EPILIPIDOME AS A NEW LEVEL OF CELLULAR REGULATION

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EPILIPIDOME AS A NEW LEVEL OF CELLULAR REGULATION

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REVIEW
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The Biosynthesis of Enzymatically Oxidized Lipids

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Enzymatically oxidized lipids are a specific group of biomolecules that function as key signaling mediators and hormones, regulating various cellular and physiological processes from metabolism and cell death to inflammation and the immune response. They are broadly categorized as either polyunsaturated fatty acid (PUFA) containing (free acid oxygenated PUFA "oxylipins", endocannabinoids, oxidized phospholipids) or cholesterol derivatives (oxysterols, steroid hormones, and bile acids). Their biosynthesis is accomplished by families of enzymes that include lipoxygenases (LOX), cyclooxygenases (COX), cytochrome P450s (CYP), and aldo-keto reductases (AKR). In contrast, non-enzymatically oxidized lipids are produced by uncontrolled oxidation and are broadly considered to be harmful. Here, we provide an overview of the biochemistry and enzymology of LOXs, COXs, CYPs, and AKRs in humans. Next, we present biosynthetic pathways for oxylipins, oxidized phospholipids, oxysterols, bile acids and steroid hormones. Last, we address gaps in knowledge and suggest directions for future work.

Keywords: biosynthesis of oxidized lipids, lipoxygenase (LOX), cyclooxygenase (COX), cytochrome P450, aldo-keto reductase (AKR), oxylipins, oxidized phospholipids, sterols and steroid hormones

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INTRODUCTION

Lipids are a key component of life. They play essential roles in membrane structure, cell signaling and energy production. Like other biomolecules, they undergo chemical modifications that expand their functional repertoire. One modification that has been steadily gaining attention is lipid oxygenation, with interest being attributed to two factors: advances in analytical methods that allow detection of oxygenated species, and the ever-growing body of literature implicating them in biological processes and disease. "Enzymatically oxidized lipids" constitute a large portion of known oxygenated lipid mediators, being bioactive lipids that are produced locally through specific

Abbreviations: 4-HNE, 4-hydroxy-2-nonenal; AA, arachidonic acid; AKR, aldo-keto reductase; ALA, α-linolenic acid; COX, cyclooxygenase; CE, cholesteryl ester; CYP, cytochrome P450; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPEP2, dipeptidase 2; EET, epoxy-eicosatrienoic acid; EPA, eicosapentaenoic acid; GGT, γ-glutamyl transpeptidase; GLA, γ-linolenic acid; GPCR, G protein-coupled receptor; HETE, hydroxy-eicosatetraenoic acid; HpETE, hydroperoxy-eicosatetraenoic acid; HSD, hydroxysteroid dehydrogenase; LA, linoleic acid; LOX, lipoxygenase; LTA_4, leukotriene A_4; lysoPL, lysophospholipid; oxPL, oxidized phospholipids; PG, prostaglandin; PL, phospholipid; PUFA, polyunsaturated fatty acid; SDR, short-chain dehydrogenase/reductase; SPM, specialized pro-resolving mediator.

biosynthetic pathways in response to extracellular stimuli. Those derived from oxygenation of polyunsaturated fatty acids (PUFA) such as arachidonic acid (AA) include prostaglandins (PG), thromboxanes and leukotrienes, which function in modulating inflammation, the immune response, and hemostasis, and are broadly termed "oxylipins" (1, 2). Oxylipins also include multiply oxygenated derivatives of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), of which specific enantiomers have been termed "specialized pro-resolving mediators" (SPM) for their proposed roles in the resolution of inflammation (3). SPMs derived from DHA include D-resolvins, maresins and protectin. On the other hand, E-resolvins are proposed to be generated from EPA but the enzymatic pathways are unclear and require further investigation. Oxylipins also constitute a core functional group on larger lipids including oxidized phospholipids (oxPL), endocannabinoids and cholesteryl esters (CE). Oxidized CEs are conversely associated with atherosclerosis progression (4), whereas enzymatically oxidized phospholipids (eoxPL) are pro-coagulant and promote a variety of innate immune actions in leukocytes and platelets (5).

Enzymatically oxidized lipids are not limited to those containing oxygenated PUFA functional groups. Oxidized derivatives of cholesterol include steroid hormones, bile acids and their oxysterol precursors. Steroid hormones regulate multiple physiological processes including metabolism (e.g., glucose homeostasis by glucocorticoids), water retention, immune function, and development of sex characteristics (6–8). Bile acids, while traditionally known for aiding in fat digestion and bilirubin excretion, are also being increasingly revealed as signaling molecules and metabolic regulators (9, 10). Similarly, accumulating evidence is revealing oxysterols as more than just bile acid intermediates with functions in signaling (11).

Enzymatic lipid oxidation is facilitated by a network of proteins that use PUFAs or sterols as substrates, specifically, lipoxygenase (LOX), cyclooxygenase (COX), and cytochrome P450 (CYP), all of which exist as several isoforms exhibiting broad substrate specificity (12–16). In general, PUFA oxygenation is initiated by COXs, LOXs, and to a lesser extent CYPs. For example, the biosynthesis of PGs is initiated by COXs, whereas the formation of leukotrienes begins with a LOX. Additionally, crossover between COX, LOX, and CYP is proposed to produce various SPMs. On the other hand, cholesterol oxidation is dominated by CYPs. For example, sidechain shortening during steroid hormone synthesis is catalyzed by CYP11A1 (P450scc). Bile acids are synthesized from cholesterol predominantly by two pathways: the neutral pathway starting with an endoplasmic reticulum resident enzyme CYP7A1, and the acidic pathway starting with CYP27A1 in mitochondria (17). CYPs account for many enzymes in both pathways, catalyzing the formation of oxysterols as well as oxygenated PUFAs. Last, aldo-keto reductases (AKR) and hydroxysteroid dehydrogenases (HSD) catalyze key redox reactions in bile acid and steroid hormone biosynthesis.

By contrast, non-enzymatically oxidized lipids are produced through uncontrolled oxidation *via* free radical mechanisms. This involves the oxidation of lipids by free radicals, followed by chain propagation and ultimately termination. Notably, during oxygenation of PUFA, active site intermediates can escape to react in an uncontrolled manner, leading to some oxidized lipids forming due to non-enzymatic rearrangements of enzymatic pathway intermediates.

Here, we provide an overview of major enzymes involved in lipid metabolism, including LOXs, COXs, CYPs and AKRs. Then we outline the biosynthetic pathways of oxylipins, oxPLs, oxysterols, bile acids and steroid hormones. Finally, we address outstanding questions and suggest directions for future work.

ENZYME FAMILIES INVOLVED IN THE BIOSYNTHESIS OF OXIDIZED LIPIDS

Lipoxygenase (LOX)

Human LOX: Isoforms and Tissue Distribution

Lipoxygenases (LOX) are a family of non-heme iron-containing dioxygenases. They catalyze the stereospecific addition of dioxygen to lipids containing a (1Z,4Z)-pentadiene group producing lipid hydroperoxides. The human genome contains six functional LOX genes, expressed in various tissues (**Table 1**). LOXs were traditionally named according to their positional specificity for arachidonic acid (AA). However, the latest characterized member eLOX3 has limited lipoxygenase activity (38), while some LOXs show preference for other PUFA. Sequence analysis of human LOXs (using UniProt entries (39)) shows that 12R-LOX (*ALOX12B*) is evolutionarily closer to 15-LOX-2 (*ALOX15B*; 48.2% identity) than 12S-LOX (*ALOX12*; 35.7% identity). Additionally, human 15-LOX-1 (*ALOX15*) exhibits dual positional specificity which is not reflected in the name (18). The lack of a robust naming system is a common cause of confusion. Thus, the use of gene names alongside enzyme names is recommended (14).

LOXs are typically constitutively expressed, except for 15-LOX-1 (*ALOX15*) which is inducible by IL-4 and IL-13 in monocyte-derived macrophages (28). Although 15-LOX-2 (*ALOX15B*) is constitutively expressed in the same cell type, its expression can be increased by cytokines, hypoxia, and lipopolysaccharide. It is possible that other constitutively expressed LOXs share this property.

Structure and Membrane Association of Mammalian LOXs

There are a limited number of published crystal structures for mammalian LOXs. Available structures of 5- and 15-LOXs show a single polypeptide chain that consists of two domains: a small β-barrel *N*-terminal domain and a larger α-helix-rich C-terminal domain containing the catalytic non-heme iron (40–42). The coordination positions of the catalytic iron are occupied by three conserved His residues, the carboxyl group of the C-terminus Ile residue, a water molecule and one last variable ligand (water, His, Asn, or Ser). Studies on mammalian LOXs found that the *N*-terminal domain is not required for catalytic activity, but instead functions in membrane binding and regulation (43, 44). The *N*-terminal domain of mammalian 5-LOXs (*ALOX5*) was found to be important for the calcium-dependent translocation from the

TABLE 1 | Human LOXs: Genes, substrates, and major expression sites.

Gene	Protein	Preferred substrate(s)	Expression sites	Refs.
ALOX12	12S-LOX	DHA & EPA > AA	Platelets, umbilical vein endothelial cells, vascular smooth muscle cells, skin epidermis	(18–22),
ALOX12B	12R-LOX	O-Linoleoyl-ω-hydroxyceramide AA & 8,11,14-eicosatrienoic acid > GLA	Hair roots, keratinocytes, B-cells, tonsil epithelial cells, bronchial epithelial cells	(23–27)
ALOX15	15-LOX-1 (12/15 LOX murine ortholog)	DHA > EPA > AA	Monocytes, macrophages, dendritic cells, eosinophils, reticulocytes, tracheal epithelium	(18, 28–32)
ALOX15B	15-LOX-2 (8-LOX murine ortholog)	DHA > EPA > AA	Macrophages, hair roots, prostate, lung, cornea, skin	(18, 28, 33),
ALOX5	5-LOX	AA & 5S-HpETE	Leukocytes, dendritic cells, mast cells, lung, placenta	(34–36)
ALOXE3	eLOX3	9R-Hydroperoxy-linoleoyl-ω-hydroxyceramide 12R-HpETE	Skin epidermis	(25, 37)

AA arachidonic acid DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; HpETE, hydroperoxy-eicosatetraenoic acid.

cytosol or nucleus (depending on cell type) to the nuclear envelope and enzyme activity (45, 46). Similar findings were reported for mammalian 15-LOXs, with the translocation from the cytosol to the cell membrane instead (47, 48). Unlike other LOXs, the activity of 5-LOX requires interaction with partner proteins 5-lipoxygenase activating protein (FLAP), coactosin-like protein (CLP) and cytosolic phospholipase A2 ($cPLA_2$) (49, 50). Additionally, 5-LOX undergoes phosphorylation at multiple sites, regulating its translocation and activity, and is allosterically activated by ATP (51–53).

On the other hand, 15-LOXs do not require accessory proteins for free FA oxygenation. Instead, 15-LOXs form a complex with a small scaffolding protein, phosphatidylethanolamine-binding protein 1 (PEPB1) (54). This complex facilitates 15-LOX activity on PL-esterified PUFA and is proposed to play regulatory roles in ferroptosis along with GPX. PEPB1 has been suggested to direct 15-LOX activity toward PL substrates when free AA is depleted (55). The crystal structure of human 15-LOX-2 (*ALOX15B*) revealed a hydrophobic loop in the *N*-terminal domain that is flanked by calcium binding sites, a feature that is absent in 5-LOX (42). Hydrogen-deuterium exchange mass spectrometry showed a decrease in H/D exchange for the hydrophobic loop, supporting a role in membrane hopping (48, 56). Both 5-LOX and 15-LOXs associate with membranes in a calcium-dependent process but they exhibit differences in activity, translocation and binding partners.

Calcium-dependent membrane association has also been shown for platelet 12S-LOX (*ALOX12*), and more recently, 12R-LOX (*ALOX12B*), and eLOX3 (19, 57). The activity of 12S-LOX (*ALOX12*) is thought to be regulated by the availability of its substrates, which are supplied through the action of phospholipases. It is currently unknown whether 12S-LOX is regulated by other mechanisms. Research on the regulation of 12R-LOX and eLOX3 is also limited, although calcium has been shown to increase the activity of mouse 12R-LOX but not eLOX3 (58).

Aside from the catalytic domain of 12S-LOX (*ALOX12*), no crystal structures are available for 12-LOXs or eLOX3. However, human platelet 12S-LOX (*ALOX12*) was characterized using small-angle x-ray scattering, showing its occurrence as a dimer in solution (59). This challenged the idea that all human LOXs exist and function as monomers. Human 5-LOX displays full activity as a monomer but forms functional dimers as well, thought to exist in equilibrium (60). Similarly, rabbit 15-LOX-1 (*ALOX15*) undergoes ligand-induced dimerization in aqueous solutions, and molecular dynamics predicts stable dimers in the presence of substrate fatty acids (61). This supports the idea of monomer-dimer equilibria in LOXs. Human 15-LOX-1 (81.1% sequence identity to rabbit) likely exhibits the same property.

In summary, all six human LOXs associate with membranes in a calcium-dependent manner. 5-LOX requires the help of protein partners for membrane translocation and activity, whereas 15-LOXs only require a partner protein to efficiently catalyze the oxygenation of PL-esterified PUFA. Less is known about the regulation of 12-LOXs and eLOX3. Dimerization and monomer-dimer equilibria have been observed in several LOX isoforms and represent a potential regulatory mechanism. Finally, complete crystal structures have yet to be reported for 12S-LOX, 12R-LOX, and eLOX3. In the case of 12S-LOX, a crystal structure would be beneficial in the rational design of inhibitors, as the enzyme functions in platelet activation.

Reaction Mechanism of LOX

The dioxygenase activity of LOX represents a highly controlled form of lipid peroxidation (**Figure 1A**). First, stereospecific hydrogen atom removal is carried out by the non-heme ferric iron [Fe(III)]. Fe(III) is not a strong enough oxidizer to abstract hydrogen directly. Thus, a mechanism involving proton-coupled electron transfer (PCET) has been proposed (62) (**Figure 1A**). In this, the electron is directly transferred to Fe(III) and the proton is picked up by the hydroxide ligand in a concerted mechanism (simultaneously), producing a lipid alkyl radical and ferrous iron [Fe(II)]. This is followed by a rearrangement of the lipid radical into the more stable conjugated diene. After that, dioxygen is introduced onto the opposite side of the removed hydrogen (antarafacially), generating a lipid peroxyl radical. Finally, the lipid peroxyl radical is reduced by Fe(II) and protonated to form a lipid hydroperoxide, through PCET. This reforms Fe(III) for another round of catalysis.

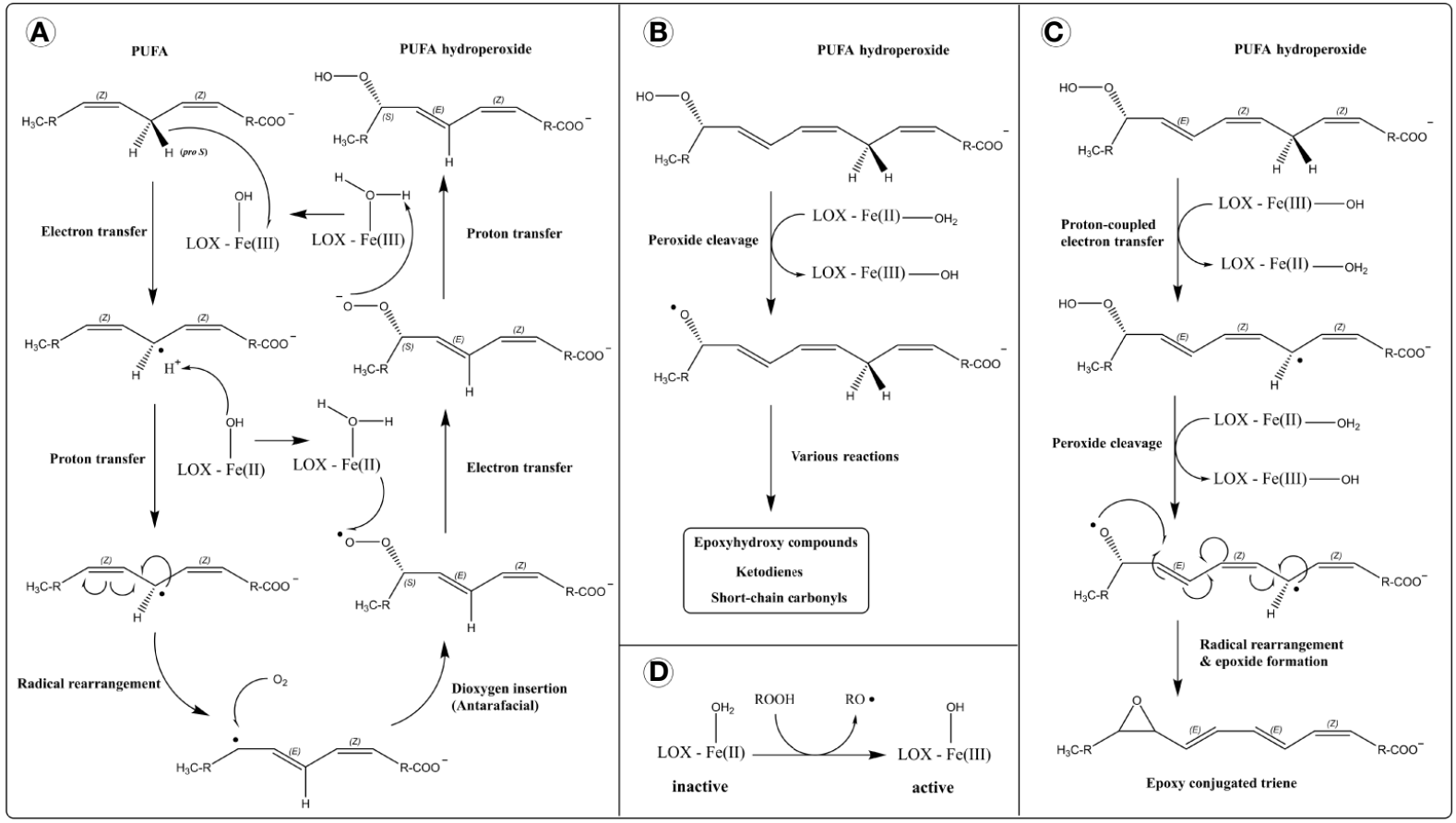

FIGURE 1 | Reaction mechanism of lipoxygenases (LOXs). **(A)** Dioxygenase activity. Hydrogen atom removal is thought to proceed through a proton-coupled electron transfer mechanism (PCET), in which the transfer of electron and proton occur simultaneously (concerted mechanism), depicted separately for simplicity. **(B)** Lipohydroperoxidase activity. **(C)** Leukotriene synthase activity. **(D)** Activation of LOX by a hydroperoxide.

The stereo and regio-specificity of lipoxygenation are determined by structural features which: (i) accommodate and position the substrate in a specific orientation, and (ii) direct radical rearrangement and oxygen insertion. These features include a U-shaped cavity that accommodates the substrate (63), a migration channel for oxygen (64), a glycine/alanine "switch" that determines stereospecificity by directing oxygen (65), and several amino acid residues that control positional specificity (66, 67). These properties are explored in-depth by Newcomer and Brash (68).

In addition to classical dioxygenase activity, LOXs catalyze other reactions that involve free-radical processes like hydrogen abstraction, homolytic bond cleavage, and radical rearrangement (69). eLOX3 exhibits a hydroperoxide isomerase activity (lipohydroperoxidase activity) which converts hydroperoxides to epoxy-alcohols and ketones (70) (**Figure 1B**). eLOX3 has restricted dioxygenase activity (38), and its hydroperoxide isomerase activity is considered its primary catalytic reaction. Also, mammalian 5-LOXs and human 15-LOX-1 (*ALOX15*) possess leukotriene A_4 synthase activity which produces epoxides from hydroperoxides (71–74). The mechanism involves the homolytic cleavage of a hydroperoxide into an alkoxy radical and hydrogen atom removal (*via* PCET) from a bis-allylic methylene carbon generating an alkyl radical. The resulting biradical is stabilized by epoxide formation (**Figure 1C**).

LOXs are inactive in their basal form with Fe(II) and require activation *via* oxidation into Fe(III) (**Figure 1D**). LOX activation is facilitated by hydroperoxides such as their dioxygenase reaction products. Although the proposed LOX catalytic cycle regenerates the ferric iron for another round of catalysis, small amounts of radical intermediates can escape the active site resulting in an incomplete catalytic cycle and requiring repeated activation of the enzyme (formation of Fe(III) by hydroperoxide) for sustained catalysis (69, 75, 76).

Some LOXs exhibit suicide inactivation, a property in which the reaction product rapidly inactivates the enzyme. Human 5-LOX is irreversibly inactivated by both of its products 5-HpETE and leukotriene A_4 (77). Similarly, rabbit reticulocyte 15-LOX (human 15-LOX-1 ortholog) is inactivated by its product 15-HpETE, and the mechanism is suggested to be through formation of reactive intermediates that covalently bind the enzyme (78). The dioxygenase reaction and the other two activities (lipohydroperoxidase and leukotriene synthase) have been proposed to inactive LOXs through different mechanisms, but the mechanistic details remain unclear (78).

LOXs Act on a Broad Range of Substrates

LOX isoforms have different substrate preferences. Substrates of mammalian 5-LOXs include AA, its hydroperoxide product 5*S*-HpETE, as well as epoxy-alcohols derived from EPA and DHA (71, 79). The former two are important in the biosynthesis of leukotrienes, while the latter two are proposed to be precursors of E- and D-series resolvins, respectively (75, 80). 15-LOXs (*ALOX15* and *ALOX15B*) accept AA, EPA, DHA, linoleic acid (LA), and γ-linolenic acid (GLA) as substrates. The preference of human 15-LOX orthologs is: DHA > EPA > AA > GLA > LA (18). One key difference is that 15-LOX-1 (*ALOX15*) possesses dual positional specificity for some of its substrates (e.g., 12 and 15-lipoxygenase activities for AA), whereas 15-LOX-2 (*ALOX15B*) exhibits singular specificity (15-lipoxygenase activity) (55). The hydroperoxide isomerase and epoxidase activities of 15-LOX-1 (*ALOX15*) are proposed to function in the synthesis of eoxins, E-series resolvins, and protectin from their respective precursors. Additionally, human 15-LOX-1 oxygenates PUFA-containing fatty amides and 2-arachidonoyl-glycerol ester (81, 82). Finally, mammalian 15-LOXs can catalyze the oxygenation of PUFA in lysophospholipids (lysoPL), PLs, CEs, and lipoproteins (83–88).

PUFA substrates of platelet 12S-LOX (*ALOX12*) include AA, EPA, DHA, and dihomo-γ-linolenic acid (DGLA) (18, 89). While no single study has compared all substrates at the same time, one determined the order of preference for the first three substrates to be: DHA > EPA > AA (18). Another found comparable kinetic parameters for EPA, AA, and DGLA as substrates (89). Both observed a singular positional specificity for 12S-LOX. LA, GLA, and α-linolenic acid (ALA) were all found to be poor substrates for 12S-LOX (18, 89, 90). Furthermore, human 12S-LOX possesses lipoxin synthase activity, which converts leukotriene A_4 into lipoxins A and B (91). The enzymatic activities of 12S-LOX are also proposed to function in the biosynthesis of hepoxilins and maresins (92, 93). Recently, 12S-LOX was shown to oxygenate 2-AA-lysoPL, proposing alternate pathways for oxylipin and oxPL biosynthesis (94).

Mammalian 12R-LOX (*ALOX12B*) and eLOX3 (*ALOXE3*) are co-expressed in skin and their functions are related. 12R-LOX efficiently catalyzes the peroxidation of AA, DGLA and GLA, and less efficiently LA, EPA and DHA (23, 24). Later, 12R-LOX was shown to oxygenate LA esterified to ω-hydroxyacyl-sphingosine more efficiently than free LA (25). The hydroperoxide product of this reaction is further converted into an epoxy-alcohol by the isomerase activity of eLOX3. These two reactions are essential for the proper formation of the water-impermeable barrier in corneocytes (25). Human eLOX3 isomerase activity has also been observed on the 12*R*- and 12*S*-hydroperoxides of AA, with the reaction occurring 2-3 times faster for the *R*-stereoisomer (95). These reactions produce hepoxilins, which play a role in skin inflammation. Curiously, mouse 12R-LOX and eLOX3 metabolize the methyl esters of AA and LA more efficiently than the unmodified FAs (58). However, human and mouse orthologs are known to exhibit differences in substrate preference and regio-specificity (95). Thus, further work is needed to assess the ability of human 12R-LOX to metabolize fatty methyl esters and whether that reaction is biologically relevant.

Several aspects of LOX enzymology remain unresolved. First, the functional implications of suicide inactivation are unknown, that is, the reason why some LOXs have not evolved to resist suicide inactivation. This suggests a biological function for this property, perhaps in regulation. Additionally, the mechanistic details of suicide inactivation are not fully understood. Second, due to the ever-increasing list of LOX substrates, it has been difficult to pinpoint biologically relevant substrates and assign

clear functions to all LOX isoforms. For example, the function of 15-LOX-2 (*ALOX15B*) in macrophages remains unclear, although it has been recently shown to regulate cholesterol levels (96). Unique substrates of each isoform (e.g., CEs for 15-LOXs, lysoPLs for 12S-LOX) might be worth investigating.

Cyclooxygenase (COX)

Human COX: Isoforms and Tissue Distribution

Cyclooxygenases (COXs), also called prostaglandin-endoperoxide synthases and prostaglandin G/H synthases, are heme-containing enzymes that possess both oxygenase and peroxidase activities. There are two COX genes in humans: *PTGS1* and *PTGS2*, which encode for COX-1 and COX-2, respectively. Traditionally, it was thought that COX-1 is constitutively expressed, whereas COX-2 is inducible in response to inflammatory signals. However, several studies suggest constitutive COX-2 expression in the brain, lungs, gut, thymus, kidneys, and blood vessels (97–99). In the vasculature, COX-2 has been demonstrated to be a key source of vascular prostacyclin (100, 101). COX-1 is ubiquitously expressed in the body, and its expression sites include blood vessels, prostate, immune cells (monocytes, T-cells), platelets, stomach, resident inflammatory cells, smooth muscles, and mesothelium of many organs (102–105). On the other hand, COX-2 is inducible in many tissues including prostate, immune cells (T-cells, B-cells, monocytes), and stomach (102–104, 106), but also constitutively expressed in some tissues as previously mentioned.

Structure of COX

The role of COXs as mediators of inflammation and their potential as drug targets were drivers for the elucidation of their structures. The crystal structure of sheep COX-1 has been solved in the presence and absence of various synthetic ligands (107–109). Similarly, there are available structures for mouse COX-2 in the presence and absence of natural and synthetic ligands (13, 110–113), as well as human COX-2 with several inhibitors (114, 115).

Both COX isoforms are homodimers, and this quaternary structure is necessary for enzymatic activity (116). Each monomer consists of three domains: An *N*-terminal epidermal growth factor (EGF)-like domain, a membrane binding domain, and a large C-terminal catalytic domain. The EGF-like domain is located at the dimer interface and potentially facilitates dimerization. It is also thought to facilitate membrane binding (117). The membrane binding domain consists of four amphipathic α-helices that insert into one face of a membrane bilayer. Both COXs are found on the luminal face of the endoplasmic reticulum (ER) and the inner and outer nuclear membranes (118). However, COX-2 is also found in the Golgi apparatus (119). The catalytic domain (in both isoforms) contains separate oxygenase and peroxidase active sites on opposite sides of the heme cofactor. The oxygenase active site is located towards the membrane binding face at the end of a hydrophobic tunnel that allows substrate entry, whereas the peroxidase active site is in a groove on the opposite face of the enzyme (120).

COXs undergo *N*-glycosylation at multiple asparagine residues. This facilitates their proper folding, and regulates the turnover of COX-2 by controlling its trafficking between the ER and the Golgi apparatus (119, 121, 122). COX-2 can also be *S*-nitrosylated by inducible nitric oxide synthase, which has been proposed to enhance its activity (123). *In vitro* experiments on recombinant human COX-1 and COX-2 demonstrated that both isoforms can be *S*-nitrosylated by a nitric oxide donor, but only COX-2 showed increased cyclooxygenase activity (124). Circular dichroism data suggest an altered, less dynamic conformation for both isoforms after *S*-nitrosylation with less α-helices, turns, and random coils, but more β-sheets. The structural change was more pronounced in COX-2 compared to COX-1. These findings provide new insight into the structural dynamics of COXs. The occurrence and biological relevance of this altered COX conformation is unexplored *in vivo* and would benefit from further investigation.

Although COXs are sequence homodimers, they are considered conformational heterodimers because one monomer functions as the catalytic subunit (E_{cat}) and the other as an allosteric regulator (E_{allo}) (125–127). For both COX isoforms, the binding of one heme molecule to E_{cat} is required for full activity. Several substrate and non-substrate FAs can bind E_{allo} to regulate the activity of E_{cat}, and the two COX isoforms exhibit differences in this regard. COX-1 is inhibited by palmitic acid, stearic acid, margaric acid and oleic acid (127), none of which are COX substrates. On the other hand, COX-2 is stimulated by palmitic acid and stearic acid (126). Substrate FAs (like AA and EPA) also bind to E_{allo} to regulate COX activity, with notable differences in the responses of the two isoforms (reviewed in (128)).

Catalytic Mechanism of COX

COXs possess two enzymatic activities: a dioxygenase activity and a peroxidase activity. The dioxygenase activity is a controlled peroxidation process (129), and the role of the enzyme is to direct hydrogen abstraction and the stereochemistry of formation of intermediates (120). Unlike LOXs where hydrogen abstraction is carried out by a non-heme associated Fe(III), hydrogen abstraction in COX is carried out by a catalytic tyrosyl radical (**Figure 2A**). The heme group oxidizes a tyrosine residue in the active site into a radical, which then abstracts a hydrogen (stereo- and regio-specifically) from the (1Z,4Z)-pentadiene group of the lipid. Using AA as an example substrate, the 13-*pro*-S hydrogen is abstracted. The resulting alkyl radical rearranges into a conjugated diene before the addition of dioxygen at C11, generating a peroxyl radical (*11R*-stereochemistry). Rotation of the peroxyl radical positions the outer oxygen atom in the correct orientation to attack the C9 carbon resulting in an endoperoxide (130). A second cyclization involving C8 and C12 generates a bicyclic ring and a radical at C15. Note that the cyclopentane ring is formed in the trans configuration (131). This differs from the non-enzymatically generated isoprostanes which occur predominantly in the cis configuration (132). Following the second cyclization, dioxygen is added onto the *si* face of the C15, forming a peroxyl radical (*15S*-stereochemistry). Hydrogen atom transfer from the active

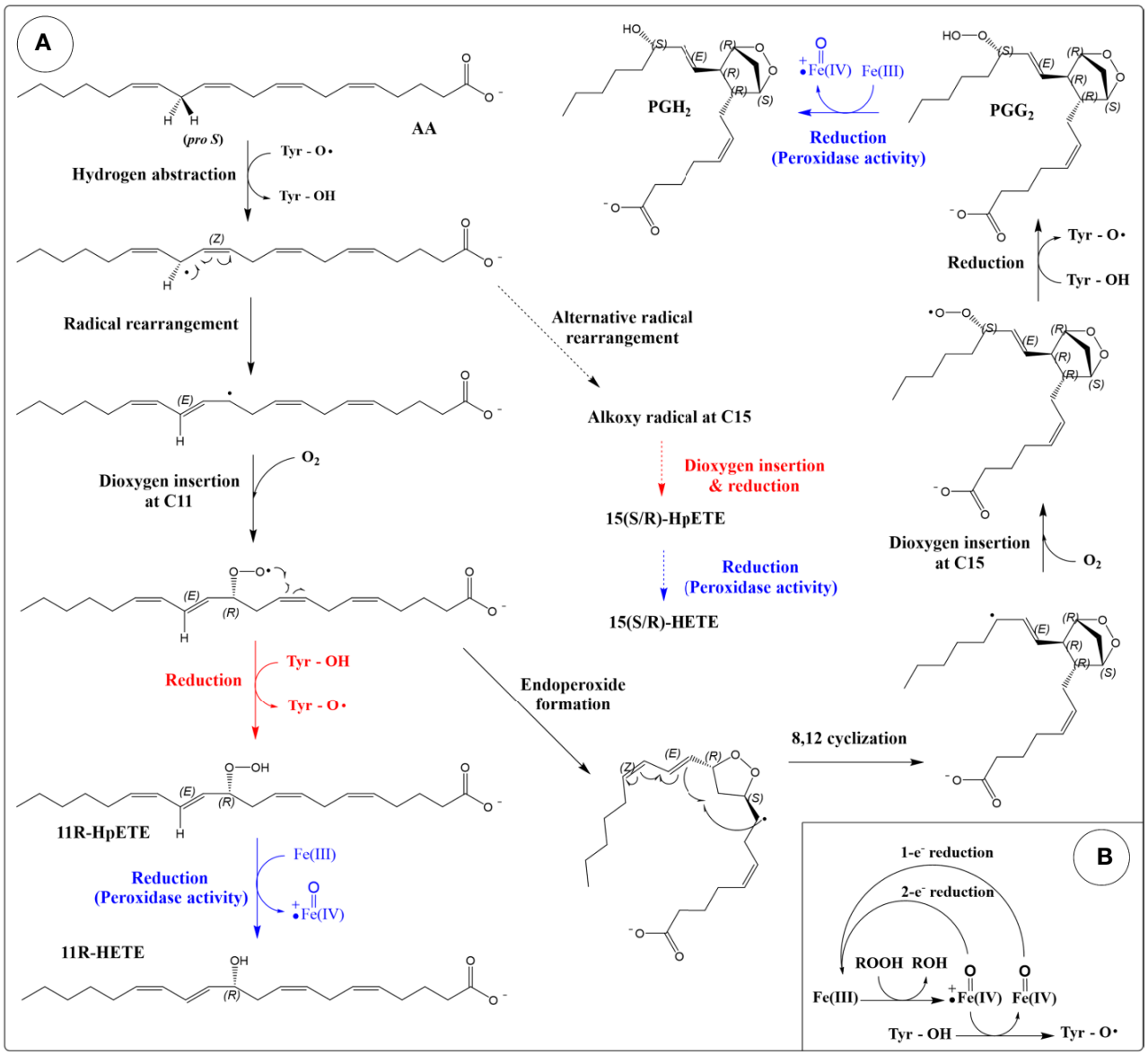

FIGURE 2 | Reaction mechanism of cyclooxygenases (COXs). **(A)** Production of eicosanoids from arachidonic acid through the dioxygenase and peroxidase activities of COX. The cyclooxygenase reaction is colored black. Peroxidase reactions are coloured blue. Reactions that produce HpETEs are colored red. Side reactions that produce 15-HETEs are depicted by dashed arrows. **(B)** The peroxidase cycle generates the Tyr radical required for hydrogen abstraction (porphyrin ring of heme not shown).

site tyrosine residue forms prostaglandin G_2 (PGG_2) and regenerates the tyrosyl radical for another round of catalysis.

The peroxidase activity of COX reduces PGG_2 into PGH_2 (**Figure 2A**). The mechanism involves a two-electron reduction of the hydroperoxide into an alcohol, with a corresponding oxidation of the heme group into the oxoferryl form (133) (**Figure 2B**). Regeneration of the heme group can be achieved by two sequential one-electron reductions. The peroxidase activity is required for the dioxygenase activity as the oxidation of the tyrosine into a radical is done by the oxoferryl-porphyrin cation radical generated during the peroxidase reaction cycle (133) (**Figure 2B**). Suicide inactivation has been described for both the dioxygenase and peroxidase activities of COX (133, 134).

The dioxygenase activity of COX can also result in a lipoxygenase-type reaction, in which one dioxygen molecule is introduced and no endoperoxide formation occurs (135, 136) (**Figure 2A**). In this case, the peroxyl radical formed after the addition of the oxygen is reduced into a hydroperoxide instead of participating in the cyclization reaction. This reaction represents an incomplete catalytic cycle and occurs as a side product. Using AA as a substrate, this lipoxygenase-type reaction (after

reduction by peroxidase) leads to the formation of 11*R*-HETE (HETE: hydroxy-eicosatetraenoic acid), 15*R*-HETE and 15*S*-HETE (minor product compared to 15*R*-HETE in both isoforms) (137). C11 and C15 are the same oxygen insertion sites in the dioxygenase (cyclooxygenase reaction), and these by-products are thought to arise from alternate conformations of substrate in the active site (138).

Aspirin (acetylsalicylic acid) is an inhibitor of COXs, and its mechanism of action involves the acetylation of a serine residue (Ser530 in COX-2) in the active site. Acetylation of COX-1 leads to complete inhibition (139), whereas the acetylation of COX-2 promotes the lipoxygenase-type reaction and the formation of 15*R*-HETE (140). It was previously thought that acetylation completely inhibits PG production by COX-2 (cyclooxygenase reaction). However, recent evidence shows that the acetylated enzyme retains that activity (15*S*-PG formation) but also more favorably forms 15*R*-PGs (141), although PGs are still minor products compared to 15*R*-HETE for acetylated-COX-2. These findings are consistent with an earlier study that reported the formation of 15*R*-PGE_2 in COX-2 Ser530 mutants (137). Thus, both acetylation and mutagenesis of Ser530 in COX-2 promote 15*R*-PG formation.

COX Isoforms Differ in Their Substrate Specificity

COXs catalyze the transformation of AA into PGH_2, which is a precursor of other PGs (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2) and thromboxane. These oxygenated AA derivatives are generated through the action of tissue-specific enzymes downstream of COX (discussed in section 3.1) and play roles in inflammation, blood flow regulation and blood clotting through interactions with specific GPCRs (142).

COXs also accept other PUFAs as substrates including DGLA, LA, ALA, EPA, and GLA (143), at least *in vitro*. An early study determined the efficiency of substrate utilization for human COXs (K_{cat}/K_m) to be AA > DGLA > LA > ALA, with ALA being a poor substrate for COX-1. EPA and GLA are poor substrates for both isoforms but they are better substrates for COX-2 than for COX-1 (143). Later studies have tested other PUFAs like eicosadienoic acid, adrenic acid, docosapentaenoic acid and DHA as COX substrates (127, 144). In general, COX-2 was found to be more efficient than COX-1 for a broader range of PUFAs. That said, AA is the preferred substrate for both isoforms, but COX-2 can oxygenate it at lower concentrations compared to COX-1 due to differences in their allosteric regulation by FAs (145).

COX-2 can also catalyze the 11*R*-, 15*R*-, 15*S*-dioxygenation and bis-oxygenation of 5*S*-HETE, forming diHETEs in the former three reactions and a di-endoperoxide product in the later reaction. However, acetylation of COX-2 shifts the specificity into favoring 15*R*-dioxygenation producing 5*S*,15*R*-diHETE (146). Additionally, COX-2 can oxygenate AA in complex lipids for example; arachidonoylethanolamide, 2-arachidonoylglycerol (2-AG), and N-arachidonoyl-glycine (147–149). Recently, 2-AA-lysoPL, and ethanolamide derivatives of EPA and DHA were also shown to be COX-2 substrates (88, 150). Oxygenation of AA-lysoPL by COX-2 generates eicosanoid-lysoPL, which can be hydrolyzed to release eicosanoids through intracellular lipases (88). The ability of COX-2 to bind and oxygenate a broader range of substrates compared to COX-1 has been attributed to a larger active site, the orientation of an Arg residue in the substrate binding pocket and amino acid residues lining the tunnel leading to the cyclooxygenase active site (13).

COX isoforms exhibit differences in expression, tissue distribution, allosteric regulation, and substrate specificity. However, some aspects of COX biochemistry are unclear. For example, the physiological functions of COX-2-derived oxygenated endocannabinoids are unclear. Additionally, COX-2 oxygenates AA-lysoPLs into eicosanoid-lysoPLs, which are proposed to function in signaling and as precursors to other mediators (88). However, the metabolism of eicosanoid-lysoPLs requires further investigation.

Cytochrome P450 (CYP)

Human CYPs: Nomenclature and Tissue Distribution

Cytochrome P450s (CYP) are a superfamily of heme-containing monooxygenases that are ubiquitous across all domains of life. There are 57 functional CYP genes in humans, and their products are further divided into 18 families and 41 sub-families based on amino acid sequence (151, 152). A robust, unified nomenclature system has been devised for CYPs, encompassing all known CYPs across living organisms (153, 154). The name includes the root "CYP", followed by a number for family, a letter for subfamily, and a gene-identifying number for isoforms. Additionally, an asterisk and a number are added at the end to denote alleles (155). Families and subfamilies are based on 40% and 55% amino acid sequence identity, respectively.

CYPs are widely distributed in mammalian tissues (156–158), with a particularly high expression in the liver, brain, kidney and lung. Intracellularly, mammalian CYPs are generally bound to mitochondrial membranes and the endoplasmic reticulum (ER; microsomes when *in vitro*) (159, 160). There is also evidence of mammalian CYPs in other compartments including the plasma membrane and the nucleus, based on *in vitro* studies on cultured cells (161–164). Some microsomal CYPs are also targeted into the mitochondria (159). In the human genome, 50 out of the 57 functional CYP genes code for microsomal CYPs, and the remaining seven for mitochondrial isoforms. Some CYPs are inducible by environmental stimuli, whereas others are constitutively expressed (152). Induction of CYP expression by environmental compounds can be through interactions with nuclear receptors, transcriptional regulatory elements, or non-coding RNAs (165).

Two major functions of CYPs are in drug metabolism and lipid metabolism. Here, we focus on lipid metabolism which involves members of most human CYP families. For an extensive review, the reader is referred to Nelson and Nebert (166).

Structure of Mammalian CYPs

Structural characterization was first carried out on bacterial CYPs, which are typically water soluble. On the other hand, mammalian CYPs are membrane-bound, and initial attempts to crystallize them were unsuccessful until a small *N*-terminal hydrophobic helical segment was replaced with a hydrophilic sequence from a related protein. The first structure to be

published was of rabbit CYP2C5, a microsomal CYP isoform that hydroxylates progesterone (167). Comparison of CYP2C5 structure with closely related microbial CYPs showed a similar folding geometry, but unique features of the mammalian enzyme were observed such as a hydrophobic surface that was proposed to play a role in membrane binding (167).

