emphasized that mechanisms underlying the pro-inflammatory as well as the anti-inflammatory and immune modulatory properties of oxPL and eoxPL are still incompletely understood and likely dependent on cell type, site of generation as well as on type and amount of the individual phospholipid species, including the repertoire of FA side chains. Studies addressing the biological properties of non-enzymatically oxPL, primarily oxPC derivatives, show that these lipids share common immune-modulatory features of eoxPL, usually HETE-PLs. Low concentrations of oxPC/eoxPL are anti-inflammatory, blocking LPS activation and maturation of dendritic cells, macrophages and endothelial cells (30,72,78,79,83,84). Proposed anti-inflammatory mechanisms involve blockade of cell-surface and soluble pattern recognition receptors (PRRs) such as TLR4, CD14 and LBP as well as activation of Nrf2, exerting potent anti-inflammatory activity (72,79,83,84). High amounts of oxPC or increased/overwhelming production of eoxPLs, on the other hand, can result in the inflammatory activation of the same cell types. Here, a shift from a blockade to an activation of TLRs as well as binding to additional PRRs such as CD36 suggestive of

partial-agonistic properties on TLR and PRR signaling has been observed(60,85,86). Also binding to G-Protein-coupled receptors such as the PAF receptor by POVPC 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). It was recently shown that high concentrations of air-oxidized PC mixtures trigger the intracellular inflammasome pathway leading to activation of caspase 11 and release of IL-1 β (88). These findings suggest that biological properties of both eoxPL and non-enzymatically generated oxPLs are highly dose- and context-dependent and that generation of eoxPLs needs to be tightly balanced to maintain homeostasis. We note that air-oxidized PC (oxPAPC) which contains 100s -1,000s of products is still often used for biological studies, including some cited above. Unfortunately, using these mixtures, it is not only unclear which specific lipid is mediating the biological effect, but also the effective concentrations of active lipids and their biological relevance are also difficult to delineate.

Emerging roles for eoxPL in ferroptosis and pathogen biology

Ferroptosis is a regulated form of necrosis, implicated in diverse processes including cell death during tissue turnover, cancer cell death and aggravation of tissue injury(89). It was recently found that ferroptosis involves the rapid and massive generation of eoxPL (mostly PE, including oxidized adrenic acid containing forms) by PL peroxidation in an iron-dependent manner(90-92). The presence of PL with long polyunsaturated ω 6 fatty acids within cellular membranes is a prerequisite for eoxPL formation during ferroptosis, and is in turn dependent on enzymes involved in PL synthesis such as acyl-CoA synthetase long-chain family member 4 (ACSL4)(90). Onset of lipid peroxidation during ferroptosis involves insufficiency and shutdown of anti-oxidative enzymes such as GPX4 as well as LOX-

mediated peroxidation that at least partially involves *ALOX15* (Figure 5). Insights into the physiological and pathological implications of this type of cell death are currently emerging.

While 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. These have been most likely acquired by horizontal gene transfer by these organisms as they display a high sequence homology to mammalian *ALOX15* (93,94). These LOXs were recently shown to generate HETE-PLs and other eoxPLs and might contribute to the pathogenicity of these pathogens e.g. by enabling immune evasion or induction of hemolysis or even ferroptosis, but this is not yet known(95,96).

The analysis of oxidized phospholipids (oxPL) using MS, historical context.

The ability to analyze oxPL/eoxPL as chemical entities emerged with the advent of modern biological MS. The first report of molecular ions generated from intact PC employed field desorption ionization(97). However, it was not until fast atom bombardment ionization (FAB) was introduced by Barber in 1981 that intact PC could be studied widely (98,99). FAB was developed in parallel with tandem MS, and represents the most widely used method of PL analysis today. With this, collisional activation (via multiple collisions with a neutral gas) is used to initiate carbon-carbon bond cleavage, forming product (daughter) ions that can be separately analyzed(100). This is also called collision-induced dissociation (CID). The terminal sector of tandem instruments then enabled determination of the mass-to-charge ratio (m/z) to be measured, providing the molecular mass of the PL. Another important advance was the development of high-performance tandem MS instruments that enabled measurement of precursor and product ions with a high mass accuracy, enabling calculation

of 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 elemental composition of fatty acid (FA), PL and oxPL ions. A detailed knowledge of PL and eicosanoid/prostaglandin gas phase chemistry has developed over the past few decades as a result of studying the MS behavior of known lipids(101,102). Thus it is now possible to characterise not only the structures, but also the mechanisms of lipid oxidation by either enzymatic or non-enzymatic pathways in cell and tissue samples using MS analysis(103).

The ionization methods most often used for FA, eicosanoid/prostaglandin, PL and oxPL/eoxPL analysis are electrospray ionization (ESI), discovered by Nobel laureate John Fenn, and matrix assisted laser desorption ionization (MALDI)(104). These were both developed in the middle 1980s. Ions generated by MALDI or ESI undergo very similar CID decomposition mechanisms. Thus understanding the ion chemistry of the oxPL is relevant to observations made by either method. ESI is widely used due to its ease of implementation, as well as the breadth of molecules that can be analyzed including nonvolatile molecules such as PL and its 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 tandem MS/MS. Chromatography is important, since it enables the separation of closely related PLs including isobaric species (with the same m/z values) and isomers, and determine their MS behavior separately. For readers wishing to learn more about tandem MS of oxidized FA, prostaglandins/eicosanoids, PL and oxPL, and for libraries of MS/MS spectra, we direct the reader to reviews on this topic and to the LIPID MAPS database and resource(101-103). In addition, LIPID MAPS have recently introduced new tools to aid the analysis of these lipids

including dedicated structure drawing tools and a computationally-generated database of oxidized PL chain composition masses.

Summary

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

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Figure Legends

Figure 1. Chemical mechanisms of lipid peroxidation. *Panel A. The underlying process of lipid peroxidation. Panel B. A simplified diagram of non-enzymatic phospholipid oxidation.*

Figure 2. Direct oxidation of phospholipids (PL) by 12/15-LOX and Indirect oxidation of PL by the Land's cycle to form eoxPL. *Panel A. Direct oxidation of membrane PLs* This is achieved by hydrogen abstraction on the *Sn2* fatty acid, usually arachidonic acid, followed by oxygen insertion and reduction, releasing a PL-hydroperoxide that is reduced by glutathione peroxidase 4 (GPX4) to form a PL-hydroxide. *Panel B. Indirect oxidation of PL.* Phospholipase A2 (PLA2) hydrolysis of an *Sn2* fatty acid releases substrate for LOX/COX oxidation, forming an oxylipin which is then

Figure 3. eoxPL support the generation of a pro-thrombotic surface on platelets, facilitating haemostasis. Activated platelets externalize aPL and eoxPL to form an electronegative surface that supports calcium-dependent binding and activation of coagulation factors, leading to thrombin formation.

Figure 4. eoxPL expressed on the surface of resident macrophages facilitate clearance of apoptotic cells. In this mechanism, MFG-E8 binds PS on apoptotic cells and macrophages, acting as a bridge to facilitate recognition and uptake by the macrophages themselves. Conversely, this prevents uptake of apoptotic cell sby inflammatory monocytes that do not generate eoxPL.

Figure 5. eoxPL formation is required for ferroptosis. Although the detailed enzymology of how eoxPL support ferroptosis is not yet fully clear, the following has been proposed.