CYPs consist of two domains: a β-sheet-rich *N*-terminal domain and a larger helix-rich C-terminal catalytic domain. The *N*-terminal domain in microsomal CYPs contains a transmembrane helix that plays a role in membrane anchoring, but that role is not exclusive as CYPs contain other regions (in the catalytic domain) that bind the membrane. This transmembrane helix is not present in mitochondrial CYPs, and their membrane binding is facilitated by hydrophobic and amphipathic regions on the surface (160, 168). The *N*-terminus also contains a signal peptide sequence for trafficking into the appropriate compartment, that in the case of mitochondrial CYPs, is cleaved during transport. The catalytic domain contains a deep cavity that houses the heme prosthetic group. A thiolate ion in a conserved cysteine residue occupies the fifth coordination position of heme, and a water molecule occupies the sixth position in the resting state. There is variable flexibility in the active site among CYPs, and a high degree of flexibility has been correlated to substrate promiscuity (169, 170). Residues in helices surrounding the active site heme position the substrate for catalysis. Additionally, in the CYP4 family, a covalent linkage has been identified between the heme group and a conserved glutamic acid residue (171), and this interaction plays a role in substrate positioning for ω-hydroxylation (hydroxylation at the methyl end of FAs) (172).

Other structural features of CYPs include access channels that allow entry of substrates from both the aqueous and membrane compartments and an exit channel for the product (160). Furthermore, oligomerization is well-documented in both microsomal and mitochondrial CYPs, which can be between subunits of the same CYP (homomeric; e.g., CYP2C2) or different CYPs (heteromeric; e.g., CYP2E1/CYP3A4 and CYP1A2/CYP2B4) (173–180). These interactions provide an additional layer of regulation and play a role in substrate specificity.

Enzymology of Mammalian CYPs

CYPs catalyze a wide range of reactions (181, 182), but the most common include C-hydroxylation, heteroatom oxidation, heteroatom dealkylation, epoxidation, and group migration (182). Many reactions of CYPs are dependent on their ability (owing to the thiolate-heme group) to catalyze the scission of dioxygen and incorporate one of the oxygen atoms into the substrate, with the other being reduced to water. These reactions require electron transfer from a donor, NADPH, to the heme iron. However, transfer of electrons from NADPH to CYPs requires protein partners: NADPH cytochrome P450 reductase in the case of microsomal CYPs, and the combined functions of adrenodoxin reductase and adrenodoxin in mitochondrial CYPs. Thus, CYPs form complexes with their redox partners, at least during catalysis. That said, not all CYP reactions require external oxygen or electron donors. Examples are isomerization reactions catalyzed by CYP5A1 (thromboxane synthase) and CYP8A1 (prostacyclin synthase) (183), two enzymes involved in PGH_2 metabolism.

A generalized reaction mechanism for CYPs (**Figure 3A**) contains the following steps: starting with the resting state, substrate recruitment displaces H_2O from the 6^{th} (axial) position of the heme Fe(III). This is followed by a 1-electron reduction from a donor forming Fe(II), O_2 binding, and another 1-electron reduction resulting in a negatively charged peroxo group. Protonation of this complex twice by nearby H_2O or amino acid residues results in the scission of the dioxygen (O-O bond) into an oxoferryl intermediate (compound I) and a water molecule (184). Compound I is thought to be the direct oxidant in many CYP oxidation reactions (185). In hydroxylation reactions, compound I abstracts a hydrogen from an alkyl group in the substrate generating a carbon radical and an iron oxygen complex, which rapidly react together forming a hydroxyl group on the substrate and regenerating the heme iron into the Fe(III) state (**Figure 3B**). Although this is a generalized reaction scheme for CYPs, the intermediates following O_2 binding until compound I have been difficult to characterize due to their instability and the exact electronic structures are not fully assigned (185).

Mammalian CYPs Play Crucial Roles in Oxidative Lipid Metabolism

CYPs are involved in the metabolism of a wide range of lipids including PUFAs and sterols (**Table 2**). Hydroxylation and epoxidation are two common CYP-mediated reactions for PUFA substrates. CYPs can hydroxylate FAs terminally (ω-hydroxylation) or midchain. Similarly, CYP-mediated epoxidation can occur on various double bonds in PUFA. CYPs exhibit variable preference with respect to the reaction type and positional specificity. CYP1-3 families catalyze epoxidation and hydroxylation reactions for FAs, whereas the CYP4 family favors hydroxylation over epoxidation, specifically ω-hydroxylation (204).

Terminal hydroxylation of AA by CYPs produces 20-hydroxyeicosatetraenoic acid (20-HETE), which functions in blood pressure regulation and water balance (205). Additionally, terminal hydroxylation functions in ω-oxidation (a catabolic pathway for FAs), the degradation of some eicosanoids (206, 207), and the proper formation of the skin permeability barrier (208). Midchain hydroxylation of PUFA produces mono-hydroxylated derivatives (HETEs in the case of AA) with various bioactivities (reviewed in (152)). Unlike LOXs and COXs which oxygenate carbons two bonds away from bis-allylic methylene carbons, CYPs can also oxygenate bis-allylic carbons (producing 7-, 10- and 13-HETE from AA), and various other positions (209–211). However, bis-allylic hydroxylation products are unstable in mildly acidic conditions and rearrange into conjugated dienes (211).

Epoxidation of AA by CYPs produce epoxy-eicosatrienoic acids (EET). However, other PUFA like LA, EPA and DHA also undergo CYP-mediated epoxidation. Epoxy derivatives of PUFAs are implicated in blood pressure regulation and inflammation (15). Their metabolism is undertaken by several epoxide hydrolases including soluble epoxide hydrolase (sEH)

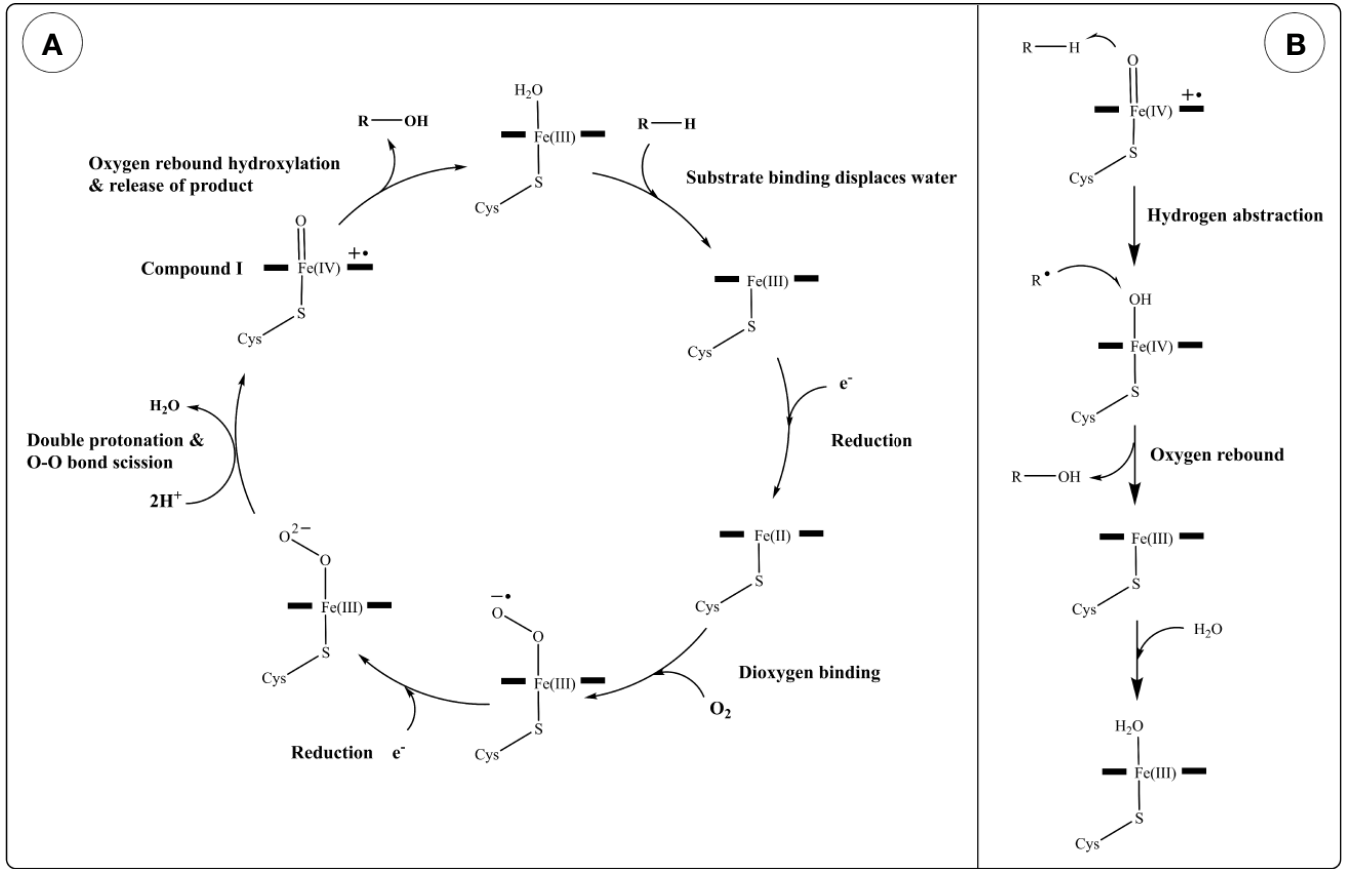

FIGURE 3 | Reaction mechanism of cytochrome P450s (CYPs). **(A)** Generalized catalytic cycle of CYPs. **(B)** Hydroxylation of a generic substrate (R–H) by compound I *via* an oxygen rebound mechanism.

TABLE 2 | Examples of human cytochrome P450s (CYPs) and their involvement in lipid metabolism.

Enzyme	Lipid substrate(s)	Reaction(s)	Refs.
CYP1A1	AA, EPA, DHA	Epoxidation & hydroxylation	(186)
CYP2C8	AA, EPA, DPA, DHA	Epoxidation & hydroxylation	(186–188)
CYP3A4	Sterols	Hydroxylation	(189–191)
CYP4A11	Lauric acid, PA	ω-Hydroxylation	(188, 192),
	AA, EPA, DPA, DHA	ω/ω-1-Hydroxylation	
CYP4F2	AA, EPA, DPA, DHA	ω/ω-1-Hydroxylation	(188)
CYP5A1 (thromboxane synthase)	PGH_2	Isomerization	(183)
CYP7A1	Cholesterol, 7-DHC	7α-Hydroxylation, epoxidation, carbonylation	(161, 193, 194)
CYP7B1	Oxysterols, steroids	7α-Hydroxylation	(195)
CYP8A1 (prostacyclin synthase)	PGH_2	Isomerization	(183)
CYP8B1	Sterols	12α-Hydroxylation	(196)
CYP11A1 (P450scc)	Cholesterol	Side-chain cleavage	(197)
CYP11B1	11-Deoxycortisol, 11-deoxycorticosterone	11β-Hydroxylation	(198)
CYP11B2	11-Deoxycorticosterone	11β-Hydroxylation, 18-hydroxylation & oxidation	(198)
CYP17A1	Pregnenolone, progesterone	17-Hydroxylation	(199)
CYP19A1 (P450arom/aromatase)	Testosterone	Aromatization	(199)
CYP21A2	Progesterone	Hydroxylation	(199)
CYP24A1	Calcitriol	24-Hydroxylation	(200)
CYP26A1	all-*trans*-Retinoic acid	Hydroxylation	(201)
CYP27A1	Sterols	(25R)26-Hydroxylation and carboxylation	(197)
CYP39A1	24-hydroxycholesterol	7α-Hydroxylation	(17)
CYP46A1	Cholesterol, desmosterol	24S-Hydroxylation, 24S-epoxidation	(197, 202)
CYP51A1	Lanosterol	14α-Demethylation	(203)

7-DHC, 7-dehydrocholesterol; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; PA, palmitic acid; PGH_2, prostaglandin H_2.

and microsomal epoxide hydrolase (mEH) (212). The action of epoxide hydrolases produces dihydroxy derivatives which possess different bioactivities from their epoxide precursors (15).

Arachidonoylethanolamide also undergoes CYP hydroxylation and epoxidation (213, 214). Similarly, CYPs catalyze epoxidation of EPA and DHA ethanolamides, producing compounds with anti-inflammatory and anti-angiogenic effects (215). These lipids can be degraded by both sEH and fatty acid amide hydrolase (FAAH) (215), suggesting a link between the CYP epoxygenase and endocannabinoid pathways. The metabolism and biological roles of these mediators is an active area of research (216).

CYPs catalyze several types of reactions in the metabolism of sterols, some of which involve multiple oxygenation/hydroxylation steps. Demethylation of lanosterol by CYP51A1 is a key reaction in cholesterol biosynthesis and involves three successive oxidations of a methyl group, resulting in its release as formic acid and the introduction of a double bond at the D ring (217). Similarly, the formation of estradiol from testosterone *via* the action of CYP19A1 (P450arom) involves the elimination of a methyl group by three successive oxidation steps and the introduction of a double bond at the A ring. Additionally, cholesterol side-chain cleavage by CYP11A1 (P450scc) involves two hydroxylation steps on different carbons followed by a C-C bond cleavage (218). This reaction produces pregnenolone which is a key intermediate in the formation of androgens, estrogens, glucocorticoids and mineralocorticoids.

CYP-mediated hydroxylation reactions are ubiquitous in cholesterol metabolism, leading to the formation of oxysterols, steroid hormones and bile acids. Key enzymes in the bile acid biosynthesis pathways are CYP7A1 (cholesterol 7α-hydroxylase), CYP8B1 (sterol 12α-hydroxylase), CYP27A1 (commonly named sterol 27-hydroxylase, but more correctly sterol (25R)26-hydroxylase) and CYP7B1 (oxysterol 7α-hydroxylase) (219, 220). Of these enzymes, CYP27A1 can both hydroxylate and carboxylate the terminal carbon of the sterol side-chain (221). Other enzymes involved in quantitatively minor bile acid biosynthesis pathways are CYP46A1 (cholesterol 24S-hydroxylase) which 24S-hydroxylates cholesterol (222, 223) and CYP3A4 which has 4β- and 25-hydroxylase activity (189, 224). CYP enzymes involved in steroid hormone biosynthesis include CYP11A (P450scc) (225), CYP17A1 (steroid 17α-hydroxylase) (226), CYP21A2 (steroid 21-hydroxylase), CYP11B1 (steroid 11β-hydroxylase), and 11B2 (aldosterone synthase) and CYP19A1 (aromatase) (198).

Thus, CYPs play indispensable roles in the metabolism of both PUFA and cholesterol. However, some human CYPs like CYP2A7 and CYP20A1 have no assigned functions and remain orphan enzymes.

Human Aldo-Keto Reductases (AKRs) and Hydroxysteroid Dehydrogenases (HSDs)

AKRs: Nomenclature, Genes, and Tissue Distribution

Aldo-keto reductases (AKRs) are a superfamily of NADPH-dependent oxidoreductases. They catalyze the reduction of carbonyl groups to alcohols. A nomenclature system has been proposed for AKRs (227). Like CYPs, AKRs are grouped into families and subfamilies based on amino acid sequence. Families have 40% sequence identity, whereas subfamilies are defined by 60% sequence similarity. The nomenclature consists of the root

"AKR" followed by a number for family, a letter for subfamily and finally a number as a unique gene identifier. The nomenclature has also been expanded to accommodate multimers (228). The older (trivial) names of AKRs are still heavily in use, which could lead to confusion. Thus, it is advised to follow the nomenclature system proposed by Jez et al. (227).

There are 15 AKRs in the human genome spanning three families and seven subfamilies. Human AKRs have variable expression and accept a wide range of substrates (**Table 3**). They are involved in the metabolism of sugars, prostaglandins and sterols, as well as the detoxification of carbonyl compounds like lipid peroxidation products. Additionally, members of family 6 (AKR6A3, AKR6A5, and AKR6A9) are constituents of voltage-gated potassium channels (250). All human AKRs have either established or proposed roles in lipid metabolism except for AKR1E2.

Human AKRs are generally cytosolic with some exceptions. AKR1B15 is found in mitochondria (236). Members of the AKR1C subfamily in the lung (1C1, 1C2 and 1C3) are also secreted in pulmonary surfactant (256). AKR1B10 is present in lysosomes and is secreted into the intestinal lumen by epithelial cells (257). AKR6 members form a complex with a plasma membrane voltage channel on the cytosolic side. Finally, rat AKR7A2 associates with the Golgi apparatus (258). Human AKR7A2 contains a similar *N*-terminal amphipathic sequence and likely associates with the Golgi apparatus.

Mammalian AKRs: Structure and Enzymology

Crystal structures have been solved for many mammalian AKRs. They comprise of a triosephosphate isomerase barrel motif $(\alpha/\beta)_8$ which has alternating α-helices and β-strands repeating eight times. The α-helices surround an internal β-barrel formed by the β-strands. The active site is located at the base of the barrel, in which the substrate and the nicotinamide head group of NADPH (cofactor) are proximally positioned for the reaction, while three flexible loops form the back of the barrel and control substrate specificity (259, 260). The structure also contains two additional helices that are not part of the barrel motif. Family 1 AKRs are monomeric, whereas families 6 and 7 form tetramers and dimers, respectively (258, 261).

AKRs catalyze the stereospecific reduction of carbonyls into alcohols. They can also catalyze the reverse oxidation reaction. However, the reducing direction is generally favored due to the abundance of reduced cofactor in the cellular environment. AKRs can use both NADPH and NADH, but NADPH is the preferred cofactor for most known AKRs as they interact with the phosphoryl group present in NADPH but not NADH (262, 263). The stereospecific nature of the reaction is due to the orientation of the NADPH and the substrate in the active site. The NADPH is bound in the anti-conformation (with respect to the ribose ring) which promotes the transfer of the 4-*pro*-R hydride (264), and the substrate is positioned perpendicular to the cofactor.

The reaction of AKRs follows an ordered bi-bi mechanism in which the NADPH cofactor binds first and leaves last. The active site of AKRs contains a highly conserved catalytic tetrad of Tyr, Lys, Asp and His. The hydride transfer is facilitated by acid-base catalysis involving the protonation state of Tyr and the other catalytic residues. In the reduction direction, the protonated form of Tyr acts as an acid by participating in proton relay

TABLE 3 | Human AKRs: Genes, expression sites, and example substrates.

Gene	Alternative protein name	Expression sites	Example substrates	Refs.
AKR1A1	Aldehyde reductase	Brain, kidney, liver, small intestine	4-HNE, acrolein, succinic semi-aldehyde, D-glucuronic acid, phospholipid aldehydes	(229, 230),
AKR1B1	Aldose reductase/Prostaglandin F synthase	Ubiquitous	Glucose, 4-HNE & its glutathione conjugate, acrolein, PGH_2, phospholipid aldehydes	(229, 230),
AKR1B10	Small intestine aldose reductase	Small intestine, colon, liver, cornea	Farnesal, retinoids, acrolein, phospholipid aldehydes	(229, 231–235),
AKR1B15	Aldo-keto reductase/3-Keto-acyl CoA reductase	Placenta, testis, adipose tissue	Androgens, estrogens, 3-keto-acyl CoA conjugates	(236)
AKR1C1	20α-HSD	Kidney, lung, liver, testis, brain	Progesterone, estrone, 5α-dihydrotestosterone, 4-HNE	(230, 237–239),
AKR1C2	Type 3 3α-HSD	Liver, brain, lung, prostate	Progesterone, estrone, 5α-dihydrotestosterone	(237, 238),
AKR1C3	Type 5 17β-HSD/Prostaglandin F synthase	Liver, lung, prostate, brain, breast, lymphocytes	Progesterone, estrone, 5α-dihydrotestosterone, PGH_2, PGD_2, 4-HNE	(238, 240–242),
AKR1C4	Type 1 3α-HSD	Liver	3-Keto-5β-sterols, 5α-dihydrotestosterone	(230, 238),
AKR1D1	Steroid 5β-reductase	Liver, placenta, brain	Δ^4-Ketosteroids, particularly bile acid intermediates	(237, 243–245),
AKR1E2	1,5-Anhydro-D-fructose reductase	Liver, testis	1,5-Anhydro-D-fructose	(246, 247)
KCNAB1	Potassium voltage-gated channel β-subunit-1 (Kvβ1)/AKR6A3	Brain, heart	Lipid peroxidation-derived aldehydes (presumed)*	(248, 249)
KCNAB2	Potassium voltage-gated channel β-subunit-2 (Kvβ2)/AKR6A5	Brain, spinal cord	Methylglyoxal, acrolein, 4-ONE, oxPL, PGJ_2*	(250–252)
KCNAB3	Potassium voltage-gated channel β-subunit-3 (Kvβ3)/AKR6A9	Brain	No data.	(253)
AKR7A2	Aflatoxin aldehyde reductase (AFAR1)	Ubiquitous	Aflatoxin B1, succinic semi-aldehyde, 4-HNE	(230, 254)
AKR7A3	Aflatoxin aldehyde reductase (AFAR2)	Liver, stomach, pancreas, kidney	Aflatoxin B1	(255),

**Evidence from work on the rat ortholog. 4-HNE, 4-hydroxy-2-nonenal; 4-ONE, 4-oxo-2-nonenal; HSD, hydroxysteroid dehydrogenase; oxPL, oxidized phospholipid; PG, prostaglandin (e.g., PGH_2, PGD_2, PGJ_2).*

with His (259) (**Figure 4A**). This polarizes the carbonyl group of the substrate, allowing it to accept a hydride ion from the cofactor. In the oxidation direction, the phenolate form of Tyr (generated through proton relay with Lys and Asp) acts as a base and abstracts a proton from the alcohol group of the substrate, facilitating hydride transfer to the cofactor (259) (**Figure 4B**).

AKR1D subfamily members catalyze the irreversible 5β-reduction of sterol double bonds into single bonds, instead of the reversible reduction of carbonyls typical of AKRs (**Figure 4C**). They possess an altered catalytic tetrad with Glu replacing His (265). This substitution allows the substrate to penetrate deeper into the active site pocket, positioning C5 close to the 4-*pro*-H of NADPH (259). The catalytic Glu residue is thought to act as a superacid, facilitating the enolization of the steroid double bond and allowing hydride transfer to C5 (259, 265).

Human AKR1B1 catalyzes the isomerization of PGH_2 to PGD_2 in the absence of cofactor, as well as the reduction of PGH_2 to $PGF_{2\alpha}$ in the presence of cofactor (266). Curiously, both of these reactions are reported to be facilitated by a catalytic triad of Lys, His and Asp, without the involvement of Tyr in catalysis, although Tyr is still required for the *p*-nitrobenzaldehyde reductase activity of the enzyme (266). Thus, both Tyr and His can participate in acid-base catalysis, at least in AKR1B1.

Involvement of Human AKRs in Lipid Metabolism

Human AKRs play roles in the detoxification of reactive carbonyls. Several AKRs metabolize lipid peroxidation-derived aldehydes like acrolein and 4-HNE (**Table 3**). Additionally, some AKRs (1A1, 1B1, 1B10, 6A5) can reduce oxidized phospholipids, particularly phospholipid aldehydes (229, 251). These activities mitigate the cytotoxicity of reactive carbonyls (240, 267–269), and in the case of family 6 AKRs are also thought to function in redox sensing (251).

Both AKR1B1 and AKR1C3 exhibit prostaglandin F synthase activity (270, 271). AKR1B1 catalyzes the reduction of PGH_2 to $PGF_{2\alpha}$. AKR1C3 catalyzes the same reaction as well as the

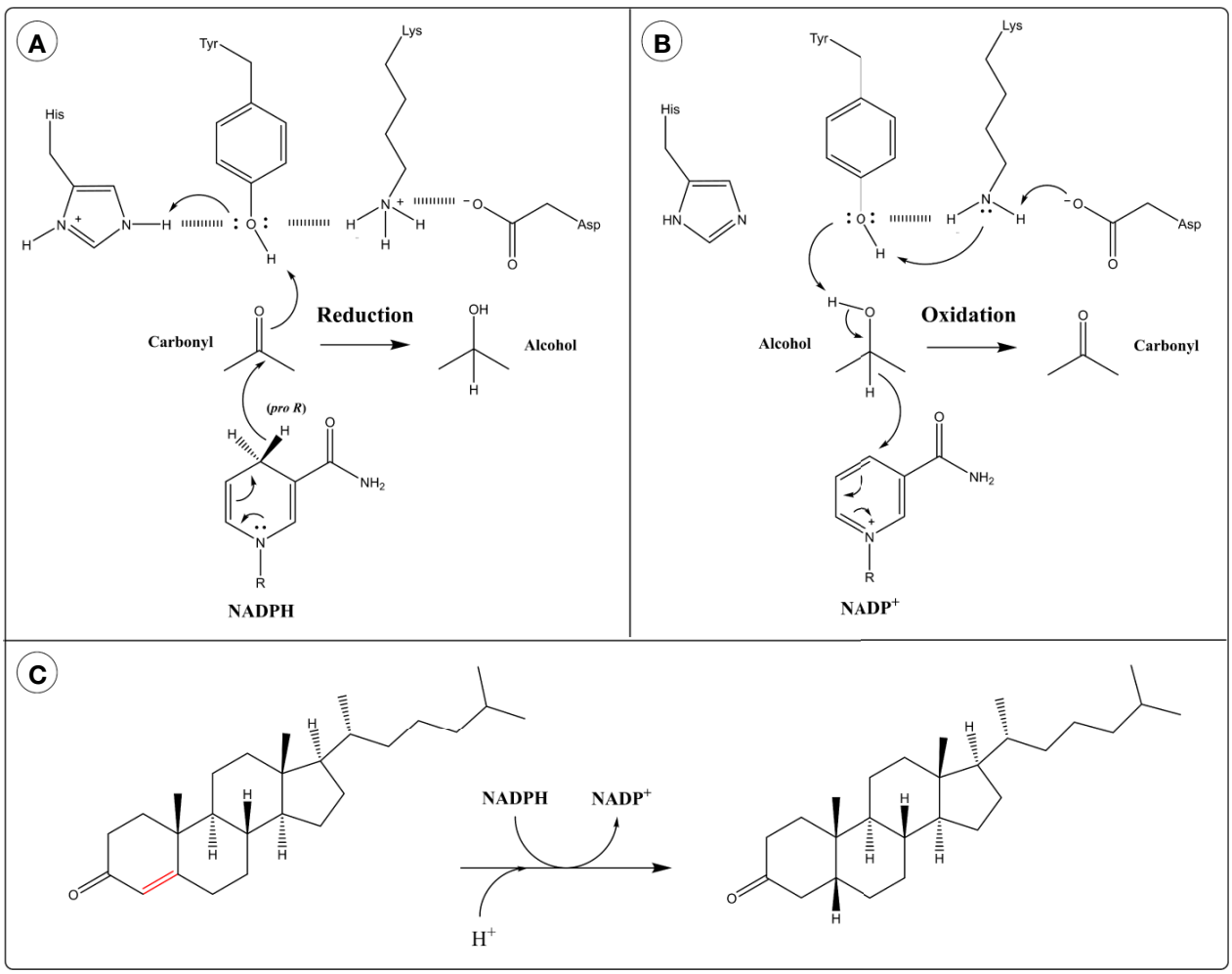

FIGURE 4 | Catalytic mechanism of aldo-keto reductases (AKRs) in the: **(A)** Reduction direction and **(B)** Oxidation direction. **(C)** 5β-Reduction of steroid double bond by AKR1D1.

reversible reduction of PGD_2 to 9α,11β-PGF_2 (241). Furthermore, AKR1B1 catalyzes the isomerization of PGH_2 to PGD_2 exclusively in the absence of NADPH as noted previously. However, the biological relevance of this activity is not clear as this isomerization reaction is also catalyzed by two prostaglandin D synthases (272), and the cellular redox status favors the reduction reaction. Both $PGF_{2\alpha}$ and its isomer 9α,11β-PGF_2 promote uterine contractions during labor (237).

Six of the 15 human AKRs are involved in sterol/steroid metabolism: AKR1B15, the four members of the AKR1C subfamily (also grouped as hydroxysteroid dehydrogenases; HSDs), and AKR1D1. AKR1B15 catalyzes the 17β-reduction of androgens and estrogens (236). AKR1C1 (20α-HSD) catalyzes the 20α-reduction of progesterone inactivating it (273), whereas AKR1C2 (Type 3 3α-HSD) possesses a 3α-dehydrogenase activity and deactivates 5α-dihydrotesterone (259). AKR1C3 (Type 5 17β-HSD) exhibits 17-ketoreductase activity and produces testosterone and estradiol from their respective precursors.

AKR1D1 and AKR1C4 (Type 1 3α-HSD) catalyze key steps in bile acid biosynthesis. AKR1D1 catalyzes irreversible 5β-reduction of a double bond in 3-ketosterols. This modification changes the geometry of the steroid nucleus from flat to twisted, with a bend in the A/B ring junction. Next, AKR1C4 catalyzes the 3α-reduction of the ketosteroid, resulting in a 3α,5β-configuration, a characteristic feature of bile acids.

Other HSDs Belong to the Short-Chain Dehydrogenase/Reductase (SDR) Family

Some HSDs belong to a different family of enzymes: the short-chain dehydrogenases/reductases (SDR). SDRs exhibit key differences from AKRs in structure, kinetics, catalytic mechanism, and reaction stereochemistry (reviewed in (274)). Of note, HSDs of the SDR family work as either ketosteroid reductases or hydroxysteroid oxidases, depending on their preference for the corresponding forms of NADP(H) or NAD(H). This contrasts with HSDs of the AKR1C family which operate in the reduction direction using (primarily) NADPH (274). Like CYPs and AKRs, a systematic nomenclature has been proposed for SDRs (275). However, the old names (especially for HSDs) remain heavily in use. HSDs of the SDR family have important functions in sterol/steroid metabolism (**Table 4**).

BIOSYNTHETIC PATHWAYS OF OXIDIZED LIPIDS

Oxygenated PUFA and Oxidized Phospholipids

Classification of Oxygenated PUFA as Eicosanoids and Docosanoids

The term "oxylipin" refers to oxygenated PUFA derivatives. It encompasses a wide range of oxidized lipids like hydroxy-, epoxy-, oxo-FAs, and endoperoxides. Oxylipins can be classified according to their precursor into eicosanoids (C20) or docosanoids (C22), etc., and this system will be used from here on. Following the classification system of the LIPID MAPS consortium, eicosanoids include prostaglandins, leukotrienes, thromboxanes, lipoxins, hepoxilins, E-resolvins, as well as hydroxy-, hydroperoxy-, epoxy-, and oxo-eicosanoids (286). Likewise, docosanoids include D-resolvins, protectins, maresins, hydroxy-, hydroperoxy-, epoxy-, and oxo-docosanoids. Structures of these lipids are available on the LIPID MAPS database (287).

Here, we focus on the biosynthesis of eicosanoids derived from AA and EPA, and docosanoids derived from DHA, as they are the most studied. Due to the broad substrate specificity of enzymes involved in lipid oxidation, PUFAs with shorter chains or different number of double bonds also undergo similar reactions. Similarly, oxygenation of endocannabinoids occurs on the PUFA moiety and follows the enzymatic mechanisms described here. Further aspects of oxygenated endocannabinoids are discussed in other reviews (216, 288).

Release of PUFA from Membranes for Oxylipin Biosynthesis

Intracellular concentration of free PUFA is tightly regulated through conjugation with coenzyme A (CoA) and subsequent esterification into lysoPL forming PL or shuttling into other pathways (like β-oxidation). PUFAs are abundant in membrane PLs, typically esterified at the *sn*-2 position. The classical pathway of oxylipin biosynthesis involves the release of PUFA from membrane PL *via* the action of lipases like phospholipase A_2 (PLA_2), phospholipase C and diacylglycerol lipase (289, 290). PLA_2 enzymes are classified into six types: secreted ($sPLA_2$), cytosolic ($cPLA_2$), calcium-independent ($iPLA_2$), platelet-activating factor

TABLE 4 | Human SDR-HSDs and their substrates/reactions.

Gene	Alternative protein names	Substrates	Reaction	Refs.
HSD3B1	Type 1 3β-HSD/Δ^{5-4} isomerase/SDR11E1	3β-Hydroxy-Δ^5-sterols	Oxidation & isomerization	(276, 277),
HSD3B2	Type 2 3β-HSD/Δ^{5-4} isomerase/SDR11E2	3β-Hydroxy-Δ^5-sterols	Oxidation & isomerization	(277, 278),
HSD3B7	Type 7 3β-HSD/SDR11E3	3β-Hydroxy-Δ^5-sterols	Oxidation & isomerization	(279)
HSD11B1	Type 1 11β-HSD/Corticosteroid 11β-dehydrogenase isozyme 1/SDR26C1	11β-Hydroxysterols (e.g. cortisol),	Oxidation	(280)
HSD11B2	Type 2 11β-HSD/Corticosteroid 11β-dehydrogenase isozyme 2/SDR9C3	11β-Hydroxysterols (e.g. cortisol),	Oxidation	(280)
HSD17B1	Type 1 17β-HSD/Estradiol 17β-dehydrogenase 1/ SDR28C1	Estrogens and androgens	Reduction	(281, 282)
HSD17B4	Type 4 17β-HSD/Peroxisomal multifunctional enzyme type 2/SDR8C1	(24R,25R)-3α,7α,12α,24-Tetrahydroxy-5β-cholestan-26-oyl-CoA	Reduction	(283)
HSD17B7	Type 7 17β-HSD/3-Ketosteroid reductase/SDR37C1	3β-Hydroxysterols, 17β-estradiol	Reduction	(284, 285)

acetylhydrolases (PAH-AH), lysosomal ($LPLA_2$), and adipose (AdPLA), and these are further divided into groups and subgroups (291, 292). $cPLA_2\alpha$ is localized in the cytosol but translocates to intracellular membranes upon calcium activation, and exhibits selectivity for phospholipids containing AA at the *sn*-2 position (293, 294). On the other hand, the release of DHA from the membrane can be facilitated by $iPLA_2\beta$ (295, 296). Last, $sPLA_2$ enzymes are involved in the release of AA, EPA and DHA (297, 298). PLA_2 enzymes are extensively reviewed by Murakami (299).

Biosynthesis of Eicosanoids

Arachidonic acid is a substrate of eicosanoids produced through the actions of COXs, LOXs, CYPs, and other downstream enzymes (**Figure 5A**). The most established of these are mono-oxygenation products of AA, DHA, and EPA and classic prostaglandins, leukotrienes, and thromboxane from COXs, LOXs, and CYPs. Most of these were described from the 1950s–90's in seminal studies, with many conducted at the Karolinska Institute in Stockholm by Sune Bergstrom, Bengt Samuelsson and colleagues. Indeed, the Nobel Prize for Physiology and Medicine was awarded for discovery of prostaglandins and related biologically active substances in 1985 to Bergstrom, Samuelsson and John Vane. More recently, research has focused on characterization of multiply oxygenated PUFAs from AA, EPA, and DHA including several which are proposed to form from transcellular sequential oxygenation by

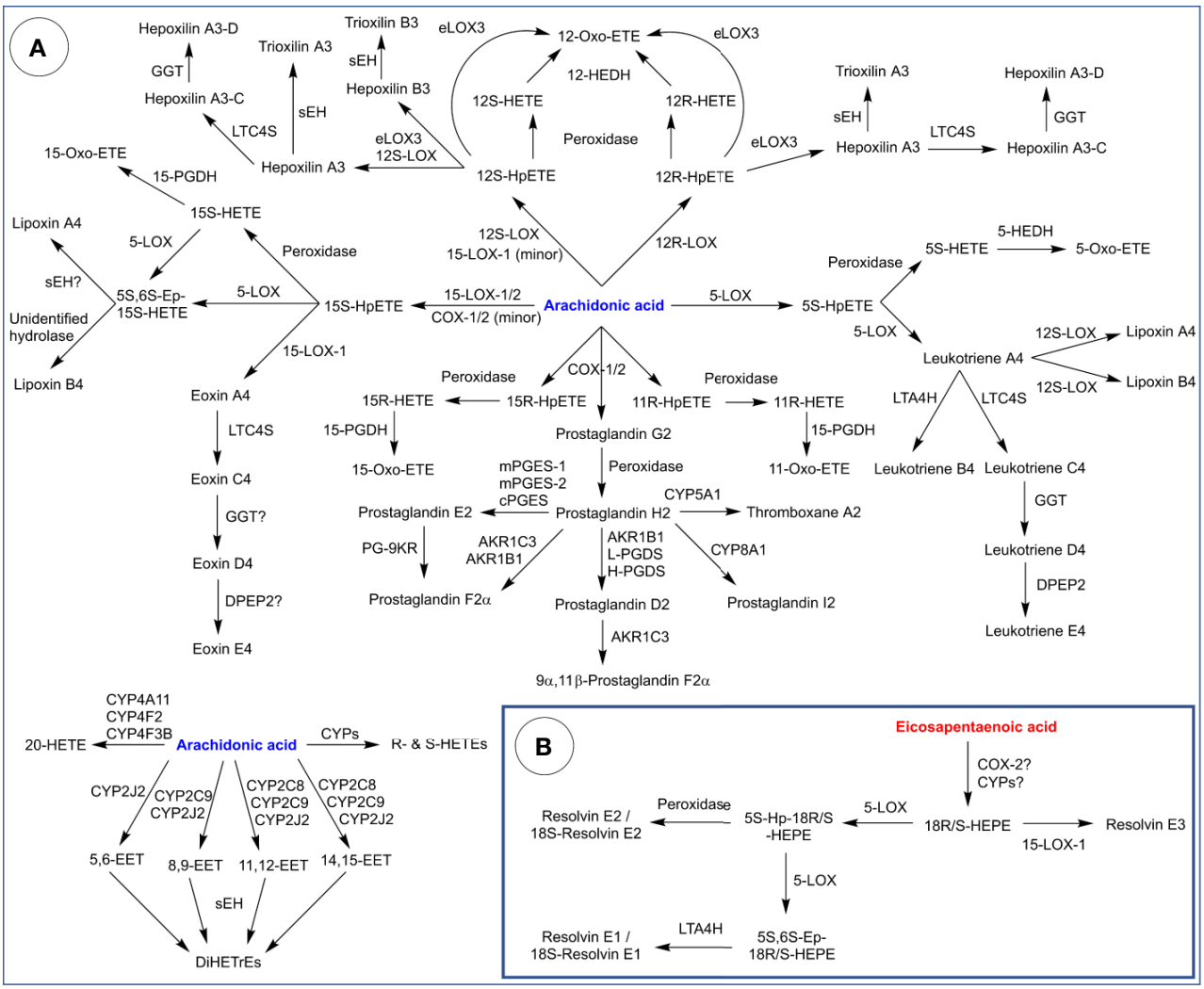

FIGURE 5 | Biosynthesis of eicosanoids derived from: **(A)** Arachidonic acid and **(B)** Eicosapentaenoic acid. HETE, hydroxy-eicosatetraenoic acid; Hp, hydroperoxy; Ep, epoxy; LTA4H, leukotriene A_4 hydrolase; LTC4S, leukotriene C_4 synthase; GGT, γ-glutamyl transpeptidase; DPEP2, dipeptidase 2; sEH, soluble epoxide hydrolase; 12-HEDH, 12-hydroxyeicosanoid dehydrogenase; PGES, prostaglandin E synthase (m: microsomal, c = cytosolic); PGDS (prostaglandin D synthase (L: lipocalin type, H: hematopoietic); 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; PG-9KR, prostaglandin 9-ketoreductase; EET, epoxyeicosatrienoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; HEPE, hydroxy-eicosapenataenoic acid.

various enzymes. However, for many of these, their specific biosynthetic pathways, enantiomeric composition and biological actions in tissues are not well understood and the levels formed are extremely low in comparison to classic PGs and monohydroxy-oxylipins. Furthermore, their bioactions often require amounts of lipids that are considerably higher than levels detected in cell and tissue samples, and thus could be considered pharmacological.

COXs convert AA into PGG_2 which is reduced into PGH_2 (**Figure 5A**). From there, PGH_2 functions as substrate for classic PGs and thromboxane, generated by tissue-specific enzymes. CYP5A1 (thromboxane synthase) is expressed in several cell types and tissues (platelets, macrophages, lung, kidney, liver) and catalyzes the isomerization of PGH_2 into thromboxane A_2, a potent vasoconstrictor and activator of platelet aggregation (300, 301). CYP5A1 also catalyzes the cleavage PGH_2 into malondialdehyde and 12-hydroxyheptadecatrienoic acid. CYP8A1 (prostacyclin synthase) is widely expressed (abundant in ovary, heart, lung, skeletal muscle, and prostate) and catalyzes the isomerization of PGH_2 into prostacyclin (PGI_2), a vasodilator and inhibitor of platelet aggregation (302).

PGH_2 can also be converted into PGD_2 through the action of AKR1B1 or PGD synthases, of which there are two isoforms: lipocalin-type PGD synthase (in the central nervous system, male genitalia, heart, cerebrospinal fluid and plasma) and hematopoietic PGD synthase (in antigen-presenting cells, mast cells and megakaryocytes) (266, 303). PGD_2 plays roles in the regulation of body temperature, sleep cycle, pain perception and the immune response (304). In the uterus, AKR1C3 catalyzes the reduction of PGD_2 into 9α,11β-PGF_2, which promotes uterine contractions (237). Similarly, AKR1C3 and AKR1B1 catalyze the reduction of PGH_2 into $PGF_2\alpha$, which also promotes uterine contractions (305). Finally, PGH_2 can be converted into PGE_2 *via* the action of PGE synthases, of which there are three isoforms: an inducible microsomal isoform (mPGES-1), a constitutive microsomal isoform (mPGES-2) and a constitutive cytosolic isoform (cPGES) (306). PGE_2 is abundant in the body and plays a complex role in immunity and inflammation (307). Additionally, PGE_2 is reduced into PGF2α by prostaglandin 9-ketoreductase (PG-9KR) (308).

COXs also produce 11*R*-, 15*R*-, and 15*S*-HpETEs as side products, all three of which can be reduced into their corresponding alcohols by peroxidase activity, which are in turn reduced into oxo-ETEs by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). Bioactivities of HETEs and oxo-ETEs are extensively reviewed by Powell and Rokach (309).