First, the formation of PL 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 anti-oxidative enzymes such as GPX4 as well as LOX-mediated peroxidation that at least partially involves 15-LOX.

Table 1. Free Radical Products that form as oxPL

| 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 | |

References

1. Halliwell, B., Gutteridge, JMC. (2015), Oxford University Press, Oxford
2. Berzelius, J., Larbok, I, Kemien, IV, Nordstrom, HA. (1827) Stockholm 1827.
3. Warburg, O. (1914) Uber die Rolle des Eisens in der Atmung des Seeigeleis nebst Bemerkungen uber einige durch Eisen beschleunigte Oxydationen. *Hoppe-Seylers Zeitschr. F. physiol. Chem.* **92**, 231-241
4. Elliott, K., Libet, B. (1944) Oxidation of phospholipid catalyzed by iron compounds with ascorbic acid. *The Journal of biological chemistry* **152**, 617-626
5. Bolland, J., Koch, HP. (1945) The course of autooxidation reactions in polyisoprenes and allied compounds. Part IX. The primary thermal oxidation of ethyl linoleate. *J Chem Soc*, 445-447
6. Holman, R. (1954) Autooxidation of fats and related substances. *Progress in lipid research* **18**, 51-98
7. Brown, S. H., Mitchell, T. W., and Blanksby, S. J. (2011) Analysis of unsaturated lipids by ozone-induced dissociation. *Biochimica et biophysica acta* **1811**, 807-817
8. Pryor, W. A. (1994) Mechanisms of radical formation from reactions of ozone with target molecules in the lung. *Free Radic Biol Med* **17**, 451-465
9. Salomon, R. G. (2012) Structural identification and cardiovascular activities of oxidized phospholipids. *Circulation research* **111**, 930-946
10. Reis, A., and Spickett, C. M. (2012) Chemistry of phospholipid oxidation. *Biochimica et biophysica acta* **1818**, 2374-2387
11. Bochkov, V. N., Oskolkova, O. V., Birukov, K. G., Levonen, A. L., Binder, C. J., and Stockl, J. (2010) Generation and biological activities of oxidized phospholipids. *Antioxidants & redox signaling* **12**, 1009-1059

12. Davis, B., Koster, G., Douet, L. J., Scigelova, M., Woffendin, G., Ward, J. M., Smith, A., Humphries, J., Burnand, K. G., Macphee, C. H., and Postle, A. D. (2008) Electrospray ionization mass spectrometry identifies substrates and products of lipoprotein-associated phospholipase A2 in oxidized human low density lipoprotein. *The Journal of biological chemistry* **283**, 6428-6437
13. Podrez, E. A., Poliakov, E., Shen, Z., Zhang, R., Deng, Y., Sun, M., Finton, P. J., Shan, L., Febbraio, M., Hajjar, D. P., Silverstein, R. L., Hoff, H. F., Salomon, R. G., and Hazen, S. L. (2002) A novel family of atherogenic oxidized phospholipids promotes macrophage foam cell formation via the scavenger receptor CD36 and is enriched in atherosclerotic lesions. *The Journal of biological chemistry* **277**, 38517-38523
14. Subbanagounder, G., Leitinger, N., Schwenke, D. C., Wong, J. W., Lee, H., Rizza, C., Watson, A. D., Faull, K. F., Fogelman, A. M., and Berliner, J. A. (2000) Determinants of bioactivity of oxidized phospholipids. Specific oxidized fatty acyl groups at the sn-2 position. *Arterioscler Thromb Vasc Biol* **20**, 2248-2254
15. Watson, A. D., Leitinger, N., Navab, M., Faull, K. F., Horkko, S., Witztum, J. L., Palinski, W., Schwenke, D., Salomon, R. G., Sha, W., Subbanagounder, G., Fogelman, A. M., and Berliner, J. A. (1997) Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *The Journal of biological chemistry* **272**, 13597-13607
16. Karvonen, J., Paivansalo, M., Kesaniemi, Y. A., and Horkko, S. (2003) Immunoglobulin M type of autoantibodies to oxidized low-density lipoprotein has an inverse relation to carotid artery atherosclerosis. *Circulation* **108**, 2107-2112

17. Tsimikas, S., Brilakis, E. S., Lennon, R. J., Miller, E. R., Witztum, J. L., McConnell, J. P., Kornman, K. S., and Berger, P. B. (2007) Relationship of IgG and IgM autoantibodies to oxidized low density lipoprotein with coronary artery disease and cardiovascular events. *Journal of lipid research* **48**, 425-433
18. Horkko, S., Bird, D. A., Miller, E., Itabe, H., Leitinger, N., Subbanagounder, G., Berliner, J. A., Friedman, P., Dennis, E. A., Curtiss, L. K., Palinski, W., and Witztum, J. L. (1999) Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *The Journal of clinical investigation* **103**, 117-128
19. Shaw, P. X., Horkko, S., Chang, M. K., Curtiss, L. K., Palinski, W., Silverman, G. J., and Witztum, J. L. (2000) Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *The Journal of clinical investigation* **105**, 1731-1740
20. Birukov, K. G., Bochkov, V. N., Birukova, A. A., Kawkitinarong, K., Rios, A., Leitner, A., Verin, A. D., Bokoch, G. M., Leitinger, N., and Garcia, J. G. (2004) Epoxycyclopentenone-containing oxidized phospholipids restore endothelial barrier function via Cdc42 and Rac. *Circulation research* **95**, 892-901
21. Cherepanova, O. A., Pidkovka, N. A., Sarmiento, O. F., Yoshida, T., Gan, Q., Adiguzel, E., Bendeck, M. P., Berliner, J., Leitinger, N., and Owens, G. K. (2009) Oxidized phospholipids induce type VIII collagen expression and vascular smooth muscle cell migration. *Circulation research* **104**, 609-618
22. Pegorier, S., Stengel, D., Durand, H., Croset, M., and Ninio, E. (2006) Oxidized phospholipid: POVPC binds to platelet-activating-factor receptor on human macrophages. Implications in atherosclerosis. *Atherosclerosis* **188**, 433-443