5-LOX converts AA into 5*S*-HpETE (**Figure 5A**), which is reduced into 5*S*-HETE through the action of a peroxidase (e.g., GPX) or converted into leukotriene A_4 (LTA_4) through the leukotriene synthase activity of 5-LOX. Various immune cells (neutrophils, monocytes, platelets) express 5-hydroxyeicosanoid dehydrogenase (5-HEDH) which converts 5*S*-HETE into 5-oxo-HETE, a potent chemoattractant for eosinophils (310). On the other hand, LTA_4 serves as a precursor for LT peptide conjugates (LTC_4, D_4 and E_4) and lipoxins (A and B). LTC_4, D_4, and E_4 are synthesized from LTA_4 through LTC_4 synthase (a gluthathione-*S*-transferase), γ-glutamyl transpeptidase (GGT) and dipeptidase 2 (DPEP2). Leukotrienes exhibit pro-inflammatory properties and are implicated in asthma and allergic reactions (311). On the other hand, lipoxins are thought to be synthesized by 12S-LOX through a transcellular pathway which involves interactions between two cell types (312). Lipoxins exhibit anti-inflammatory properties and are proposed to play a role in wound healing and tissue homeostasis (313). Alternatively, LTA_4 can be hydrolyzed by leukotriene A_4 hydrolase (LTA4H) into LTB_4, which attracts neutrophils (314).

15-LOXs converts AA into 15*S*-HpETE (**Figure 5A**), which can be reduced into 15S-HETE then oxidized into 15-oxo-ETE. 15*S*-HpETE and 15*S*-HETE are also substrates for 5-LOX, which produces a 5*S*,6*S* epoxy intermediate that can be hydrolyzed into lipoxins by unidentified hydrolases. Additionally, 15*S*-HpETE is proposed to be a precursor for eoxin A_4 (a 14,15 leukotriene), which is converted into peptide conjugates (eoxin C_4, D_4, and E_4) similar to leukotrienes (315). Conversion of eoxin A_4 into C_4 is thought to involve LTC4S. Enzymes that catalyze the following two steps leading to eoxin D_4 and E_4 are unidentified, presumably a GGT isoform and DPEP2. Eoxins are produced in eosinophils, mast cells and airway epithelial cells, and have been shown to possess pro-inflammatory properties (316).

12*S*-HpETE and 12*R*-HpETE are produced by 12S-LOX and 12*R*-LOX, respectively (**Figure 5A**). These hydroperoxides can be reduced to their corresponding alcohols *via* a peroxidase, and both alcohols further oxidized into 12-oxo-ETE by 12-hydroxyeicosanoid dehydrogenase (12-HEDH) (309). Both hydroperoxides are also precursors for hepoxilins, which are epoxy-alcohols. eLOX3 in skin converts 12*R*-HpETE into either hepoxilin A_3 or 12-oxo-ETE. On the other hand, 12*S*-HpETE can be converted into hepoxilin A_3 or B_3 by either 12S-LOX or eLOX3, but eLOX3 also generates 12-oxo-ETE (70, 317). Hepoxilins A_3 and B_3 are hydrolyzed by sEH into trioxilins A_3 and B_3, respectively (318). Hepoxilin A_3 can also be conjugated with glutathione *via* LTC4S producing hepoxilin A_3-C, which can be converted into a hepoxilin A_3-D by GGT (319). The occurrence of hepoxilin A_3-E (cysteinyl conjugate) has also been proposed but not confirmed. Hepoxilin A_3 is proposed to regulate mucosal inflammation by recruiting neutrophils across the epithelial junction into the gut lumen (320).

CYPs generate various midchain *R*- and *S*-HETEs as well as 20-HETE through hydroxylation of AA (**Figure 5A**). Alternatively, epoxyeicosatrienoic acids (EET) are produced from AA through epoxidation of its double bonds. Major mammalian CYP epoxygenases include CYP2C8, CYP2C9, and CYP2J2 (15). EETs are further metabolized by sEH (and potentially other epoxide hydrolases) into dihydroxyeicosatrienoic acids (DiHETrE). Note that EPA and other PUFA are also targets of CYP-mediated reactions, which generate the corresponding hydroxy-, epoxy-, and dihydroxy- derivatives.

"E-resolvins", another class of eicosanoids, are proposed to be generated from EPA (**Figure 5B**). COX-2 and CYPs have both been proposed to act on EPA to produce 18*R*-hydroxyeicosapentaenoic acid (18*R*-HEPE), which is in turn a proposed precursor for lipids

termed resolvins E1, E2 and E3 (E-resolvins). It is known that the acetylation of COX-2 shifts its activity into favoring the lipoxygenase-type reaction over the cyclooxygenase activity, and that acetylated COX-2 generates 18*R*-HEPE from EPA (321). However, acetylated COX-2 is not a physiologically relevant form, and 18*R*-HEPE could be potentially generated through other means. COX-2 is theoretically capable of generating 18*R*-HEPE from EPA in a lipoxygenase-type reaction followed by peroxidase activity. However, whether the generation of 18*R*-HEPE from EPA occurs *in vivo* and in sufficient amounts without COX-2 acetylation is unknown. Microbial CYPs have also be proposed as sources of 18*R*-HEPE during infection. E-resolvins are proposed to be produced through a transcellular mechanism involving microbial and mammalian cells (322). Alternatively, mammalian CYPs are also potential sources of 18*R*-HEPE, although the exact isoforms involved are unknown.

18*R*-HEPE can be metabolized by 5-LOX into a 5*S*-hydroperoxy intermediate and further into a 5*S*,6*S*-epoxy intermediate (323). The former is proposed to be converted into resolvin E2 by a peroxidase, whereas the latter is proposed to be hydrolyzed into resolvin E1 by LTA4H (324). 18*S*-analogues of resolvin E1 and E2 have also been described and are proposed to be generated from 18*S*-HEPE through a similar pathway (**Figure 5B**), although the enzymatic sources of 18*S*-HEPE (aside from acetylated COX-2) are also unknown. 18*R*-HEPE is also proposed to be a precursor to two stereoisomers collectively called resolvin E3 generated by 15-LOX-1 (325). E-resolvins are proposed to exhibit anti-inflammatory effects and promote the resolution of inflammation (325, 326).

Both lipoxins and E-resolvins have been classified as specialized pro-resolving mediators (SPMs), a term which currently describes families of oxygenated PUFA metabolites with proposed roles in the resolution of inflammation and tissue regeneration generated at extremely low levels in biological samples.

Biosynthesis of Docosanoids

Oxygenation of DHA by LOXs and CYPs forms oxygenated docosanoids (hydroxy-, epoxy-, and dihydroxy derivatives). DHA is also the precursor to several classes of multiply oxygenated docosanoids, including additional SPMs which have been named maresins, D-resolvins, and protectins (**Figure 6**). Maresin biosynthesis has been proposed to start with action of 12S-LOX, generating 14*S*-hydroperoxy-DHA (14*S*-HpDoHE) then a 13*S*,14*S*-epoxy intermediate. Hydrolysis of this epoxy intermediate by sEH or an unidentified hydrolase is proposed to generate maresin 2 and maresin 1, respectively (93, 327). The biosynthesis of protectin and D-resolvins both begin with 15-LOX-1 which generates a 17*S*-hydroperoxide (17*S*-HpDoHE). Further conversion of this hydroperoxide into a 16*S*,17*S*-epoxy intermediate by 15-LOX-1 followed by hydrolysis (unidentified hydrolase) is proposed to generate protectin D1 (328). Alternatively, 17*S*-HpDoHE could be reduced into an alcohol by a peroxidase, which then serves as a precursor for D-resolvins. In this route, 5-LOX is proposed to generate two distinct products hydroperoxides (4*S* or 7*S*), which

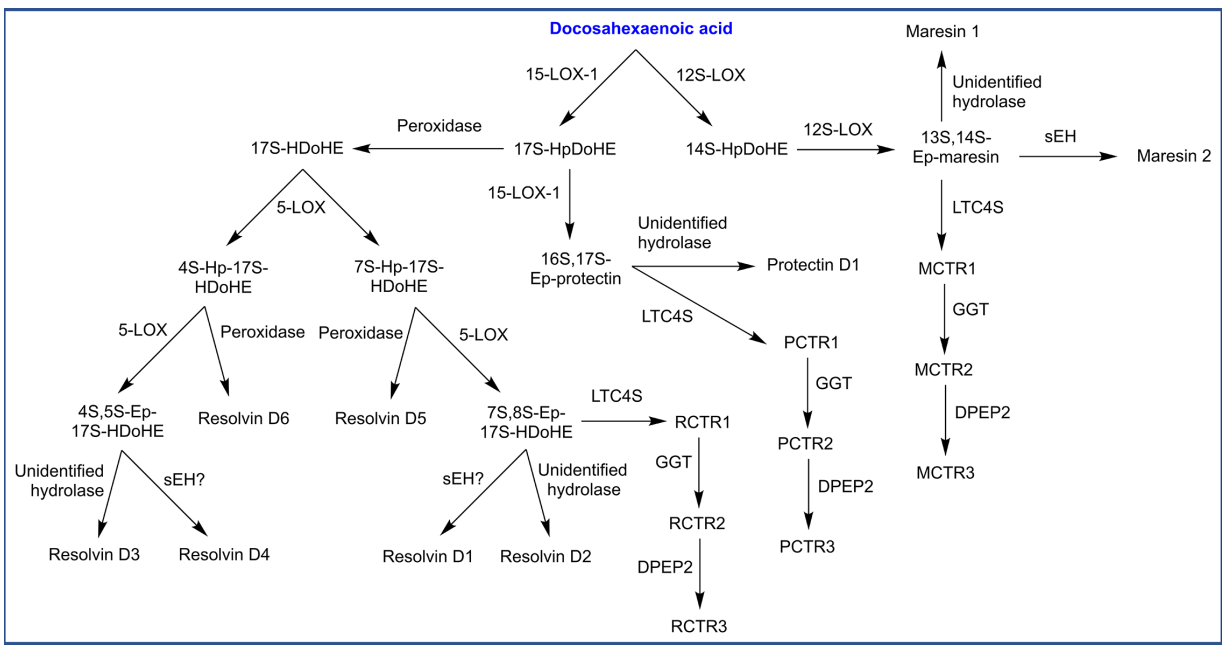

FIGURE 6 | Biosynthesis of specialized pro-resolving mediators (SPMs) derived from docosahexaenoic acid. HDoHE, hydroxy-docosahexaenoic acid; Hp, hydroperoxy; Ep, epoxy; CTR, conjugate in tissue regeneration (M: maresin, P: protectin, R: resolvin); sEH, soluble epoxide hydrolase; LTC4S, leukotriene C_4 synthase; GGT, γ-glutamyl transpeptidase; DPEP2, dipeptidase 2.

undergo further transformations leading to several D-resolvins (**Figure 6**). Additionally, epoxide intermediates in the previously described pathways are proposed to undergo glutathione conjugation by LTC4S, generating peptide conjugates of maresin, protectin and D-resolvin named MCTR1, PCTR1, and RCTR1, respectively (CTR = conjugate in tissue regeneration). These conjugates are proposed to be metabolized by GGT and DPEP2, similar to leukotrienes (329).

Oxygenation of Esterified PUFA and Biosynthesis of Oxidized Phospholipids

The classical pathway for the biosynthesis of oxidized phospholipids starts with the release of PUFA from membranes through the action of PLA_2. PUFA are then oxygenated by the enzymes discussed earlier. This is followed by acylation of oxygenated PUFA with CoA and esterification into a lysoPL *via* the action of an *sn*-2 acyltransferase.

The generation of PL-esterified eicosanoids is well-documented in mammalian cells such as epithelial, endothelial and immune cells (330). HETE-PLs are acutely generated by activated neutrophils, platelets and monocytes (331–333). Similarly, PGD_2 and PGE_2 generated from COX-1-derived PGH_2 in activated platelets are rapidly incorporated into PE-lysoPLs (334). Also, EET-PLs have been detected in rat liver (335). For most of these, the mechanism of formation requires endogenous generation of an oxylipin, which is then rapidly esterified into membrane PL pools (e.g., on a timescale of minutes) *via* Lands cycle enzymes (336).

15-LOXs also contribute to the formation of oxPL by two other pathways. The first involves direct oxygenation of membrane PLs (331, 337). In the second pathway, 15-LOXs oxygenate the PUFA moiety of CEs, then hydrolysis of oxygenated PUFA from CE liberates oxygenated PUFA which can be esterified to lysoPL (87).

Recent studies found that COX-2, 15-LOX-2, and platelet 12S-LOX can catalyze the oxygenation of 2-AA-lysoPL released by $iPLA_2\gamma$ in response to calcium ionophore stimulation (88, 94). These reactions generate 2-eicosanoid-lysoPLs which are proposed to be a source of free eicosanoids as well as acting as signaling mediators themselves. 2-eicosanoid-lysoPLs can also be converted into oxPLs through the action of *sn*-1 acyltransferase (338). Cytochrome *c* has been recently identified as a plasmalogenase, and is proposed to be a source of 2-eicosanoid-lysoPLs under conditions of oxidative stress (339). These findings describe novel pathways for the biosynthesis of oxPLs (**Figure 7**). Further research is required to assess the contribution of these pathways to the formation of oxPLs and other mediators *in vivo*.

Biosynthesis of Oxysterols, Bile Acids, and Steroid Hormones

Oxysterols

Oxysterols are formed in the first steps of cholesterol metabolism: they are oxidized forms of cholesterol and also of its precursors (340). 7α-Hydroxycholesterol (7α-HC) is formed from cholesterol by CYP7A1 and represents the first metabolite in the neutral pathway of bile acid biosynthesis (**Figure 8**) (219, 220). (25R)26-Hydroxycholesterol (26-HC), more commonly called 27-hydroxycholesterol, and 3β-hydroxycholest-5-en-(25R)26-oic acid (3β-HCA) are both formed from cholesterol by CYP27A1 and are the first members of the acidic or alternative pathway of bile acid biosynthesis (219–221). While CYP7A1 is an endoplasmic reticulum and liver specific protein, CYP27A1 is mitochondrial and expressed in many tissues. CYP46A1 is almost exclusively expressed in neurons, its function is to maintain cholesterol balance in the brain, converting cholesterol from a molecule unable to pass the blood brain barrier to 24S-hydroxycholesterol (24S-HC), a more polar molecule which can cross the barrier (222, 223, 341). CYP11A1, like CYP27A1, is a mitochondrial inner membrane protein. It is highly expressed in steroidogenic tissue and will oxidize cholesterol to pregnenolone in a three step process involving 22R-hydroxycholesterol (22R-HC) and 20R,22R-dihydroxycholesterol as intermediates (20R,22R-diHC) (342, 343) (**Figure 9**). The pathways of bile acid biosynthesis are discussed in more detail below.

While most "primary" oxysterols are formed from cholesterol in CYP catalyzed reactions, cholesterol 25-hydroxylase (CH25H), the dominating enzyme that generates 25-hydroxycholesterol (25-HC) is an exception, in that is not a CYP, but a member of a family of enzymes that utilize di-iron cofactors to catalyze the hydroxylation (344). CH25H is expressed in activated immune cells and 25-HC has both anti-bacterial and anti-viral activities (345–348). 25-HC is metabolized by CYP7B1 to 7α,25-diHC, a ligand to the GPCR Epstein Barr virus induced gene 2 (EBI2 or GPR183). 7α,25-diHC acts as a chemoattractant to GPR183 expressing immune cells (349, 350). An alternative route to 7α,25-diHC production is through CYP3A4 oxidation of 7α-HC (190), while the same enzyme has also been reported to act as a second cholesterol 25-hydroxylase and also a 4β-hydroxylase of cholesterol (189, 351).

Oxysterols can also be formed from cholesterol precursors in reactions catalyzed by CYP enzymes (202, 352). These reactions may be important in patients suffering from inborn errors of cholesterol biosynthesis such as Smith-Lemli-Opitz syndrome (SLOS, 7-dehydrocholesterol reductase deficiency) and desmosterolosis (3β-hydroxysterol-Δ^{24}-reductase deficiency) or where there is very high expression of sterol hydroxylases, e.g., CYP7A1 in cerebrotendinous xanthomatosis, where CYP27A1 is deficient.

Bile Acid Biosynthesis

There are two quantitatively major and at least five minor pathways of bile acid biosynthesis and all pathways involve multiple oxidation reactions (219, 220, 353–358). Besides CYP enzymes, key oxidation reactions are carried out by hydroxysteroid dehydrogenase (HSD) members of the short chain dehydrogenase/reductase (SDR) family and reductions by aldo-keto reductases (AKR) enzymes. One of the minor pathways that results in the formation of 3β,5α,6β-trihydroxycholan-24-oic acid involves of cholestane-3β,5α,6β-triol which is likely formed *via* the cholesterol peroxidation product 5,6-epoxycholesterol (219, 354).

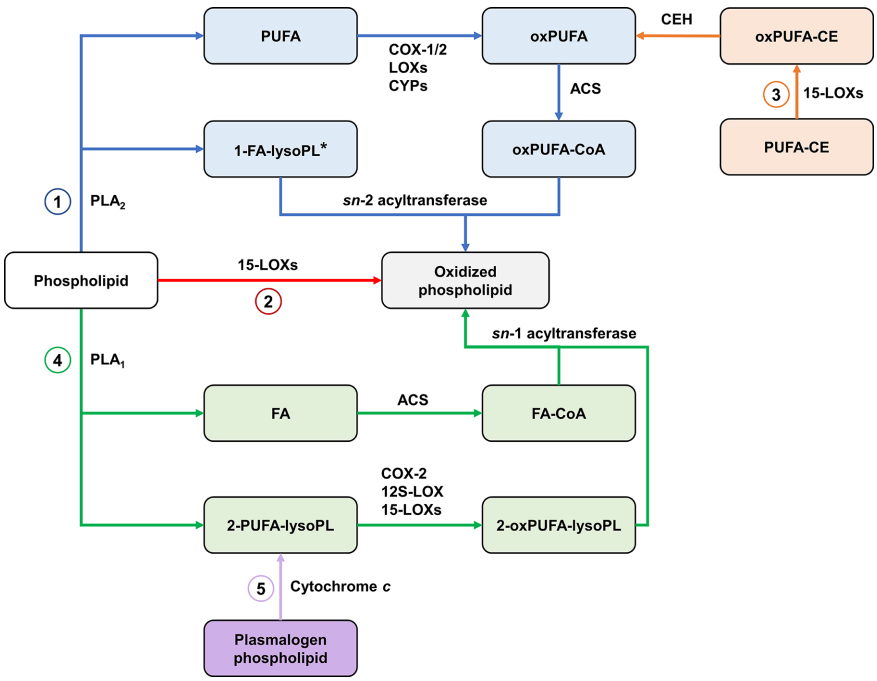

FIGURE 7 | Biosynthetic pathways of oxidized phospholipids. **(1)** The classical pathway involves the action of PLA_2 on membrane phospholipids, releasing *sn*-2 PUFA which are oxygenated by cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450s (CYPs) then re-esterified. *Oxygenated PUFA can be also be esterified with plasmalogen lysophospholipids **(2)** Direct oxygenation of membrane phospholipids by 15-LOXs. **(3)** 15-LOX-mediated oxygenation of PUFA in cholesteryl esters followed by hydrolysis of oxygenated PUFA provides substrates for the classical pathway. **(4)** An alternative pathway involves the action of PLA_1, forming 2-PUFA-lysophospholipids which are oxygenated by COX-2, 12S-LOX, and 15-LOXs then re-esterified with FA. **(5)** Cytochrome *c* releases 2-PUFA-lysophospholipids by cleaving the vinyl ether bond in plasmalogen phospholipids, providing substrates for the alternative pathway. PLA, Phospholipase A; oxPUFA, oxygenated PUFA; ACS, acyl-CoA synthase; CE, cholesteryl ester; CEH, neutral cholesterol ester hydrolase; FA, fatty acid/acyl; lysoPL, lysophospholipid.

While the acidic pathway may be most important in infants (359), at later stages of life the neutral pathway is dominant. This is initiated by CYP7A1 oxidation of cholesterol to generate 7α-HC in the liver. Further oxidation may occur at C-12 by CYP8B1 to generate 7α,12α-dihydroxycholesterol (7α,12α-diHC), which may be preceded or succeeded by oxidation and isomerization of the 3β-hydroxy-5-ene structure to a 3-oxo-4-ene by HSD3B7, giving 7α-hydroxycholest-4-en-3-one (7α-HCO) and 7α,12α-dihydroxycholest-4-en-3-one (7α,12α-diHCO), respectively (**Figure 8**). A general feature of bile acid biosynthesis is that many of the enzymes involved in the pathways accept multiple substrates resulting in variations in the order of reactions depending on the tissue in which they proceed (360). The next steps involve A-ring reductions which may be succeeded or preceded by (25R)26-hydroxylation and (25R)26-carboxylation to ultimately give 3α,7α,12α-trihydroxy-5β-cholanestan-(25R)26-oic acid. The A-ring reductions are carried out by AKR1D1 and AKR1C4, while CYP27A1 carries out the (25R)26-oxidations. Side-chain shortening of the cholestanoic acid proceeds in the peroxisome through the CoA-thioester formed by bile acid Co-A synthetase (BACS, *SLC27A5*) or very long chain acyl-CoA synthetase (VLCS, *SLC27A2*). Following C-25 racemization by α-methylacyl-CoA racemase (AMACR) the next oxidation involves the introduction of Δ^{24} double bond by the enzyme acyl-CoA oxidase 2 (ACOX2). The Δ^{24} double bond is then hydrated by D-bifunctional protein (DBP), which then oxidizes the C-24 hydroxy to a C-24 ketone *via* HSD17B4 activity. The resulting product 3α,7α,12α-trihydroxy-24-oxo-5β-cholestan-(25R)26-oyl-CoA is then oxidized by the enzyme peroxisomal thiolase 2 (SPCx) to the thioester of cholic acid ready for conjugation with glycine, taurine, or hydrolysis to the free acid by peroxisomal acyl-CoA thioesterase (ACOT) (219, 220).

The acidic pathway starts with (25R)26-hydroxylation and (25R)26-carboxylation of cholesterol by CYP27A1 and this and many other steps may proceed extrahepatically (**Figure 8**). Most of the enzymes involved in the neutral pathway are also involved in the acidic pathway although not in the same order (361). An exception is CYP7A1, which is replaced by CYP7B1 as the 7α-hydroxylase in the acidic pathway. CYP7B1 is expressed in many tissues, not just liver (362, 363), and unlike CYP7A1 uses side-chain oxysterols as its substrate. The acidic pathway mostly generates chenodeoxycholic acid rather than cholic acid, so CYP8B1 has minor involvement. The order of A-ring reduction and side-chain cleavage can be reversed in the acidic pathway with the formation of bile acid intermediates possessing 3-oxo-4-ene or 3β-hydroxy-5-ene functions.

Steroid Hormone Biosynthesis

The classical steroid hormones aldosterone (mineralocorticoid), cortisol (glucocorticoid), testosterone (male sex hormone) and 17β-estradiol (female sex hormone) are all formed from

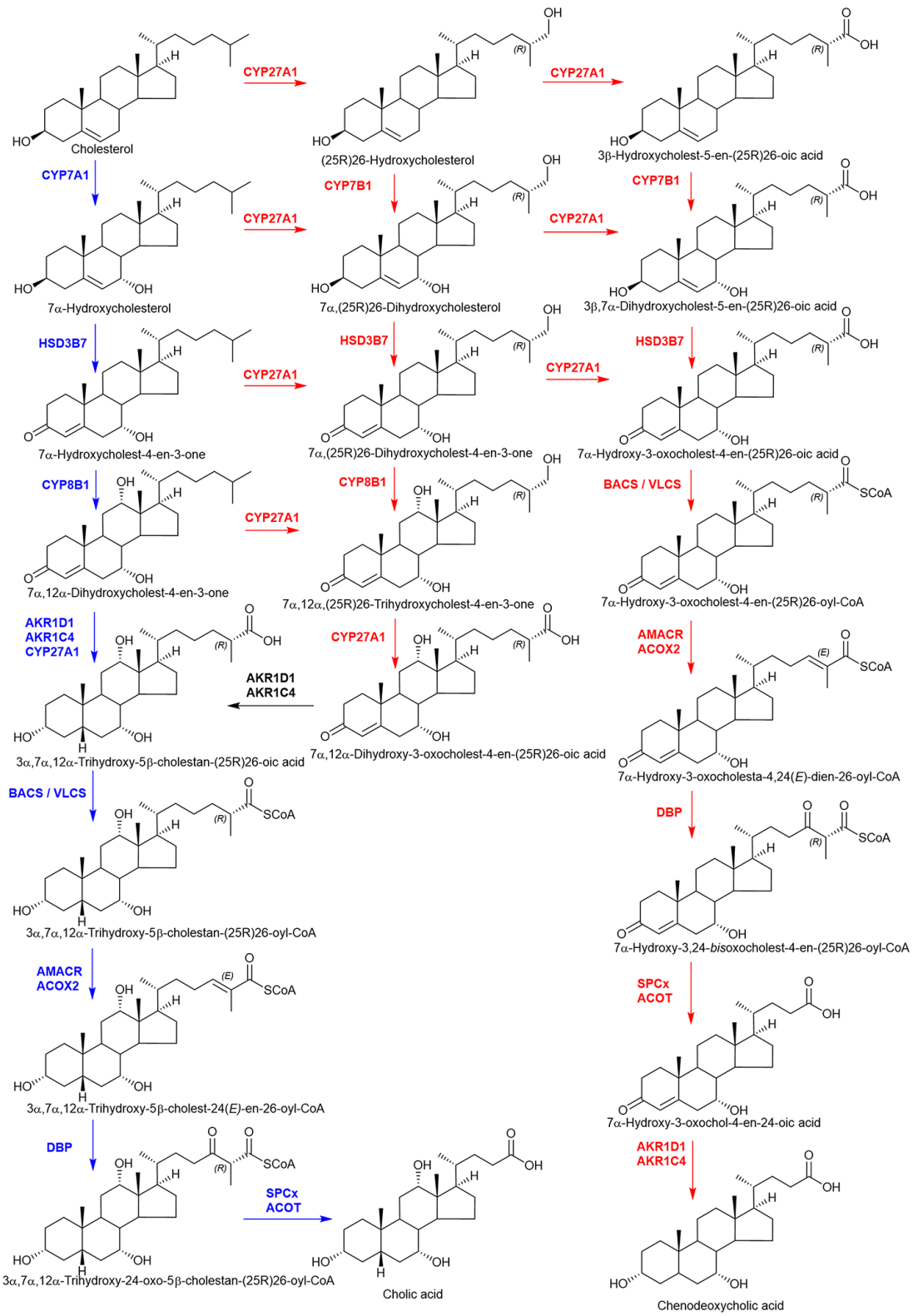

FIGURE 8 | A simplified view of the major bile acid biosynthesis pathways. The "neutral" pathway (highlighted in blue) starts with 7α-hydroxylation of cholesterol by CYP7A1, the "acidic" pathway with (25R)26-hydroxylation then (25R)26-carboxylation of cholesterol by CYP27A1. In the "acidic" pathway (highlighted in red) CYP7B1 is the 7α-hydroxylase.

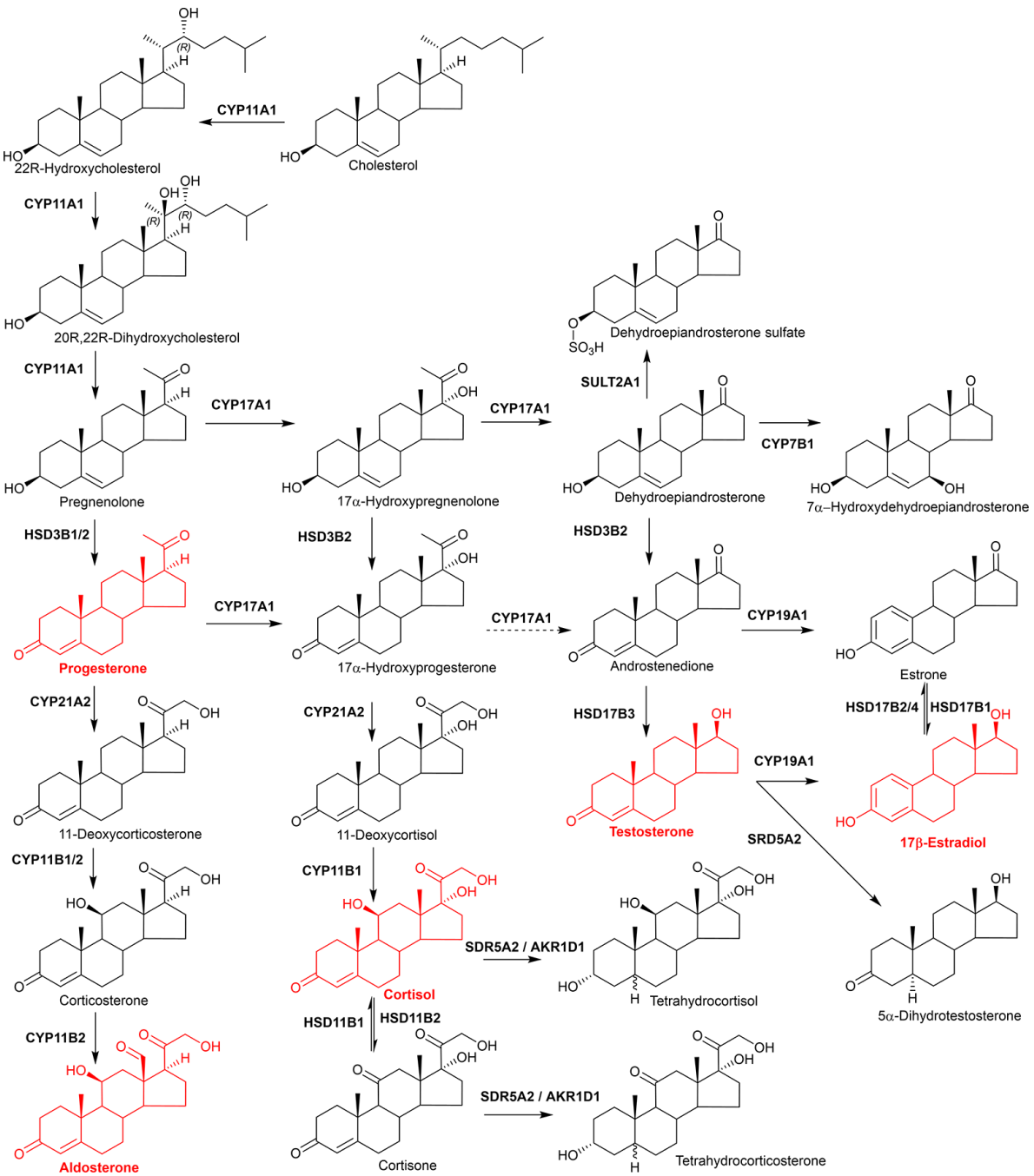

FIGURE 9 | Simplified view of steroid hormone biosynthesis. Highlighted in red are the classical steroid hormones, progesterone, aldosterone, cortisol, testosterone, and 17β-estradiol.

cholesterol through multiple oxidation reactions (**Figure 9**) (364, 365). Pregnenolone formed *via* CYP11A1 oxidation of cholesterol represents an intermediate between oxysterol and steroid hormone biosynthesis. It is oxidized by HSD3B2 to progesterone on the pathway to aldosterone or by CYP17A1 to 17α-hydroxypregnenolone on the route to cortisol. Both these pathways use CYP21A2 as a C-21 hydroxylase and CYP11B enzymes as the 11β-hydroxylase. Note, HSD3B1 is the enzyme which generates progesterone from pregnenolone in the placenta, when progesterone acts as the hormone of pregnancy. Further oxidation of 17α-hydroxypregnenolone by CYP17A1 leads to dehydroepiandrosterone on the road to testosterone and17β-estradiol. In this pathway additional oxidations and reductions are carried out by HSD3B2 and

HSD17B3, respectively, while CYP19A1 is required to generate estrogens. Besides being synthesized in the adrenal gland and sex organs it is noteworthy that steroids can be synthesized in the brain and are then named neurosteroids (366, 367). A more general term for brain steroids which may be synthesized in the brain or imported from the periphery and exert rapid non-genomic effects is neuroactive steroids. It is beyond the scope of this review to discuss steroid hormone biosynthesis and metabolism in greater detail and the reader is directed to the excellent reviews of Shackleton and colleagues (364, 368–370).

CONCLUDING REMARKS

Enzymatically oxidized lipids are derivatives of PUFA or cholesterol with critical functions in cellular and physiological processes as signaling mediators and hormones. Their biosynthesis is highly regulated and carried out by enzymes that include LOXs, COXs, CYPs, and AKRs. Advances in our understanding of these enzymes have led to the discovery of novel lipid mediators and their biosynthetic routes. Many functional aspects of these enzymes and their products remain unclear, requiring further investigation. These include elucidating the function of 15-LOX-2 (ALOX15B) in macrophages, the regulatory mechanisms of 12-LOXs and eLOX3, the mechanistic details of transcellular biosynthesis, the origin of 18-HEPE required for E-resolvin biosynthesis, the biological functions of orphan CYPs, and the bioactivities/functions of oxygenated endocannabinoids. The relative physiological importance of some multiply oxygenated PUFA mediators, which are generated in extremely low amounts, also needs to be further clarified. This is also true for multiply oxygenated derivatives of cholesterol.

Research on oxygenated PUFA and oxysterols has been carried out largely in parallel, and both areas have benefited greatly from advances in analytical methods. However, there are still major questions to be answered in terms of their proposed roles in human disease including atherosclerosis and neurodegeneration. Here, we presented biosynthetic pathways of oxygenated PUFA and oxysterols, highlighting their (known) functions to show the diversity of products but also to draw connections between the two groups. An integrated approach encompassing the analysis of both lipid groups could be useful in examining the etiology of disease. Despite their involvement in the progression of disease such as atherosclerosis and neurodegenerative disease, the two lipid groups are rarely analyzed together. We propose that oxygenated PUFA and oxysterols are more connected than previously thought, especially as 15-LOX is being increasingly recognized as a regulator of cholesterol metabolism. The immune and nervous systems are both of particular interest as major sites of cholesterol metabolism and 15-LOX expression. Further work on the enzymology of 15-LOX with cholesteryl substrates could lead to the discovery of novel oxidized lipids. For example, 15-LOX oxygenation of PUFA esters of oxysterols could generate novel lipids that link both classes directly in cell types that possess the enzymatic machinery for oxysterol and oxylipin biosyntheses (e.g., macrophages). An alternative biosynthetic route could be through the esterification of oxylipins with free oxysterols. That said, the detection of these proposed molecules could be challenging due to low abundance and sensitivity to alkaline conditions commonly used in analysis. Finally, the substrate promiscuity of many of the enzymes involved in production of oxidized lipids provides a technical challenge for dissection of (patho)physiological function of specific oxidized lipids. Clearly, there is plenty of scope for ongoing exploration of the molecular, cellular, and physiological functions of oxidized lipids.

AUTHOR CONTRIBUTIONS

AH planned the manuscript. AH, WG, and YW wrote the manuscript with input from AF and VO. All authors contributed to the article and approved the submitted version.

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From Inert Storage to Biological Activity—In Search of Identity for Oxidized Cholesteryl Esters

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Esterification of cholesterol is a universal mechanism to store and transport large quantities of cholesterol between organs and tissues and to avoid toxicity of the excess of cellular cholesterol. Intended for transport and storage and thus to be inert, cholesteryl esters (CEs) reside in hydrophobic cores of circulating lipoproteins and intracellular lipid droplets. However, the inert identity of CEs is dramatically changed if cholesterol is esterified to a polyunsaturated fatty acid and subjected to oxidative modification. Post-synthetic, or epilipidomic, oxidative modifications of CEs are mediated by specialized enzymes, chief among them are lipoxygenases, and by free radical oxidation. The complex repertoire of oxidized CE (OxCE) products exhibit various, context-dependent biological activities, surveyed in this review. Oxidized fatty acyl chains in OxCE can be hydrolyzed and re-esterified, thus seeding oxidized moieties into phospholipids (PLs), with OxPLs having different from OxCEs biological activities. Technological advances in mass spectrometry and the development of new anti-OxCE antibodies make it possible to validate the presence and quantify the levels of OxCEs in human atherosclerotic lesions and plasma. The article discusses the prospects of measuring OxCE levels in plasma as a novel biomarker assay to evaluate risk of developing cardiovascular disease and efficacy of treatment.

Keywords: cholesteryl ester, oxidized, macrophage, atherosclerosis, cardiovascular disease, biomarker, inflammation, toll-like receptor 4

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INTRODUCTION

Cholesterol esterification is a mechanism the body uses to store and transfer cholesterol, while at the same time to avoid cellular toxicity of the excess of unesterified (often called free) cholesterol. However, oxidation of a cholesteryl ester (CE) drastically changes the part CE plays, from a subdued supporting actor to a contender for the leading role. The script, in other words, the specific physiological or pathological context, defines if oxidized CE (OxCE) plays a villain or the hero.

Unesterified cholesterol is an essential component of cellular membranes, where it plays both structural and signaling roles, the latter *via* regulation of lipid rafts and binding to many transmembrane proteins. In the nervous system, cholesterol is a major component of myelinated sheath of many nerve fibers. Cholesterol is also a precursor for biosynthesis of steroid hormones and bile acids. Thus, no wonder that cholesterol homeostasis is under tight control to ensure proper cellular and systemic functions. Dysregulation of cholesterol metabolism underlies

many pathologies, from cardiovascular disease (CVD) to neurodegenerative disorders to cancer (1, 2). In homeostatic state, cellular cholesterol content is tightly controlled by balancing *de novo* synthesis, uptake of lipoproteins, export to extracellular milieu, and storage (2–4). The strategy for storage and transport of amphipathic cholesterol molecules is their esterification to fatty acids and tight packaging of the resulting hydrophobic CEs in the core of intracellular lipid droplets or circulating lipoproteins (**Figure 1**—Transport and Storage).

Inside the cells, after a threshold level in cellular cholesterol mass has been reached, excess cholesterol is esterified in the ER by the enzyme acyl CoA cholesterol acyltransferase (ACAT), and the newly synthesized CEs are stored in lipid droplets. To re-enter the cellular pathway, CE is hydrolyzed by neutral CE hydrolase (NCEH). Alternatively, lipid droplets are packaged into autophagosomes and fuse with lysosomes, where the CE is hydrolyzed by lysosomal acid lipase (LAL), generating unesterified cholesterol for delivery to cellular membranes or for export (5). Cellular CEs undergo a continual cycle of hydrolysis and re-esterification with a half-life of about 24 h (6, 7).

In circulation, lower density lipoproteins transport CEs from digestive organs to tissues, and high-density lipoprotein (HDL) returns excess cholesterol in the form of CE back to the liver. In brief, very-low-density lipoprotein (VLDL), packing triglycerides (TGs) and CEs, undergoes intravascular remodeling by shedding TGs and transitions into low-density lipoprotein (LDL), which is the most CE-rich lipoprotein in circulation. LDL is internalized by many cell types and thus delivers CEs to tissues. Serving the opposite function, HDL gathers excess of unesterified cholesterol

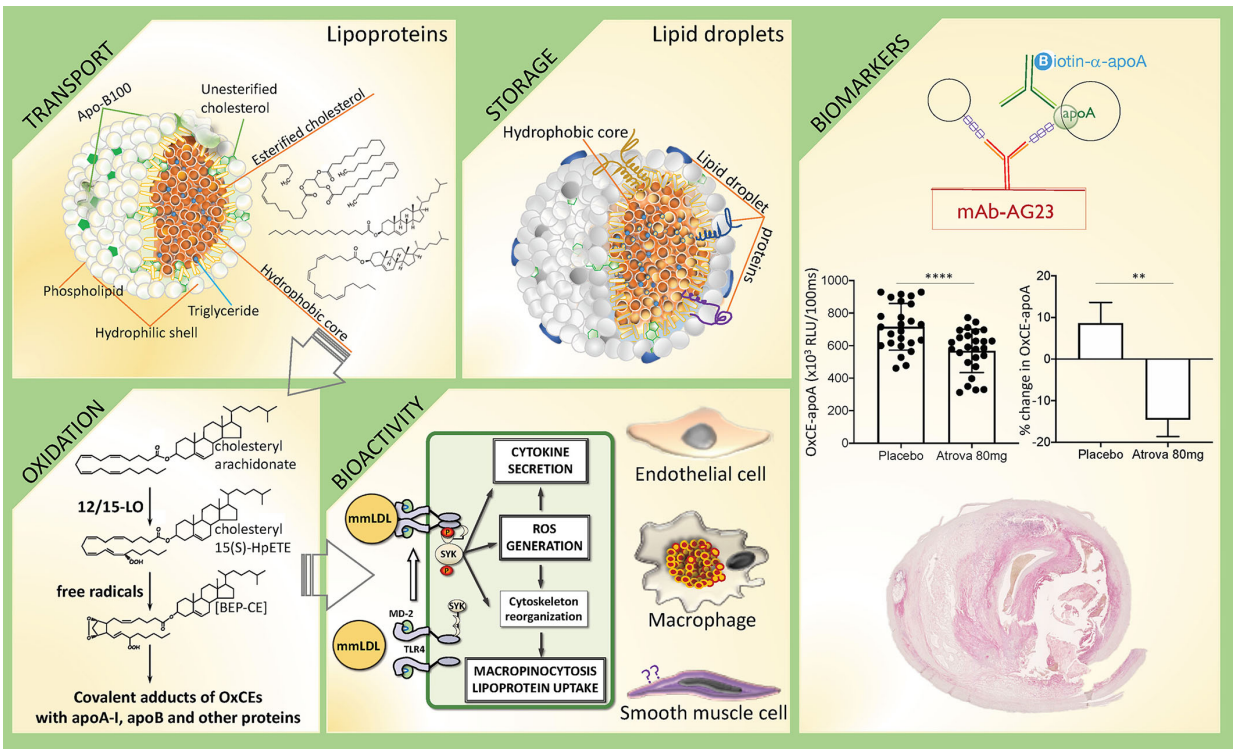

FIGURE 1 | In search of CE identity—from inert storage to bioactivity and CVD biomarker. This diagram illustrates different biological processes that involve CEs, using examples described in text, and potential biomarker applications of detecting OxCEs in plasma and atherosclerotic plaques. **Transport:** Cholesteryl esters (CEs) together with triglycerides (TGs) populate the hydrophobic core of circulating lipoproteins, serving to deliver cholesterol and fatty acids to organs. Depicted is the low-density lipoprotein (LDL), a major CE-transporting lipoprotein in blood. Shown are representative structures (from top to bottom) of a TG, a CE with saturated fatty acyl [cholesteryl palmitate], and a CE with polyunsaturated fatty acyl (PUFA) [cholesteryl arachidonate], the latter is susceptible to oxidation. **Storage:** Intracellular lipid droplets predominantly store either CEs, like in macrophage foam cells in atherosclerotic lesions, or TGs, like in adipocytes. **Oxidation:** PUFA-CEs are the preferential substrate for 12/15-lipoxygenase (12/15-LO). Shown is cholesteryl 15(S)-HPETE, the product of 12/15LO-mediated oxidation of cholesteryl arachidonate, which in turn is oxidized to more complex products, like BEP-CE. Hydroperoxide, endoperoxide and aldehyde groups in OxCEs are reactive and can covalently modify proteins. **Bioactivity:** In one example of OxCE biological activity, BEP-CE and minimally modified LDL (mmLDL), which carry many OxCE molecules, activate an MD-2/TLR4/SYK pathway in macrophages, resulting in ROS generation, inflammatory cytokine secretion, and macropinocytosis-mediated LDL uptake and foam cell formation. OxCEs also activate endothelial cells, but their effects on vascular smooth muscle cells have not been studied. **Biomarkers:** Antibodies against OxCE-protein adducts stain human atherosclerotic lesions and recognize a fraction of ApoB and ApoA-I lipoproteins that carry OxCE in human plasma. In one example, an immunoassay measuring levels of OxCE-apoA lipoproteins detects reduced levels of this potential biomarker in subjects after treatment with atorvastatin compared to placebo. The artwork in this figure uses panels originally published in the Journal of Lipid Research. Gonen et al. A monoclonal antibody to assess oxidized cholesteryl esters associated with apoA-I and apoB-100 lipoproteins in human plasma. *J Lipid Res* 2019; 60:436-445. © The American Society for Biochemistry and Molecular Biology.

from extrahepatic tissues in order to move it back to the liver. The HDL-associated enzyme lecithin-cholesterol acyltransferase (LCAT) catalyzes the esterification of free cholesterol with a fatty acyl transferred from the *sn-2* position of phosphatidylcholine (PC), resulting in the formation of a CE. In addition, there is a bidirectional exchange of CEs and TGs between HDL and the apoB-containing lipoproteins VLDL and LDL, mediated by the CE transfer protein (CETP) in plasma (8, 9). In plasma of healthy human subjects, approximately 70% of cholesterol molecules are esterified and reside in lipoprotein cores (10, 11).