23. Qiao, J., Huang, F., Naikawadi, R. P., Kim, K. S., Said, T., and Lum, H. (2006) Lysophosphatidylcholine impairs endothelial barrier function through the G protein-coupled receptor GPR4. *Am J Physiol Lung Cell Mol Physiol* **291**, L91-101
24. Thomas, J. P., Geiger, P. G., Maiorino, M., Ursini, F., and Girotti, A. W. (1990) Enzymatic reduction of phospholipid and cholesterol hydroperoxides in artificial bilayers and lipoproteins. *Biochimica et biophysica acta* **1045**, 252-260
25. Tyurina, Y. Y., Poloyac, S. M., Tyurin, V. A., Kapralov, A. A., Jiang, J., Anthonymuthu, T. S., Kapralova, V. I., Vikulina, A. S., Jung, M. Y., Epperly, M. W., Mohammadyani, D., Klein-Seetharaman, J., Jackson, T. C., Kochanek, P. M., Pitt, B. R., Greenberger, J. S., Vladimirov, Y. A., Bayir, H., and Kagan, V. E. (2014) A mitochondrial pathway for biosynthesis of lipid mediators. *Nat Chem* **6**, 542-552
26. Clark, S. R., Guy, C. J., Scurr, M. J., Taylor, P. R., Kift-Morgan, A. P., Hammond, V. J., Thomas, C. P., Coles, B., Roberts, G. W., Eberl, M., Jones, S. A., Topley, N., Kotecha, S., and O'Donnell, V. B. (2011) Esterified eicosanoids are acutely generated by 5-lipoxygenase in primary human neutrophils and in human and murine infection. *Blood* **117**, 2033-2043
27. Maskrey, B. H., Bermudez-Fajardo, A., Morgan, A. H., Stewart-Jones, E., Dioszeghy, V., Taylor, G. W., Baker, P. R., Coles, B., Coffey, M. J., Kuhn, H., and O'Donnell, V. B. (2007) Activated platelets and monocytes generate four hydroxyphosphatidylethanolamines via lipoxygenase. *J Biol Chem* **282**, 20151-20163
28. Thomas, C. P., Morgan, L. T., Maskrey, B. H., Murphy, R. C., Kuhn, H., Hazen, S. L., Goodall, A. H., Hamali, H. A., Collins, P. W., and O'Donnell, V. B. (2010) Phospholipid-esterified eicosanoids are generated in agonist-activated human platelets and enhance tissue factor-dependent thrombin generation. *J Biol Chem* **285**, 6891-6903

29. O'Donnell, V. B., and Murphy, R. C. (2012) New families of bioactive oxidized phospholipids generated by immune cells: identification and signaling actions. *Blood* **120**, 1985-1992
30. Morgan, A. H., Dioszeghy, V., Maskrey, B. H., Thomas, C. P., Clark, S. R., Mathie, S. A., Lloyd, C. M., Kuhn, H., Topley, N., Coles, B. C., Taylor, P. R., Jones, S. A., and O'Donnell, V. B. (2009) Phosphatidylethanolamine-esterified eicosanoids in the mouse: tissue localization and inflammation-dependent formation in Th-2 disease. *The Journal of biological chemistry* **284**, 21185-21191
31. Morita, E., Schroder, J. M., and Christophers, E. (1990) Production of 15-hydroxyeicosatetraenoic acid by purified human eosinophils and neutrophils. *Scand J Immunol* **32**, 497-502
32. Heydeck, D., Thomas, L., Schnurr, K., Trebus, F., Thierfelder, W. E., Ihle, J. N., and Kuhn, H. (1998) Interleukin-4 and -13 induce upregulation of the murine macrophage 12/15-lipoxygenase activity: evidence for the involvement of transcription factor STAT6. *Blood* **92**, 2503-2510
33. Brinckmann, R., Schnurr, K., Heydeck, D., Rosenbach, T., Kolde, G., and Kuhn, H. (1998) Membrane translocation of 15-lipoxygenase in hematopoietic cells is calcium-dependent and activates the oxygenase activity of the enzyme. *Blood* **91**, 64-74
34. Morgan, L. T., Thomas, C. P., Kuhn, H., and O'Donnell, V. B. (2010) Thrombin-activated human platelets acutely generate oxidized docosahexaenoic-acid-containing phospholipids via 12-lipoxygenase. *Biochem J* **431**, 141-148
35. Takahashi, Y., Glasgow, W. C., Suzuki, H., Taketani, Y., Yamamoto, S., Anton, M., Kuhn, H., and Brash, A. R. (1993) Investigation of the oxygenation of phospholipids by the porcine leukocyte and human platelet arachidonate 12-lipoxygenases. *Eur J Biochem* **218**, 165-171

36. Schnurr, K., Belkner, J., Ursini, F., Schewe, T., and Kuhn, H. (1996) The selenoenzyme phospholipid hydroperoxide glutathione peroxidase controls the activity of the 15-lipoxygenase with complex substrates and preserves the specificity of the oxygenation products. *J Biol Chem* **271**, 4653-4658
37. Serhan, C. N., and Brezinski, M. E. (1991) Selective incorporation of 15-HETE in phosphatidylinositol: agonist-induced deacylation and transformation of stored HETEs by human neutrophils. *Adv Prostaglandin Thromboxane Leukot Res* **21A**, 105-108
38. Spector, A. A. (2009) Arachidonic acid cytochrome P450 epoxygenase pathway. *Journal of lipid research* **50 Suppl**, S52-56
39. Spector, A. A., and Norris, A. W. (2007) Action of epoxyeicosatrienoic acids on cellular function. *Am J Physiol Cell Physiol* **292**, C996-1012
40. Bernstrom, K., Kayganich, K., Murphy, R. C., and Fitzpatrick, F. A. (1992) Incorporation and distribution of epoxyeicosatrienoic acids into cellular phospholipids. *The Journal of biological chemistry* **267**, 3686-3690
41. Aldrovandi, M., Hammond, V. J., Podmore, H., Hornshaw, M., Clark, S. R., Marnett, L. J., Slatter, D. A., Murphy, R. C., Collins, P. W., and O'Donnell, V. B. (2013) Human platelets generate phospholipid-esterified prostaglandins via cyclooxygenase-1 that are inhibited by low dose aspirin supplementation. *Journal of lipid research* **54**, 3085-3097
42. Aldrovandi, M., Hinz, C., Lauder, S. N., Podmore, H., Hornshaw, M., Slatter, D. A., Tyrrell, V. J., Clark, S. R., Marnett, L. J., Collins, P. W., Murphy, R. C., and O'Donnell, V. B. (2017) DioxolaneA3-phosphatidylethanolamines are generated by human platelets and stimulate neutrophil integrin expression. *Redox biology* **11**, 663-672

43. Klett, E. L., Chen, S., Yechoor, A., Lih, F. B., and Coleman, R. A. (2017) Long-chain acyl-CoA synthetase isoforms differ in preferences for eicosanoid species and long-chain fatty acids. *Journal of lipid research* **58**, 884-894
44. Slatter, D. A., Aldrovandi, M., O'Connor, A., Allen, S. M., Brasher, C. J., Murphy, R. C., Mecklemann, S., Ravi, S., Darley-Usmar, V., and O'Donnell, V. B. (2016) Mapping the Human Platelet Lipidome Reveals Cytosolic Phospholipase A2 as a Regulator of Mitochondrial Bioenergetics during Activation. *Cell Metab* **23**, 930-944
45. O'Connor, A., Brasher, C. J., Slatter, D. A., Meckelmann, S. W., Hawksworth, J. I., Allen, S. M., and O'Donnell, V. B. (2017) LipidFinder: A computational workflow for discovery of lipids identifies eicosanoid-phosphoinositides in platelets. *JCI Insight* **2**, e91634
46. Lauder, S. N., Allen-Redpath, K., Slatter, D. A., Aldrovandi, M., O'Connor, A., Farewell, D., Percy, C. L., Molhoek, J. E., Rannikko, S., Tyrrell, V. J., Ferla, S., Milne, G. L., Poole, A. W., Thomas, C. P., Obaji, S., Taylor, P. R., Jones, S. A., de Groot, P. G., Urbanus, R. T., Horkko, S., Uderhardt, S., Ackermann, J., Vince Jenkins, P., Brancale, A., Kronke, G., Collins, P. W., and O'Donnell, V. B. (2017) Networks of enzymatically oxidized membrane lipids support calcium-dependent coagulation factor binding to maintain hemostasis. *Sci Signal* **10**, ean2787
47. Morgan, A. H., Hammond, V. J., Morgan, L., Thomas, C. P., Tallman, K. A., Garcia-Diaz, Y. R., McGuigan, C., Serpi, M., Porter, N. A., Murphy, R. C., and O'Donnell, V. B. (2010) Quantitative assays for esterified oxylipins generated by immune cells. *Nat Protoc* **5**, 1919-1931
48. Kuhn, H., Heydeck, D., Hugou, I., and Gniwotta, C. (1997) In vivo action of 15-lipoxygenase in early stages of human atherogenesis. *The Journal of clinical investigation* **99**, 888-893

49. Slatter, D. A., Percy, C. L., Allen-Redpath, K., Gajsiewicz, J. M., Brooks, N. J., Clayton, A., Tyrrell, V. J., Rosas, M., Lauder, S. N., Watson, A., Dul, M., Garcia-Diaz, Y., Aldrovandi, M., Heurich, M., Hall, J., Morrissey, J. H., Lacroix-Desmazes, S., Delignat, S., Jenkins, P. V., Collins, P. W., and O'Donnell, V. B. (2018) Enzymatically oxidized phospholipids restore thrombin generation in coagulation factor deficiencies. *JCI Insight* **3**, e98459
50. Uderhardt, S., Ackermann, J. A., Fillep, T., Hammond, V. J., Willeit, J., Santer, P., Mayr, M., Biburger, M., Miller, M., Zellner, K. R., Stark, K., Zarbock, A., Rossaint, J., Schubert, I., Mielenz, D., Dietel, B., Raaz-Schrauder, D., Ay, C., Gremmel, T., Thaler, J., Heim, C., Herrmann, M., Collins, P. W., Schabbauer, G., Mackman, N., Voehringer, D., Nadler, J. L., Lee, J. J., Massberg, S., Rauh, M., Kiechl, S., Schett, G., O'Donnell, V. B., and Kronke, G. (2017) Enzymatic lipid oxidation by eosinophils propagates coagulation, hemostasis, and thrombotic disease. *J Exp Med* **214**, 2121-2138
51. Miller, Y. I., and Shyy, J. Y. (2017) Context-Dependent Role of Oxidized Lipids and Lipoproteins in Inflammation. *Trends Endocrinol Metab* **28**, 143-152
52. Boullier, A., Friedman, P., Harkewicz, R., Hartvigsen, K., Green, S. R., Almazan, F., Dennis, E. A., Steinberg, D., Witztum, J. L., and Quehenberger, O. (2005) Phosphocholine as a pattern recognition ligand for CD36. *Journal of lipid research* **46**, 969-976
53. Greenberg, M. E., Li, X. M., Gugiu, B. G., Gu, X., Qin, J., Salomon, R. G., and Hazen, S. L. (2008) The lipid whisker model of the structure of oxidized cell membranes. *The Journal of biological chemistry* **283**, 2385-2396

54. Wong-Ekkabut, J., Xu, Z., Triampo, W., Tang, I. M., Tieleman, D. P., and Monticelli, L. (2007) Effect of lipid peroxidation on the properties of lipid bilayers: a molecular dynamics study. *Biophysical journal* **93**, 4225-4236
55. van Leyen, K., Duvoisin, R. M., Engelhardt, H., and Wiedmann, M. (1998) A function for lipoxygenase in programmed organelle degradation. *Nature* **395**, 392-395
56. Hammond, V. J., Morgan, A. H., Lauder, S., Thomas, C. P., Brown, S., Freeman, B. A., Lloyd, C. M., Davies, J., Bush, A., Levonen, A. L., Kansanen, E., Villacorta, L., Chen, Y. E., Porter, N., Garcia-Diaz, Y. M., Schopfer, F. J., and O'Donnell, V. B. (2012) Novel keto-phospholipids are generated by monocytes and macrophages, detected in cystic fibrosis, and activate peroxisome proliferator-activated receptor-gamma. *The Journal of biological chemistry* **287**, 41651-41666
57. Falls, L. A., Furie, B., and Furie, B. C. (2000) Role of phosphatidylethanolamine in assembly and function of the factor IXa-factor VIIIa complex on membrane surfaces. *Biochemistry* **39**, 13216-13222
58. Tavoosi, N., Davis-Harrison, R. L., Pogorelov, T. V., Ohkubo, Y. Z., Arcario, M. J., Clay, M. C., Rienstra, C. M., Tajkhorshid, E., and Morrissey, J. H. (2011) Molecular determinants of phospholipid synergy in blood clotting. *The Journal of biological chemistry* **286**, 23247-23253
59. Clark, S. R., Thomas, C. P., Hammond, V. J., Aldrovandi, M., Wilkinson, G. W., Hart, K. W., Murphy, R. C., Collins, P. W., and O'Donnell, V. B. (2013) Characterization of platelet aminophospholipid externalization reveals fatty acids as molecular determinants that regulate coagulation. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 5875-5880
60. Mancek-Keber, M., Frank-Bertoncelj, M., Hafner-Bratkovic, I., Smole, A., Zorko, M., Pirher, N., Hayer, S., Kralj-Iglic, V., Rozman, B., Ilc, N., Horvat, S., and Jerala, R.

- (2015) Toll-like receptor 4 senses oxidative stress mediated by the oxidation of phospholipids in extracellular vesicles. *Sci Signal* **8**, ra60
61. Hiltunen, T., Luoma, J., Nikkari, T., and Yla-Herttuala, S. (1995) Induction of 15-lipoxygenase mRNA and protein in early atherosclerotic lesions. *Circulation* **92**, 3297-3303
62. Yla-Herttuala, S., Rosenfeld, M. E., Parthasarathy, S., Glass, C. K., Sigal, E., Witztum, J. L., and Steinberg, D. (1990) Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 6959-6963
63. Cyrus, T., Witztum, J. L., Rader, D. J., Tangirala, R., Fazio, S., Linton, M. F., and Funk, C. D. (1999) Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. *The Journal of clinical investigation* **103**, 1597-1604
64. Huo, Y., Zhao, L., Hyman, M. C., Shashkin, P., Harry, B. L., Burcin, T., Forlow, S. B., Stark, M. A., Smith, D. F., Clarke, S., Srinivasan, S., Hedrick, C. C., Pratico, D., Witztum, J. L., Nadler, J. L., Funk, C. D., and Ley, K. (2004) Critical role of macrophage 12/15-lipoxygenase for atherosclerosis in apolipoprotein E-deficient mice. *Circulation* **110**, 2024-2031
65. Zhao, L., and Funk, C. D. (2004) Lipoxygenase pathways in atherogenesis. *Trends Cardiovasc Med* **14**, 191-195
66. Leitinger, N., Tyner, T. R., Oslund, L., Rizza, C., Subbanagounder, G., Lee, H., Shih, P. T., Mackman, N., Tigyi, G., Territo, M. C., Berliner, J. A., and Vora, D. K. (1999) Structurally similar oxidized phospholipids differentially regulate endothelial binding of