This abridged description of CE metabolism illustrates complex pathways involved in keeping CE locked away in hydrophobic cores of lipid droplets and lipoproteins for storage and safe passage through the body—that is until CE undergoes oxidation and becomes biologically active.

CE Oxidation

The acyl chain in a CE can be derived from a saturated, monounsaturated or polyunsaturated fatty acid (PUFA). The most common PUFA-CEs are cholesteryl linoleate [CE(18:2)], arachidonate [CE(20:4)], and docosahexaenoate [CE(22:6)]. The PUFAs are more susceptible to oxidation than cholesterol due to the presence of a weaker C-H bond at the bis-allylic position and will therefore be oxidized preferentially (12). The hydrogen atoms are easily abstracted from the bis-allylic positions of PUFAs to form a lipid radical - the first intermediate of enzymatic or non-enzymatic lipid peroxidation (13, 14). This article will be largely focused on the OxCEs with oxidized acyl chain and non-oxidized sterol, with a brief discussion of oxysteryl-containing OxCEs.

The enzyme 12/15-lipoxygenase (mouse 12/15-LO is highly homologous to human and rabbit 15-LO) differs from other PUFA-oxidizing enzymes like cyclooxygenases in that its preferential substrate is a CE and not a free fatty acids or a phospholipid (PL) (12, 15). In a test-tube reaction of LDL oxidation by rabbit 15-LO, even when the LDL particle is loaded with free linoleic acid, cholesteryl linoleate constitutes the major 15-LO substrate (15). However *in vivo*, 12/15-LO is an intracellular enzyme and LDL is an extracellular lipoprotein, so it was at first puzzling how CEs were oxidized by 12/15-LO. According to one suggested mechanism (16, 17), LDL binds to macrophage LDL receptor related protein-1 (LRP-1), which in turn induces 12/15-LO translocation from the cytosol to the cell membrane and mediates CE transport from LDL to the cell membrane, where it becomes oxidized by 12/15-LO, as well as the return of an OxCE to the LDL. It is unknown if any of lipid droplet-associated proteins can mediate a similar CE exchange and if CE oxidation can occur on the surface of intracellular lipid droplets.

Products of 12/15-LO–mediated CE oxidation are vulnerable to subsequent oxidation in free radical reactions, forming numerous and complex isoprostane OxCE products, with up to 6 oxygen atoms inserted in the molecule of cholesteryl arachidonate (18, 19). Among these polyoxygenated CE products, molecules with a bicyclic endoperoxide group (18–20), such as cholesteryl (9,11)-epidioxy-15-hydroperoxy-(5*Z*,13*E*)-prostadienoate (abbreviated as BEP-CE for the presence of bicyclic endoperoxide and hydroperoxide groups), have biological activities and can covalently modify proteins, including apolipoproteins, as discussed below. OxCEs can also decompose to produce highly reactive end products, like malondialdehyde (MDA) or 4-hydroxy-2-nonenal (4-HNE), which in turn covalently modify proteins and phosphatidylethanolamines (21, 22). These posttranslational modifications profoundly affect protein function.

In addition, intracellular OxCE hydrolysis and subsequent re-esterification of an oxidized fatty acyl chain can produce oxidized PL (OxPL) in the cell (12). In wild type but not 12/15-LO–deficient murine macrophages, radioisotope-labeled cholesteryl linoleate and cholesteryl arachidonate, either intracellular or as part of LDL, were oxidized by the macrophage 12/15-LO, and the oxidized fatty acyls in OxPL molecules originated from the OxCEs (12).

Esterification of Oxysterols

Oxysterols, derived from either enzymatic or non-enzymatic oxidation of cholesterol, are bioactive and play important regulatory roles (23, 24). Similar to esterification of cholesterol, esterification of oxysterols is mediated by ACAT in cells and LCAT in plasma, as well as by lysosomal phospholipase A2 (25, 26). Esterification of oxysterols in plasma shifts their distribution away from albumin to LDL and HDL (27), where approximately 95% of plasma esterified oxysterols are found (28–31).

OxCE Trafficking

Transport of OxCEs in circulation and their uptake by cells occur *via* the same pathways that traffic non-oxidized CEs. HDL carries 85% of total plasma CE hydroperoxides. While HDL and LDL carry approximately equal numbers of CE hydroperoxide molecules per particle, the CEs in HDL on a per lipid basis are over 20-fold "more oxidized" than those in LDL (32). Lipid peroxidation products in HDL are increased due to direct oxidation, transfer from LDL to HDL or by enzymatic re-esterification from OxPL by LCAT (9, 33, 34). CETP does not distinguish between CE and OxCE and mediates exchange of OxCE between HDL and LDL at the same rate as it transfers CE (9). Oxidized cholesteryl linoleate in which the fatty acyl moiety is oxidized to a hydroperoxide moves readily from HDL to hepatoma cells in serum-free medium (35), and the rate of SR-BI-mediated OxCE uptake by cells is approximately 9 times faster than that of non-oxidized CE and at least 40 times faster than the uptake of a whole HDL lipoprotein (36, 37). Because of the presence of hydrophilic, oxygen-containing groups in OxCE molecules, they become amphipathic and more mobile and presumably less confined to hydrophobic cores of lipoproteins or lipid droplets.

Biological Activity of OxCE

There is an important feature of OxCEs that sets them apart from other oxidized lipids—a combination of reactive and/or functional oxidation moieties in the fatty acyl chain, which we already discussed, with cholesterol. Unmodified cholesterol is a major regulatory molecule for many proteins. The cholesterol recognition/interaction amino acid consensus (CRAC) motif and

its reverse version CARC are present in many transmembrane proteins and are essential for their function (38). In model binding experiments, cholesterol is often replaced with an acidic short-chain CE, cholesteryl hemisuccinate (39), suggesting that cholesterol esterification does not significantly affect its interaction with CRAC/CARC motifs. However, we have not seen direct experimental or modeling comparison of cholesterol and long-chain CE or OxCE binding to these domains. And we are unaware of studies of interaction between transmembrane proteins, like GPCRs, with OxCEs, which are bifunctional—carrying both unmodified cholesteryl and fatty acyl oxidation moieties.

In addition to transmembrane cholesterol-binding proteins, there are non-membrane proteins that have hydrophobic pockets where cholesterol docks, CETP and Niemann-Pick disease, type C2 protein (NPC2) being the most characterized proteins in this class. Another cholesterol-binding protein is MD-2 (40). MD-2 is the LPS-binding co-receptor for TLR4, an obligatory component for LPS-induced TLR4 activation and signaling. MD-2 has a β-cup fold structure composed of two antiparallel β sheets forming a hydrophobic pocket, with positively charged residues located near the opening rim of the pocket (41–43). Fatty acyl chains of LPS dock into the hydrophobic pocket, and negatively charged phosphate groups of LPS bind positively charged residues at the pocket opening. Likewise, in the molecule of cholesterol, a hydrocarbon chain together with the steroid form an elongated hydrophobic structure, which docks in the hydrophobic pocket of MD-2, and a hydroxyl group linked to the other side of the steroid stabilizes cholesterol at the positively charged entrance to the pocket. Test-tube experiments confirm that MD-2 binds cholesterol (40). Furthermore, cholesterol is found associated with the MD-2 immunoprecipitated from human plasma or from mouse atherosclerotic lesions (40). It is unlikely that unesterified cholesterol binding to MD-2 activates TLR4 because there is no moiety in the MD-2-bound cholesterol that would interact with TLR4, however, such a moiety is present in the cholesteryl esterified to a fatty acyl–CE. The hypothesis that an oxygenated fatty acyl chain in OxCE provides additional interaction surfaces, which, in combination with cholesteryl anchoring in the MD-2 hydrophobic pocket, provide sufficient interfaces for OxCE-induced MD-2-TLR4 binding, remains untested.

Although structural determinants are not yet elucidated, OxCE, and specifically BEP-CE, indeed induces MD-2 recruitment to TLR4 and TLR4 dimerization (44). Interestingly, MyD88, a TLR4 adaptor which mediates the bulk of LPS effects, minimally contributes to macrophage responses to minimally modified LDL (mmLDL), a major carrier of OxCE. Instead, spleen tyrosine kinase (SYK) has been identified as a kinase, which is recruited to TLR4 and mediates the majority of mmLDL- and OxCE-induced effects in macrophages (45–47). This dichotomy between LPS- and OxCE-mediated TLR4 responses attests, in addition to the pattern-recognition character of TLR4, to the TLR4 functional selectivity, similar to biased agonism of GPCRs (48).

The SYK-dependent activation of TLR4 by mmLDL and OxCE results in profound cytoskeleton changes in macrophages, including actin polymerization, cell spreading, membrane ruffling and macropinocytosis (44, 46, 49) (**Figure 1**—Bioactivity). Macropinocytosis is a robust mechanism of OxLDL, mmLDL, and native LDL uptake by macrophages and foam cell formation. In addition, mmLDL induces PLCγ, PKC and NOX2-dependent ROS production, which regulates expression of RANTES (CCL5), IL-1β, and IL-6 (45). NOX-2 also regulates mmLDL-induced expression of MCP-1 (CCL2), TNFα, MIP-2 (CXCL2), and MIP-1α (CCL3) (45). *Tlr4*$^{-/-}$ primary macrophages fail to respond to mmLDL or OxCE (44, 46, 49). Remarkably, in *in vitro* experiments, mmLDL and low-dose LPS, imitating subclinical endotoxemia observed in patients with the metabolic syndrome, synergize to produce higher levels of inflammatory cytokines. Although published data point to pro-inflammatory effects of OxCE, a more extensive literature on biological effects of OxPL describes both pro-and anti-inflammatory effects depending on the disease or pathological condition context (21). Thus, we cannot exclude the possibility of context-dependent, anti-inflammatory effects of OxCE, but this requires further research.

In contrast to the biological activity of fatty acyl-oxidized OxCE, esterification of oxysterols largely serves to curtail their biological activity (50). However, in neurons, ACAT-mediated esterification of 24(S)-hydroxycholesterol results in the formation of atypical lipid droplets and neurotoxicity (51). These findings suggest cell type and context dependent effects of esterification of oxysterols.

In addition to free lipid OxCE, cholesteryl fatty acyl hydroperoxides or endoperoxides (like in BEP-CE) can make covalent adducts with proteins and thus affect their function and/or produce novel protein-OxCE epitopes. For example, cholesteryl hydroperoxyoctadecadienoate (HPODE) forms covalent adducts with PDGF, TGFβ, and bFGF and inactivates them (52). In contrast, cholesteryl HPODE does not modify EGF (52), implying specificity of OxCE-protein modification, however, determinants of this specificity remain unclear. Similarly, remain unclear the exact mechanisms of cholesteryl HPODE and cholesteryl 9-oxononanoate (9-ON) induced activation of PKC and ERK1/2 in endothelial cells, which results in expression of fibronectin connecting segment-1 and enhanced adhesion of monocytes to endothelial cells (53). Cholesteryl 9-ON induces expression of both TFG-β and TGF-β receptor type I in human U937 promonocytic cells. This effect is mediated by ERK1/2 and potentially is involved in sustaining vascular remodeling in atherosclerosis (54). Cholesteryl HPODE, but not HPODE-containing OxPL, has been identified as an active component that induces PPARα-dependent expression of CD36 in human monocyte-derived macrophages (55). It is also possible that in the experimental systems employed in the above experiments, cholesteryl HPODE underwent further oxidative modifications, resulting in more complex products, which in turn evoked biological activity different from that of an initial hydroperoxide.

OxCE in Human Atherosclerotic Lesions

How relevant is the biological activity of OxCE to the pathogenesis of atherosclerosis? Definitive studies for OxCE are yet to be conducted but if any guide, recent work from Witztum's group has demonstrated that constitutive expression of the single-chain E06 antibody, which neutralizes atherogenic effects of OxPL, in $Ldlr^{-/-}$ mice significantly reduces atherosclerosis and its co-morbidities (56, 57). In the absence of similar work targeting OxCE, we can only hypothesize that the substantial presence of OxCE in atherosclerotic lesions may have an atherogenic effect. Indeed, using advanced mass spectrometry techniques helped identify many OxCE species in human atherosclerotic lesions, estimating that, on average, 23% of cholesteryl linoleate, 16% of cholesteryl arachidonate and 12% of cholesteryl docosahexaenoate are oxidized (58). In a different study, OxCEs have accounted for 11 to 92% of the CE-PUFA pool in atherosclerotic plaques from different subjects (59). BEP-CE has been detected in human atherosclerotic lesions as well (44).

The studies cited in the previous paragraph identified free lipid OxCEs. It is technically challenging to construct a mass spectrometry method that would detect covalent OxCE adducts to proteins. However, these new epitopes can be detected with antibodies raised against the OxCE moiety independent of a protein, which has been covalently modified by this OxCE. For example, a monoclonal antibody raised against proteins modified with cholesteryl 9-ON has been shown to stain atherosclerotic lesions (60). Studies in our lab have produced a new monoclonal antibody that recognizes an OxCE epitope on modified proteins. To ensure the independence of an OxCE epitope recognition from the protein carrier, mice were immunized with OxCE-KLH and the antibody was selected against OxCE-BSA. The resulting antibody, AG23, bound OxCE-modified KLH, BSA, apoA-I, and a 6-amino acid peptide (61). The OxCE used for covalent modification of these proteins was a product of cholesteryl arachidonate oxidation with 2,2'-azobis (2,4-dimethylvaleronitrile) in an atmosphere of oxygen, which predominantly produced BEP-CE, but other complex oxidation products were present as well (44). The AG23 immunoreactivity was abundant in human carotid endarterectomy specimens, demonstrating the presence of OxCE epitopes in atherosclerotic lesions (**Figure 1**—Biomarkers) and suggesting relevance of OxCE to the pathogenesis of human CVD (61).

OxCE in Human Plasma as a Biomarker for CVD

As with detection of OxCE in atherosclerotic tissue, early studies employing biochemical and mass spectrometry techniques reported a wide range of CE hydroperoxides in human plasma, from 3 to 920 nmol/L (62, 63), with hydroperoxides of cholesteryl linoleate and cholesteryl arachidonate as the major oxidation products (32, 64–66). Plasma levels of CE hydroperoxides have been significantly increased on day 1 and peaked at day 5 after subarachnoid hemorrhage, returning to normal levels on days 7 and 9 (67). This temporal sequence correlates well with the known time course of cerebral vasospasm, which typically has its onset between 5 and 7 days after subarachnoid hemorrhage. Using a targeted lipidomic approach to quantify multiple classes of OxCE, Yin's group tested plasma samples from 49 CVD patients and observed a significant elevation of multiple oxidation products of cholesteryl arachidonate and cholesteryl linoleate in plasma of patients with myocardial infarction compared to that of control and other CVD groups. These results suggest release of OxCEs from the raptured atherosclerotic plaque (68).

The AG23 mAb against OxCE described in the preceding section has been used to develop a new ELISA method to measure OxCE associated with apoA-I or apoB-100 lipoproteins in human plasma (61). This assay measures levels of lipoproteins that have at least one OxCE epitope. Measuring OxCE-apoB and OxCE-apoA in plasma samples from PROXI, a randomized parallel-arm double-blind placebo-controlled trial in which human subjects received placebo or a statin treatment for 16 weeks, we demonstrated that the OxCE-apoA levels were significantly lower in subjects treated with atorvastatin than in the placebo group, independent of the apoA-I levels (**Figure 1**—Biomarkers) (56). Testing larger cohorts of human subjects with different conditions and treatment regimens will determine if this particular OxCE assay will become a useful diagnostic and/or prognostic CVD biomarker.

CONCLUSIONS AND UNRESOLVED QUESTIONS

Biological activity and biomarker potential of OxCEs remain understudied. In part, this is due to a common perception of CEs as inherently inert intracellular "storage" and lipoprotein "transport" lipids, which is certainly the case for CEs with saturated fatty acyls. However, there exist mechanisms for oxidative modification of CEs with polyunsaturated fatty acyls, producing a multitude of OxCEs, which exhibit biological activity as free lipid and can covalently modify proteins. The unique feature of OxCEs is that they contain both an oxidized fatty acyl, which is often reactive and/or makes OxCEs less hydrophobic and thus more mobile, and the unmodified cholesteryl, which binds to and modulates function of many membrane and soluble proteins. In this article, we reviewed how cholesterol binding to MD-2 makes OxCE an agonist for TLR4, resulting in inflammatory and lipid accumulation responses in macrophages. Future studies will elucidate whether OxCE can interact with GPCRs, which potentially may have broad implications. More work is also needed to understand biological effects of esterified oxysterols, which seem to be tissue and pathology context dependent. The spectrum of proteins in plasma and in tissues that have OxCE covalent adducts remains unexplored, as remain poorly understood determinants of the OxCE specificity in covalent modification of proteins. The development of new antibodies that recognize OxCE-protein covalent complexes independent of a protein carrier will be instrumental in answering these

questions. However, there remains a challenge of characterization of the exact chemical structure of OxCE-protein covalent adducts; where mass spectrometry methods are insufficient, co-crystallization of antibodies with their OxCE antigens may become a productive approach. Initial studies using OxCE-specific antibodies, as illustrated in this article, are promising but examination of larger cohorts of subjects with CVD and possibly other conditions are needed to fully evaluate the diagnostic and prognostic potential of OxCE as a new biomarker.

AUTHOR CONTRIBUTIONS

AG and YM wrote this manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Oxidation-Specific Epitopes in Non-Alcoholic Fatty Liver Disease

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An improper balance between the production and elimination of intracellular reactive oxygen species causes increased oxidative stress. Consequently, DNA, RNA, proteins, and lipids are irreversibly damaged, leading to molecular modifications that disrupt normal function. In particular, the peroxidation of lipids in membranes or lipoproteins alters lipid function and promotes formation of neo-epitopes, such as oxidation-specific epitopes (OSEs), which are found to be present on (lipo)proteins, dying cells, and extracellular vesicles. Accumulation of OSEs and recognition of OSEs by designated pattern recognition receptors on immune cells or soluble effectors can contribute to the development of chronic inflammatory diseases. In line, recent studies highlight the involvement of modified lipids and OSEs in different stages of the spectrum of non-alcoholic fatty liver disease (NAFLD), including inflammatory non-alcoholic steatohepatitis (NASH), fibrosis, and hepatocellular carcinoma. Targeting lipid peroxidation products shows high potential in the search for novel, better therapeutic strategies for NASH.

Keywords: oxidative stress, innate immunity, non-alcoholic fatty liver disease, lipid peroxidation, steatohepatitis (NASH), oxidation-specific epitopes

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INTRODUCTION

In parallel with the global epidemic obesity, the prevalence and incidence of non-alcoholic fatty liver disease (NAFLD) has greatly increased over time and continues in doing so, making it the most common liver disease worldwide (1). Nowadays, the prevalence of NAFLD is estimated around 25% in Western countries. NAFLD covers a histological spectrum of liver conditions with varying degrees of liver injury and scarring in individuals who do not consume alcohol in harmful quantities and was first recognized as distinct entity about 40 years ago. NAFLD ranges from excessive hepatic lipid accumulation alone (steatosis) that might progress into non-alcoholic steatohepatitis (NASH), which is characterized by inflammatory cell infiltrates. NASH increases the risk for further liver fibrosis, cirrhosis and hepatocellular carcinoma, ultimately requiring liver transplantation (2). In fact, progressive NASH is the second ranked cause for liver transplantation, directly following alcoholic liver disease (3). Although the diagnosis for NAFLD or NASH can be suspected based on ultrasound imaging and abnormal levels of liver enzymes in serum, NASH can only be definitely diagnosed by liver biopsy and evaluation of a pathologist. While lifestyle interventions and associated weight loss can improve steatosis alone, sustained effects are difficult to obtain and the presence of inflammation makes prognosis considerably worse. Further, although multiple drugs and combination therapies are under investigation, currently no truly effective treatment exists (4, 5). The progression from steatosis alone into NASH can be explained by the so-called "two-hit

hypothesis," in which lipid accumulation would represent the first hit, rendering the tissue more prone for a second hit (such as oxidative stress and lipid peroxidation) triggering inflammation and liver damage. However, this concept changed into a more "multiple parallel hits" theory, in which different molecular triggers simultaneously cause steatosis and liver injury (6–8). More recently, metabolic associated fatty liver disease (MAFLD) has been suggested as a novel term reflecting current knowledge about disease pathology more precisely (9). In this review, we use NASH as term for describing more advanced fatty liver disease compared to steatosis alone in NAFLD.

The occurrence of oxidative stress reactions and the accumulation of reactive oxygen species are common features observed in different stages of the NAFLD spectrum (10, 11). On one hand, excess exposure and build-up of free fatty acids in hepatocytes cause lipotoxicity and damage to mitochondria, and stimulates the release of pro-inflammatory cytokines and microvesicles (12). Further, the activation of non-parenchymal cells, especially resident Kupffer cells, together with the recruitment of infiltrating monocytes and neutrophils to the liver contributes to inflammation *via* the release of cytokines, chemokines, nitric oxide, and reactive oxygen species (2). Importantly, although reactive oxygen species are produced normally as by-products of cellular metabolism, they can attack different vital macromolecules such as proteins, lipids, and nucleic acids (DNA/RNA). This results in different oxidative damage products such as protein carbonyls, lipid peroxides, and 8-hydroxy-2′-deoxyguanosine (8-OH-dG), respectively. These products are often used as biomarkers of oxidative stress and have all been associated with NAFLD/NASH (reviewed in (13)). In order to overcome inappropriate levels of reactive oxygen species, we depend on the scavenging capacity by antioxidants in our system. Relevantly, in parallel to increased oxidative stress, reduced systemic levels of antioxidants such as glutathione or vitamin E, as well as lower anti-oxidative enzyme activity has been documented during NAFLD (13–15). In this review, we will particularly focus on the oxidation of membrane lipids, a process called lipid peroxidation, and their involvement in steatohepatitis (16).

Besides affecting their biological function, peroxidation of lipids also results in the generation of several degradation end-products which can further modulate the normal properties of proteins and lipids. Furthermore, highly reactive aldehydes generated in the process of lipid peroxidation modify self-molecules and form antigenic adducts, known as oxidation specific epitopes (OSEs), which are bound by various receptors of the immune system to alert the host and promote their removal to prevent inflammatory effects (17, 18). However, accumulation of OSEs and their insufficient clearance triggers sterile inflammation. Major carriers of OSEs are cells undergoing cell death (apoptosis), extracellular vesicles, and damaged lipoproteins, such as oxidized low-density lipoproteins (OxLDL). Owing to their biological activities, OSEs and their immune recognition are involved in a wide variety of physiological and pathological processes, including atherosclerosis (19–21) and several autoimmune diseases such as systemic lupus erythematosus (22). Here, we provide an overview of studies linking OSEs and their immune recognition to NASH and potential implications in the field of fatty liver disease.

OSE Generation

Various mechanisms cause lipid peroxidation, particularly polyunsaturated fatty acids (PUFAs), which involves both enzymatic and non-enzymatic mechanisms (23). The enzymatic processes of lipid peroxidation include the activation of lipoxygenases, myeloperoxidases, cyclo-oxygenases, and cytochrome p450. On the other hand, non-enzymatic oxidation is done by free radicals, which are indirectly generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and nitric-oxide synthases. Both enzymatic and non-enzymatic mechanisms result in the generation of lipid-hydroperoxide molecules (LOOH), which then decompose. As part of this degradation process a large variety of secondary products including malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and the remaining core aldehyde of oxidized phospholipids (OxPL) are produced (24, 25). These remaining end-products of lipid peroxidation can further propagate oxidative damage as a result of defective clearance and as such, have been considered as downstream mediators of oxidative stress. Notably, measuring lipid peroxidation degradation products, especially MDA and 4–HNE, by the frequently used 2–thiobarbituric acid reaction (TBAR) assay, is an established method for assessing oxidative stress (16).

PUFA-containing phospholipids are particularly prone to oxidative damage (16, 23). Hence, the major membrane phospholipid phosphatidylcholine is very susceptible to free-radical-induced oxidation, resulting in the exposure of hydrophilic phosphocholine (PC) headgroups and the generation of a complex mixture of OxPL and their terminal degradation products (26). Especially, oxidation of the PUFA chain at the sn–2 position of phosphatidylcholine results in its degradation and the formation of reactive PUFA fragmentation products such as MDA. Further, the oxidation of cholesterol and cholesteryl esters can take place, resulting in structural changes and altered biological activities (27, 28). Owing to their biological activities and pro-inflammatory potential, the formation of lipid peroxidation-derived adducts can be viewed as post-translational modifications generating neo-epitopes which are now recognized as a type of danger-associated molecular pattern (DAMP) (20). Importantly, several of these lipid-derived adducts have been documented and implicated in a wide variety of pathologies, including NAFLD and NASH (18, 29, 30).

OSE Presence in NAFLD

Oxidation of LDL is thought to contribute to fatty liver disease progression by multiple mechanisms, including the formation of OSEs (31, 32). As mentioned before, OSEs are present on apoptotic cells, OxLDL, and microvesicles, components that all have been shown to be associated with NAFLD. Here, we provide several lines of evidence that indicate the presence and importance of different lipid peroxidation products in the onset of NASH (see **Figure 1**).

Among the different aldehydes that can be formed as secondary products during lipid peroxidation, MDA and 4-HNE are the most extensively studied and both are associated with different stages of fatty liver disease. A significant correlation between hepatic 4-HNE adducts and the stage of fibrosis has been described (33), and

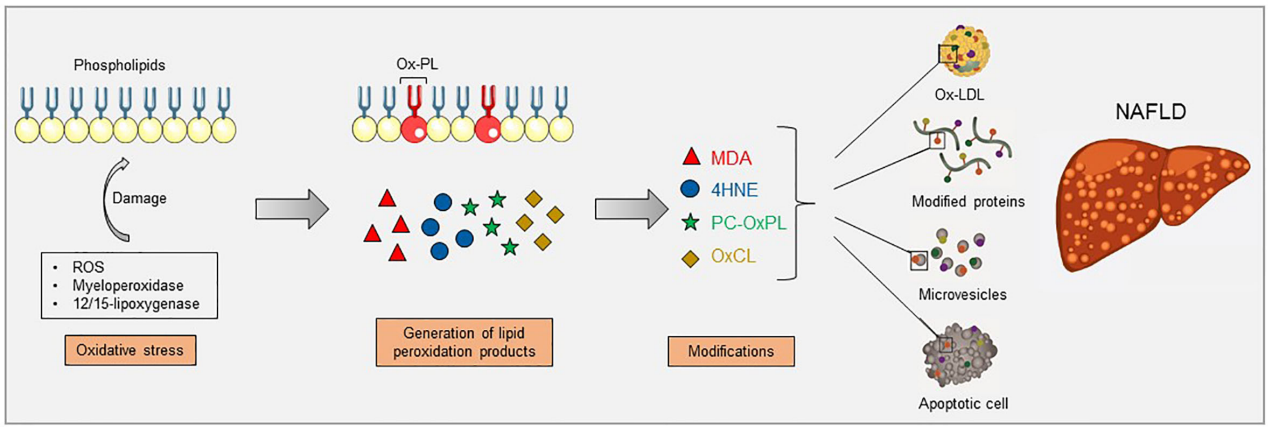

FIGURE 1 | Increased oxidative stress causes lipid peroxidation, which can occur *via* enzymatic reactions, such as myeloperoxidase and 12/15-lipoxygenase, and non-enzymatic reactions, such as reactive oxygen species (ROS). Lipid peroxidation of membrane phospholipids results in their fragmentation and the generation of breakdown products which can further modify free amino groups of proteins and lipids, forming covalent adducts and creating oxidation-specific epitopes (OSEs), including malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), phosphocholine on oxidized phospholipids (PC-OxPL), and oxidized cardiolipin (OxCL). These epitopes are carried by oxidized low-density lipoproteins (OxLDL), modified proteins, microvesicles, and apoptotic cells, aspects that have been shown to be present during NAFLD. This figure was produced using Servier Medical Art (Servier, www.servier.com, licensed under a Creative Commons Attribution 3.0 Unported Licence).

increased mitochondrial 4-HNE–protein adducts during NASH development have been reported (34). Multiple studies have indicated increased amounts of MDA, as measured by the TBAR assay, and OxLDL levels in human NAFLD and NASH patients in comparison to control subjects (15, 35–39). In addition, the presence of MDA adducts in livers has been documented for several different experimental rat and mouse models of NAFLD and NASH (40–43). In line with these publications, work from our group indicated the presence of MDA adducts in livers of human NASH (44), as well as during steatohepatitis in hypercholesterolemic *Ldlr*$^{-/-}$ mice on high-fat high-cholesterol diet (45), a murine model resembling human lipid profiles of NAFLD (46). Using *in vitro* and *in vivo* approaches, we showed that MDA epitopes detectable in hepatic inflammation act as sterile mediators of inflammation *via* their stimulation of cytokine secretion by macrophages and promotion of leukocyte recruitment (45).

Recently, Sun et al. reported elevated amounts of PC-OxPLs in the liver and circulation during NASH compared to controls using various murine models and human subjects (47). In humans, increased plasma and liver PC-OxPL was more closely associated with NASH rather than steatosis, suggesting their importance during disease progression. Mechanistically, OxPLs induced mitochondrial damage and ROS accumulation, partly *via* modification of the anti-oxidative enzyme manganese superoxide dismutase (MnSOD/SOD2), thereby blocking its activity.

Work from our group demonstrated that various OSEs are carried by circulating extracellular vesicles (48), a heterogeneous population of small membrane-deliminated structures including exosomes and microvesicles. Importantly, the release of extracellular vesicles has been identified as a result of hepatocyte lipotoxicity and has been implicated in NAFLD (49). Indeed, toxic lipid classes are abundantly present in circulation of NASH patients and stressed/damaged hepatocytes as a result of treatment with lipotoxic lipids causes the release of large amounts of extracellular vesicles (50). Besides, microvesicles have been proposed as potential biomarkers for chronic liver diseases (51). Microvesicles, potentially hepatocyte-derived, have been shown to stimulate pro-inflammatory responses *via* the activation of immune cells and stellate cells in the liver and thus might link lipotoxicity and lipid peroxidation to inflammation and fibrosis, major components of NASH. More specific microvesicle profiling, including assessment of biological properties and cell-cell interaction, will enable us to better understand the role of OSE-carrying microvesicles during NASH development and progression.

Another phospholipid component prone to oxidation is cardiolipin, which is almost exclusively present at the inner mitochondrial membrane. Cardiolipin is importantly involved in mitochondrial energetics and mitochondrial dependent steps of apoptosis. Hence, oxidized cardiolipin (OxCL) has been associated with mitochondrial dysfunction. Relevantly, increased cardiolipin levels are reported in experimental NASH models as well as human NAFLD (52–54). Moreover, patients with NAFLD have increased circulating IgG antibody titers towards OxCL compared to healthy controls (55), further supporting the presence of OxCL during NAFLD.

Innate Immune Recognition of OSEs

As described above, lipid peroxidation and its end-products are associated with both structural and functional alterations of these macromolecules. In order to protect us from potential detrimental effects of accumulated altered self-molecules, the human body requires recognition mechanisms to provide effective clearance. Indeed, OSEs are believed to represent a class of DAMPs which can stimulate both adaptive and innate immune responses, dependent on recognition by pattern recognition receptors (PRR) (21, 56). Detailed characterization of various OSEs and the innate immune responses towards them indicated that OSEs are recognized by both cellular and soluble PRRs, which we will discuss in this section in view of steatohepatitis (see **Figure 2**).

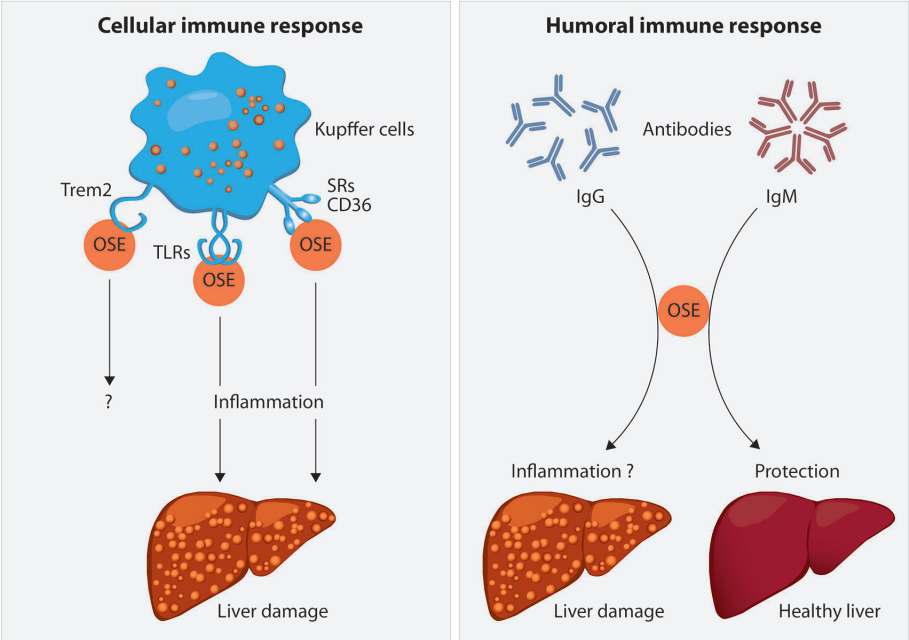

FIGURE 2 | Oxidation-specific epitopes (OSEs) act as danger-associated molecular patterns (DAMPs) which are recognized by different pattern recognition receptors (PRR) as part of the cellular immune response towards OSEs. Receptors known to bind to certain lipid peroxidation adducts are the family of scavenger receptors (SR) such as CD36, Toll-like receptors (TLRs) and the triggering receptor expressed on myeloid cells 2 (TREM2). In the liver, the presence of SR and TLRs on Kupffer cells and their uptake of modified lipids has been shown to cause inflammation, thereby leading to liver damage during NAFLD progression. The functional role of TREM2 during NAFLD has not been addressed yet. Moreover, B cell-derived antibodies are able to recognize OSEs. Whereas increased anti-OSE IgG titers are associated with NASH and fibrosis, potentially *via* triggering pro-inflammatory responses, IgM antibodies targeting OSE are found to be protective against NASH, both by neutralizing the pro-inflammatory effects of OSE and by enhancing the clearance of dying cells.

Cellular Receptors

As part of the innate immune response triggered by OSEs, PRR present on the surface of immune cells, such as the scavenger receptor family, recognize and internalize oxidized but not native LDL particles (57). As such, macrophages mediate the phagocytosis of oxidatively altered molecules, triggering inflammatory cascades *via* the secretion of pro-inflammatory chemokines and cytokines such as CC-chemokine ligand 1 (CCL1), CCL2, and CCL5, thereby promoting increased recruitment of monocytes which further contribute to the production of pro-inflammatory mediators as well as oxidative damage (58). Among the family of scavenger receptors, CD36, SR-A1, and SR-A2 are the best described and most relevant receptors for OxLDL uptake as macrophages lacking CD36, SR-A1, and SR-A2 have 75–90% reduced binding and degradation of OxLDL (59). In addition, we demonstrated that bone marrow-derived macrophages from mice lacking CD36 or SR-A1 secreted less CXCL1 compared to wildtype cells after stimulation with malondialdehyde-acetaldehyde (MAA) epitopes, the immunodominant subset of MDA epitopes (45). Similar to lipid-laden foam cells as occurring during atherosclerotic lesions, Kupffer cells, resident macrophages of the liver, show a foamy appearance after the enhanced uptake of modified lipoproteins during NAFLD (60). Importantly, we found that hematopoietic SR-A1 and/or CD36 deficiency protects against hepatic steatosis and inflammation in hyperlipidemic *Ldlr*$^{-/-}$ mice fed an atherogenic diet, indicating that SR-mediated uptake of modified lipids is at least in part responsible for diet-induced inflammation in the liver (61). Further studies indicated that OxLDL uptake by macrophages is associated with abnormal intracellular trafficking, resulting in lysosomal trapping of OxLDL, cholesterol crystal formation, and dysfunctional lysosomes (31). Similar to cholesterol crystals, stimulating J774 macrophages with MDA-modified hen egg lysozyme (MDA-HEL) caused lysosomal rupture (62). Therefore, one might hypothesize that OSE-induced lysosomal damage might account for inflammatory responses and apoptosis during diet-induced liver disease. Indeed, human NASH patients have increased circulatory levels of cathepsins, lysosomal enzymes responsible for breakdown of internalized products, indicating lysosomal leakage (63). Taken together, ample evidence supports the involvement of scavenger receptor-mediated uptake of modified lipids in NAFLD.

Toll-like receptors (TLRs) are another class of PRR taking part in the response to damaged lipoproteins, capable of binding to a variety of different pathogen-associated molecular patterns (PAMPs), including bacterial and viral components, as well as DAMPs. Additionally, certain TLRs have been shown to recognize—as part of a multimeric complexes with other PRRs—OxPL, OxLDL, and other OSEs, thereby mediating pro-inflammatory signals (18). Particularly TLR2, TLR4, and TLR6 have been shown to respond to OSEs, i.e. oxidized cholesteryl esters (OxCE) and OxPL on the surface of extracellular vesicles appear to be ligands for TLR4 (29, 64). In relation to NAFLD, the

involvement of certain TLRs in disease progression has been described, such as TLR2, TLR4, TLR7, and TLR9 (65). For instance, absence of TLR4 in *Ldlr*$^{-/-}$ mice on atherogenic diet has been shown to protect against triglyceride accumulation in the liver (66) and features of NASH were ameliorated in TLR4 deficient mice receiving methionine choline-deficient (MCD) diet (67). Further, activation of TLR7 signaling was found to reduce lipid accumulation and autophagy in hepatocytes, thereby preventing NAFLD progression. Interestingly, 4-HNE and MDA potentially inhibit this process, thereby aggravating disease onset (68). Nevertheless, a direct link between OSE-TLR signaling and fatty liver disease development is missing as most studies focus on the recognition of bacterial ligands.

More recently, the triggering receptor expressed on myeloid cells 2 (TREM2), belonging to the immunoglobulin superfamily and mainly expressed on myeloid cells, has been shown to bind and recognize lipoproteins and apolipoproteins, including ApoE, LDL, and MDA-LDL particles (69), suggesting TREM2 might also recognize OSEs. Upon cleavage of TREM2 by A Disintegrin And Metalloprotease (ADAM) 10 and ADAM17, soluble TREM (sTREM2) is released (70). Whether sTREM has similar binding capacities as TREM2 is currently not known. Interestingly, single-cell RNA sequencing (scRNAseq) studies have identified the presence of *Trem2* expressing macrophages in several lipid-mediated diseases, including obesity (71) and atherosclerosis (72). Importantly, also in relation to liver damage during fatty liver disease, the emergence of so-called NASH-associated macrophages (NAMs) (73) and scar-associated macrophages (SAMs) (74), that are characterized by high *Trem2* expression, has been described. *Xiong et al.* found more hepatic *Trem2* expression in human and murine NASH and described that increased *Trem2* expression in livers of NASH patients is positively correlated with AST and ALT levels (73). These findings are further supported by another study, which indicated more *Trem2* expressing macrophages in the liver of human subjects with cirrhosis based on scRNAseq analysis of CD45+ cells (74). As the receptor has been reported to bind MDA-LDL, these data suggest a potential novel mechanism of OSE-induced immune recognition. Nevertheless, functional studies assessing the role of TREM2 during the onset of NAFLD are currently missing and more specific insights in recognition of different OSEs by TREM2 and its soluble form are needed.

Soluble Receptors

Besides recognition by receptors present on the cell surface, OSEs represent targets for several soluble PRRs that also include secreted forms of cellular PRR. One soluble protein capable of binding OSEs is C-reactive protein (CRP), an acute-phase reactant made in the liver. Originally, CRP was identified as the plasma component binding PC located on the capsular polysaccharide of *Streptococcus pneumoniae*. Only later, CRP was also identified to bind PC-OxPL on OxLDL and dying cells, indicating CRP to respond to a common OSE of microbial origin or derived from lipid peroxidation (75). Although some discrepancies exist over the relationship between CRP levels in NAFLD, most studies report increased circulatory CRP levels, which is a known marker for systemic inflammation, during NAFLD (76). One could hypothesize that changes in CRP levels are correlated with oxidative stress and/or lipid peroxidation products, thereby affecting study outcomes. However, whether CRP itself has a functional role in NAFLD development is unclear.