- monocytes and neutrophils. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 12010-12015
67. Pidkovka, N. A., Cherepanova, O. A., Yoshida, T., Alexander, M. R., Deaton, R. A., Thomas, J. A., Leitinger, N., and Owens, G. K. (2007) Oxidized phospholipids induce phenotypic switching of vascular smooth muscle cells in vivo and in vitro. *Circulation research* **101**, 792-801
68. Subbanagounder, G., Wong, J. W., Lee, H., Faull, K. F., Miller, E., Witztum, J. L., and Berliner, J. A. (2002) Epoxyisoprostane and epoxycyclopentenone phospholipids regulate monocyte chemotactic protein-1 and interleukin-8 synthesis. Formation of these oxidized phospholipids in response to interleukin-1beta. *The Journal of biological chemistry* **277**, 7271-7281
69. Bolick, D. T., Orr, A. W., Whetzel, A., Srinivasan, S., Hatley, M. E., Schwartz, M. A., and Hedrick, C. C. (2005) 12/15-lipoxygenase regulates intercellular adhesion molecule-1 expression and monocyte adhesion to endothelium through activation of RhoA and nuclear factor-kappaB. *Arterioscler Thromb Vasc Biol* **25**, 2301-2307
70. Kadl, A., Meher, A. K., Sharma, P. R., Lee, M. Y., Doran, A. C., Johnstone, S. R., Elliott, M. R., Gruber, F., Han, J., Chen, W., Kensler, T., Ravichandran, K. S., Isakson, B. E., Wamhoff, B. R., and Leitinger, N. (2010) Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. *Circulation research* **107**, 737-746
71. Zhao, J., Maskrey, B., Balzar, S., Chibana, K., Mustovich, A., Hu, H., Trudeau, J. B., O'Donnell, V., and Wenzel, S. E. (2009) Interleukin-13-induced MUC5AC is regulated by 15-lipoxygenase 1 pathway in human bronchial epithelial cells. *Am J Respir Crit Care Med* **179**, 782-790

72. Rothe, T., Gruber, F., Uderhardt, S., Ipseiz, N., Rossner, S., Oskolkova, O., Bluml, S., Leitinger, N., Bicker, W., Bochkov, V. N., Yamamoto, M., Steinkasserer, A., Schett, G., Zinser, E., and Kronke, G. (2015) 12/15-Lipoxygenase-mediated enzymatic lipid oxidation regulates DC maturation and function. *The Journal of clinical investigation* **125**, 1944-1954
73. Stein, K., Stoffels, M., Lysson, M., Schneiker, B., Dewald, O., Kronke, G., Kalff, J. C., and Wehner, S. (2016) A role for 12/15-lipoxygenase-derived proresolving mediators in postoperative ileus: protectin DX-regulated neutrophil extravasation. *J Leukoc Biol* **99**, 231-239
74. Tani, Y., Isobe, Y., Imoto, Y., Segi-Nishida, E., Sugimoto, Y., Arai, H., and Arita, M. (2014) Eosinophils control the resolution of inflammation and draining lymph node hypertrophy through the proresolving mediators and CXCL13 pathway in mice. *FASEB J* **28**, 4036-4043
75. Uderhardt, S., Herrmann, M., Oskolkova, O. V., Aschermann, S., Bicker, W., Ipseiz, N., Sarter, K., Frey, B., Rothe, T., Voll, R., Nimmerjahn, F., Bochkov, V. N., Schett, G., and Kronke, G. (2012) 12/15-lipoxygenase orchestrates the clearance of apoptotic cells and maintains immunologic tolerance. *Immunity* **36**, 834-846
76. Yamada, T., Tani, Y., Nakanishi, H., Taguchi, R., Arita, M., and Arai, H. (2011) Eosinophils promote resolution of acute peritonitis by producing proresolving mediators in mice. *FASEB J* **25**, 561-568
77. Dioszeghy, V., Rosas, M., Maskrey, B. H., Colmont, C., Topley, N., Chaitidis, P., Kuhn, H., Jones, S. A., Taylor, P. R., and O'Donnell, V. B. (2008) 12/15-Lipoxygenase regulates the inflammatory response to bacterial products in vivo. *Journal of immunology* **181**, 6514-6524

78. Bluml, S., Kirchberger, S., Bochkov, V. N., Kronke, G., Stuhlmeier, K., Majdic, O., Zlabinger, G. J., Knapp, W., Binder, B. R., Stockl, J., and Leitinger, N. (2005) Oxidized phospholipids negatively regulate dendritic cell maturation induced by TLRs and CD40. *Journal of immunology* **175**, 501-508
79. Bretscher, P., Egger, J., Shamshiev, A., Trotsmuller, M., Kofeler, H., Carreira, E. M., Kopf, M., and Freigang, S. (2015) Phospholipid oxidation generates potent anti-inflammatory lipid mediators that mimic structurally related pro-resolving eicosanoids by activating Nrf2. *EMBO Mol Med* **7**, 593-607
80. Emerson, M. R., and LeVine, S. M. (2004) Experimental allergic encephalomyelitis is exacerbated in mice deficient for 12/15-lipoxygenase or 5-lipoxygenase. *Brain Res* **1021**, 140-145
81. Kronke, G., Katzenbeisser, J., Uderhardt, S., Zaiss, M. M., Scholtysek, C., Schabbauer, G., Zarbock, A., Koenders, M. I., Axmann, R., Zwerina, J., Baenckler, H. W., van den Berg, W., Voll, R. E., Kuhn, H., Joosten, L. A., and Schett, G. (2009) 12/15-lipoxygenase counteracts inflammation and tissue damage in arthritis. *Journal of immunology* **183**, 3383-3389
82. Kronke, G., Reich, N., Scholtysek, C., Akhmetshina, A., Uderhardt, S., Zerr, P., Palumbo, K., Lang, V., Dees, C., Distler, O., Schett, G., and Distler, J. H. (2012) The 12/15-lipoxygenase pathway counteracts fibroblast activation and experimental fibrosis. *Ann Rheum Dis* **71**, 1081-1087
83. Bochkov, V. N., Kadl, A., Huber, J., Gruber, F., Binder, B. R., and Leitinger, N. (2002) Protective role of phospholipid oxidation products in endotoxin-induced tissue damage. *Nature* **419**, 77-81
84. von Schlieffen, E., Oskolkova, O. V., Schabbauer, G., Gruber, F., Bluml, S., Genest, M., Kadl, A., Marsik, C., Knapp, S., Chow, J., Leitinger, N., Binder, B. R., and

- Bochkov, V. N. (2009) Multi-hit inhibition of circulating and cell-associated components of the toll-like receptor 4 pathway by oxidized phospholipids. *Arterioscler Thromb Vasc Biol* **29**, 356-362
85. Hazen, S. L. (2008) Oxidized phospholipids as endogenous pattern recognition ligands in innate immunity. *The Journal of biological chemistry* **283**, 15527-15531
86. Imai, Y., Kuba, K., Neely, G. G., Yaghubian-Malhami, R., Perkmann, T., van Loo, G., Ermolaeva, M., Veldhuizen, R., Leung, Y. H., Wang, H., Liu, H., Sun, Y., Pasparakis, M., Kopf, M., Mech, C., Bavari, S., Peiris, J. S., Slutsky, A. S., Akira, S., Hultqvist, M., Holmdahl, R., Nicholls, J., Jiang, C., Binder, C. J., and Penninger, J. M. (2008) Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell* **133**, 235-249
87. McIntyre, T. M. (2012) Bioactive oxidatively truncated phospholipids in inflammation and apoptosis: formation, targets, and inactivation. *Biochimica et biophysica acta* **1818**, 2456-2464
88. Zandoni, I., Tan, Y., Di Gioia, M., Broggi, A., Ruan, J., Shi, J., Donado, C. A., Shao, F., Wu, H., Springstead, J. R., and Kagan, J. C. (2016) An endogenous caspase-11 ligand elicits interleukin-1 release from living dendritic cells. *Science* **352**, 1232-1236
89. Stockwell, B. R., Friedmann Angeli, J. P., Bayir, H., Bush, A. I., Conrad, M., Dixon, S. J., Fulda, S., Gascon, S., Hatzios, S. K., Kagan, V. E., Noel, K., Jiang, X., Linkermann, A., Murphy, M. E., Overholtzer, M., Oyagi, A., Pagnussat, G. C., Park, J., Ran, Q., Rosenfeld, C. S., Salnikow, K., Tang, D., Torti, F. M., Torti, S. V., Toyokuni, S., Woerpel, K. A., and Zhang, D. D. (2017) Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell* **171**, 273-285
90. Doll, S., Proneth, B., Tyurina, Y. Y., Panzilius, E., Kobayashi, S., Ingold, I., Imler, M., Beckers, J., Aichler, M., Walch, A., Prokisch, H., Trumbach, D., Mao, G., Qu, F.,

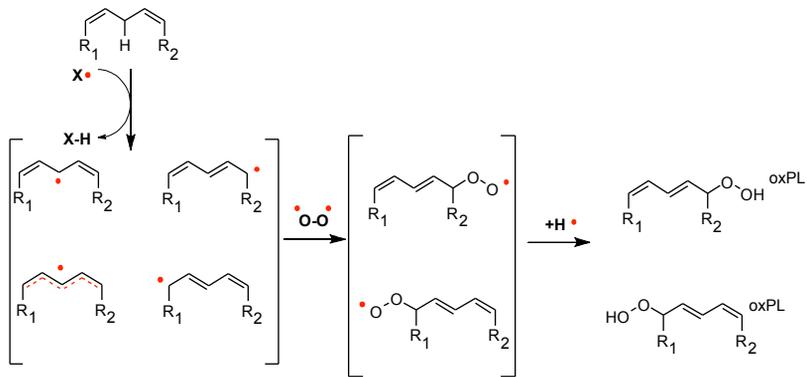
- Bayir, H., Fullekrug, J., Scheel, C. H., Wurst, W., Schick, J. A., Kagan, V. E., Angeli, J. P., and Conrad, M. (2017) ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. *Nat Chem Biol* **13**, 91-98
91. Friedmann Angeli, J. P., Schneider, M., Proneth, B., Tyurina, Y. Y., Tyurin, V. A., Hammond, V. J., Herbach, N., Aichler, M., Walch, A., Eggenhofer, E., Basavarajappa, D., Radmark, O., Kobayashi, S., Seibt, T., Beck, H., Neff, F., Esposito, I., Wanke, R., Forster, H., Yefremova, O., Heinrichmeyer, M., Bornkamm, G. W., Geissler, E. K., Thomas, S. B., Stockwell, B. R., O'Donnell, V. B., Kagan, V. E., Schick, J. A., and Conrad, M. (2014) Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nature cell biology* **16**, 1180-1191
92. Kagan, V. E., Mao, G., Qu, F., Angeli, J. P., Doll, S., Croix, C. S., Dar, H. H., Liu, B., Tyurin, V. A., Ritov, V. B., Kapralov, A. A., Amoscato, A. A., Jiang, J., Anthonymuthu, T., Mohammadyani, D., Yang, Q., Proneth, B., Klein-Seetharaman, J., Watkins, S., Bahar, I., Greenberger, J., Mallampalli, R. K., Stockwell, B. R., Tyurina, Y. Y., Conrad, M., and Bayir, H. (2017) Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis. *Nat Chem Biol* **13**, 81-90
93. Bannenberg, G. L., Aliberti, J., Hong, S., Sher, A., and Serhan, C. (2004) Exogenous pathogen and plant 15-lipoxygenase initiate endogenous lipoxin A4 biosynthesis. *The Journal of experimental medicine* **199**, 515-523
94. Vance, R. E., Hong, S., Gronert, K., Serhan, C. N., and Mekalanos, J. J. (2004) The opportunistic pathogen *Pseudomonas aeruginosa* carries a secretable arachidonate 15-lipoxygenase. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 2135-2139
95. Aldrovandi, M., Banthiya, S., Meckelmann, S., Zhou, Y., Heydeck, D., O'Donnell, V. B., and Kuhn, H. (2018) Specific oxygenation of plasma membrane phospholipids by

- Pseudomonas aeruginosa lipoxygenase induces structural and functional alterations in mammalian cells. *Biochimica et biophysica acta* **1863**, 152-164
96. Banthiya, S., Pekarova, M., Kuhn, H., and Heydeck, D. (2015) Secreted lipoxygenase from Pseudomonas aeruginosa exhibits biomembrane oxygenase activity and induces hemolysis in human red blood cells. *Arch Biochem Biophys* **584**, 116-124
 97. Wood, G. W., and Lau, P. Y. (1974) Analysis of intact phospholipids by field desorption mass spectrometry. *Biomed Mass Spectrom* **1**, 154-155
 98. Lehmann, W. D., and Kessler, M. (1983) Fatty acid profiling of phospholipids by field desorption and fast atom bombardment mass spectrometry. *Chem Phys Lipids* **32**, 123-135
 99. Clay KL, W. L., Murphy RC. (1983) Interlaboratory reproducibility of fast atom bombardment mass spectral data. *Biomed Mass Spectrom* **10**, 489-494
 100. Shukla, A. K., and Futrell, J. H. (2000) Tandem mass spectrometry: dissociation of ions by collisional activation. *J Mass Spectrom* **35**, 1069-1090
 101. Murphy, R. C., Barkley, R. M., Zemski Berry, K., Hankin, J., Harrison, K., Johnson, C., Krank, J., McAnoy, A., Uhlson, C., and Zarini, S. (2005) Electrospray ionization and tandem mass spectrometry of eicosanoids. *Anal Biochem* **346**, 1-42
 102. Pulfer, M., and Murphy, R. C. (2003) Electrospray mass spectrometry of phospholipids. *Mass Spectrom Rev* **22**, 332-364
 103. Murphy, R. C. (2015) *Mass Spectrometry of Lipids: Molecular Analysis of Complex Lipids. New Developments in Mass Spectrometry. Chapter 5*, Royal Society of Chemistry, London

104. Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., and Whitehouse, C. M. (1989)
Electrospray ionization for mass spectrometry of large biomolecules. *Science* **246**,
64-71