Further, MDA epitopes have been found to be targets of proteins involved in the complement machinery, which exerts a key function in the maintenance of tissue homeostasis *via* immune surveillance and managing clearance of metabolic waste and dead cells (77). Besides the C3 cleavage product C3a (78), a pro-inflammatory anaphylatoxin, we identified that complement factor H (CFH) recognizes and binds MDA epitopes. CFH is a regulator of complement activity as it inhibits the alternative pathway by preventing C3 cleavage. Whereas increased activation of the complement system is reported in NAFLD (79), the specific levels of CFH mRNA were found downregulated in patients with NASH in line with protein levels of CFH in the liver (80). Therefore, low CFH expression might contribute to the harmful effects of lipid peroxidation products in fatty liver disease. In addition, our group has shown that genetic variants of one of the MDA-binding sites of CFH influence the capacity of CFH to bind MDA (81). Further, we recently demonstrated that genetic deletions of complement factor H-related protein 1 (CFHR1), which was previously shown to bind to MDA-LDL (82), and 3 (CFHR3) affects the ability of plasma CFH to bind MDA surfaces. Moreover, purified CFHR1 and CFHR3 competes with CFH for binding to MDA-epitopes (83). Our findings indicate the influence of genetic variations within the CFH/CFHR1/CFHR3 locus on the recognition and binding of OSEs, thereby affecting outcome in diseases associated with oxidative stress and aging such as NAFLD. Further molecular and genetic studies are needed to elucidate the involvement of CFH and CFHR proteins in relation to OSE recognition during fatty liver disease.

Natural antibodies are pre-existing germline-encoded antibodies that are already present at birth and of which the occurrence does not depend on external antigens, as they can be found in germfree mice. Natural antibodies are predominantly of the IgM type, with a broad specificity to various pathogens, but which are also able to recognize endogenous antigens, such as OxPL and adducts formed by end products of the lipid peroxidation process (84). Besides their protective function in the first line defense against invading microbes, natural IgM antibodies maintain homeostasis *via* the clearance of dying cells and metabolic waste products. Indeed, MDA-specific natural IgM recognize apoptotic cells as well as microvesicles carrying MDA epitopes (48). Natural IgM antibodies are produced by B1 cells, which are widely studied in the field of atherosclerosis and believed to exert protective effects *via* the production of natural IgM antibodies (85). In line with these findings in cardiovascular disease, we found that patients with NAFLD have lower levels of IgM targeting OSEs than healthy individuals (44). In addition, we demonstrated that IgM plasma titers towards an immunological mimotope of MDA, P1 mimotope, inversely correlate with signs of obesity, systemic inflammation, and liver injury. Further studies by others and us indicating the protective role of OSE-IgM in the course of NAFLD are described later on in the section about OSE as targets for treatment options.

Taken together, OSE represent DAMPs that initiate certain immune responses upon recognition by several cellular and soluble PRRs as part of the innate immune system. Besides that, adaptive immune reactions can also be triggered by OSEs.

Adaptive Immune Recognition of OSEs

In contrast to innate immunity, adaptive immunity is acquired throughout life as a direct consequence of exposure to external antigens. This way, an immense repertoire of highly specific receptors is generated, resulting in lifelong immune memory, which is mediated by B cells and the antibodies they secrete as well as various T cell subsets. The role of adaptive immunity in the progression of NAFLD, including anti-OSE responses, has recently been reviewed elsewhere [see (30)]. Hence, we will only briefly describe OSE-specific B-cell mediated adaptive responses during NAFLD.

In addition to the involvement of anti-OSE IgM in NAFLD, IgG antibodies targeting OSE have been shown to be elevated in ~40% of adults diagnosed with NAFLD or NASH (55) and in 60% of children with NASH (86). Particularly, IgG antibodies against the cyclic MAA adduct are increased in adults with NAFLD or NASH (55). Further, anti-OSE IgG levels are correlated with the degree of lobular inflammation and are an independent predictor of fibrosis. In contrast to natural IgM, anti-OSE IgG are thought to be produced by B2 cells, suggesting the involvement of specific acquired immunity by different B cell subsets in NASH (87). In line, increased IgG titers were found to be associated with increased maturation of liver B2 cells to plasma cells in diet-induced NAFLD in rats as well as in MCD-induced NASH in mice (88). As such, increased B2 cells might be involved in NASH by promoting pro-inflammatory signals and antigen presentation to CD4 T cells and as a source of pathogenic IgG antibodies. The potential opposing effects of B1 and B2 cells (decreased IgM and elevated IgG titers) are similar to observations during atherosclerosis (89). Hence, further studies investigating the role of B1 and B2 cell subsets and their implications in NASH are needed to potentially identify novel therapy options in which specific B cell subsets are targeted.

OSE as Targets for the Treatment of NAFLD

As oxidative stress is one of the best characterized triggers for liver inflammation during NASH, makes it an attractive target for intervention to prevent disease progression. Although work by us and others point to the potential of targeting lipid peroxidation products and immune recognition of OSEs to ameliorate the inflammatory response occurring in NASH, these studies only involve the usage of different murine models. So far, evidence from human studies in which OSEs or immunity towards OSEs are directly targeted for the treatment of NAFLD and NASH is lacking. Nevertheless, multiple studies exploited the effect of nutrients and antioxidants to reduce oxidative stress and liver damage. The protective effects of vitamin E provided promising results (90, 91), and subsequently the effect of vitamin E supplementation was tested in NASH adults (PIVENS trial) and NAFLD children (TONIC trial). Both studies demonstrated a favorable effect by decreasing NAS score and stimulating resolution of inflammation, but did not affect fibrosis (92, 93). Importantly, these studies used very high doses of vitamin E (400–800 IU) while the daily-recommended intake is around 22.4 IU, thereby raising some concerns regarding increased risk for unwanted side-effects. Interestingly, a recent study found an inverse correlation between vitamin E intake and serum MDA levels among women with NAFLD and NASH (39), indirectly supporting the idea to target MDA adducts in NASH. Besides vitamin E, several other approaches such as vitamin C have been proposed to reduce oxidative stress. Although vitamin C intake has been reported to be inversely correlated with NAFLD severity (94), sufficient data to support the clinical importance is still lacking and more studies in human subjects are necessary to identify modes of action to reduce reactive oxygen species and limit lipid peroxidation.

Using a transgenic $Ldlr^{-/-}$ mouse expressing a functional single-chain variable fragment of E06, a natural antibody capable of neutralizing OxPLs, targeting PC-OxPLs protected against several aspects of NASH, including lipid accumulation, inflammatory responses, fibrotic scarring, hepatocyte cell death, and progression to hepatocellular carcinoma, further supporting the causal role of OxPLs in the pathogenesis of NASH (47). In addition, E06 administration has been shown to protect against liver injury in a mouse model of ischemic reperfusion *via* the blockage of TLR4-mediated neutrophil activation by extracellular vesicles carrying OxPLs (95).

In line with lower anti-OSE IgM titers in human NAFLD (44), an increase in B1-derived natural IgM with specificity for OxLDL resulted in a better outcome for liver disease in atherogenic diet-fed $Ldlr^{-/-}$ mice deficient for the sialic acid-binding immunoglobulin-like lectin G (Siglec-G), a negative regulator of B1 cells and OSE-specific IgM. Further, since PC epitopes as found on OxLDL are also present on the cell wall of *S. pneumoniae*, cross-reactivity exists between PC epitopes from OxLDL and this microbe (96). Similar to findings in experimental atherosclerosis (97), immunization with heat-inactivated *S. pneumonia* increased specific anti-PC IgM titers and protected $Ldlr^{-/-}$ mice from diet-induced steatohepatitis (98). Immunized mice fed a Western-type diet presented less foamy Kupffer cells, reduced inflammatory cell infiltrates and less cholesterol crystals in the lysosomes of Kupffer cells compared to mice without immunization. Moreover, we previously showed that *in vivo* neutralization of endogenously generated MDA epitopes by intravenous administration of a specific MDA antibody (LR04) results in reduced liver inflammation in $Ldlr^{-/-}$ mice (45). Taken together, these studies indicate that anti-OSE IgM expansion is protective and suggest a beneficial role for B1 cells in the course of NAFLD. Therefore, identifying additional epitopes that are recognized by protective antibodies would identify novel antigens that could be useful in the development of stable peptides that might work as vaccine against NAFLD and NASH. One such example peptide might be the P1 mimotope for MDA identified by us (99). Future studies investigating the potential use of this P1 peptide in ameliorating NASH hold great promise for future applications. More studies involving human participants are needed to translate murine findings and elucidate potential protective mechanisms to identify novel treatment targets for patients with chronic fatty liver disease.

DISCUSSION

Evolutionary developed specialized immune recognizers of OSEs, both cellular and humoral, enables the human body to

deal with key housekeeping functions such as the removal of cell debris, dying cells and damaged molecules. Heightened lipid levels and higher oxidative stress as seen during the development and progression of NASH is accompanied with increased generation and burden of lipid peroxidation end products and OSEs. Under such circumstances of excess OSE formation and/or dysfunctional removal, the immune system gets activated, generating inappropriate amounts of chemokines and pro-inflammatory cytokines, and subsequently the development and propagation of chronic inflammatory diseases such as NASH become manifest.

Of note, multiple unresolved questions remain in our understanding of the mechanisms by which OSE-induced immunity can contribute to NASH and its resolution. For instance, as bacterial dysbiosis and the role of the gut-liver axis has been widely accepted in the field of multiple chronic lipid-mediated diseases including NASH (100), one could question the potential direct effect of OxLDL and OSEs on intestinal communities. Moreover, it is not entirely clear yet how B cell-mediated antibody responses towards lipids are mediated in the gut and how these can effect liver disease manifestation. Further, as discussed in this review, although studies indicating the beneficial effect of IgM associated immune response towards OSE, all these data come from experimental mouse models. Translational studies further supporting the importance of this field in the human clinical condition during NASH are still limited. Nevertheless, it can be envisioned that gaining insights in the role of OSEs and OSE-reactive immunity in maintaining homeostasis and in controlling inflammatory responses will ultimately identify new treatment targets that can be explored to modulate NAFLD progression and inflammatory states in general.

AUTHOR CONTRIBUTIONS

TH and CB wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Formation of Oxidatively Modified Lipids as the Basis for a Cellular Epilipidome

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While often regarded as a subset of metabolomics, lipidomics can better be considered as a field in its own right. While the total number of lipid species in biology may not exceed the number of metabolites, they can be modified chemically and biochemically leading to an enormous diversity of derivatives, many of which retain the lipophilic properties of lipids and thus expand the lipidome greatly. Oxidative modification by radical oxygen species, either enzymatically or chemically, is one of the major mechanisms involved, although attack by non-radical oxidants also occurs. The modified lipids typically contain more oxygens in the form of hydroxyl, epoxide, carbonyl and carboxylic acid groups, and nitration, nitrosylation, halogenation or sulfation can also occur. This article provides a succinct overview of the types of species formed, the reactive compounds involved and the specific molecular sites that they react with, and the biochemical or chemical mechanisms involved. In many cases, these modifications reduce the stability of the lipid, and breakdown products are formed, which themselves have interesting properties such as the ability to react with other biomolecules. Publications on the biological effects of modified lipids are growing rapidly, supporting the concept that some of these biomolecules have potential signaling and regulatory effects. The question therefore arises whether modified lipids represent an "epilipidome", analogous to the epigenetic modifications that can control gene expression.

Keywords: phospholipids (PL), oxidation, nitration, oxysterols (cholesterol oxidation products), free radicals, hypochlorous acid (HOCl)

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INTRODUCTION

The oxidation of lipids and lipid-like substances has been known for centuries, and has been widely regarded as an undesirable effect: in foods, lipid oxidation leads to the development of rancidity and acrid flavors, while in materials such as rubber it causes loss of elasticity and perishing (1). In biology, where lipids have important structural, nutritional, and signaling roles, the adventitious, radical oxidation of lipids in cells and tissues was for many years also be regarded as a detrimental process, for example disrupting cell membranes and causing cytotoxicity (**Figure 1A**) (2, 3). On the other hand, in the 1950s the structure of prostaglandins was elucidated and found to result from peroxidation of arachidonic acid [reviewed by (4)]; subsequently, thromboxanes and leukotrienes were also realized to be derived from hydroperoxyeicosatetraenoates (HPETEs) (5).

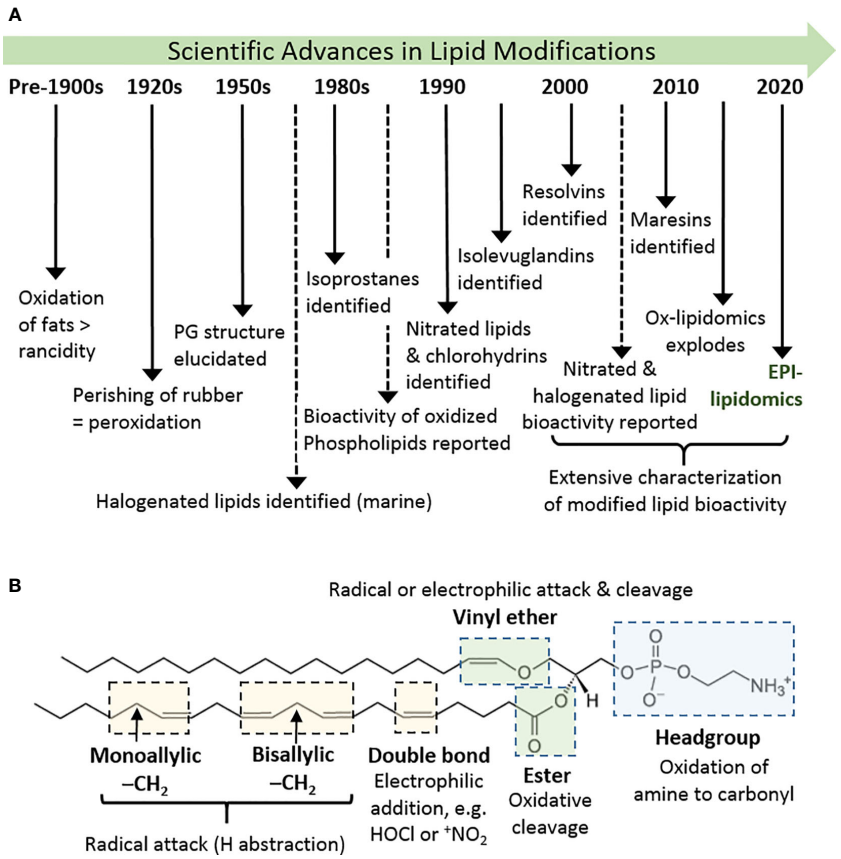

FIGURE 1 | History and basics of lipid oxidation. **(A)** Diagrammatic timeline of research into lipid oxidation identifying key discoveries and concepts. **(B)** Major site sites of attack in phospholipids and types of reaction that can occur there, using 1-(1-octadecenyl)-2-arachidonoyl-sn-glycero-3-phosphoethanolamine as an example. Other phospholipids containing these or analogous chemical groups show similar susceptibility.

These enzymatically generated non-esterified lipid products were recognized as important signalling molecules in the cardiovascular and immune systems, and therefore as important therapeutic targets (6). Consequently, there was much interest in their enzymatic production by cyclooxygenases, lipoxygenases, and cytochrome P450-dependent enzymes (7), a topic that continues to be of interest and is reviewed elsewhere in this issue. Later, the non-enzymatic formation of analogous compounds (F_2-isoprostanes) was discovered (8) and, in parallel, evidence began to emerge that non-enzymatic oxidation products of fatty acids esterified in phospholipids also had biological activities (9). While initial studies reported detrimental effects in atherosclerosis, soon it was noted that some of these compounds were able to block immune receptors and prevent damaging immune responses, e.g. in sepsis (10). The years from 2000 onwards witnessed an explosion in the identification of non-enzymatic lipid modifications and resulting biological effects. A wide variety of additional oxidation product families were identified, including isolevuglandins, nitrated and halogenated fatty acids or phospholipids, oxysterols and halogenated sterols, as well as the discovery of resolvins (11) and maresins (12) from oxidation products of the omega-3 fatty acids eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA). In most cases, a strong driver in their discovery has been the elucidation of biological signalling effects and, as the field has evolved, it has become clear that certain modified lipid species have beneficial effects in specific circumstances; in many cases, we also have an understanding of the mechanisms involved. Thus, oxidatively modified lipids are now well-established as mediators of biological processes (2, 13–16).

CHEMICAL PROPERTIES OF LIPIDS THAT ENABLE MODIFICATIONS

Lipids are a hugely diverse chemical group, but the lipid species most prevalent in biological systems, and especially in mammalian cells, are free fatty acids, ceramides, phospholipids (including phosphatidylglycerols and sphingomyelin), mono-, di- and tri-acylglycerols, and sterols. The lipid structure determines the nature and likelihood of oxidative modifications to it, but reactive oxidizing compounds also demonstrate different specificities (17). The chemical moieties most typically susceptible to oxidative attack and modifications are shown in

Figure 1B. In general, these are electron-dense regions of the molecules (double bonds), or ones where the bonds are polarized and can be broken with lower energy input.

The site of attack that leads to the widest range of modifications and oxidation products is the fatty acyl chain. Although fully saturated hydrocarbon chains can be attacked by high energy oxidants, e.g. ozone and triplet oxygen, higher numbers of double bonds increase the susceptibility to radical attack, as hydrogen atoms can more easily be abstracted from bis-allylic carbon atoms (17). On the other hand, mono-unsaturated fatty acyl chains react readily with non-radical oxidants, such as hypochlorous acid (18). In sphingomyelins, the sphingosine moiety appears to be the main site of modification, at least by hydroxyl radicals, reflecting the presence of a C-C double bond (19). Likewise, in cholesterol the mono-unsaturated B ring is readily oxidized, although enzymatic oxidation of the tail also occurs (20–22).

In phospholipids, fatty acyl chains are connected to the glycerol backbone by 3 different types of bond: ester bonds, ether bonds (in alkanyl phospholipids), or vinyl ether bonds (in alkenyl phospholipids, also called plasmalogens). The ether or vinyl ether bonds occur mostly commonly at the SN-1 position of the glycerol. The ester bonds are most common biologically and can be hydrolyzed enzymatically, for example by phospholipase A_1 or A_2, which results in formation of lysophospholipids. These have altered biological properties and can be considered as biological mediators. In contrast, vinyl ether bonds are susceptible to attack by radicals (23) and electrophilic oxidants (24), forming oxidant-dependent products. Phospholipid headgroups containing an amine group can also undergo oxidation, although the quaternary ammonium structure of phosphocholine is resistant; changes in headgroup structure are likely to impact significantly on the phospholipid function within the cell membrane (25, 26).

TYPES OF LIPID MODIFICATIONS

The variety of sites of modification in lipids present the basis for the large range of products that can be formed (27), but this is expanded by the type of oxidant that causes the modification and the stability or otherwise of the initial product. This aspect will be explored in the following sections to illustrate the potential for diversity in modified lipids. **Figure 2** provides an overview of the key types of products.

Peroxidation of Fatty Acyl Chains Caused by Free Radical Attack

Whether enzymatic or non-enzymatic lipid modification is considered, radical attack leads to the widest range of products, largely because of the unstable nature of the initial oxidation products, their potential for rearrangement, and subsequent breakdown or fragmentation. For hydrocarbon chains, radical attack involves the abstraction of a hydrogen to form a carbon-centered radical, and leads to formation of a peroxide by incorporation of molecular oxygen (28). The potential for rearrangement at carbon radical stage depends on the degree and nature of unsaturation in the local area; for example, whether it is a conjugated system.

Hydrogen abstraction at bis-allylic carbons is favored, although it can also occur at allylic sites. This makes polyunsaturated fatty acyl chains such as linoleate (1 bis-allylic carbon); linolenate acid (2 bis-allylic carbons), arachidonate (3 bis-allylic carbons), eicosapentenoate (4 bis-allylic carbons), and docosahexenoate (5 bis-allylic carbons) increasingly susceptible to peroxidation, which can occur at multiple sites (3, 17). As the extent of modification by oxygen increases, the complexity of the oxidation product set increases, and their stability decreases. The initial product is a peroxyl radical, which can either react intramolecularly to form an endoperoxide in which the molecule retains an unpaired electron, or it can abstract a hydrogen from an adjacent molecule to form a hydroperoxide, concomitantly initiating the chain reaction of lipid peroxidation.

Endoperoxide formation is central in the formation of a number of bioactive oxidized lipid families, including the isoprostanes (29). Rearrangement of the endoperoxide results in formation of 5-membered ring structures, such as cyclopentenone rings, which are present in isoprostanes and their enzymatic analogues prostaglandins (30). Alternatively, the hydrocarbon chain can be cleaved to form the highly reactive compounds isolevuglandins, which are di-aldehydes (31, 32). Similar reactions also result in formation of the lipid oxidation breakdown product malondialdehyde.

In contrast, the hydroperoxides are relatively stable, and can be detected in biological samples following organic extraction and storage at -20°C or lower (33). Hydroperoxides can be reduced through the action of phospholipid-dependent glutathione peroxidase (GPx4), which converts the hydroperoxide to an alcohol (34, 35), although a mechanism for removing the –OH moiety to regenerate the hydrocarbon chain is not currently known. Hydroperoxides can also be converted to epoxides through homolytic cleavage of the hydroperoxide to form an alkoxyl radical, which attacks the adjacent carbon atom (36).

Either peroxyl radical, endoperoxides, or hydroperoxides can undergo intra-molecular reactions leading to the fragmentation of the carbon chain, which usually generates an aldehyde at one or both sides of the cleavage site. This process is responsible for the formation of a variety of lipid peroxidation breakdown products, of which the best-known example is 4-hydroxynonenal, in parallel with the corresponding chain-shortened phospholipid (37). These products can subsequently be metabolized by enzymes of the aldoketoreductase (AKR) and aldehyde dehydrogenase families, involving either reduction to alcohols or oxidation to carboxylic acids (38–40), thus generating further product diversity. An idea of the extent of the possible diversity can be obtained by considering that addition of two molecular oxygens to arachidonate can yield a family of 64 F_2-isoprostanes, when stereoisomers are included (41). Moreover, fragmentation of oxidized phospholipids can yield multiple breakdown products, and analysis is challenging as ones from different parent lipids may be isomeric or isobaric, as observed by liquid chromatography tandem mass spectrometry (42).

Analogous reactions can also take place on cholesterol and sphingolipid chains. Radical oxidation of cholesterol yields a

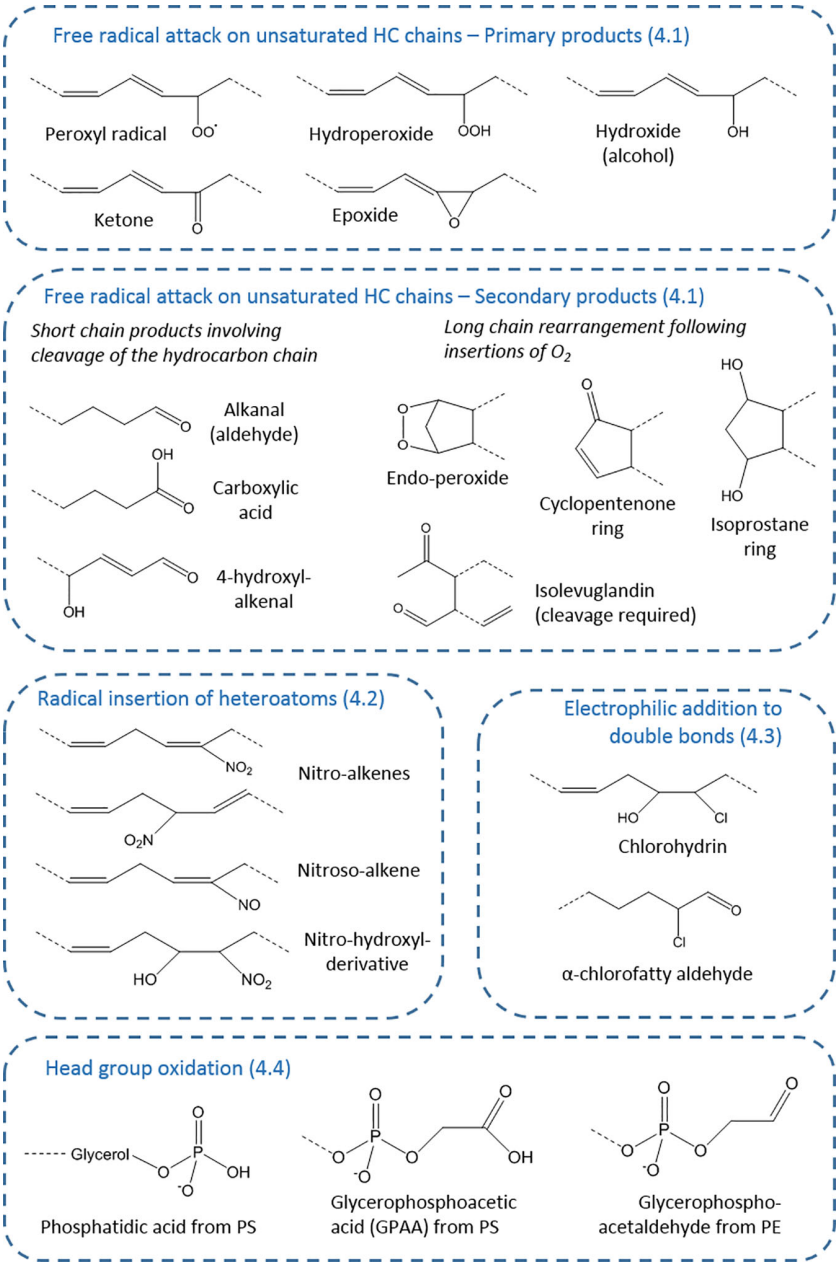

FIGURE 2 | Types of oxidative modifications on fatty acyl chains. The products are organized according to section of the article (numbered), showing the wide variety of chemical structures possible. These chemical moieties can occur on esterified or non-esterified fatty acyl chains, or cholesterol, and for each generic structure many distinct compounds (isomers and stereoisomers) may exist—for example, 64 in the case of isoprostanes—as well as analogous compounds from starting lipids with different chain length and unsaturation.

family of oxysterols modified on the B-ring, including 7-hydroxycholesterol, 7-keto cholesterol, 5-hydroperoxycholesterol, 5,6-epoxycholesterol and cholestane-3,5,6- triol (43). In contrast, enzymatic oxidation catalyzed by cytochrome P450 enzymes (e.g. CYP27A1, CYP46A1) tends to hydroxylate the saturated hydrocarbon tail, although 7a-hydroxycholesterol is formed by CYP7A1 (20). Carotenoids contain conjugated polyunsaturated chains and are also highly susceptible to oxidative attack; carotene oxidation products reported include cyclized hydroxy- and keto-containing as well as aldehydes resulting from chain cleavage (44, 45). Oxidation of sphingosylphosphorylcholine has been observed to form hydroxyl and keto derivatives on the sphingosine chain (19).

The ability of radicals to initiate hydrogen abstraction varies. Hydroxyl radical ($OH^{\bullet}$) is one of the most reactive radicals formed in biological systems, and readily causes lipid

peroxidation (46). In contrast, superoxide, a radical produced by certain NADPH oxidases, is relatively poor at initiating lipid peroxidation, and hydrogen peroxide is unable to do this in the absence of transition metal ions that support Fenton chemistry to generate hydroxyl radicals (17); transition metals such as copper, manganese and iron readily undergo one-electron (radical) reactions. Similarly, the non-radical anion peroxynitrite ($ONOO^-$) does not cause hydrogen abstraction directly, although it is reactive and can be converted to nitrogen-containing radicals such as nitrogen dioxide that do, and also reacts with carbon dioxide to form carbonate radicals ($CO_3^{\bullet-}$) that enhance peroxidation (47). Although a radical, nitric oxide (NO) is a better reductant than oxidant in biological systems (48). Radical nitrogen species can also be generated by the neutrophil enzyme myeloperoxidase; as well as its conventional non-radical product hypochlorous acid, it is able to oxidize nitric oxide to form the radical NO_2, and can also oxidize other compounds, for example tyrosine to yield tyrosyl radicals (49, 50). Oxygen itself is a di-radical and can initiate the direct peroxidation of dry lipid monolayers *in vitro* (auto-oxidation), but this process may not be biologically relevant as the oxygen concentration in cell membranes is much lower than air.

Modification of Fatty Acyl Chains by Radical Nitrogen Species

As well causing peroxidation, reactive nitrogen radicals can also cause nitration and nitroxidation of unsaturated fatty acyl chains, and the resulting nitrated lipids have important biological functions, for example as anti-inflammatory agents and stress signaling molecules in both animals and plants (51–53). The formation of nitrogen-containing oxidized lipid derivatives was first documented in the mid-1990s (54) and was rapidly followed by further mechanistic studies of nitration reactions (55). Radical-initiated nitration can occur by two distinct mechanisms. The first requires hydrogen abstraction by a radical followed by addition of NO_2 at the carbon-centred radical, in a mechanism analogous to lipid peroxidation. Under acidic conditions, peroxynitrite is converted to peroxynitrous acid (ONOOH), which decomposes to form $OH^\bullet$ and NO_2; thus hydroxyl radical initiates the hydrogen abstraction followed by addition of NO_2 to nitrate the hydrocarbon chain, forming a nitro-lipid (51, 56). The radical $NO^\bullet$ could also undergo a radical condensation with the carbon-centred radical, which would result in lipid nitrosylation. NO_2 can also react directly with one of the carbons in the double bond to form a nitroalkane radical, and if the NO_2 concentration is high a second nitration can occur to yield a di-nitro species. Subsequent loss of nitrous acid (HNO_2) leads to nitro-alkenes, and substitution with water can form nitrohydroxy lipids (51). As with oxidation products resulting from free radical attack, the molecular rearrangements of nitro-lipids allow a wide variety of positional and stereochemical isomers to be formed, for example on phosphatidylserine (57), cardiolipin (58), phosphatidylcholine, and phosphatidylethanolamine (59). Nitrated fatty acids have been detected in human plasma, suggesting that they are biologically relevant lipid products (60). Nitration of unsaturated fatty acids can also occur by non-radical electrophilic substitutions, as described in the following section.

Electrophilic Attack by Non-Radical Species

Unsaturated fatty acids and fatty acyl chains of phospholipids can be oxidatively modified in a non-radical manner *via* electrophilic addition of oxidants to double bonds. For example, addition of the reactive nitronium ion (NO_2^+), usually from a polarized nitronium carrier such as nitronium hexafluorophosphate, generates nitroalkenes (51), although it is not clear that such a mechanism is biologically relevant. In contrast, electrophilic addition of hypohalous acids to unsaturated lipids is better established, with more evidence for its occurrence *in vivo*. Hypohalous acids include hypochlorous acid (HOCl), hypobromous acid (HOBr), and hypoiodous acid (HOI) and are produced mainly by phagocytes (18). The main source of HOCl is the neutrophil enzyme myeloperoxidase; this enzyme has a higher Km for bromide than chloride, but the higher biological chloride levels mean that HOCl is the major product (61, 62). Eosinophil peroxidase is a related enzyme that is highly selective for HOBr production (61).

Hypohalous acids can add across double bonds in unsaturated fatty acyl chains to form halohydrins: the products on mono-unsaturated chains (e.g. mono-chlorohydrins) are fairly stable, but reaction with poly-unsaturated chains leads to a large number of products through rearrangement by loss of water or loss of chlorine, with the possibility of further reactions in the presence of high concentrations of HOCl (7). Chlorohydrins of fatty acids (adjacent hydroxy and chloro groups) have been detected in clinical conditions such as acute pancreatitis and sepsis (63, 64). Hypohalites can also attack vinyl ether bonds in plasmalogen phosholipids, which causes cleavage to form a lysophospholipid and releases an α-halo-fatty aldehyde (24, 65). This contrasts with radical attack of plasmalogens, which yields fatty aldehydes (23). It has most commonly been reported for HOCl, and α-chloro hexadecanal and α-chloro octadecanal have been detected in plasma of patients with cardiovascular disease (66) and sepsis (67, 68), but bromo-fatty aldehydes can also be formed (69). HOCl can react with the double bond in cholesterol to form 5-chloro-6-hydroxy-cholesterol and its isomer (70); these were reported in cell membranes (71) and subsequently myeloperoxidase-derived chlorine was reported to form a family of chlorinated sterols (72). HOCl can react with β-carotene and shows overlap in the products formed by free radical cleavage (73). Thus although the variety of halogenated products is less than that from radical oxidation, it still adds substantially to the modified lipid family.

Modifications of Phospholipid Headgroups

Although attention tends to focus on hydrocarbon chain oxidation, amine-containing phospholipid head groups can be attacked both by radicals and electrophilic oxidants. The photooxidation of phosphatidylethanolamines (PE) has been demonstrated to cause loss of ethanolamine to form

phosphatidic acid; interestingly, glycation by reaction with the amine enhanced the propensity for oxidation and led to oxidative cleavages in the glucose unit (**Figure 2**) (74, 75). The ethanolamine head group can also be modified by reaction with isolevuglandins (76), illustrating the complexity of effects of phospholipid oxidation, and such products have been detected in cells (77). Radical oxidation of phosphatidylserine (PS) typically yields glycero-3-phosphoacetic acid (GPAA) *via* oxidative deamination (78, 79), whereas glycero-3-phosphoacetaldehyde and glycero-3-phosphonitrile were observed following reaction with HOCl (80). These modifications are important as the head groups play key roles in membrane structure and function, as well as cell signaling.

DISCUSSION

It is clear that oxidative modifications of lipids are legion, resulting a substantial expansion in the variety and properties of lipids. Many of the oxidized, nitrated, and chlorinated products show altered biological activities, including toxicity, altered proliferation, differentiation, pro-inflammatory, anti-inflammatory and barrier protective effects, *via* diverse signalling pathways to affect gene expression or other regulatory processes. In this sense, the modifications offer a chemical/biochemical mechanism to alter cell behaviour in both beneficial and deleterious ways, and to some extent meet the concept of an epilipidome. There is a close analogy to the recent shift in thinking on "reactive oxygen species (ROS)" as potentially beneficial signalling compounds, rather than agents of destruction (81, 82). On the other hand, the modifications underlying epigenetics are reversible and enzyme-catalyzed, offering clear evidence that they are a regulatory process. The recent concept of epi-proteomics also depends on the principle of reversibility: many post-translational modifications are enzymatically controlled and reversible, e.g. phosphorylation, and histone acetylation (83, 84). In contrast, the same cannot be said of lipid oxidation. While some enzymes are specific for lipid oxidation products, such as GPx4, aldoketo reductases and aldehydes dehydrogenases, these constitute metabolism rather than direct reversibility. On this basis, the epilipidome would function in the sense of a metabolic loop, involving formation and degradation *via* distinct pathways. It is also worth bearing in mind that reactive lipid oxidation products exert at least some of their effects *via* covalent interactions with proteins in the form of post-translational modification known as lipoxidation, and these reactions are chemically reversible (85). In view of the wide variety of cellular effects reported for modified lipids, as well as its role in ferroptosis (86, 87) and inflammatory diseases (88), it is important to continue to explore their potential as an epilipidome, including aspects of reversibility and enzyme interaction. This will require development of new technologies to handle the large datasets of modified lipids that form the epilipidome (89).

AUTHOR CONTRIBUTIONS

CMS is the sole author and therefore responsible for all aspects of this article.

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The Skin Epilipidome in Stress, Aging, and Inflammation

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Lipids are highly diverse biomolecules crucial for the formation and function of cellular membranes, for metabolism, and for cellular signaling. In the mammalian skin, lipids additionally serve for the formation of the epidermal barrier and as surface lipids, together regulating permeability, physical properties, acidification and the antimicrobial defense. Recent advances in accuracy and specificity of mass spectrometry have allowed studying enzymatic and non-enzymatic modifications of lipids—the epilipidome—multiplying the known diversity of molecules in this class. As the skin is an organ that is frequently exposed to oxidative-, chemical- and thermal stress, and to injury and inflammation, it is an ideal organ to study epilipidome dynamics, their causes, and their biological consequences. Recent studies uncover loss or gain in biological function resulting from either specific modifications or the sum of the modifications of lipids. These studies suggest an important role for the epilipidome in stress responses and immune regulation in the skin. In this minireview we provide a short survey of the recent developments on causes and consequences of epilipidomic changes in the skin or in cell types that reside in the skin.

Keywords: skin, ultraviolet, inflammation, stress, oxidized phospholipid, epilipidome, aging, senescence

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INTRODUCTION

The lipidome of keratinocytes (KC), the dominant cell type of the basal layer of the epidermis is made up mainly of phospholipids, cholesterol, and triacylglycerides. Differentiation of living KC into dead corneocytes, a controlled cell death process that continuously renews the epidermal barrier (1), drastically changes the KC's lipid composition several times during the process. The last living (granular) epidermal layer contains cells with lamellar bodies containing glucosylceramides, phospholipids, and sphingomyelin which are further metabolized to produce the stratum corneum (SC) lipids, a mixture of free fatty acids (FFAs), cholesterol and ceramides (2, 3). The SC lipids form the lipid matrix, a flexible connection of low water permeability between the corneocytes which remain from terminal differentiation (4) and the FFAs contribute to the required acidification of the SC (5). Part of the surface lipids derive from the sebum, a mixture of TAG, wax esters, squalene and FFA, produced by holocrine secretion of terminally differentiating cells of the sebaceous gland, a lipid producing skin appendage. Most biological consequences of epilipidomic modification take

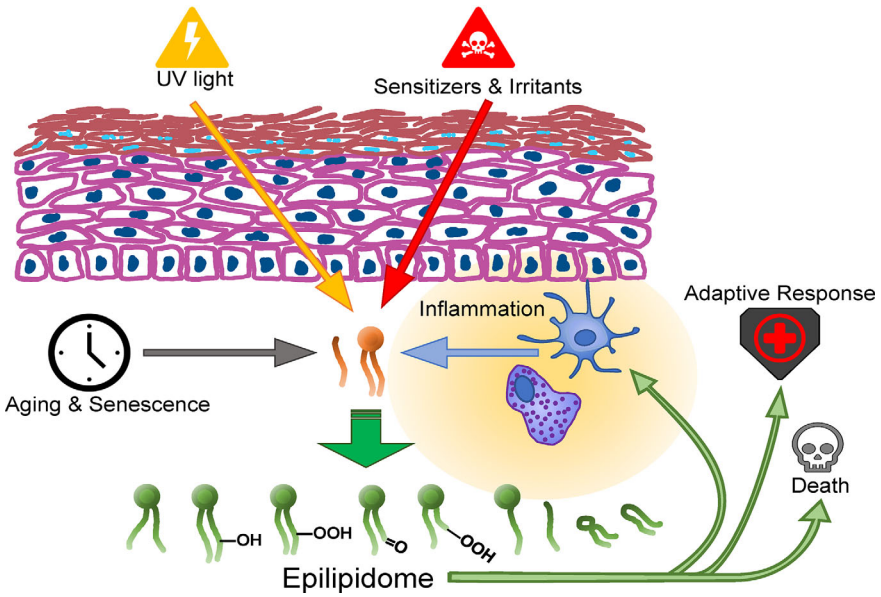

FIGURE 1 | Formation routes and action spectrum of modified lipids in the skin.

place in the living layers of the epidermis or in the dermal compartment underneath; nonetheless SC lipids are susceptible to modifications. Some of these modifications are ROS-mediated (squalene oxidation), while others depend on enzymatic cascades, as for example in the formation of the lipid envelope where hydroxyl ceramides are esterified to corneocyte proteins by specific transglutaminases.

Modification of The Skin Epilipidome by Ultraviolet Radiation

The best-studied oxidative modifier of skin lipids is solar radiation and wavelength bands thereof, which are used alone or in combination with photoactive chemicals as therapy for various skin diseases. The action of UV radiation (UVR) on human skin depends on wavelength and can induce acute inflammation-, immunosuppression, or cell death (6). The latter is elicited by combining UVR with photoactive drugs to specifically target cancer- or immune system cells. UVR can cause both enzymatic and non-enzymatic modification of lipids. The long-wavelength UVA (320–400 nm) oxidizes lipids in absence of enzymes (7, 8) but also shorter wavelength radiation can non-enzymatically generate oxidized lipids *via* free radical mechanisms (9). Cholesterol, phospholipids, free fatty acids, and squalene are targets for non-enzymatic lipid oxidation and yield bioactive products. Enzymatic synthesis of oxidized lipids, most prominently eicosanoids and related oxidized polyunsaturated fatty acids (PUFAs) results from UV activation of phospholipases, lipoxygenases and cyclooxygenases (10–12). Most of the work on enzymatic generation of eicosanoids [rev. in (10)] has been done on the response to clinically relevant short wavelength UVB irradiation. This may lead to an underestimation of non-enzymatic effects to solar UV exposure which are mostly elicited by longer wavelength radiation. Similarly biasing may be that UV-regulated eicosanoids (and related FA derived mediators) are investigated mainly in their free form, while a large fraction of the modified FA may be presently attached to more complex lipids.

Previously it was observed that the UVA-photo-oxidation of PUFA esterified to phospholipids is more efficient than photo-oxidation of the same PUFA in the free form, probably due to increased UVA induced singlet oxygen generation in the PL esterified configuration of the PUFA (13). Indeed, Leung et al. found in HaCaT cells exposed to UVA little effect on n-6 PUFA and their non-enzymatic oxidation products immediately after exposure (14) but detected elevation of enzymatically modified hydroxides of docosahexaenoic acid (DHA). The authors conclude that HaCaT cells required 24 h to return to PUFA homeostasis.