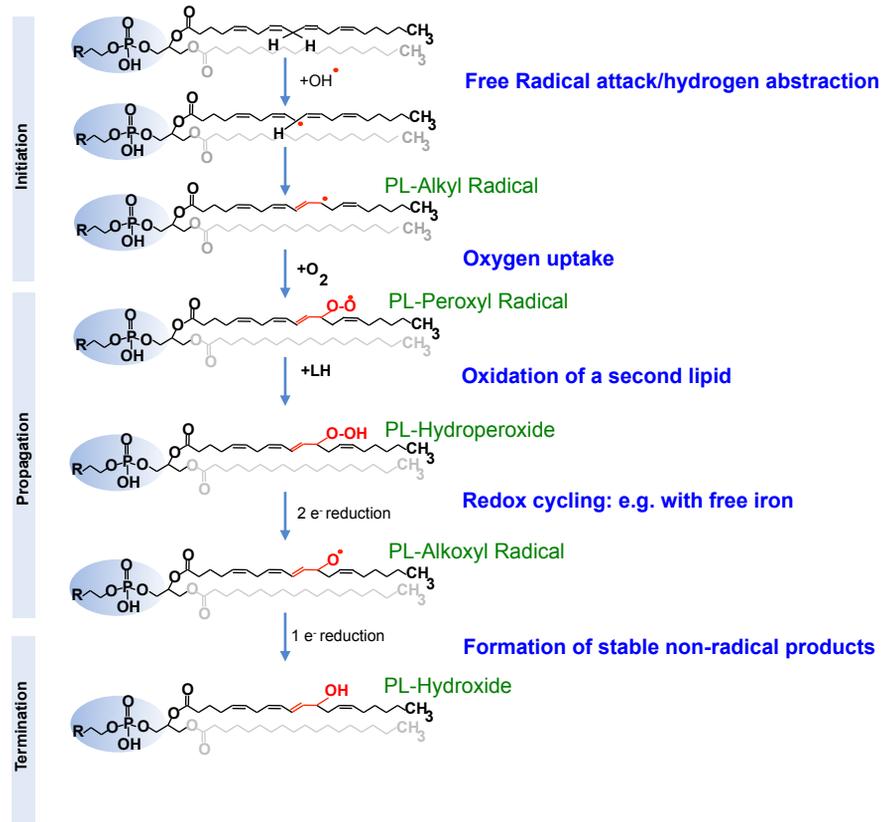
Figure 1

A



The process of hydrogen abstraction and oxygen addition that occurs during lipid oxidation, whether enzymatic or non-enzymatic is the same

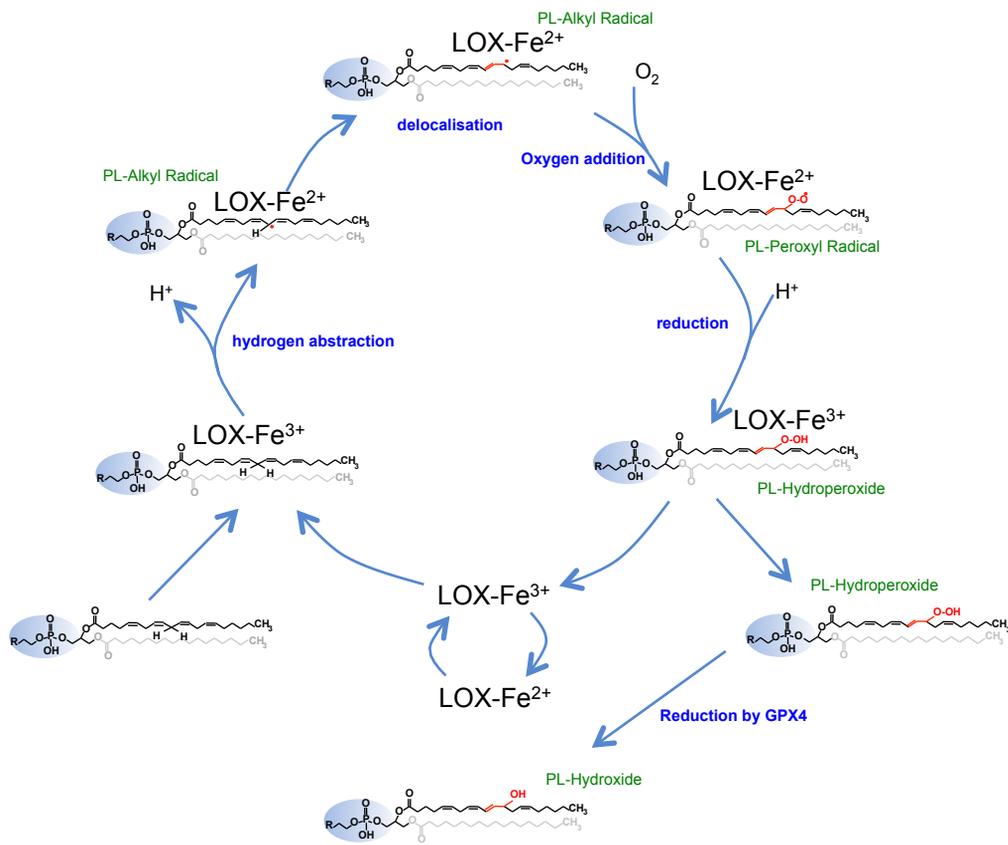
B



Examples of non-enzymatic oxidation of PL

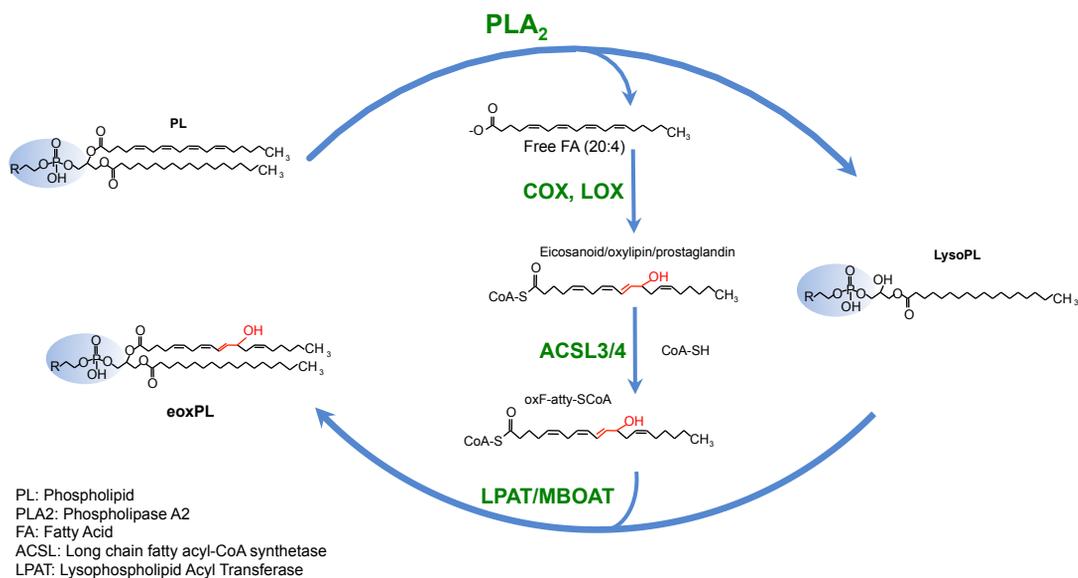
Figure 2

A



Lipoxygenase oxidation of PL (direct oxidation)