In primary human dermal fibroblasts, our group identified more than 500 features corresponding in retention properties to polar and oxidized phosphatidylcholines (PCs) that were induced immediately after irradiation with UVA (15), and also in primary human keratinocytes we found significant elevation of 173 OxPC species immediately after irradiation. In both cell types, the elevated species comprised also non-enzymatic PUFA-PC oxidation products such as PC-hydroperoxides and hydroxides, di-carboxylic and carbonyl group containing PC species. In the keratinocyte investigation we found that even at the high UVA-1 fluence of 40 J/cm^2 the cells recover, and most lipid species return to baseline levels within 24 h, insofar as the KC appear to limit especially the amount of highly reactive carbonyl containing lipids. The restoration of phospholipid redox (or epilipidome)

homeostasis involves the antioxidant response, autophagy, the unfolded protein response and, as recent findings suggest, the transcriptional regulator NUPR1 (16). Conversely, in vitro oxidized PUFA-PC are potent inducers of autophagy and Nrf2 (17, 18). These are mechanisms and signaling pathways that can be assigned to the protective, pro-resolving spectrum of oxidized phospholipid action. At the same time these lipid extracts or *in-vitro* oxidized PAPC preparations contain phospholipids with known pro-inflammatory activity and highly reactive carbonyl compounds (19, 20). A detailed investigation of the quantities of individual lipid species and localization of the lipids, their functional groups and their adducts will be next steps for elucidating the biological net effect of epilipidomic modifications on (phospho-) lipids through oxidative stressors in the skin. Elaborate mass spectrometric methods are required for structural analysis of aldehyde adducts to proteins [rev in (21)]. Because even as antibodies to protein-lipid adducts and the dinitrophenylhydrazine method to investigate protein carbonylation are widely used, lipid oxidation products and especially malondialdehyde can show not only high diversity in the type of modification of proteins (and thereby yielding very different epitopes) (22), but also interfere with the detection of other adducts (23).

The dietary intake of fatty acids affects the systemic and cutaneous composition of systemic free fatty acids and the composition of phospholipids to which these fatty acids are dynamically esterified. It also affects the potential enzymatic and non-enzymatic oxidation products that will form after UV exposure. Supplementation with eicosapentaenoic acid (EPA) and a subsequent UV exposure led to a shift in the UVA induced eicosanoids that were recovered from skin suction blisters from arachidonic acid metabolites (prostaglandin E2 and 12-HETE) towards EPA metabolites (prostaglandin E3 and 12-hydroxy-eicosapentaenoic acid, respectively) which have less pro-inflammatory activity (24). When administering docosahexaenoic acid (DHA) to cultured fibroblasts, we observed an elevation of DHA-containing phospholipids which were highly susceptible to photo-oxidation. Only in Nrf2 deficient cells this increased oxidation susceptibility led to increased expression of inflammation markers. Therefore, both the type of UV-induced lipid signaling mediator and the cell's capability to limit peroxidation may determine the epilipidomic effect on UV mediated inflammation regulation. UV not only can enzymatically generate immunomodulatory platelet activating factor (PAF), but PAF-like lipids can also result from free radical action on phospholipids. PAF and PAF-like lipids relay both acute inflammatory and delayed immunosuppressive UV effects, and potentially elicit systemic signals by releasing microvesicles from KC (25).

The effects of UV exposure are not restricted to cellular lipids. Also the sebum is susceptible to modification. Hydroperoxides of squalene generated by UV exposure have been identified *in vitro* and *in vivo* (26, 27), and as squalene is a major component of the epidermal surface lipids, its peroxidation products including also reactive aldehydes (28) were proposed as sensors conveying metabolic and inflammatory responses to UV radiation (29). One study even suggested that corneocyte dust containing high levels of oxidized squalene may be a relevant environmental irritant (30). The full spectrum of immunomodulatory actions of (UV-) oxidized squalene and other sebaceous lipids is discussed in (31), where the epidermal NLRP3 inflammasome is suggested as the cellular component that senses and relays inflammatory signaling.

An amplification of photo-damage is elicited by photosensitizers in photo(dynamic) therapy. Porphyrins and their derivatives have hydrophobic properties that locate them to membranes of target cells, allowing to kill those with light through photosensitized ROS generation. At the same time, this treatment leads to massive oxidation of (phospho) lipids (32), and it remains to be elucidated whether oxidized lipids interfere with- or contribute to the therapeutic efficacy. Lipotoxicity upon oxidative stress is mainly exerted by aldehydolipids and was reviewed in (33). In the skin context, the OxPL POVPC was toxic in melanocytes in the micromolar range (32), at which (34) we detected this lipid after exposure to physiologic fluences of UVA in other cell types (15).

THE SKIN EPILIPIDOME IN INFLAMMATION

The two major chronic inflammatory skin diseases associated with impaired barrier function, psoriasis and atopic dermatitis (AD), affect composition and ordering of the epidermal barrier lipids and composition of basal epidermal, dermal, and systemic lipids [reviewed in (10, 35, 36)]. Metabolites attributable to the epilipidome are regulated and likely contribute to the disease, but functional data are yet limited. 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HODE) were significantly elevated in plasma samples from psoriatic patients, as was 7-hydroxycholesterol. In skin biopsies from the same patients the free and esterified levels of 8- and 12-hydroxy-eicosatetraenoic acids (8- and 12 HETE) and 9- and 13-HODE were accordingly elevated, but also eicosanoids with known anti-inflammatory properties (37). First data where resolvin D1 was applied on patient KC and reduced interleukin synthesis by these cells indicate that small pro-resolving mediators of the epilipidome that are topically applied or generated *in situ* could be useful for the treatment of psoriasis (38). At the same time the pro-inflammatory components of the epilipidome likely contribute to the inflammation. Interestingly, a phospholipase that is transferred *via* exosomes to Langerhans cells seems to process psoriasis specific antigens (39). Thus, clear spatial localization of lipid metabolites, *e.g.* with high resolution mass spectrometric imaging and detailed functional studies are needed to fully understand the contribution of the epilipidome in psoriasis.

In the sera of juvenile AD patients, leukotriene B4 (LTB4), thromboxane 2 (TXB2), prostaglandins, HETE and HODE were found elevated, and lipidomic analysis could distinguish between clinically relevant subgroups of patients with high *versus* low immunoglobulin E levels (40). Among the distinguishing markers lysophosphatidyl-ethanolamine (18:2), thromboxane b 2 (TXB2), and 11-, 12-dihydroxyeicosatrienoic acid (DHET) can

be attributed to the epilipidome. TXB2 and 11, 12-DHET were found elevated in skin tissue lipid samples in a comparable study (41), that came to the conclusion that the ratio of pro-inflammatory to pro-resolution mediators was increased in the patients, especially PPARalpha agonistic oxidized lipids. These, especially 12-HETE mediate inflammation and disturb differentiation in AD organotypic skin models (42). Further research will elucidate the contribution of non-enzymatically formed isoforms or mimetics to the downstream signaling of these enzymatically generated mediators in skin inflammation. Agonism or signaling *via* prostaglandin receptors, PPARs, and pattern recognition receptors (PRR) through ROS mediated changes to lipids in other context has been reported (43–45).

MODIFICATIONS OF THE SKIN EPILIPIDOME BY EXPOSURE TO AGING, CHEMICAL IRRITANTS, DRUGS, AND OTHER STRESSORS

Highly reactive lipid oxidation products and their adducts to other macromolecules accumulate in the skin that prematurely aged due to sun exposure (46, 47). However, also chronologic aging of the skin at the cellular level and senescence of cells are similarly associated with lipoxidizing redox events, for example ROS accumulation in mitochondrial dysfunction and in senescence related chronic inflammation (48). The skin's cellular composition as well as the synthetic and metabolic fidelity changes during the mammalian lifespan, and these changes leave traces in the skin's lipidome and epilipidome. Those epilipidomic changes introduce a novel, autonomous layer of signaling for complex exposure–response relationships (49) in cellular stress, aging, and inflammation. Recently, elevated leukotriene generation was identified as a feature of senescent fibroblasts that promotes lung fibrosis (50), and we found compatible changes in the oxidized phospholipidome of senescent dermal fibroblasts (51).

The skin is exposed to temperature fluctuations, which likely affects the dynamics of enzymatic- and ROS-mediated epilipidomic modifications. One study monitored barrier lipids of acne and control patients over the course of a year, together with trans-epidermal water loss (TEWL) measurements and assessment of acne severity. The authors found that in acne-affected skin the ceramide species Cer[NH] and Cer[AH] were significantly reduced. This effect was greatest in winter and correlated with the highest TEWL measurements. Ceramide species with 18-carbon species of 6-hydroxysphingosine appeared to be most significantly reduced, an example of the diverse consequences that oxidative modification of lipids has in epidermal barrier function (52). Compatible with the latter finding, a (redox-) lipidomic study (53) on SC lipids from volunteers receiving glucocorticosteroids (GC) identified that the barrier damage, which is a side effect of GC therapy, was associated with reduction of ceramides with an esterified omega-hydroxy acyl chain. Furthermore, anti-cancer chemotherapy can affect the skin epilipidome, shown in a murine melanoma model, where chemotherapy generated, probably due to ROS generation, PAF-receptor agonistic lipids which negatively affected anti-tumor immunity (54). In murine epidermis exposed to the carcinogenic chemical irritant 12-O-tetradecanoylphorbol 13-acetate (TPA), we found strong epilipidome modification. Phospholipid hydroperoxides were elevated three days after the last treatment, and we found that peroxiredoxin 6 is an important regulator of epidermal lipid (per) oxidation *in vivo* (55). Cigarette smoke (CS) is a lifestyle-related environmental stress for the skin, and exposure of KC to CS increases the formation of carbonyl (4-hydroxy-2-nonenal; 4-HNE) adducts which likely result in part from lipid oxidation (56), and the immunosuppressive PAF-like lipids (57). A novel therapeutic option for dermatological wound- and inflammation management is the directed application of beams of cold atmospheric plasma (CAP) which contains highly dynamic matter, to tissue (58). One consequence when this treatment is applied to surface lipids is a massive change in the skin epilipidome (59), and it remains to be investigated whether epilipidomic changes contribute to the efficacy of the treatment which appears to involve activation of the antioxidant response (60).

Whereas most of the studies discussed so far have investigated the modification of fatty acid residues, Maciel and colleagues reported that the radical generating 2,20-azobis(2-amidinopropane) dihydrochloride (AAPH) modifies the headgroup of phosphatidylserines in cultured keratinocytes, adding an additional layer of complexity and novel potential biological consequences to the epilipidome (61). Beyond the oxygen-mediated modifications to lipids, the complexity of the epilipidome can be increased by sulfonation of lipids (62) nitration and nitroxidation of phospholipids, observed *in vivo* in diabetes models and under metabolic stress [Rev. in (63)] and several nitro- and nitroso modifications of unsaturated PC and PS have been characterized (64). Nitro fatty acids were also found in dermal fibroblasts upon virus infection and impaired interferon gamma signaling (65) by modulating the palmitoylation of the adaptor molecule stimulator of IFN genes (STING) which led to inhibition of interferon release, and the authors suggested the pharmacological potential of these lipids in diseases caused by abnormally high STING activity.

DISCUSSION AND OUTLOOK—CONNECTION OF THE EPILIPIDOME WITH OTHER NON-CANONICAL REGULATORS AND LOCALIZATION OF EPILIPIDOMIC MODIFICATIONS WITHIN THE SKIN

Although the importance of the epilipidome for the regulation of cellular processes is clearly evidenced (66), little is known about its interaction with other non-canonical regulators of cell fate ("epi-omics"), such as the epigenome, epitranscriptome, epiproteome or epimetabolome. As all of these "epi-omics" are influenced by oxidative stress, it is well conceivable that oxidized lipids further

exacerbate the effects of the original redox stressor. For example, 4-HNE is formed by lipid peroxidation and is highly reactive towards cysteine, lysine and histidine residues. Thereby, protein adducts are formed which do not only impinge on the epiproteome (67), but also on the epigenome through covalent modification of histones. Histones are common advanced lipoxidation endproducts (ALEs), and some of them are associated with human disorders, such as systemic lupus erythematosus or Alzheimer's disease (68). ALE formation impairs the interaction of histones with DNA and consequently leads to increased vulnerability of exposed DNA stretches to oxidative stress (69, 70). Similarly, chromatin reader, writer and eraser enzymes might be covalently modified by oxidized lipids and thereby their function might be altered. Besides histone acetylation, the epigenome is shaped by methyltransferases, adding methyl groups to bases of DNA. The metabolite S-adenosyl-methionine (SAM) might represent an important link between the different layers of "epi-omics", because it acts as the universal methyl group donor for most DNA, RNA, lipid, and protein methylation reactions. Phospholipid methylation is the major consumer of SAM and SAM availability in cells is limited. Thus, changes in the methylation of phospholipids strongly reflect on methylation reactions of other substrates. Ye and colleagues provided evidence for this phenomenon by demonstrating that loss of phospholipid methylation causes hypermethylation of histones as well as of the major phosphatase PP2A (71). In contrast to DNA methylation, chemical modifications of different RNA species came into focus only recently (72), and might be subject to similar redox- and metabolism-based connections with the epilipidome (73–75). Moreover, RNA modifications were already implicated in the interaction of specific RNA molecules with lipid bilayers (76). N6-adenosine methylation of ribosomal RNA (rRNA) by METL-5 represents an interesting example for a complex crosstalk between the different layers of "epi-omics" in *Caenorhabditis elegans*. Methylation of A_{1717} on 18S rRNA enhances selective ribosomal binding and translation of CYP-29A3 mRNA. This enzyme is required for oxidation of eicosapentaenoic acid to eicosanoids and modulates heat stress resistance (77). Oxidized lipids might also directly influence selective protein synthesis through oxidation of ribosomal proteins (78). Since the synthesis of post-translational protein modifications, such as glycosylations, is tightly synchronized with translation, the epiproteome might be regulated by the epilipidome as well.

The novel gold standard methods for redox- and other epilipidomic investigations are typically based on high resolution mass spectrometry (HRMS), often in combination with chromatographic separation and require intensive bioinformatic post-processing. These methods and their application on the lipidome, redoxlipidome and especially the skin are the topic of recent reviews that are suggested to the reader (35, 66, 79–85). The emerging technology of mass spectrometry-based imaging (MSI) has the unique feature to reveal the distribution of analytes within a tissue allowing the detection, localization and identification of multiple lipid species in an area of interest. Ionization techniques like secondary ion mass spectrometry (SIMS) (86), matrix assisted-laser desorption/ionization (MALDI) or desorption electrospray (DESI) (87) are the methods of choice allowing sensitive measurements. One tissue section can be used for consecutive measurements in positive and negative ion modes depending on the lipid class under investigation (88). However, low concentrations and ion suppression effects can lead to low ion intensities making lipid identification difficult. However, low signal intensities in respect to concentration levels of lipid peroxidation products or method-inherent ion suppression effects makes lipid identification by tandem MS often infeasible and HRMS (*i.e.* Fourier Transform Ion Cyclotron or Orbitrap) is indispensable. The novelty of MSI in the context of skin research is reflected by the limited number of publications available. Few papers focusing on sample preparation (89), few studies are available giving a general overview of lipid changes in skin during wound healing (90), in reconstructed skin equivalents (91) studying lipid profiles over time and in *ex vivo* human skin samples (92). Worth mentioning is research on the effect of topically applied compounds on lipid changes in the skin (93, 94). Despite the promising future of MS imaging, limitations have to be considered and challenges have to be met. One limitation is the rather low spatial resolution achieved with most instruments. (Nano)DESI provides spatial resolutions of approximately 40 to 100 μm, and conventional MALDI measurements can be carried out at pixel sizes down to 10 μm, still larger than most mammalian cells. As a result, each pixel represents the average lipid profile of maybe multiple cells and not of individual cells within the tissue. Reducing the spot size to a single cell level is therefore one of the most important endeavors in MSI research and instrument development (95). SIMS on the one hand has the potential to measure at a few nm spot size (approximately 30 nm), easily reaching cellular levels. However, SIMS is not a soft ionization technique, fragmenting lipid species and providing only lipid class information by head group analysis but not the full molecular information one is usually striving for. MALDI on the other hand is allowing the detection of intact lipid species at rather low resolution, being therefore the most often used method so far. But MALDI shows different ionization efficiencies for different lipid classes, making a comprehensive analysis for the entire lipidome a challenge, choosing the appropriate matrix is key (96). In summary, combining a multimodal approach at high spatial and mass resolution information on the skin's epilipidome with immunohistological features of individual cells, their activation- and differentiation state, their metabolic configuration and their (epi-) transcriptome will be an important task in the imminent future that will help elucidate the contribution of the epilipidome to skin biology (**Figure 1**).

AUTHOR CONTRIBUTIONS

FG, MD, and MS wrote the manuscript. CK provided visualization. All authors contributed to the article and approved the submitted version.

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Pain Control by Targeting Oxidized Phospholipids: Functions, Mechanisms, Perspectives

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Within the lipidome oxidized phospholipids (OxPL) form a class of chemically highly reactive metabolites. OxPL are acutely produced in inflamed tissue and act as endogenous, proalgesic (pain-inducing) metabolites. They excite sensory, nociceptive neurons by activating transient receptor potential ion channels, specifically TRPA1 and TRPV1. Under inflammatory conditions, OxPL-mediated receptor potentials even potentiate the action potential firing rate of nociceptors. Targeting OxPL with D-4F, an apolipoprotein A-I mimetic peptide or antibodies like E06, specifically binding oxidized headgroups of phospholipids, can be used to control acute, inflammatory pain syndromes, at least in rodents. With a focus on proalgesic specificities of OxPL, this article discusses, how targeting defined substances of the epilipidome can contribute to mechanism-based therapies against primary and secondary chronic inflammatory or possibly also neuropathic pain.

Keywords: oxidized phospholipids, TRP channel, ion channel, analgesia, pain therapy, nociception, therapeutic antibody, mimetic peptide

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INTRODUCTION

Pain is the result of molecular and psycho-behavioural elements triggered by tissue damage (1). In developed countries, the estimated prevalence of chronic pain is about 20% (2, 3). In the new, eleventh international classification of diseases (ICD-11), primary chronic pain has been classified as an independent disease, like low back pain or fibromyalgia, and separated from syndromes where pain is secondary to another underlying disease, like in osteoarthritis or rheumatoid arthritis (4). To improve the quality of life, an effective pain treatment has major impact. Reports by the National Institutes of

Abbreviations: 4-HNE, 4-hydroxynonenal; 13-HODE, 13-hydroxy-10E,12Z-octadecadienoic acid; 9-HODE, 9-hydroxy-10E-,12Z-octadecadienoic acid; ApoA-I, Apolipoprotein A-I; D-4F, D-amino-4-phenylalanine mimetic peptide of Apolipoprotein A-I; E06, E06 monoclonal antibody; H_2O_2, Hydrogen peroxide; HDL, High density lipoprotein; LDL, Low density lipoprotein; LPC, Lysophosphatidycholin; OxLDL, Oxidized low density lipoprotein; OxPAPC, Oxidized 1-palmitoyl-2-arachidonyl-sn- glycero-3-phosphorylcholine; OxPL, Oxidized phospholipids; PEIPC, 1-palmitoyl-2-(5,6)-epoxyisoprostane E2-sn-glycero-3-phosphocholine; PGPC, 1-palmitoyl-2-glutaryl phosphatidylcholine; POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine; ROS, Reactive oxygen species; TLR-4, Toll-like receptor 4; TRPA1, Transient receptor potential ankyrin type 1; TRPC5, Transient receptor potential canonical type 5; TRPV1, Transient receptor potential vanilloid type 1.

Health indicate that opioids have been the most prescribed drugs for pain treatment in the US leading to the opioid overdose crisis uncovered in 2017/18. In addition, over the counter drugs like ibuprofen can provoke severe side effects such as gastrointestinal bleedings. Hence, a better understanding of pain-inducing mechanisms as well as the development of novel targets for acute and chronic inflammatory pain treatment is essential.

Pain can be initiated and maintained by a subpopulation of primary sensory neurons, the nociceptors, which have their cell bodies in dorsal root ganglia (5, 6). According to the *International Association for the Study of Pain* (IASP), nociceptors are defined as: 'A high-threshold sensory receptor of the peripheral somatosensory nervous system that is capable of transducing and encoding noxious stimuli' (7). Peripheral branches of these pseudounipolar dorsal root ganglion neurons sense physical and chemical stimuli. After passing the dorsal root ganglion, central branches transmit the sensory information to the spinal cord. Nociceptive dorsal root ganglion neurons mostly have small-diameter cell bodies and are primarily responsible for slow pain sensation evoked by noxious stimuli (6). Chronic pain often results from temporary to permanent changes in the signaling cascades responsible for nociception. This leads to prolonged and enhanced transmission of nociceptive signals from the periphery to the central nervous system. For instance, the local inflammatory environment can sensitize nociceptors, increase the spontaneous action potential firing rate, and facilitate the responsiveness to endogenous or exogenous, proalgesic irritants (8).

Recent research on lipids points toward its new role in pain signaling. Molecular components that act as pro- and analgesic factors, are found within the epilipidome. When looking at lipids in a hierarchical order (**Figure 1A**), compound lipids such as the ubiquitous phospholipids or glycerophospholipids, both critically important for integrity and function of all cellular membranes (9), are identified as upstream pain-inducing metabolites (10, 11). Phospholipids carry unsaturated fatty acids making them accessible for oxidation, nitration, and subsequent oxidative degradation. Chemical, non-enzymatic production of oxidized phospholipids (OxPL) leads to diverse biologically active OxPL species (proalgesic metabolites are indicated in **Figure 1B**). Besides non-enzymatic oxidation of phospholipids, enzymatic activity, for instance by lipoxygenases, also regulates OxPL abundance (9, 12, 13). Experimental evidence, mostly in preclinical rodent models, has corroborated the view that OxPL contribute to many diseases, including diverse pain syndromes, thus, making them attractive for a broad range of therapeutic approaches (**Figure 2**).

This review focuses on the biology of oxidized phospholipids (specifically in pain syndromes) and summarizes recent data in preclinical rodent pain models that show how targeting the

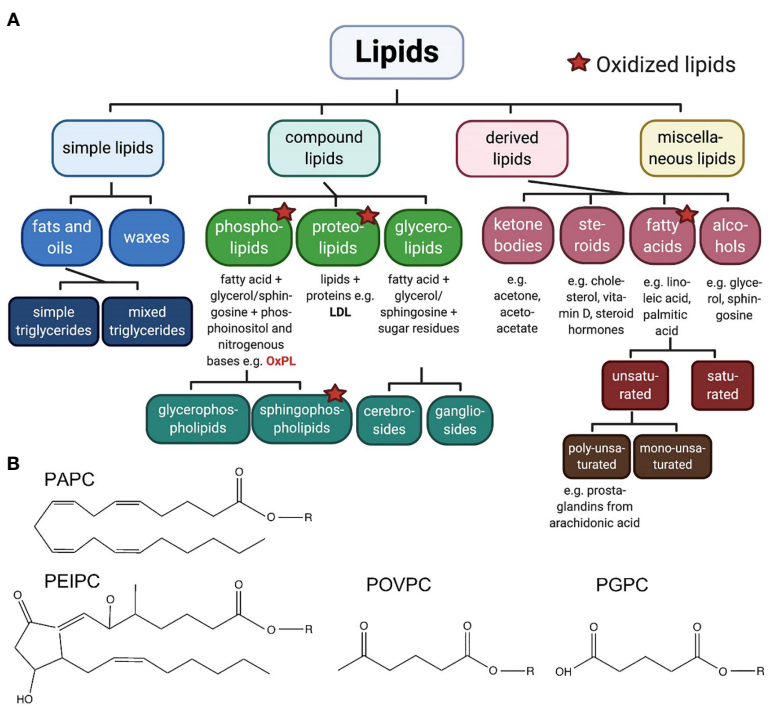

FIGURE 1 | **(A)** Classification of lipids. The large group of lipids can be divided in four groups with respective subgroups. Oxidized phospholipids, pain-inducing, natural metabolites, are discussed in this review. Created with biorender.com©. **(B)** Pain-related oxidized phospholipids. The unoxidized PAPC consists of a 1-palmitoyl-sn-glycero-3-phosphocholine backbone (R) and a linear, arachidonic tail of 20 carbon atoms including four double bonds. Oxidation of this phospholipid generates fragments such as POVPC and PGPC. In both molecules, the arachidonic tail is shortened to C5. Both molecules carry an aldehyde group or a carboxyl group, respectively. In addition, PEIPC is generated from PAPC by formation of a bond between C8 and C12, within the arachidonic tail, by reduction of two double bounds and additional oxygenation as well as radical formation.

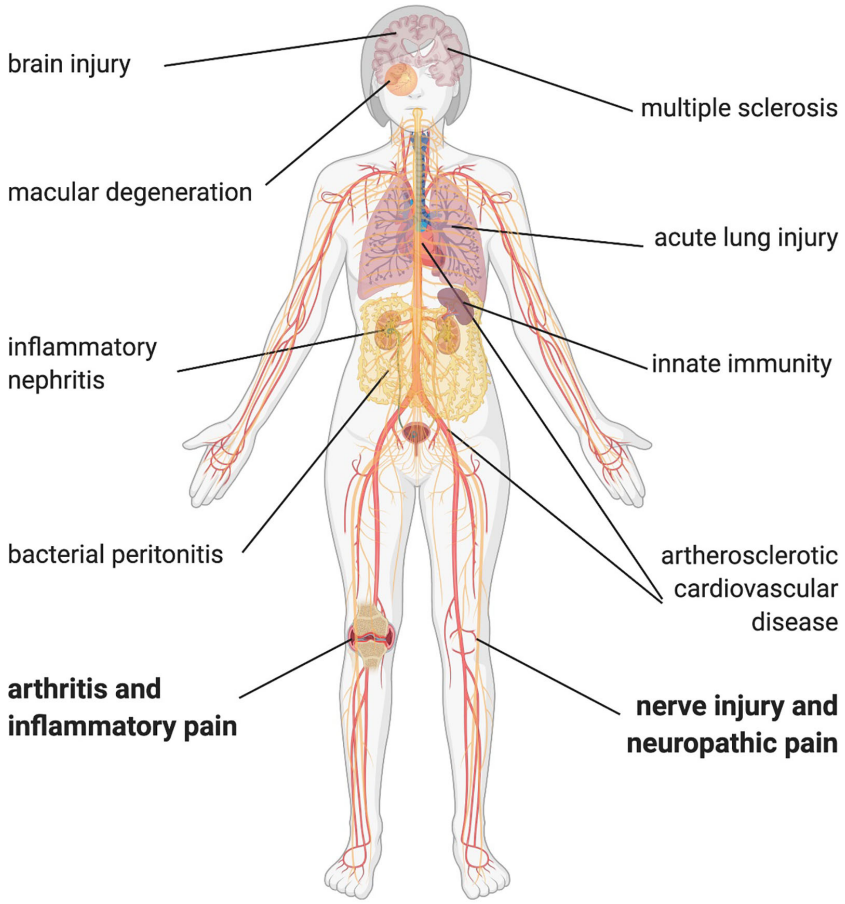

FIGURE 2 | OxPL contributing to disease pathophysiology. OxPL can be found in several tissue affected by inflammatory diseases throughout the body. Most of the evidence comes from preclinical models, but especially in atherosclerotic cardiovascular disease and multiple sclerosis, there is evidence of OxPL in human tissue. Created with biorender.com©.

biological activity of OxPL can control pain or can even contribute to natural pain resolution.

OXIDIZED PHOSPHOLIPIDS ARE LINKED TO INFLAMMATORY CONDITIONS AND PAIN

Research on inflammatory pain in the early years focused on stable biomolecules like prostaglandins and peptides/proteins such as cytokines which trigger the action potential firing of nociceptors (8). Recently, works by our group and others have identified OxPL as proalgesic compounds in preclinical pain models (10, 11, 14, 15). Mechanistically, the highly reactive, transient, endogenous irritants directly activate ion channels on nociceptive C-fiber neurons. This function is different to the sensitizing effects provoked by typical inflammatory mediators (10, 11). Ion channels, like transient receptor potential ankyrin 1 (TRPA1) or voltage-gated sodium channels like $Na_V1.9$, are exciting pharmacological targets for pain relief. Inhibiting ion channel function can stop effectively the transmission of nociceptive signals toward the central nervous system, devoid of central nervous system side effects. Therapeutic strategies against OxPL-mediated pain aim to reduce their direct excitatory function on nociceptors.

Acute and chronic inflammation can cause a variety of pain states. By affecting many different organs and the contribution to chronification of pain, inflammation is hindering pain resolution. Immune cells continuously produce reactive oxygen species (ROS), a source of highly reactive hydroxyl radicals. The reactions of ROS with phospholipids in plasma membranes and in lipoproteins lead to a continuous and even self-perpetuating production of OxPL (16). For instance, in inflammatory and neuropathic conditions such as arthritis or sciatic nerve axotomy, levels of a variety of reactive lipids rise in the plasma and in dorsal root ganglion neurons (17–19). Furthermore, levels of reactive lipids correlate positively with nocifensive behavior in mice. In endometriosis oxidized LDL (OxLDL) levels increase and subsequently occurring fragments such as prostaglandins evoke pain. In addition, OxLDL correlate with symptom severity in patients with fibromyalgia (20, 21). However, oxidized LDL levels remain comparable in patients with and without polyneuropathy due to type 2 diabetes (22). Notably,

OxPL levels also rise in many other diseases (13). These include classical inflammatory diseases like peritonitis, nephritis, lung injury and multiple sclerosis (**Figure 2**). Most of the evidence comes from preclinical and clinical studies in cardiovascular disease (12, 23, 24). Many other bioactive lipids, e.g., eicosanoids (prostaglandins, leukotrienes, resolvins; see **Figure 1**) also rise already during the early acute phase of inflammation. It is obvious that both OxPL and eicosanoids co-exist in acute pain. It might be that OxPL, when generated non-enzymatically, appear a little bit earlier than eicosanoids. As some eicosanoids such as prostaglandin E2 are pro-inflammatory and are able to sensitize nociceptors and OxPL-mediators (e.g., $Na_V1.9$), it might be beneficial to target non-enzymatic OxPL production as well as cyclooxygenases as early as possible.

OXPAPC, A MIXTURE OF PAIN-INDUCING OXPL METABOLITES

Oxidation of PAPC (1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine) generates long-chain and fragmented oxidized PAPC (OxPAPC) products. These include POVPC (1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine), PGPC (1-palmitoyl-2-glutaryl-sn-glycero-3-phospho-choline) and PEIPC (1-palmitoyl-2-5,6-epoxyisoprostane E2-sn-glycero-3-phosphatidyl-choline) (**Figure 1B**) (13). Commercially available OxPAPC is a mixture of molecules that is produced by air oxidation of synthetic PAPC. This OxPAPC-formulation serves as model substance for investigating OxPL functions in pain (10, 11).

OxPL can also be produced by enzymatic reactions, e.g., by lipoxygenases, cyclooxygenases, cytochrome P450 and cytochrome *c*. Enzymatic OxPL synthesis leads to a more precise formation of individual, often truncated, oxidized lipids (25). An enzymatic modification of PAPC has also been reported (26). How enzymatic production of phospholipids contributes to pain is not known yet, but it can well be that especially secreted lipoxygenases promote local, continuous and long-lasting OxPL production. From a mechanistic point of view, enzymatic OxPL production might even contribute to pain sensation in case of bacterial infections, because microbes such as Pseudomonas aeruginosa, a critical human pathogen responsible for many healthcare-associated infections, secrete lipoxygenases to produce OxPL (27).

OxPAPC as well as its corresponding metabolites excite nociceptors and pain behaviour in different models for inflammatory pain (10, 11, 14, 15, 28, 29). Specific OxPAPC components such as PGPC and POVPC as well as the OxPL degradation product lysophosphatidylcholine (LPC) are increased in chloroform-based lipid extracts from inflamed tissue by MALDI-TOF (10). Highly reactive, long-chain OxPL, such as PEIPC or POVPC, degrade to transient products or rearrange to more stable metabolites. These degradation products also show biological activity and include molecules such as LPC, the oxidation end product PGPC, the fragmented product 4-hydroxynonenal (4-HNE), or prostaglandin 15d-PGJ2 (10, 30). The ladder is a remote metabolite of OxPAPC. In addition to oxidative processes, enzymatic reactions, e.g., by phospholipase A2, facilitate the metabolization of OxPL species. The fast metabolization of the compounds produces new physiologically relevant, bioactive signaling molecules. Important is that many of the OxPL metabolites generated under inflammatory conditions can induce pain by acting as acute excitants.

OXPL-MEDIATED SIGNAL TRANSDUCTION

The transient receptor potential channels TRPA1 and TRPV1 are excitatory ion channels mediating OxPL effects (**Figure 3**). They are polymodal, ionotropic receptors detecting noxious stimuli including chemical (e.g., capsaicin) or physical stimuli (e.g., heat) (5). Both cation channels are highly expressed in sensory neurons. When activated, the channels mediate pain behaviour and promote inflammation by triggering the increased release of neurotransmitters, inflammatory mediators and neuropeptides (8). Behavioural studies in knock-out mice and *in vitro* experiments on cultured small-diameter dorsal root ganglion neurons revealed that TRPA1 is the main target of OxPAPC and its components PGPC and POVPC. Much higher concentrations of the OxPL compounds are needed to stimulate TRPV1 responses and the induced activation of TRPC5, another TRP channel involved in pain mediation, is only short-lasting (10, 11, 31).

When applied rapidly and locally to cultured small-diameter neurons the prototypical OxPL compound PGPC induces calcium spikes – an effect mediated by TRPA1, but not TRPV1 (29). This is not easy to understand but points to a different mechanism of how OxPL activate TRP channels. For instance, TRPA1 activation by OxPL *via* an interaction with cysteines in the TRP N-terminus requires an electrophilic substance, similar to activation by the mustard oil compound AITC or 4-HNE or electrophilic OxPAPC compounds (28, 32, 33). Electrophilic compounds such as 4-HNE covalently bind *via* Michael addition and Schiff base formation to histidine, lysine, and cysteine residues, a key mechanism of TRPA1 activation. However, in an OxPAPC-hTRPA1 peptide binding study, no adduct formation and direct thiol oxidation were detected, suggesting that OxPAPC act as two-electron oxidants. However, Kelch-like ECH-associated protein 1 (Keap1), the oxidative stress sensor, is activated by the same electrophilic compounds as TRPA1 including OxPAPC (34).

Due to its chemical properties, the prototypical OxPL PGPC, a stable but non-electrophilic, acid oxidation end-product of OxPAPC, must use a different mechanism to activate TRPA1 (29). PGPC and other oxidized phospholipids rapidly integrate into the lipid bilayer of plasma membranes and thereby modulate functional properties of lipids and proteins (35). At least some of the OxPL compounds or even other members of the epilipidome probably induce rearrangements of the plasma membrane to mechanically activate TRPA1 and maybe even TRPV1 (36–38). When co-expressed, TRPA1 function can be increased *via* TRPV1-induced calcium influx. Though, an interaction of TRPA1 and TRPV1 might contribute to the rather complex OxPAPC action (see below).

Fast calcium imaging and patch-clamp recording revealed additional relevant ion channels that are involved in the signal

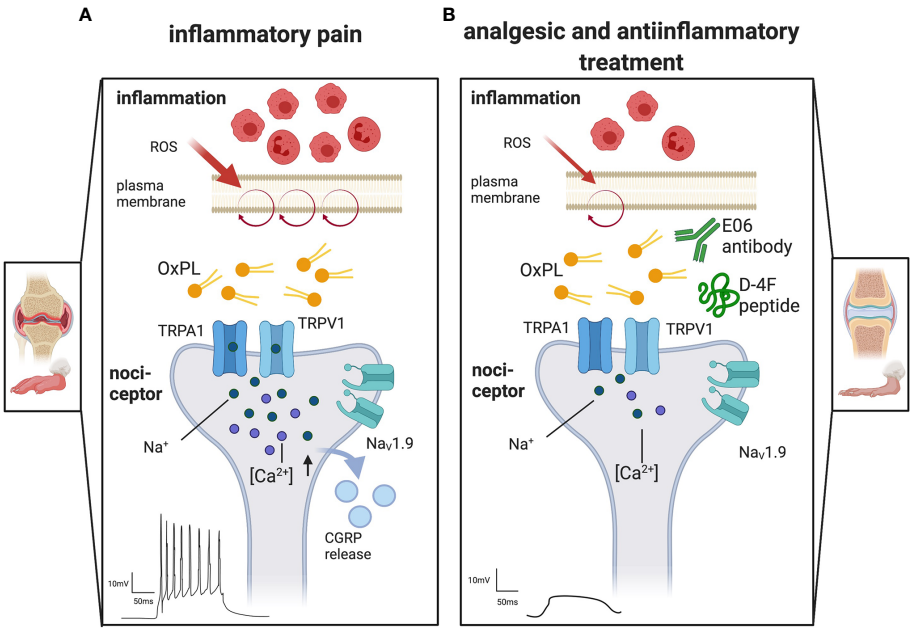

FIGURE 3 | The proalgesic role of oxidized phospholipids and therapeutic options to target OxPL. Display of representative terminals of nociceptors, the first neuron in the pain pathway, found in peripheral tissue. **(A)** Inflammation attracts immune cells invading the tissue, e.g., in arthritis. Especially neutrophils and macrophages release reactive oxygen species (ROS). They oxidize lipids like those found in the plasma membrane (purple circle). The resulting oxidized phospholipids (OxPL) activate non-selective, excitatory ion channels like transient receptor potential ankyrin 1 (TRPA1) or transient receptor potential vanilloid 1 (TRPV1). Activation of TRP channels leads to a subsequent activation of voltage gated sodium channels (Na_V) and the release of proalgesic molecules like calcitonin gene related peptides (CGRP). Action potentials are induced and propagated along the pain pathways, resulting in pain perception. **(B)** Treatment with monoclonal E06 antibodies or with the ApoA-I mimetic peptide D-4F scavenge OxPL and thereby reduce nociceptor firing, pain and inflammation. Created with biorender.com©.

transduction of OxPL stimuli (29). The unique low-threshold sodium channel $Na_V1.9$ is of outstanding importance. In nociceptors, $Na_V1.9$ is switched on under inflammatory conditions, by coincident signaling pathways (29, 39). When active, Nav1.9 potentiates acute OxPL responses and increases the excitability of neurons (29). This even induces calcium spikes in nociceptors that can be blocked with Ω -conotoxin, a high-affinity blocker of N-type voltage-gated calcium channels (29, 40). This is remarkable as N-type calcium channels mediate rather strong calcium influx signals. Activity-dependent calcium influx is often upstream of cellular plasticity effects such as activity-dependent axon growth (40) and is able to stimulate long-term changes in gene expression. It will be interesting to find out whether this calcium influx is involved in gene expression changes or axon sprouting upstream of nociceptor sensitization. Also, downstream, the stimulation of TRP channels with OxPAPC is sufficient to release calcitonin gene-related peptide (CGRP), a potent vasodilator and mediator of peripheral nociceptor sensitization. This indicates that OxPL are not only excitants, but also contribute indirectly to neurogenic inflammation (10, 15).

In summary, OxPL are nociceptor excitants and trigger signaling cascades that are able to induce long-term changes in nociceptors. Interrupting acute OxPL signaling might support pain resolution in transient and chronic inflammatory and possibly also neuropathic pain.

OxPL act locally and acutely on nociceptors. Biochemically, proalgesic OxPL are produced in all cellular membranes. It is likely that OxPL act autocrine as well paracrine. But, OxPL are also highly enriched in apolipoproteins. OxPL-rich apolipoproteins can also be found in the cerebrospinal fluid in the brain, spinal cord and dorsal root ganglion. Therefore, it is theoretically possible that OxPL contribute systemically to certain pain syndromes, maybe *via* a dysbalanced OxPL/apolipoprotein/cholesterol axis.

ADDITIONAL OXIDIZED LIPIDS INVOLVED IN PAIN PERCEPTION

Besides the long-chain OxPL, other lipid metabolites being generated in inflammation evoke nocifensive behaviour in rodents. For instance, fragmented products of OxPAPC such as eicosanoids and prostaglandins are downstream of phospholipase A2-mediated conversion of arachidonic acid. Oxidized metabolites of the arachidonic and linoleic acids increase upon systemic stimulation mice with nerve growth factor, a most important neurotrophic factor involved in pain signaling (41, 42). In sterile inflammation, evoked by physical burn as well as UVB-mediated sunburn, derivates of oxidized linoleic acid increase (43, 44). Lysophosphatidic acid 18:1 and lysophosphatidyl choline 16:0 and 18:1, additional metabolites deriving from phosphatidylcholine precursors, mediate neuropathic pain (45–48). In a mouse model of chronic inflammation,

oxidized linoleic acid-derived mediators increase in the central nervous system (49). The complex mode of actions in algesia are highlighted by modulation of small G-proteins such as GPR132 in addition to direct ion channel activation (18). These findings indicate that a variety of alterations of the lipid signaling cascade have deciphered promising approaches for future pain treatment.

TARGETING OXIDIZED PHOSPHOLIPIDS TO TREAT PAIN SYNDROMES

Advances in lipidomics give the opportunity to identify lipids that may serve as biomarkers or treatment targets. Based on the idea that proalgesic OxPL are preferentially produced locally and non-enzymatically, OxPL neutralization was thought to be a feasible strategy to target the proalgesic function of OxPL, at least in rodent pain models (10, 14, 15). Targeting enzymatic OxPL production with small molecule inhibitors, for instance oxazolone-derived compounds for lipoxygenase inhibition, is antinociceptive (50). It could well be that co-treatment with OxPL scavengers together with small molecule lipoxygenase blockers can synergistically even enhance the efficiency of pain control.

THERAPEUTIC EFFECTS OF MONOCLONAL E06 ANTIBODIES FOR PAIN TREATMENT

Oxidation of the phosphatidylcholine headgroup creates "neo"-self-antigens that are recognized by the acquired immune system. Distinct chemical structures and the conformation of lipids are indispensable for the formation of pattern recognising molecules such as antibodies. Anti-OxPL antibodies were originally discovered in preclinical models for atherosclerosis and pneumonia where they substantially increased under inflammatory conditions and (51, 52). One class of antibodies, the monoclonal E0 antibodies, were isolated from apoE-deficient mice, their eponym. Due to its high affinity to the oxidized head group of phosphatidylcholines, the monoclonal antibody (mAb) E06, also known as T15, got striking attention (53–55). Due to its unique properties, mAb E06 became an often-used tool to investigate OxPL abundance in various inflammatory diseases like acid-induced lung injury, arteriosclerosis, bacterial peritonitis, multiple sclerosis, inflammatory nephritis, and age-related macular degeneration (52, 56–60). Furthermore, the antibody was employed in immunohistochemistry or ELISA for OxPL binding studies. Notably, the mAb E06 also has therapeutic potential: passive immunization with E06 protects against atherosclerotic plaque development and inflammatory arthritis (61, 62).

In a model of inflammatory pain using complete Freund's adjuvant (CFA), an E06-based competitive binding assay allowed for quantification of OxPAPC species in freshly isolated tissue. These experiments revealed rather high amounts of mAb E06 reactive OxPL in inflamed tissue (15, 28). These observations raised the idea that the pro-inflammatory OxPL function could also be blocked by antigen neutralization with the mAb E06. Indeed, locally applied E06 allowed to control pain behaviour in preclinical pain models (15, 28). Intraplantar application of E06 even ameliorated hypersensitivity in CFA-induced hind paw inflammation and collagen-induced arthritis (10). Furthermore, E06, but not its isotype control IgM, reduced the levels of low mass metabolization products of OxPAPC, like lysophosphatidylcholine (LPC 16:0).

In order to nail down specific mediators interacting with E06, TRPA1 and TRPV1 ligands were tested. *In vitro*, E06 prevented 4-HNE, OxPAPC, AITC, but not capsaicin-induced calcium influx in HEK-293 cells expressing recombinant TRPA1 and TRPV1. Furthermore, E06 could also inhibit corresponding TRPA1 and TRPV1 responses in dorsal root ganglion neurons. Mechanical hypersensitivity induced by intraplantar OxPAPC, or irritants like AITC or 4-HNE, was also prevented by local E06 application (15, 28). This was not due to binding of 4-HNE itself, but rather the formation of E06-reactive OxPLs after local irritant injection (15, 54).

Recently, in a series of very elegant experiments, the single-chain, non-immunogenic variable fragment of E06 (E06-scFv) has been expressed in transgenic mice. These experiments showed a striking potential of E06 to ameliorate atherosclerosis, non-alcoholic steatohepatitis, and high fat-induced bone loss (63–65). In summary, E06 is an exciting tool to scavenge ROS-induced downstream mediators and is a promising therapeutic for inflammatory diseases and inflammatory pain.

D-4F, AN APOA-I MIMETIC PEPTIDE SHOWS ANALGESIC POTENTIAL

OxPL are typically transported by apolipoproteins, which can provide anti-atherogenic and anti-inflammatory properties. In order to mimic this function, apolipoprotein A-I (ApoA-I)-mimetic peptides have been developed. Especially the peptide D-4F, a small peptide of 2.3 kDa, shows considerable anti-inflammatory features *in vitro* and in animal models (66, 67). In addition, multiple doses of oral D-4F lower the HDL inflammatory index in high-risk coronary heart disease patients (68). D-4F has high affinity to OxPL – actually several magnitudes higher than ApoA-I. Apo-mimetic peptides, however, are not specific for OxPAPC: the binding capacity of D-4F to PGCP, POVPC, PEIPC and 1-(palmitoyl)-2-(5-keto-6-octene-dioyl) phosphatidylcholine (KOdiaA-PC) is substantially higher than for PAPC or other non-oxidized lipids, cholesterol, or oxidized lipids like 5(S)-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (HPETE). Hence, the peptide D-4F is a well-suited tool to scavenge and neutralize free OxPL (67). As OxPL are involved in many diseases (**Figure 2**) and due to the fact that D-4F mimic a natural and common mechanism, it was suggested that these peptides could have a rather broad therapeutic potential. However, albeit the basic concept is rather old, mimicking the beneficial role of HDL/Apo-A1 or OxPL scavenging with mimetic peptides has not reached the clinic yet.

D-4F blocks H_2O_2, 4-HNE, and OxPAPC, but not capsaicin- or AITC-induced calcium influx in TRPA1- or TRPV1-expressing HEK293 cells or dorsal root ganglion neurons (10, 14). Apart from OxPL scavenging, other mechanisms of function are discussed for D-4F. It may be that 4-HNE, a lipid peroxidation downstream

product, is the functional target of D-4F. 4-HNE could possibly bind to the lysine residues in D-4F and consecutively being inactivated (14). Indeed, in a murine asthma model, 4-HNE production was decreased after D-4F treatment. Alternatively, D-4F stimulates cholesterol efflux from cellular membranes, a mechanism altering lipid rafts and thereby nociceptors excitability (69, 70). Similar mechanisms have been observed in experiments with the ApoA-I binding protein which reduces lipid raft abundance *via* increased removal of excess cholesterol, thereby preventing allodynia in different preclinical models (71).

When D-4F is applied systemically, it inhibits capsaicin-, OxPAPC- and 4-HNE-evoked hypersensitivity in rodents (14, 28). In these experiments, D-4F was more effective against mechanical than thermal hypersensitivity. In preclinical models of inflammation like collagen-induced arthritis and CFA-induced hind paw inflammation, systemic D-4F reverses mechanical and thermal hypersensitivity as paw edema (14, 28).

In summary, D-4F is an analgesic substance ameliorating inflammatory pain. Whether it is beneficial in other painful diseases like traumatic nerve injury or chemotherapy-induced neuropathy needs further evaluation. Likewise, its exact mechanisms of function other than OxPAPC scavenging, e.g., interference with cholesterol metabolism like ABC transporters, and its potential in clinical trials have to be examined in the future.

CONCLUSION

This review highlights oxidized phospholipids as novel targets for the treatment of acute and prolonged inflammatory pain. New technological developments allow an in-depth classification of oxidized lipids within the epilipidome and, thereby, the identification of innovative biomarkers and druggable targets. Detection of undiscovered compounds by oxidized lipidomics provide new insights into pain-generating mechanisms. Research shows that endogenous OxPL, generated under inflammatory conditions, activate well-studied pain pathways like the TRPA1 signaling cascade. Deciphering their modes of action improve the development of pharmacological tools like the monoclonal antibody E06 and the ApoA-1 mimetic peptide D-4F as promising new therapeutic options. E06 antibodies as well as ApoA1 are physiologically present in our body. This may point to the body's own potential to handle pain and give a better understanding of the fundamental mechanisms of OxPL physiology. Insights might open new gates for pain management by strengthening natural mechanisms of pain resolution. Proven biological compatibility of D-4F in humans opens the possibility of local or systemic application of D-4F for patients with inflammatory pain, in the near future.

As our understanding of anti-OxPL therapy in pain has only been tested in preclinical rodent models in a few studies, mostly by a few research groups (10, 11, 14, 15, 29), a broad evaluation of the OxPL biology and anti-OxPL therapy by the science community is essentially needed.

AUTHOR CONTRIBUTIONS

BO drafted, wrote, and revised the manuscript. AB revised the manuscript. RB conceptualized, wrote, and revised the manuscript. HR created the graphics, wrote, and revised the review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Redox Epiphospholipidome in Programmed Cell Death Signaling: Catalytic Mechanisms and Regulation

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A huge diversification of phospholipids, forming the aqueous interfaces of all biomembranes, cannot be accommodated within a simple concept of their role as membrane building blocks. Indeed, a number of signaling functions of (phospho)lipid molecules has been discovered. Among these signaling lipids, a particular group of oxygenated polyunsaturated fatty acids (PUFA), so called lipid mediators, has been thoroughly investigated over several decades. This group includes oxygenated octadecanoids, eicosanoids, and docosanoids and includes several hundreds of individual species. Oxygenation of PUFA can occur when they are esterified into major classes of phospholipids. Initially, these events have been associated with non-specific oxidative injury of biomembranes. An alternative concept is that these post-synthetically oxidatively modified phospholipids and their adducts with proteins are a part of a redox epiphospholipidome that represents a rich and versatile language for intra- and inter-cellular communications. The redox epiphospholipidome may include hundreds of thousands of individual molecular species acting as meaningful biological signals. This review describes the signaling role of oxygenated phospholipids in programs of regulated cell death. Although phospholipid peroxidation has been associated with almost all known cell death programs, we chose to discuss enzymatic pathways activated during apoptosis and ferroptosis and leading to peroxidation of two phospholipid classes, cardiolipins (CLs) and phosphatidylethanolamines (PEs). This is based on the available LC-MS identification and quantitative information on the respective peroxidation products of CLs and PEs. We

focused on molecular mechanisms through which two proteins, a mitochondrial hemoprotein cytochrome *c* (cyt *c*), and non-heme Fe lipoxygenase (LOX), change their catalytic properties to fulfill new functions of generating oxygenated CL and PE species. Given the high selectivity and specificity of CL and PE peroxidation we argue that enzymatic reactions catalyzed by cyt *c*/CL complexes and 15-lipoxygenase/phosphatidylethanolamine binding protein 1 (15LOX/PEBP1) complexes dominate, at least during the initiation stage of peroxidation, in apoptosis and ferroptosis. We contrast cell-autonomous nature of CLox signaling in apoptosis correlating with its anti-inflammatory functions *vs.* non-cell-autonomous ferroptotic signaling facilitating pro-inflammatory (necro-inflammatory) responses. Finally, we propose that small molecule mechanism-based regulators of enzymatic phospholipid peroxidation may lead to highly specific anti-apoptotic and anti-ferroptotic therapeutic modalities.

Keywords: regulated cell death, apoptosis, ferroptosis, phospholipid peroxidation, redox lipidomics, cytochrome *c*, cardiolipin, lipoxygenase

The real reason for not committing suicide is because you always know how swell life gets again after the hell is over. ***Ernest Hemingway***

If you're going through hell, keep going. ***Winston Churchill***

SIGNALING BY POLYUNSATURATED LIPIDS: AUTOCRINE, PARACRINE, AND ENDOCRINE TYPES

Billions of years of evolution created and optimized mechanisms for efficient translation of genomic information into thousands of finely tuned protein machines (1) and perfected functional interactions between the proteins through sophisticated multi-leveled signaling systems (2). Lipids of biological membranes constitute a critical part of this complicated signaling network (3). The metabolic coordination requires that the flow of signaling information proceeds with optimized levels of fidelity and speed. Conservative estimates indicate that the number of proteins in the human proteome is on the order of 10^5-10^6 (4, 5). Thus it is not surprising that the diversity of signaling lipids coordinating multiple protein-protein and lipid-protein interactions within and between subcellular organelles, cells, and tissues may be even greater resulting in the possible $>10^6$ of individual molecular species in the lipidome. Engagement of membrane phospholipids (PLs) in the signaling process occurs *via* their biochemical modifications leading to the appearance of small amounts of "unusual" PL molecules such as their hydrolysis or peroxidation products (6). This review is focused on oxidatively modified (phospho)lipids as the signaling entities. Among them are well known lipid mediators represented by oxygenated free polyunsaturated fatty acids (PUFA) as well as oxygenated PUFA esterified into different classes of membrane phospholipids (PLs). The latter group will be the subject of the current review.

Cultural beliefs of successful societies have led to the common opinion that suicidal elimination is not the necessary way to resolve life conflicts that may encompass transient dark episodes within an otherwise bright present and even more wonderful future. This optimistic view has been expressed in many statements by politicians, writers and other artistic celebrities (including those by W. Churchill and E. Hemingway quoted above). On the molecular level, however, the ruthlessness of life/death elimination decisions is frequently a necessary attribute of the high fidelity of cell populations and their adaptive adjustments. The suicidal programs of cell death are genetically pre-determined and deciphering their specific mechanisms represents one of the emerging fields of cell biology. The lipid-derived signals may act within a given cell (autocrine signaling), affect cells within the surrounding neighborhood (paracrine signaling) or act on remote targets using the circulatory system for the transportation of death signals (endocrine signaling). Currently, more than a dozen regulated death programs have been identified in cells that accumulate excessive amounts of the (geno) toxic materials and hence are recognized by the surveillance machinery as irreparably damaged. It is believed, but not proven, that peroxidation of polyunsaturated lipids (PUFA-lipids) has been associated with the initiation and execution of many, if not all, of these programs (7). In spite of these general associations, neither the specific roles of peroxidized PLs in the fulfillment of the programs nor their chemical identity have been identified. Notable exceptions are apoptosis and ferroptosis, two programs for which the progress in redox lipidomics has resulted in the deciphering of death signals.

REDOX DEATH SIGNALS IN APOPTOSIS AND FERROPTOSIS

The structural core of biological membranes is formed by the bilayer of PLs—amphipathic molecules with long lipophilic

hydrocarbon chains and water-soluble polar heads. The hydrophobic/hydrophilic balance of phospholipids dictates the organization of the bilayer in which lipophilic chains interact with each other while the polar head-groups are localized at interface with the aqueous phase. In PLs, the chains represent fatty acids covalently attached to two sites of the glycerol backbone whereas the third position is occupied by phospho-base that may be a non-charged zwitter-ion (when the negative charge of the phosphate is compensated by a positive charge) or carry a negative charge (when an extra negatively charged group is present). Fatty acyls of PLs may have no double bonds (saturated) or contain one (mono-unsaturated) or several methylene-interrupted double bonds [polyunsaturated PLs (PUFA-PL)]. While unsaturated PLs may be biosynthesized both in anaerobic and aerobic conditions, the huge diversity of PUFA-PLs is characteristic for different domains of aerobic life (8).

One of the most popular concepts explaining the presence of diversified PUFA-PLs in the lipid bilayer relates to their function as a regulator of membrane fluidity necessary for the rapid diffusion and conformational flexibility of membrane proteins (9). In spite of its attractive simplicity, this concept does not explain the huge molecular variety of PUFA-PLs. Indeed, contemporary lipidomics detects 10^3–10^4 individual molecular species of major classes of phospholipids in cells and tissues. This is a conservative estimate of the species with differing masses. Indeed, with the two acyls/PL molecule and a menu of >30 commonly found fatty acids the number of possible isomeric species of "two-legged" PLs should be close to 10^3. However, for "three-legged" tri-glycerides this estimate would yield 10^4 species and for "four-legged" mitochondrial cardiolipins (CL) – $>10^5$ molecular species.

One of the prominent features of PUFA-PLs is their susceptibility to peroxidation *via* free radical mechanisms (10). These mechanisms may be comprised of enzymatic systems for the activation of oxygen and/or lipid substrates or occur non-enzymatically (see below). As the general schema of peroxidation includes abstraction of hydrogen from bis-allylic positions, PLs with multiple (four-six) double-bonds, are preferred substrates, particularly for non-enzymatic free radical reactions (10, 11). The primary product of the peroxidation process generates hydroperoxy-PLs (HOO-PLs). These products are not stable and readily undergo secondary decay reactions leading to a variety of electrophilic aldehydic-, keto-, hydroxy-derivatives as well as cyclic compounds (10). In terms of lipid diversification, this adds another order of magnitude to the possible number of individual phospholipids, thus bringing it to 10^5 species. It should be noted, however, that the measurable amounts of peroxidized PLs in healthy cells and tissues is markedly lower than their non-oxidized parent PLs (12). This is partly due to the fact that only a fraction of PUFA-PLs are involved in peroxidation and also to the high reactivity of the secondary electrophilic decay products toward nucleophilic sites in proteins (13). As a result, the life-time of these products in "free" form may be relatively short as they form lipid-protein adducts. However, the levels of these products may increase many-fold in conditions associated with cell injury and death. These reactive secondary intermediates of PUFA-PL peroxidation represent the proximate entities affecting functions of numerous proteins (14). Given that the formation of these adducts is, in a way, a reaction of protein "lipidation" that may dramatically change the distribution and functional characteristics of the affected proteins, it has been hypothesized, although not proven, that electrophilic products of PL peroxidation and their adducts with specific proteins represent the proximate "gateways" of cell's demise in regulated cell death programs.

TYPES OF REGULATED CELL DEATH: INVOLVEMENT OF LIPID PEROXIDATION AND POSSIBLE INVOLVEMENT OF PL-OOH

Since the time of the first detailed description of regulated cell death almost five decades ago, about a dozen different programs have been identified (15). The best described programs include apoptosis, necroptosis, pyroptosis, ferroptosis, entotic cell death, netotic cell death, parthanatos, lysosome-dependent cell death, autophagy-dependent cell death, alkaliptosis, and oxeiptosis (15). The majority of them have been qualified as responses to different types of stresses causing irreversible changes not only to one particular cell but also representing a high-risk threat to the entire community of surrounding cells. Among the different causative factors, oxidative stress has been universally identified as one of the leading mechanisms engaged early at the initiation or later during the execution stages of the death programs (15) (**Table 1**).

Given the vague definition of what exactly "oxidative stress" means, attempts have been made to connect the death programs with specific pathways and manifestations of the aberrant redox metabolism. Due to the high sensitivity of PUFA-PL to oxidative modifications, lipid peroxidation (LPO) has been considered as one of the common denominators of programmed cell death (57, 58). However, the specific role and mechanisms of LPO in the pathways leading to cell demise remain poorly defined. This is due, to a large extent, to difficulties in the analysis of highly diversified and very low abundance LPO products (12). These technological problems were resolved with the advent of high-resolution liquid-chromatography-mass spectrometry (LC-MS) based redox lipidomics (6, 12) with its capability to detect, identify and quantitatively characterize a variety of PL oxidation products. While the application of this technology may provide important information in any of the known death pathways, so far the significant results have been obtained mostly for two death programs—apoptosis and ferroptosis (16, 21, 34, 36).

Numerous studies of PL peroxidation in model chemical and biochemical systems using different initiating agents (e.g., azo-initiators of peroxyl radicals, Fe-ascorbate dependent generators of HO• radicals) demonstrated that the

TABLE 1 | Involvement of lipid peroxidation in the execution of regulated cell death programs.

Death type	Target	Stimuli	Lipid peroxidation	
			Implicated but not evidenced by LC/MS (References)	Implicated with evidence by LC/MS (References)
Apoptosis	Intrinsic pathway	STS; rotenone; ActD; Hyperoxia; NAO/light; g-IR; TBI; stretch		Kagan et al. (16); Tyurin et al. (17); Tyurina et al. (18, 19); Huang et al. (20); Mao et al. (21); Belikova et al. (22); Bayir et al. (23); Ji et al. (24)
	Extrinsic pathway	Anti-Fas	Jiang et al. (25); Serinkan et al. (26) (evidence by HPLC)	Wiernicki et al. (7)
Ferroptosis	System X_c^-	Erastin; IKE; sorafenib	Dixon et al. (27); Yang et al. (28); Larraufie et al. (29); Louandre et al. (30)	Gaschler et al. (31)
	GPX4	RSL3; with aferin A; $FINO_2$; ML162; Smoke/COPD; BAY-87-2243	Dixon et al. (27); Yang et al. (32); Basit et al. (33)	Kagan et al. (34); Doll et al. (35); Wenzel et al. (36); Kapralov et al. (37); Dar et al. (38); Hassannia et al. (39); Gaschler et al. (31); Yoshida et al. (40); Wiernicki et al. (7)
	Glutamate-cysteine ligase	BSO	Yang et al. (32)	
	Glutathione-S-transferase	Artesunate	Eling et al. (41); Lisewski et al. (42)	
	FSP1	FSP1ko/RSL3	Bersuker et al. (43); Doll et al. (44)	
	iNOS	iNOS kd/RSL3		Kapralov et al. (37)
	Iron oxidation	$FINO_2$		Gaschler et al. (31)
	Other	TBI; P. aeruginosa; viral infection; AKI; heart transplants	Matsushita et al. (45)	Kenny et al. (46); Wenzel et al. (36); Dar et al. (38); Li et al. (47)
Necroptosis	GSH depletion	Hemin; gallic acid Myocardial infarction (RIP3)	Laird et al. (48); Chung et al. (49) Ghardashi Afousi et al. (50)	
	GPX4	GPX4 ko	Canli et al. (51)	
		TNF-α		Wiernicki et al. (7)
Pyroptosis	Caspase-11	Gasdermin-D	Kang et al. (52); Chen et al. (53)	
	Other	HIRI; CI; LPS/ATP; sevoflurane	Zhang et al. (54); Liang et al. (55); Li et al. (56)	Wiernicki et al. (7)

γ-Irradiation, γ-IR; staurosporine, STS; actinomycin D, ActD; nonyl-acridine orange, NAO; acute kidney injury, AKI; buthionine sulfoximine, BSO; imidazole ketone erastin, IKE; traumatic brain injury, TBI; hepatic ischemia-reperfusion injury, HIRI; cerebral ischemia, CI.

susceptibility to oxidative modification is largely defined by the number of double bonds in the fatty acid residues (59). As a result, PLs with hexa-, penta-, and tetra-enoyl residues are the predominant peroxidation substrates as compared to doubly or triply unsaturated PLs. Notably, the nature of the polar part of the PL molecules was not influential as a factor determining vulnerability to oxidation. In sharp contrast, redox lipidomics studies of programmed death associated peroxidation has determined that there is a high selectivity of the process toward specific classes of PLs. Execution of the intrinsic apoptotic program revealed a high selectivity toward peroxidation of mitochondrial CLs whereby the species with C18:2 represented the major substrates (16, 21). Notably, CL molecular species containing more PUFA residues remained non-oxidized. Moreover, in hetero-acylated CL species, oxidation of C18:2 residues occurred preferentially even when C22:5 and C22:6 residues remained non-oxidized within the same molecule (**Figure 1**).

Ferroptosis-associated LPO was also highly selective toward a specific PL class—PUFA-containing phosphatidylethanolamines (PE) (34). Interestingly, two types of PE-molecular species with C20:4 and C22:4 displayed the highest sensitivity toward oxidative modification. In terms of positional specificity, the 15^{th} position in C20:4 and the 17^{th} position in C22:4 were the preferred oxidation sites. Importantly, these PE oxidation products exerted predictive features of ferroptosis biomarkers and displayed pro-ferroptotic activity upon co-incubations with target cells (34). A recent study demonstrated that not only di-acyl-phospholipids but also PUFA-plasmalogens (ether-phospholipids), synthesized in peroxisomes, underwent peroxidation in ferroptosis (60). Downregulation of ether phospholipids was associated with the increased resistance of cancer (carcinoma) cells to ferroptosis *in vivo*.

Selectivity and specificity of the PL peroxidation process in two different cases of apoptotic and ferroptotic (non-apoptotic) regulated cell death suggest a possible involvement of enzymatic catalytic mechanisms. Indeed, two different metalloproteins, a hemoprotein cytochrome *c* (cyt *c*) (**Figure 2A**) and a non-heme Fe-protein, 15-lipoxygenase (15LOX) (**Figure 2B**), have been identified as the highly likely enzymes initiating the peroxidation process in apoptosis and ferroptosis, respectively (16, 34). In both cases, the selectivity of the enzymatic peroxidation mechanisms is achieved due to the formation of lipid-protein or protein-protein complexes as described below.

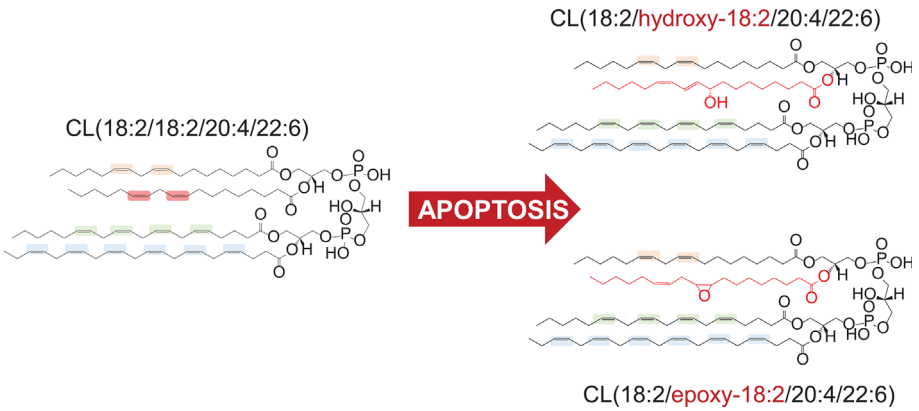

FIGURE 1 | Preferential peroxidation of C18:2 residue in hetero-acylated cardiolipin (CL) molecule in apoptosis.

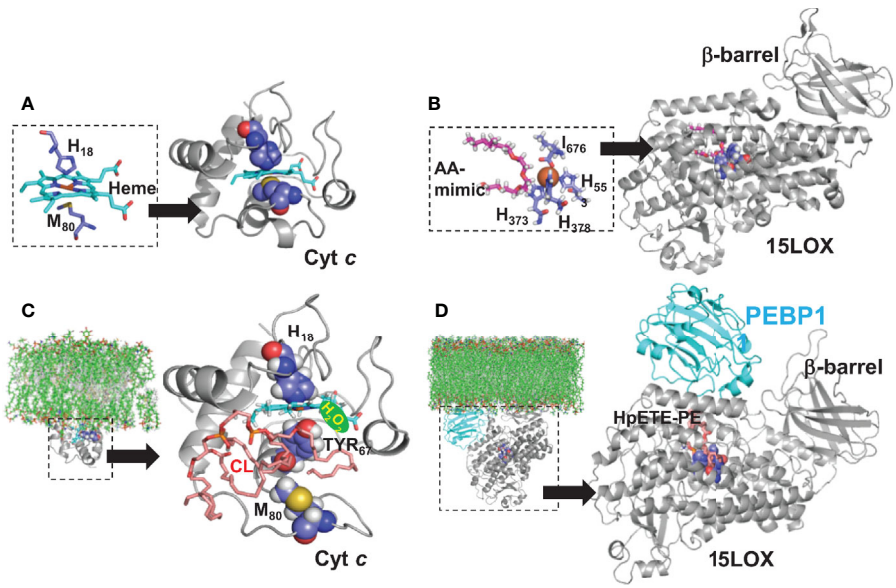

FIGURE 2 | Structural models of phospholipid peroxidizing Fe-proteins. **(A)** Structure of cytochrome *c* (pdb ID 1hrc) (61) shown in ribbon diagram, with the heme molecule (in cyan) and the Cyt C residues (H18 and M80) highlighted in space filling representation. The inset shows the coordination of the Heme molecule by the Cyt C residues, including H18 and M80. **(B)** Structure of 15LOX Ipdb ID 4nre) (62), also shown in ribbon diagram, with the arachidonic mimic (AA) shown in magenta, stick representation. The catalytic site region is shown in detain in the inset. The b-barrel, which interfaces with the membrane, is labelled. **(C)** Structure of cyt *c*-cardiolipin complex. The residue M80 which coordinates the heme has moved away from the Heme molecule, leading to an unfolded cyt *c* conformation. This unfolded conformation, which was obtained from an earlier study (16) was used to dock a cardiolipin molecule (shown in pink). **(D)** Structure of 15LOX/PEBP1-HpETE-PE complex. The model of the complex, proposed by us (36) is shown in ribbon diagram. The PEBP1 (shown in cyan) is docked onto the 15LOX, and this complex model, was used to dock HpETE-PE molecule (shown in pink). The ligand docking for both cyt *c* and 15LOX/PEBP1 complex was performed by SMINA (63). The insets in **(C, D)** depict the interfacing of peroxidase complex and membrane bilayer. The models for the protein-membrane complexes were built using the Orientation of Proteins in Membrane webserver (https://opm.phar.umich.edu/ppm_server) which calculates translational and rotational position of membranes and proteins from their three-dimensional structures.

ENZYMATIC AND NON-ENZYMATIC LIPID PEROXIDATION MECHANISMS; CATALYTIC ROLE OF IRON

PUFA residues of lipids are believed to be highly susceptible to oxidative modification by oxygen (10, 64). This process, LPO, proceeds *via* the formation of radical intermediates. The rate limiting stage is the initial formation of radicals that can further propagate the overall process. Therefore, the peroxidation rate is very low in the absence of catalysts. While there are many different radical initiators—physical factors like irradiation or chemical agents, like compounds spontaneously decomposing to

form carbon-centered radicals—the most important biological peroxidation catalysts are transition metals, particularly iron (Fe) (65). Therefore, the levels of redox active free Fe-ions or "loosely bound" Fe in low molecular weight complexes are strictly controlled in cells and biological fluids (**Figure 3A**). Catalytic Fe of active enzymes is regulated by the protein structure. Fe required for these catalytic functions is delivered to the respective protein clients—Fe-sulfur proteins, hemoproteins, and non-heme Fe-proteins—by several specialized protein chaperons (66). Quantitatively, hemoproteins represent the most abundant endogenous source of Fe and catabolic degradation of these proteins accompanied by the release of ferrous ions (Fe^{2+}) is operated by heme oxygenases (67). Specialized ferroxidases convert (Fe^{2+}) to (Fe^{3+})—the form suitable for the intracellular iron storage by ferritin (**Figure 3**).

Normally regulated Fe-catalyzed reactions are enzymatic. However, under special conditions such as Fe-overload, excessive catabolism of Fe-proteins or diseases, aberrant non-enzymatic Fe-driven lipid peroxidation by poorly controlled low molecular Fe-complexes may occur, becoming excessive or even overwhelming (68). These reactions commonly proceed *via* the production of reactive oxygen species (ROS), particularly HO• radicals, generated in Fenton/Haber-Weiss reactions or *via* the formation of lipid radicals generated during decomposition of organic hydro peroxides, including lipid hydroperoxides (69) (**Figure 3B**). As cell death programs are based on regulated mechanisms, the associated LPO is initiated by a selective and specific enzymatic process (**Figures 3C, D**). Interestingly, the enzymes involved in the production of lipid death signals *via* peroxidation mechanisms during apoptosis and ferroptosis—cyt *c* and 15LOX—usually are involved in different biological functions. For example, cyt *c* is shuttling electrons between mitochondrial respiratory complexes III and IV in the intermembrane space (70). 15LOX is a dioxygenase catalyzing the formation of oxygenated lipid mediators from free PUFA, particularly free arachidonic acid [AA or eicosatetraenoic (ETE) acid 20:4] (71). Upon the initiation of the cell death program, these enzymes change their properties/functions and switch their activity to the peroxidation of PLs. The transformation of enzymatic activity occurs due to protein interaction with other molecules—a mitochondria-specific PL molecule, CL, in the case of cyt *c* (**Figure 2C**), and the protein PEBP1 in the case of 15LOX (**Figure 2D**).

Cyt *c*-Catalyzed Peroxidation of Cardiolipins

Cyt *c* is a small mitochondrial intermembrane space hemoprotein (MW about 12.5 kD, 104 amino acids) (72). As a transporter of electrons, cyt *c* utilizes hexa-coordinated heme whereby the Fe has four coordination bonds with a protoporphyrin IX and His_{18} and Met_{80} at the proximal and distal sides as the fifth and sixth iron ligands (73). Participation in the execution of the apoptotic death program is a recently established important function of cyt *c*. There are two pro-apoptotic processes that depend on cyt *c*: i) apoptosome formation and ii) CL peroxidation (16, 74, 75). These two seemingly unrelated roles of cyt *c* may, in fact, be closely linked to each other. Cyt *c* that is released from mitochondria into the cytosol interacts with the apoptotic protease-activating factor 1 (Apaf-1) to form the apoptosome, thus initiating the activation of caspase-9 and downstream caspases (75). The outer mitochondrial membrane (OMM) is permeable to small molecules—co-factors, small peptides, etc.—that get readily released through the pores

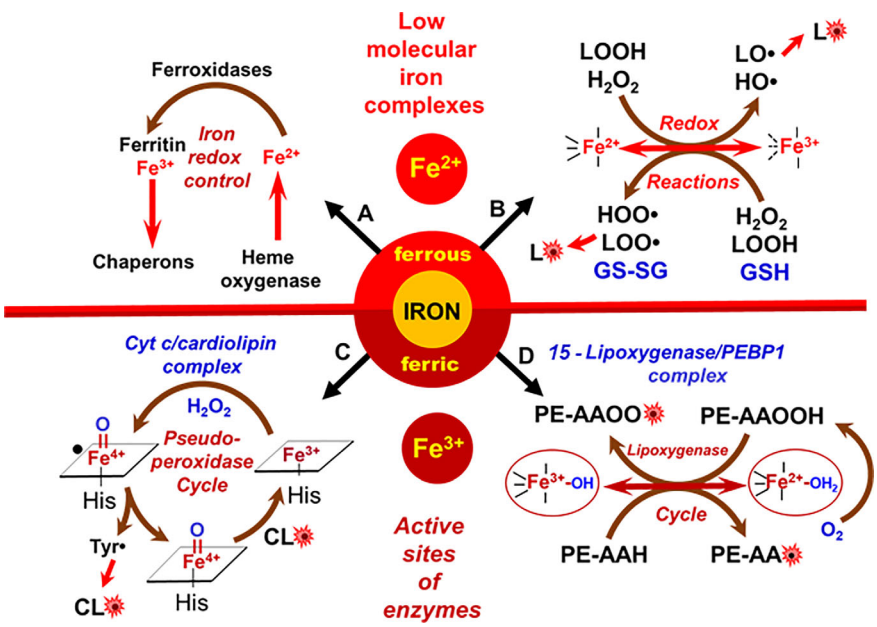

FIGURE 3 | Metabolic redox pathways of iron in cells. **(A)** Tight control of redox-active iron in cells prevents its participation in peroxidation reactions; **(B)** redox activity of low molecular iron complexes (labile iron); **(C)** oxidation of CL in cyt *c*/CL complexes; **(D)** formation of lipid radicals by the catalytic site of 15LOX/PEBP1 complex.

with the size limit of ~2nm in diameter (76). As the average diameter of the cyt *c* globule is ~4nm, it is normally retained within the intermembrane space (77). It has been hypothesized that products of CL peroxidation may accumulate in the OMM where they can get oxidized and became involved in the production of pores with diameters >4nm that will facilitate the release of cyt *c* from the intermembrane space into the cytosol.

The accumulation of irreparable defects in mitochondria triggers their elimination through a special type of autophagy, mitophagy (78). This is a multistage process in which the signaling by a mitochondria-specific CL plays a prominent role. Normally confined almost exclusively to the matrix leaflet of the inner mitochondria membrane (IMM), CL undergoes several trans-membrane migrations to the mitochondrial surface (the outer leaflet of the OMM) (79, 80). Externalized CL binds microtubule-associated proteins 1A/1B light chain 3B (LC3) (79), one of the central executioners of autophagy. Timely and successful elimination of damaged organelles *via* activation of mitophagy is a pro-survival mechanism (81). However, incomplete autophageal digestion of injured mitochondria with dysregulated electron transport represents a peril to the entire community of surrounding cells. As a result, the entire cell undergoes apoptotic elimination.

The transition from the pro-survival mitophagic to the apoptotic death program relies on a new interaction of cyt *c* with CL (which becomes possible during CL migration from IMM to OMM). Noteworthy, formation of the cyt *c*/CL complex causes a strong negative shift of its redox potential (by ~400 mV) such that cyt *c* can no longer act as an electron acceptor from complex III, and function as an electron carrier in the respiratory chain (82). This results in the elevated production of superoxide anion-radicals and their dismutation to H_2O_2. The latter can be used as a source of oxidizing equivalents for a peroxidase reaction, provided that an inadvertent peroxidase activity is present in the microenvironment. The formation of cyt *c*/CL complexes offers this opportunity.

Evidently both pools of CL facing the intermembrane space—in the outer leaflet of the IMM and the inner leaflet of the OMM—bind cyt *c* to form a peroxidase complex activated by the available H_2O_2. Deprotonated CL phosphate groups can electrostatically interact with eight positively charged lysine residues of cyt *c*, particularly $Lys_{72/73}$ (74). The electrostatic binding is followed by strong hydrophobic interactions between PL acyl chains and non-polar regions of the protein (83). There are different views on the degree of the protein conformational changes induced by CL. While some of the data have been interpreted as the evidence for dramatic protein unfolding and denaturation ("molten globule"), recent multidimensional solid-state NMR results favors the model in which only minimal structural rearrangements take place whereby the hydrophilic milieu at the membrane interface stabilizes a native-like fold, but also leads to localized flexibility at the membrane-interacting protein face (84). One way or another, CL induced changes weaken and/or disrupt the coordination bond between heme-iron and Met_{80} (**Figure 2C**). Other amino acids can interact with the distal position of heme iron but they are not strong ligands and can be replaced by small molecules, including H_2O_2 and FA-OOH (73). Overall, the catalytic site of the cyt *c*/CL complex adopts peroxidase activity triggered by the available H_2O_2 or HOO-PUFA. If the iron in the cyt *c*/CL is in ferrous state, H_2O_2 oxidizes it into ferric form thus completing the conversion of cyt *c*/CL into a peroxidase. H_2O_2 leads to the formation of highly reactive oxoferryl porphyrin-π-cationic radical (compound I), which can oxidize various substrates (**Figure 3C**). In the case of pseudo-peroxidases, like cyt *c*/CL, compound I oxidizes protein amino acids to form a protein-immobilized radical (most likely tyrosyl) thus designating the emergence of compound II. Tyr_{67} is located in closest proximity to the heme group of cyt *c* and mediates oxidation of CL (**Figure 2C**) (85). The lower "pro-oxidant" capacity of compound II suggests that it is an unlikely candidate to further oxidize protein amino acids but it can readily abstract bis-allylic hydrogens from PUFA-CL (86). While other negatively charged lipids, like PIPs, PG, and PS, can also activate cyt *c* into a peroxidase, nevertheless the effectiveness of these alternative peroxidase complexes of cyt *c* with PLs is markedly lower than that of cyt *c*/CL complexes. Notably, the most abundant non-charged PLs of mitochondria, PC and PE, neither form peroxidase complexes with cyt *c* nor undergo peroxidation during apoptosis (16). Lipid hydroperoxides are orders of magnitude more effective in initiating CL peroxidation by cyt *c*/CL complexes than H_2O_2 (87). This indicates that accumulation of small amounts of HOO-CL may strongly stimulate the peroxidation process. Indeed, progressive acceleration of CL peroxidation in the presence of CL-OOH has been experimentally confirmed (74). The described role and specific features of CL peroxidation by cyt *c*/CL complexes may inform the mechanism-based design of small molecule anti-apoptotic regulators with therapeutic potential as described below (88, 89).

15LOX-Catalyzed Peroxidation of Phosphatidylethanolamines in Ferroptosis

The execution of ferroptosis includes the Fe-dependent production and accumulation of ox-PUFA-PL (27, 90). Theoretically, both an enzymatic mechanism as well as a random free radical reaction may be engaged in this process. As *sn2*-15-HpETE-PE has been identified as a selective and specific product eliciting pro-ferroptotic activity, it is reasonable to assume that an enzymatic mechanism should be, at least in part, enacted in ferroptosis. Among several possible redox-catalyzing Fe-proteins, 15LOX has been proposed as the likely candidate (34). Mammalian LOXes are a family of non-heme iron containing dioxygenases that effectively catalyze oxidation of one or more 1,4-cis,cis-pentadiene segments of free PUFA. A typical U-shaped PUFA binding channel is organized such that the oxidizable bis-allylic carbon is juxtaposed to Fe. The LOX nomenclature—5LOX, 8LOX, 12LOX, 15LOX—is based on the ETE carbon position that is oxidized by the enzyme's Fe (91, 92). The highly organized catalytic site contains Fe^{3+} with five coordination bonds occupied by the protein's amino acids and the sixth position interacting with the hydroxide (in the native enzyme) or water (in the intermediate) (**Figure 3D**). Fe^{3+}-OH abstracts hydrogen from

PUFA and yields a carbon-centered radical and Fe^{2+}-OH_2 (93). Thus formed lipid radical (L•) interacts with molecular oxygen (O_2) delivered to the catalytic site through a special channel (94) which controls the production of the peroxyl radical at the catalytic site. The completion of the catalytic cycle is achieved *via* the hydrogen transfer from Fe^{2+}-OH_2 to the peroxyl radical resulting in the formation of the lipid hydroperoxide.

Normally, 15LOX effectively oxidizes free ETE as a preferred substrate to generate 15-hydroperoxy-eicosatetraenoic acid (15HpETE) (71). Among the members of the LOX family, 15LOX is uniquely organized to also catalyze peroxidation of esterified PUFA, particularly membrane PLs (95). ETE-phosphatidylethanolamines (ETE-PE) represent one of the preferred substrates for 15LOX leading to the production of 15-HpETE-PE (96). The catalytic efficiency of 15LOX toward ETE-PE is relatively low. However, its formation has dramatic consequences as 15-HpETE-PE has been identified as a pro-ferroptotic signal (34). Paradoxically, a number of phenolic compounds and aromatic amines effectively prevent ferroptotic death but are poor 15LOX inhibitors (97). It has been hypothesized that 15LOX alone is not sufficient for the production of pro-ferroptotic death signals but there may be an additional factor modifying the enzymatic properties of 15LOX under ferroptotic conditions. Indeed, this factor has been identified as a scaffold protein, PE-binding protein-1 (PEBP1) (36). PEBP1 was shown to form a complex with 15LOX in which allosteric changes in 15LOX permit the entry and positioning of ETE-PE (**Figure 4**) in a way that the enzymatic activity toward specific oxidation of the ETE-residue increases two-fold (99). Participation of 15LOX/PEBP1 in the generation of pro-ferroptotic PEox death signals was demonstrated in a number of different types of cultured and primary cells as well as *in vivo* in airway epithelial cells in asthma, kidney epithelial cells in renal failure, cortical and hippocampal neurons in brain trauma (100), and in intestinal epithelial cells after total body irradiation (101). Given the demonstrated necro-inflammatory consequences of ferroptosis (102), the specific features of 15LOX/PEBP1 complexes offer an exciting opportunity for the design of ferroptosis-specific small molecule regulators with profound implications for human disease. It should be noted, however, that alternative enzymatic mechanisms of peroxidation of PUFA-PE may be involved in triggering ferroptosis. A recent study identified a NADPH-dependent oxidoreductase (possibly with a partner isoform of cytochrome P450), as an essential activator of pro-ferroptotic phospholipid peroxidation in several types of cancer cells with low levels of 15LOX expression (103). The sensitivity of cells to pro-ferroptotic stimulation was decreased after downregulation of the oxidoreductase but not of 15LOX. In contrast, in cells with high levels of 15LOX expression (e.g., after stimulation of human airways epithelial cells by inducers of Th2 responses), 15LOX KD caused a strong suppression of ferroptosis (36).