B



Indirect oxidation by Land's remodelling pathway

Figure 3

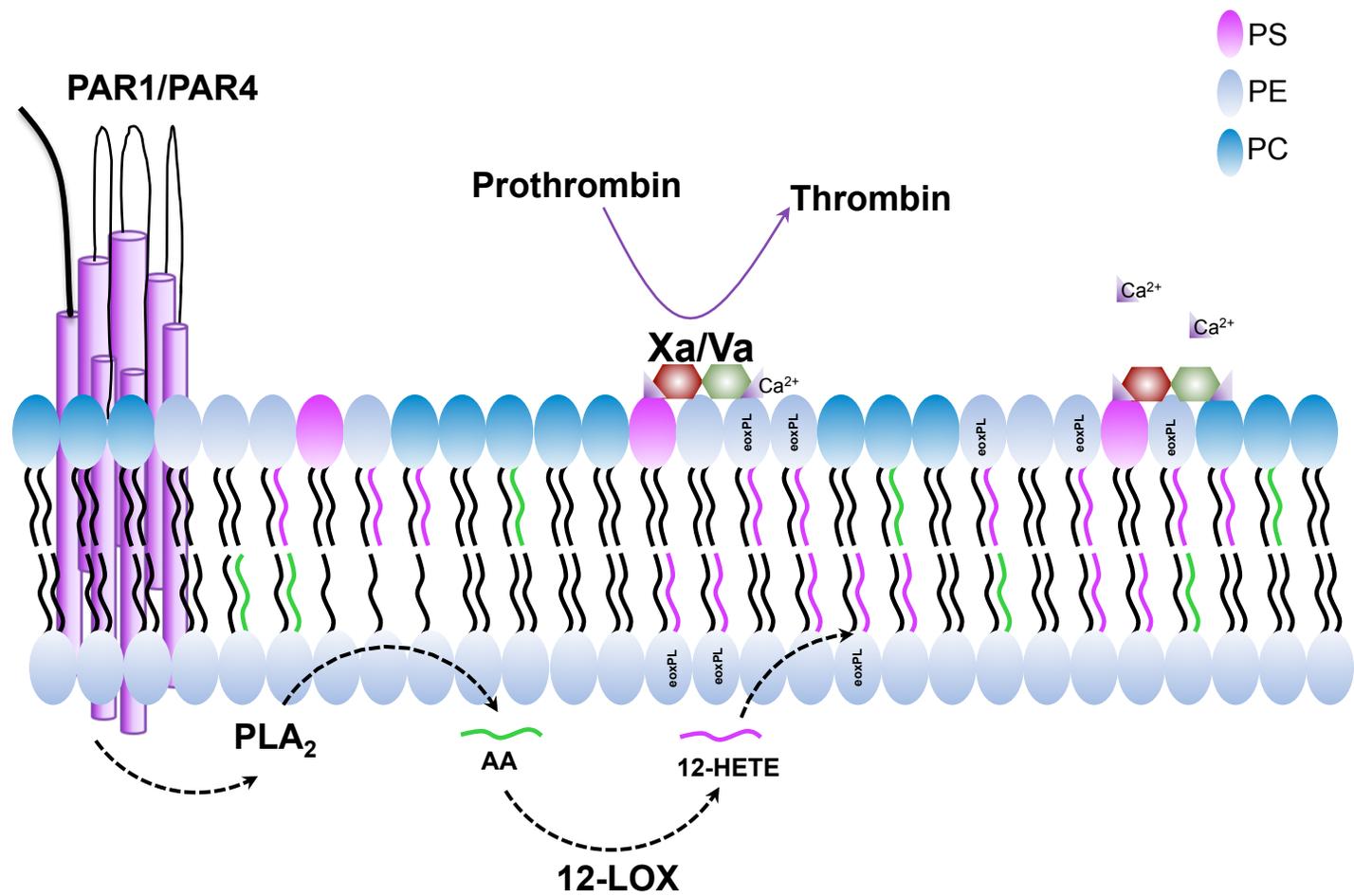


Figure 4

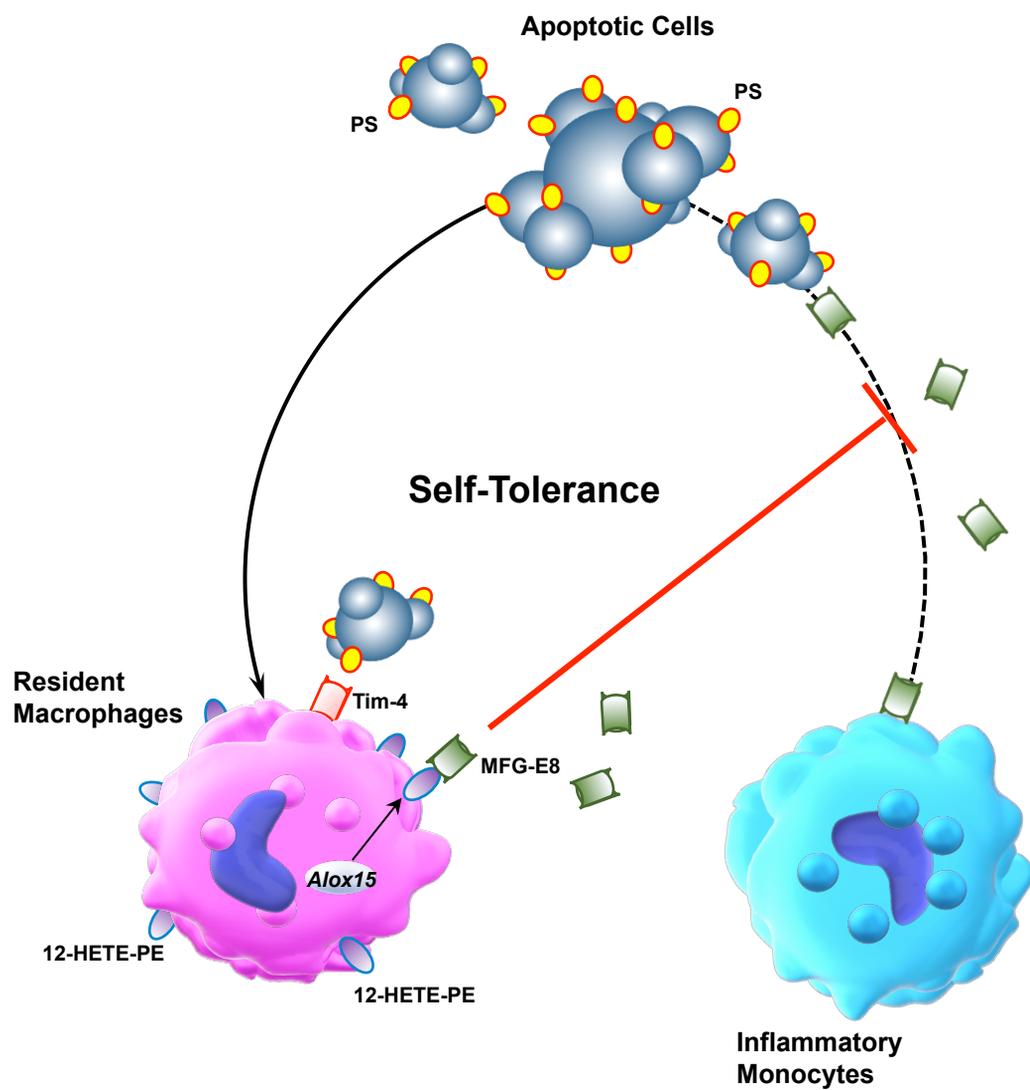


Figure 5