Non-Enzymatic Lipid Peroxidation by "Loosely Bound" Fe-Complexes

Under extreme circumstances where the strict control of Fe is lost, Fe can display its redox activity mostly *via* participation in Fenton/Haber-Weiss reactions leading to the formation of ROS. The major biologically impactful event in these reactions is the reductive splitting of H_2O_2 by Fe^{2+} to yield highly reactive HO• radicals capable of initiating the oxidation process. H_2O_2 reacts poorly with most biological molecules due the high activation energy barrier that must be overcome (104). The rate constant of the reaction of H_2O_2 with free iron is low

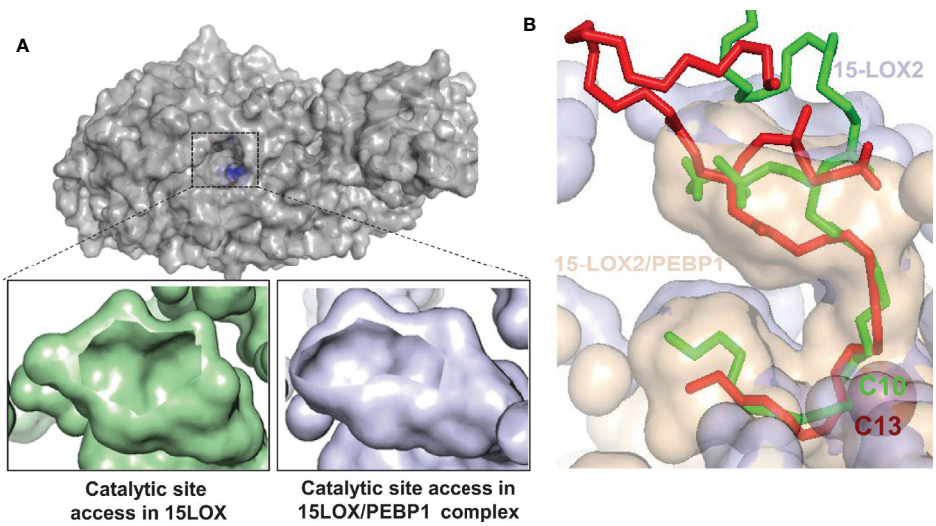

FIGURE 4 | Allosteric modification of lipid binding in the 15LOX/PEBP1 complex. **(A)** Surface representation of 15LOX, viewed from top, showing the entrance to the catalytic site, the residues of which are highlighted in blue (top panel). The opening of the entrance site is reduced in the 15LOX/PEBP1 complex (bottom panel, right), compared to that in 15LOX alone (bottom panel, left). **(B)** The binding of sn1-18:0/sn2-20:4-PE onto 15LOX alone (red) and 15LOX/PEBP1 complex (green), showing the position of the nearest carbon at the catalytic iron. In 15LOX alone, this carbon is C10, leading to peroxidation at C13, where is in the complex it is C13, which leads to peroxidation at C15. These figures were adapted from (98).

($< 10^2\ M^{-1}s^{-1}$), however, Fe-ligation may accelerate the reaction by several orders of magnitude (up to $10^4\ M^{-1}s^{-1}$). In the context of lipid peroxidation, HO• radicals avidly attack lipid molecules to produce carbon-centered lipid radicals (69) which, in the presence of O_2, are converted into peroxyl radicals (LO_2•). The latter are much less reactive toward lipids and the abstraction of a hydrogen atom from the oxidizable lipid molecules represents the "most difficult" initiating event. The easiest "victims" of RO_2• are bis-allylic positions in PUFA—hence their number in PUFA is the major factor defining the oxidizability of lipids.

Random free radical chemical reactions are driven by the reactivity of the participating reagents—radicals and oxidation substrates and enzyme-imposed structural factors which do not limit the peroxidation process. Consequently, detection of selectivity of the peroxidation that deviates from this principle of oxidizability governed by the number of bis-allylic sites can be viewed as a strong argument against the participation of a free radical chemical reaction in the overall peroxidation process. Importantly, lipid peroxidation occurring during apoptosis and ferroptosis is highly specific not only with regards to classes of PLs—CL and PE. Indeed, linoleoyl (C18:2)-CLs represent the major substrates of pro-apoptotic peroxidation in mitochondria. Further, arachidonoyl (C20:4)- and adrenoyl (C22:4)-PE species, rather than more polyunsaturated docosapentaenoyl- and docosahexanoyl-PE species, are predominantly peroxidized in cells undergoing ferroptosis.

Another important difference between enzymatic and non-enzymatic LPO is that the latter is mostly driven by ferrous iron —in contrast to cyt c or 15LOX-dependent processes where ferric iron is the major catalytic species. In contrast, ferrous iron is markedly more effective in decomposing lipid hydroperoxides, the reaction leading to the production of secondary oxidatively truncated electrophilic products of LPO that can modify proteins and change their structure and functions. This is yet another controversy in understanding the leading role of Fe-dependent enzymatic *vs.* non-enzymatic reactions of LPO.

PRIMARY AND SECONDARY LIPID PEROXIDATION PRODUCTS

LPO—enzymatic or non-enzymatic—has a radical-mediated reaction in its nature. Radical intermediates are very short lived and cannot be directly detected by conventional high resolution analytical protocols such as LC-MS. The primary molecular products of LPO are hydroperoxides and they represent the first opportunity for the LC-MS based quantitative characterization. The analysis of lipid hydroperoxides has become a formidable task for several reasons. First, their chemical and metabolic instability and thermolabile nature most often results in the formation of secondary products, some of which are susceptible to degradation. Second, the sheer number of lipid signaling molecules is staggering which translates directly into a plethora of potential LPO products that, in many circumstances, occur at very low levels. Finally, while lipid hydroperoxides are the initial/primary products of lipid oxidation, secondary products such as aldehyde, ketone, hydroxy, and epoxide products add to the heterogeneity and complexity of the signaling language. Thus, this rich signaling language should include not only full-length peroxidation products, but also truncated PLs, cyclized PLs as well as fragments of oxidized fatty acyl chains resulting from secondary reactions of lipid hydroperoxides (34, 105).

Lipid hydroperoxides are present in very low steady-state concentrations and are unstable due to their cleavage to yield, dependent on the reducing or oxidizing environment, new alkoxyl- or peroxyl- radicals, which readily decompose into secondary products (34). Indeed, many studies have focused on small reactive lipid fragments, such as malondialdehyde, 4-hydroxynonenal, etc. that can act as secondary downstream reactive products in a variety of cell death mechanisms and can covalently modify proteins [3]. However, this will depend on whether the reactive group (a reactive aldehyde for example) resides with the "leaving" short lipid fragment or remains with the truncated parent PL. Either of these two groups of reactive products can potentially react with nucleophilic amino acids, such as histidine, cysteine, and lysine that reside in cellular proteins. It has been well established that the process of PL peroxidation generates electrophilic species that are able to modify proteins and change their structure, activity and function (106). As PEs are preferentially peroxidized during ferroptotic death, it is likely that the truncated reactive parent PL is necessary for driving the downstream effects of ferroptosis (**Figure 5**). Indeed, small reactive lipid fragments can be formed from any polyunsaturated (phospho)lipid class, hence will not be specific for ferroptosis. By forming conjugates with a protein, lipidation by truncated reactive parent PLs will undoubtedly change the hydrophobic-hydrophilic balance, likely changing the distribution of the proteins into membranes (107–109) where they can form dreadful oligomeric pores.

Peroxidation reactions catalyzed by cyt *c*/CL complexes yield a highly diversified set of oxidized CL species with hydroperoxy-, hydroxy-, epoxy-, and oxo-functionalities (110). These CL peroxidation products are similar to the CLox signals detected in cells during execution of the intrinsic mitochondria-mediated apoptotic program triggered in cells by actinomycin D (16), staurosporine (17), and ionizing radiation (22, 111). Given that oxidizable PUFA-CLs are found exclusively in mitochondria, it is not surprising that oxidatively modified CL species, particularly mono-oxygenated C18:2-containing CLs, have been associated with the execution of the apoptotic death program (21). It should be noted, however, that a variety of CLox species, including hydroperoxy-, epoxy-, and oxo-CLs are detectable in cells and tissues *in vitro* and *in vivo*. For instance, CLox containing hydroxy-, epoxy-, oxo-, and hydroperoxy-functionalities have been detected in the small intestine of mice exposed to total body irradiation (110, 112, 113). Similarly, hydroxy- and hydroperoxy-CL species were detected in the brain of mice after traumatic injury (114). Notably, prevention of apoptosis by a mitochondrial electron acceptor, XJB-125, was associated with decreased levels of CLox in the peri-contusional zone of the traumatized brain.

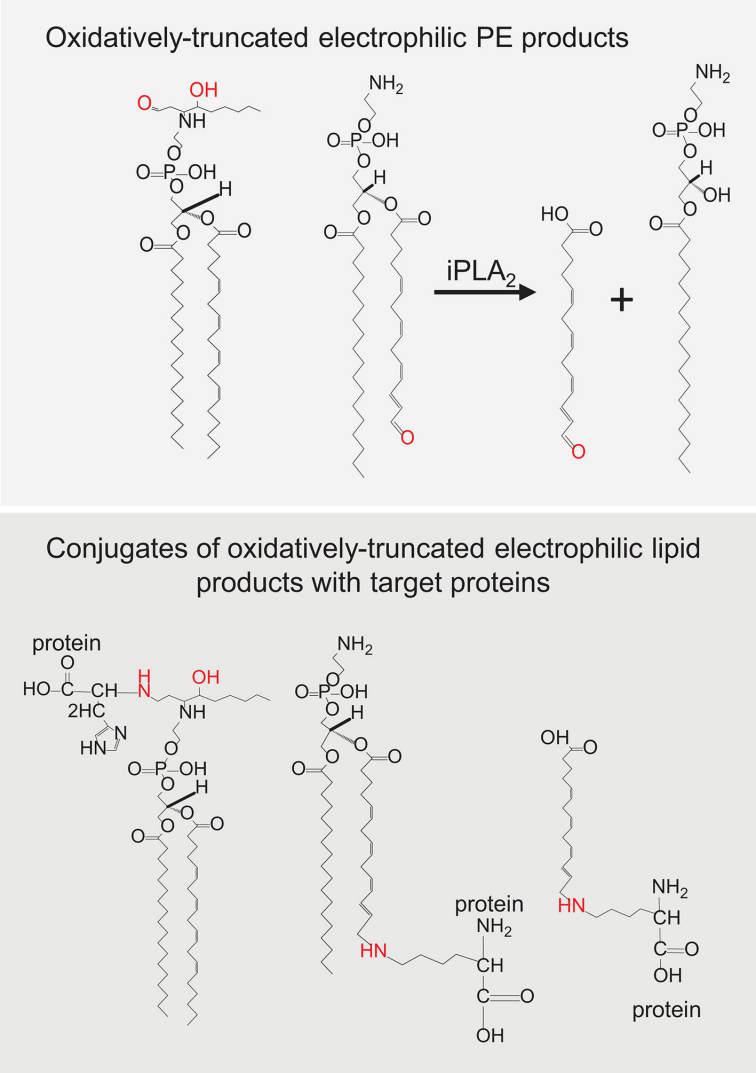

FIGURE 5 | Oxidatively truncated electrophilic products formed from phosphatidylethanolamine (PE) and their conjugates with target proteins.

It is possible that oxidatively truncated CLox species with high electrophilic potential can be formed in mitochondria. These products can readily attack nucleophilic sites in proteins and form protein adducts that will affect mitochondrial function and damage their integrity (115). Oxidatively truncated CL species can be formed in cyt *c*/CL catalyzed reactions *in vitro* (**Figure 6**) and were detected *in vivo* in the ileum of mice exposed to total body irradiation (**Figure 7**). While it is possible that truncated CLox products can modify a number of mitochondrial proteins, including those involved in the execution of mitochondria-dependent cell death programs (e.g., apoptosis), this work has not been accomplished and represents a goal for future investigations. The isolation and determination of the site of lipidation on proteins directly participating in the execution of cell death in apoptosis and ferroptosis is a formidable task. However, lipidated proteins, and more importantly lipidated peptides generated from protein digests, should impart a dominant hydrophobic characteristic whereby the peptides should be highly retained on reverse-phase solid supports. Adjusting and modifying gradients for extremely hydrophobic species and/or applying different chromatographic solid supports should aid in the separation and identification of lipidated species.

INTRACELLULAR LOCALIZATION OF LIPID PEROXIDATION CENTERS

Apoptosis

The execution of the intrinsic apoptotic program is initiated in mitochondria as it requires the oxidation of CLs (117). Formation of the peroxidase cyt *c*/CL complexes (16, 118) along with the H_2O_2-producing interruption in electron transport causes accumulation of

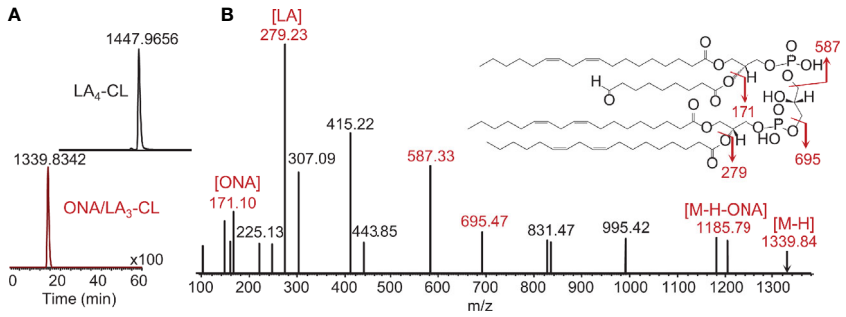

FIGURE 6 | Enzymatic peroxidation of polyunsaturated CL by cyt c yields a variety of products (12, 110), including oxidatively truncated molecular species. Shown are LC-MS results identifying the production CL molecular species containing 9-oxo-nanoic acid. **(A)** Profiles of tetralinoleyl cardiolipin (LA_4-CL, upper panel) with m/z 1,447.9656 and 9-oxo-nanoyl (ONA)/LA_3-CL with m/z 1,339.8342 (lower panel); **(B)** MS^2 fragmentation pattern and structural formulae (inset) of molecular ion with m/z 1,339.8342. MS^2 analysis reveals the fragments with m/z 1,185.79, m/z 695.47, and 587.33 produced due to the loss of oxidatively truncated residue as well as to di-linoleoyl-glycerol phosphatidate and diacylglycerol phosphatidate containing linoleic acid (LA) and its oxidatively truncated residue. Further MS^2 fragmentation of ion with m/z 587 yield ions with m/z 307 and m/z 415. The fragments corresponding to ONA (m/z 171) and LA (m/z 279) were detected as well. ONA, 9-oxo-nonanoic acid; LA, linoleic acid.

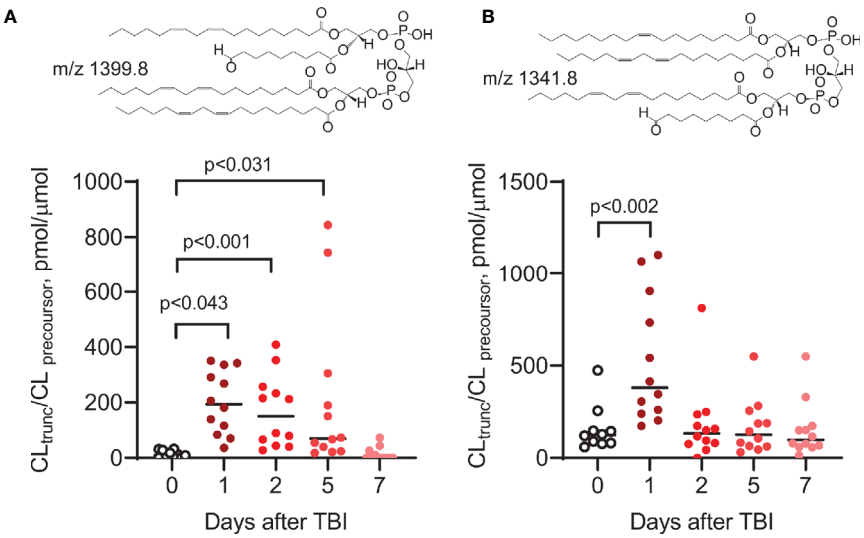

FIGURE 7 | Peroxidized CLs, including oxidatively truncated species, are produced in the small intestine (ileum) of mice *in vivo* after total body irradiation (9.5 Gy) (110, 116). Two truncated CL species, ONA/LA_3-CL **(A)** and OA/LA_2/ONA-CL **(B)**, have been identified by MS^2/MS^3 fragmentation analysis (ONA, 9-oxo-nonanoic acid; LA, linoleic acid; OA, oleic acid). As previously described (110, 112, 113, 116), the levels of peroxidized CL (including oxidatively truncated CL species containing ONA) are elevated after irradiation. Insets: structural formulas of ONA/LA_3-CL **(A)** and OA/LA/ONA-CL **(B)**.

CLox leading to the release of cyt *c* into the cytosol and apoptosome formation (75). In the cytosol, the released cyt *c* can bind an anionic phospholipid, phosphatidylserine (PS), located in the inner (cytosolic) leaflet of the plasma membrane. This complex also displays peroxidase activity that can trigger PS oxidation and accumulation of oxidized PS (PSox) (119, 120). As a membrane-disrupting agent, PSox can act as a "non-enzymatic scramblase" leading to the appearance of both PS and PSox molecules on the cell surface (120, 121). Both lipids serve as universal "eat-me" signals in efferocytosis, whereby PSox is more efficiently recognized by professional phagocytes (122–124). Thus, two separate cyt *c*-dependent oxidation mechanisms utilizing CL and PS are activated at different stages of apoptosis and generate signals with two distinctive functions. CLox is involved in the intracellular signaling at the initiation stage of apoptosis and leading to the release of cyt *c* from mitochondria, whereas PSox is an important part of inter-cellular communications regulating efferocytotic clearance of apoptotic cells by professional phagocytes (80, 125).

Ferroptosis

Intracellular localization of LPO and participation of different organelles in the generation of ferroptotic death signal remains an important but still controversial issue (126, 127). Fluorescence-based measurements using a reagent selectively

reacting with lipid hydroperoxides, Liperfluo, indicate that the endoplasmic reticulum (ER) is the major site of ferroptosis initiation (127). Both 15LOX and low molecular weight Fe-complexes are found in the ER compartment suggesting that both enzymatic initiation and non-enzymatic cleavage of HOO-PLs can take place in the ER (128, 129). The ER can also promote ferroptotic peroxidation indirectly *via* induction of autophagy driving the degradation of several important regulators of lipid peroxidation such as ferritin, lipid droplets and glutathione peroxidase 4 (GPX4) (130, 131). Given the likely engagement of mitochondria in the ferroptotic peroxidation, it is possible that mitochondria-associated ER membranes (MAMs) are the immediate locales where the LPO initiating events predominantly occur (132, 133).

Mitochondria are likely to be directly and/or indirectly involved in pro-ferroptotic LPO. There are several lines of evidence favoring the mitochondrial participation: i) GPX4 is localized in the intermembrane space of mitochondria, whereas 15LOX is associated with the mitochondrial membrane (94, 134–137), ii) a significant part (~40% of total phospholipids) of the major LPO substrate, PUFA-PE, is synthesized and present in this organelle (138), iii) they play a crucial role in cellular iron homeostasis, iv) they act as the major source of pro-oxidant ROS as well as a variety of molecular species of phospholipid hydroperoxides, *v)* they undergo dramatic morphological changes during ferroptosis (27). While strongly supportive, these characteristics make mitochondria a plausible but not penultimately proven universal participant of ferroptosis (139–141). It has been shown that cells incapable of generating mito-ROS due to depletion of mitochondrial DNA or with dramatically lowered levels of mitochondria (eliminated by mitophagy) did not demonstrate decreased sensitivity to ferroptosis (31). A highly effective mitochondria-targeted and hydrophobic radical scavenger, TPP-tagged MitoQ, was less effective than its non-targeted derivative (142). However, another mitochondria-targeted nitroxide, XJB-5-131, suppressed ferroptosis better than non-targeted nitroxides (143).

As an integral part of cell catabolism, lysosomes can, under some circumstances, indirectly participate in the ferroptotic program *via* autophagy e.g., by digesting Fe-containing proteins and releasing Fe used in pro-ferroptotic machinery (144, 145). However, it appears that lysosomal LPO is not critical for ferroptosis, as prevention of the accumulation of ferroptosis inhibitor ferrostatin-1 (Fer-1) in lysosomes, caused a stronger inhibition of ferroptosis (31). Evidently, the plasma membrane may be a target for the ferroptotic program rather than a part of its execution machinery and phospholipid peroxidation products may participate in the late destructive stages of the cell's demise. In line with this, only minimal amounts of Fer-1 were detectable in the plasma membrane during execution of ferroptosis (146, 147).

Non-Cell Autonomous Features of Lipid Signaling in Ferroptosis *vs.* Apoptosis

Preservation of plasma membrane integrity and formation of apoptotic bodies which are engulfed and removed by phagocytes are typical hallmarks of apoptosis. This process prevents spillover of cellular contents and makes apoptosis a non-inflammatory, cell-autonomous phenomenon. Opposite to apoptosis, ferroptotic cell death is "spread," in a synchronized way, suggesting a direct cell-to-cell communication for the delivery of death signals (38, 148–150). The nature of potential death signals was revealed by LC-MS based redox lipidomics in a number of experiments, including those with the induction of ferroptosis by exogenous pLoxA, and included the oxidation of exogenous ETE-PE yielding 15-HpETE-PEs. Results of these experiments demonstrated the synchronous character of the spreading of cell death. The fluorescence of Liperfluo interacting with 15-HpETE-PEs demonstrated propagation of these products among the neighboring cells suggesting that they can serve as a death signal initiating ferroptosis (38). The non-cell autonomous nature of ferroptosis may be associated with intercellular communications stimulating immune and metabolic responses (38).

REGULATION OF LIPID PEROXIDATION IN APOPTOSIS AND FERROPTOSIS AND ITS SPECIFICITY

The oxidation of CL during apoptosis occurs very early during the time when its molecular reactions leading to the initiation of apoptosis remain located in a restricted space inside of mitochondria whereby its inhibition provides an effective target for preventing apoptosis. This suggestion was confirmed by the experimental results demonstrating, that genetic depletion of CL-synthase as well as deficiency of cyt c or mutation of its Tyr_{67} residue result in an increased resistance of cells to apoptosis (20, 151–153). Several low molecular weight compounds preventing CL oxidation by inhibiting of peroxidase activity of cyt c have been designed and tested, including mitochondria-targeted electron scavengers and stable nitroxides radicals (116, 154–156).

Regulation of LPO which play crucial roles in the development of ferroptosis is a key approach in preventing ferroptotic cell death. One of the most important defenders is glutathione peroxidase 4 (Gpx4), converting toxic PL hydroperoxides to non-toxic alcohols by using GSH as the reducing *substrate* (142). Direct inactivation of GPX4 by small molecular weight compounds interacting with selenocysteine such as RSL3, ML162, ML210, $FINO_2$ as well as indirect inhibition by deprivation of GSH were effective in inducing ferroptosis (28, 126, 157) (**Table 1**). One of the prerequisites for ferroptosis is the presence of ETE-PE, the substrate for the production of the death signal, HpETE-PE. Enzymes participating in the synthesis of ETE-PE such as ACSL4 responsible for the formation of CoA-derivatives of ETE and LPCAT3 catalyzing the esterification of CoA-ETE into lyso-PE, act upstream of GPX4 and are important ferroptosis regulators. Experimental data demonstrated a direct correlation between their expression and sensitivity to ferroptosis (34–36). GPX4 and ACSL4 double-KO cells have the ability to overcome the deadly effect of GPX4 deficiency and do not undergo ferroptosis (35).

Some cells contain additional enzymatic mechanisms such as inducible nitric oxide synthase (iNOS)/NO• which can interact with 15LOX and/or lipid radicals and neutralize them (37). The anti-ferroptotic effect of NO• involves the inhibition of 15-LOX-dependent oxidation of ETE-PE and neutralization of HpETE-PE as well as secondary lipid radicals formed during cleavage and oxidative truncation of this molecule, thus preventing their toxic effects. Increased expression of iNOS/NO• in M1 macrophages/microglia or addition of NO• donors to the M2 macrophages not expressing iNOS lead to their high resistance to ferroptosis. Due to its ability to neutralize the formation of HpETE-PE, iNOS can inhibit ferroptotic cell death acting upstream of GPX4.

Nrf2/NFE2L2 transcription factor is activated as a feedback loop to protect cells from LPO generated during ferroptosis and it is one of the key players in the protection of cells from ferroptosis. After dissociation from its complex with its negative regulator Keap1, Nrf2 translocates to the nucleus where it activates the transcription of target genes (158, 159). NRF2 inhibits ferroptosis through the regulation of hundreds of genes, including genes participating in the regulation of glutathione, GPX4 expression, iron, mitochondrial function and lipid metabolism (159, 160).

Unique Role of Thiols

Reduced glutathione (GSH), the most prevalent non-protein thiol and the major intracellular antioxidant, plays an important role in maintaining a tight control over the redox status and cellular defense against LPO. However, the specific mechanisms of action of GSH in apoptosis and ferroptosis are different in spite of the fact that GSH depletion is a common feature of both extrinsic and intrinsic apoptosis (161). GSH depletion creates redox instability promoting the activation of signaling pathways leading to the initiation of apoptosis. One of the possible mechanisms activated by GSH depletion involves the deterioration of mitochondrial function. It appears that GSH deficiency promotes pro-apoptotic effects of other inducers; by itself GSH depletion is not sufficient to initiate apoptosis (162).

In ferroptosis, GSH is required as a substrate for proper functioning of GPX4. A decline in the GSH contents leads to the accumulation of pro-ferroptotic PUFA-PLox. The cystine/glutamate antiporter (system xc^-) represents the main route for extracellular transport of cystine serving as an essential precursor for the synthesis of GSH. The xc^-/GSH/GPX4 axis is the crucial controlling mechanism of ferroptosis and its inhibitors (e.g., erastin, imidazole ketone erastin, sulfasalazine, etc.) are classified as "class 1" ferroptosis-inducing compounds to distinguish them from RSL3 and other direct inhibitors of GPX4 classified as "class 2" (163).

Thiol-Independent Regulation of Lipid Signaling

A recently discovered protein FSP-1—formerly known as mitochondrial flavoprotein AIFM2—was found to inhibit ferroptosis in a GPX4/GSH-*independent* way. Accordingly, FSP1 knockout cells display a higher sensitivity to ferroptosis inducers whereas overexpression of FSP1 correlates with a higher resistance to ferroptosis in multiple tumor cell lines. The mechanisms of FSP-1 anti-ferroptotic effects involve its ability to serve as an oxidoreductase catalyzing the NAD(P)H-dependent reduction of CoQ to ubiquinol (43, 44).

Similar to vitamin E, a lipophilic radical-trapping antioxidant CoQ10 is a very effective inhibitor of ferroptosis both *in vitro* and *in vivo* (164, 165). The redox biochemistry of CoQ and vitamin E are intertwined: the more powerful antioxidant, vitamin E (tocopherol), neutralizes lipid radicals by donating an electron and can be regenerated back to its reduced form by CoQ-OH (166–169). This recycling of vitamin E is maintained by FSP1 that maintains control of the reduced CoQ-OH. In addition to FSP-1, cells contain several oxidoreductases capable of reducing CoQ and vitamin E, hence be involved in the GSH/GPX4 independent inhibition of ferroptosis. Additional work in this area will elucidate the role of several oxidoreductases such as NAD(P)H: (quinone acceptor) oxidoreductase 1 (NQO1) (170), cytochrome *b*5 reductase (CyB5R), in CoQ/vitamin E anti-ferroptotic regulations (171).

MECHANISM-BASED REGULATORS OF ENZYMATIC PHOSPHOLIPID PEROXIDATION AS SPECIFIC ANTI-APOPTOTIC AND ANTI-FERROPTOTIC AGENTS

Contemporary understanding of the role, contribution and mechanisms of PL peroxidation as drivers of apoptotic and ferroptotic death programs, leads to the design of new classes of specific small molecule inhibitors with a potential for their application as therapeutic agents. To emphasize the importance of the structural and functional organization of the enzymatic complexes directly involved in the production of PL death signals, we will re-iterate their relevant and most important features in this section.

Anti-Apoptotic Regulators

Oxidation of mitochondrial CL by the cyt *c*/CL peroxidase complex is an early and critical step in apoptosis signaling. CL oxidation facilitates the release of cyt *c* from mitochondria to the cytosol and participates in apoptosome formation. CL depletion disrupts the function of various IMM enzymatic complexes (80, 172). Regulating enzymatic CL oxidation is a promising LPO-focused anti-apoptotic therapeutic strategy. CL is mitochondria-specific and asymmetrically localized, being overwhelmingly localized and enriched (≤25% of total PL) at the IMM inner leaflet. To interact with intermembrane cyt *c*, CL is first flipped to the IMM outer leaflet. The mechanisms of the overall process leading to CL externalization remain enigmatic. Several candidate proteins contribute to loss of CL

asymmetry following injury—such as PL scramblase-3 (PLS3) that facilitates transposition of CL from the inner-to-outer IMM leaflets and other transporters implicated in IMM-to-OMM CL transport [e.g., mitochondrial nucleoside diphosphate kinase (NDPKD), adenine nucleotide translocator (ANT), uncoupling proteins (uCP), creatine phosphokinase (CPK), and truncated-BH3 interacting domain death agonist (t-Bid)] (173, 174).

Interaction between CL and cyt *c* is mediated by electrostatic forces between positively charged lysines in cyt *c* and CL's two negative phosphate groups (74, 175). Binding of CL induces: **1**) distortion of cyt *c*'s heme-associated Trp_{59} and reduction in cyt *c*'s hydrophobic core volume; **2**) disruption of cyt *c*'s Met_{80}-Fe bond, lead to a mixture of penta-coordinated and His_{33}/His_{26} hexa-coordinated heme (176); **3**) a decreased Fe(II)/Fe(III) couple redox potential (~400 mV more negative than native cyt *c*) abolishing its electron shuttling activity while gaining peroxidase activity (177, 178); and **4**) opening of cyt *c*'s heme crevice enabling substrate access to the newly formed peroxidase active site (153, 179). The newly formed cyt *c*/CL peroxidase substrate specificity leans toward organic peroxides, like CLox. However, given the complex's proximity to mitochondrial H_2O_2 sources (ETC complexes and TCA dehydrogenase) and electrostatic affinity for unoxidized CL, the primary substrate of the cyt *c*/CL complex is CLox (180, 181). Liberation of CL's oxidized acyl chains through the action of phospholipases (e.g., $iPLA_2\gamma$) yields a suite of immune activating signaling molecules—such as hydroxy-octadecadienoic acids (HODEs), hydroperoxyoctadecadienoic acids (HpODEs), and hydroxy-eicosatetraenoic acids (HETEs) (182, 183).

Small molecule therapies targeting CL peroxidation hold promise as a specific anti-apoptotic interventions. However, the administration of non-targeted global antioxidants (e.g., vitamin E, CoQ10, or nitroxyl radicals) proved ineffective at improving survival and CLox-associated apoptosis in acute injury models. Poor localization of the therapeutic compounds to the pathogenic target sites, low activity with the target substrate, and/or deleterious off-target effects on ROS signaling may have led to this failure (184). These limitations may all be overcome, to varying degrees, by specifically targeting and enriching antioxidant and electron scavengers, like nitroxide, to the IMM. XJB-5-131 and JP4-039 are comprised of a hemigramicidin (HS) peptide conjugated to TEMPO (114). These compounds attenuate lesion volume and improve behavioral deficits following experimental brain injury. Mechanistically, XJB-5-131 serves as an electron scavengers, preventing $O_2^{\cdot-}$ and H_2O_2 formation and limiting fuel for the cyt *c*/CL peroxidase. Alternatively, mitochondrial-targeted imidazole-conjugated fatty acids (TPP-ISA, TPP-IOA) coordinate the hepta-coordinated form of the cyt *c*/CL complex, impeding CL access and oxidation (**Figure 8**) (80). Therapies may also promote CL lipidome remodeling into a less oxidizable, mono-unsaturated form using mitochondria targeted oleic or stearic acid derivatives (185). While perhaps less viable for application in acute injury, this remodeling approach has shown potential in chronic neurodegenerative disease models (114). Development of new mitochondria-targeted molecules that suppress CL peroxidation, as well as the optimization of the pharmacodynamic/pharmacokinetic properties of existing ones may lead to promising ant-apoptotic therapeutic modalities.

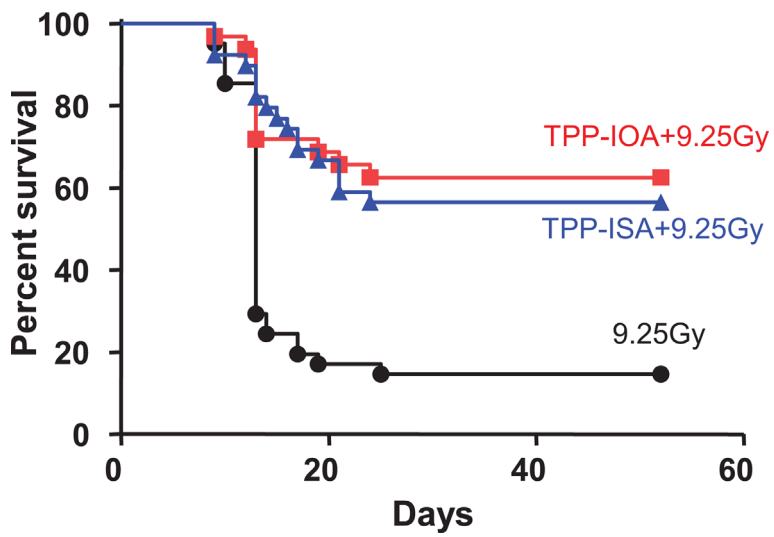

FIGURE 8 | Radiation protection and mitigation by TPP-IOA and TPP-ISA. C57BL/6NTac female mice were exposed to total body irradiation to a dose of 9.25 Gy using a cesium source (n = 31–35 mice per group). The mice were irradiated and injected i.p. with TPP-IOA or TPP-ISA (5 mg per kg body weight in 100 µl of water containing 25% ethanol) 10 min after irradiation. $P < 0.0001$ (a two-sided log-rank test)—TPP-IOA or TPP-ISA injected and exposed to total body irradiation mice *vs.* mice exposed to total body irradiation only. These figure was adapted from (116).

Anti-Ferroptotic Regulators

Most anti-ferroptosis strategies target LPO in one way or another —whether they promote cellular lipidome remodeling into a less oxidizable form, inhibit LOXs, or serve as lipophilic radical trapping agents. As indicated above, many of the ferroptotic inhibitors exhibit both LOX inhibition and radical trapping capacity, although to varying degrees (186, 187). The lack of appropriate analytical tools hindered the understanding of the precise mechanism of these molecules. With regards to the LOX inhibitors, their anti-ferroptotic effectiveness is often higher than the LOX inhibitory activity (97). Notably, the EC_{50} values for LOX inhibition have been calculated exclusively based on the suppression of free HpETE production by LOX alone. The rate of the HpETE production by 15LOX is many-fold higher than that of HpETE-PE. Because the latter, but not free HpETE, act as pro-ferroptotic signals, the meaning of these estimates in the context of ferroptosis is dubious (34). Assuming that the 15LOX/PEBP1 complex is the generator of pro-ferroptotic HpETE-PE signals (36, 188), assessments of small molecule radical scavengers have to be performed in this system. A recent study conducted with Fer-1, known to act as good radical scavenger, demonstrated that the EC_{50} value for Fer-1 in inhibiting the HpETE-PE production by the 15LOX/PEBP1 complex is considerably lower than its effective cytoprotective concentration in cells triggered to undergo ferroptosis (98). Moreover, the Fer-1-driven ferroptosis suppression was mainly realized through its enzymatic inhibitory capacity (98). It should be noted that the radical trapping reagents could have off-target effects that may decrease the (phospho)lipid peroxide load of the cellular system. These effects may be helpful to mitigate diseases involving multimodal mechanisms of cell death as exemplified by traumatic brain injury and inflammatory disease triggered by total body gamma-irradiation (36, 46, 189). At the same time, highly specific inhibitors could be beneficial for diseases in which ferroptosis is the predominant pathogenic mechanism.

CONCLUDING REMARKS

PLs are the major building blocks of the membrane bilayer and their structural role is indispensable and fundamental to compartmentalization and interfacing of thousands of processes in cells and their organelles. In this capacity, macroscopic characteristics of the PL assembles such as fluidity, flexibility, lateral and trans-membrane diffusion are essential for the function of membranes as interactive barriers. Interconnected with this is the signaling by membrane phospholipids that is associated with their post-synthetic modifications. Most commonly this occurs *via* oxidative transformations of polyunsaturated PL resulting in the production of a huge variety of new molecular species of lipids, integrated into a new concept of the *epilipidome*. The epilipidome includes not only oxidatively modified lipids but also a huge variety of their adducts with proteins thus merging with the epi-proteome, post-translationally modified proteins. Signaling by individual lipids and lipoprotein adducts comprising the epilipidome/epiproteome is broadly employed in almost each of the myriads of life-defining activities of cells and cell communities. This type of signaling is also essential for coordination of regulated cell death programs. Although these programs may be viewed as suicidal, their strict control and harmonized execution defines the borderline between health and disease. Therefore, deciphering the signaling phospholipid language of these programs is one of the central areas of research not only in cell biology but also in many fields of biomedicine.

While lipid peroxidation has been attributed to essentially all known death programs, its specific mechanisms and role have been clearly defined for only two of them—apoptosis and ferroptosis. Formation of free radical intermediates in the course of LPO occurs both during regulated enzymatic and random non-enzymatic processes. As a result of this, a broad spectrum of agents of different classes with high hydrogen/electron-donating capacities, particularly phenolic compounds and aromatic amines, may be effective in blocking the LPO component of death programs. This has created an optimistic view that many of these compounds may lead to "anti-suicidal" cell-based therapies. The indiscriminative nature of this strategy —likely affecting multiple vital biochemical reactions proceeding *via* free radical intermediates—may be associated with low effectiveness and serious side effects. Free radical scavengers/sacrificial antioxidants/chain-breaking antioxidants—have been designed and developed and ultimately tested in numerous clinical trials in more than three dozens of diseases. Disappointingly, the results of these were uniformly negative. This strategic mistake in searches for a "magic antioxidant bullet" should be avoided in the design of new anti-apoptotic and anti-ferroptotic therapeutic agents—the lesson has to be learnt. Future generations of small molecule regulators of *regulated* cell death program therapies must consider their selective and specific mechanisms, hence, to be precise and highly discriminative. In line with this, distinctive inhibitors/regulators have to be developed for controlling individual cell death programs—as has been discussed above.

AUTHOR CONTRIBUTIONS

VEK conceived the idea and wrote the manuscript. YYT, IIV, IHS, and VAT prepared the figures and wrote part of the manuscript. AAK, AAA, TSA FBC, AL, MWE, JSG, DHB, RKM, AKS, HB, and AAS wrote part of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Dooming Phagocyte Responses: Inflammatory Effects of Endogenous Oxidized Phospholipids

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Endogenous oxidized phospholipids are produced during tissue stress and are responsible for sustaining inflammatory responses in immune as well as non-immune cells. Their local and systemic production and accumulation is associated with the etiology and progression of several inflammatory diseases, but the molecular mechanisms that underlie the biological activities of these oxidized phospholipids remain elusive. Increasing evidence highlights the ability of these stress mediators to modulate cellular metabolism and pro-inflammatory signaling in phagocytes, such as macrophages and dendritic cells, and to alter the activation and polarization of these cells. Because these immune cells serve a key role in maintaining tissue homeostasis and organ function, understanding how endogenous oxidized lipids reshape phagocyte biology and function is vital for designing clinical tools and interventions for preventing, slowing down, or resolving chronic inflammatory disorders that are driven by phagocyte dysfunction. Here, we discuss the metabolic and signaling processes elicited by endogenous oxidized lipids and outline new hypotheses and models to elucidate the impact of these lipids on phagocytes and inflammation.

Keywords: oxidized phospholipids, oxPAPC, inflammation, immunometabolism, inflammasome, atherosclerosis, lung, COVID-19

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INTRODUCTION

Immune cells are strategically distributed in the body and react rapidly to internal and external cues, thereby controlling tissue homeostasis. In particular, phagocytes such as macrophages play a key role not only against pathogen invasions, but also in organ function. Macrophages regulate remodeling and maturation of synapses during brain development (1), as well as bone formation (2), electrical conduction in cardiomyocytes (3), gastrointestinal motility (4) and insulin sensitivity (5), among others. Thus, perturbations in the biology of these cells, or in the quality of their responses, have a profound impact on the etiology and development of several pathologies. Classically, phagocytes respond to stress stimuli, which trigger inflammatory programs and eliminate the source of stress, and/or support adaptation mechanisms. The persistence and accumulation of stress signals may lead to the exacerbation and persistence of inflammation, and thus to tissue dysfunction. Endogenous oxidized phospholipids have been shown to function as stress signals that may profoundly impact the activity of innate immune phagocytes.

The arachidonic acid-containing phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) is a constituent of the plasma membrane of every cell type (6)), lung surfactant (7–9), and circulating lipoproteins (10). PAPC reacts with oxygen on the *sn-2* chain to create a mixture of oxidized phospholipids, collectedly referred to as "oxPAPC". Although exogenous acute administration of oxPAPC before the encounter with an inflammatory moiety reduces the subsequent immune response both *in vitro* and *in vivo* (11–13), endogenous production and accumulation of oxPAPC during pathophysiological conditions are strictly associated with the onset of a detrimental chronic inflammation. In fact, oxPAPC accumulates in apoptotic cells (14–16), microparticles released by activated or dying cells (17, 18), oxidized low density lipoproteins (oxLDLs) (19) and oxidized pulmonary surfactant (20). oxPAPC also actively modulates cellular signaling processes, and contributes to the initiation and amplification of inflammation in atherosclerosis (21), lung injury and viral infections (20), non-alcoholic steatohepatitis (NASH) (22), colitis (23), leprosy (24), UV-irradiated skin (25), myocardial and hepatic ischemia (17, 18, 26), multiple sclerosis (27, 28) and inflammatory pain (29, 30).

In this review, after an overview of the capacity of lipids to modify several signaling processes, we focus on the role of *endogenous non-enzymatically oxidized phospholipids* (oxPLs) such as oxPAPC, in sustaining and enhancing inflammatory disorders. In particular, we discuss how oxPLs modulate pro-inflammatory responses in immune cells, with special attention on the crosstalk between metabolic and signaling pathways in phagocytes; we discuss how oxPAPC affects the pathophysiology of inflammatory diseases such as atherosclerosis and lung infections.

LIPIDS MODULATE CELLULAR SIGNALING PROCESSES

Lipids not only serve a structural role in membranes and function as a source of energy, but they are able to modulate cellular signaling processes. This last task is performed *via* several mechanisms, which are not mutually exclusive.

Alteration of the relative abundance of lipid species that constitute the cellular "lipidome" (31) is one of such mechanisms. Changes in the lipid composition of the plasma membrane can modify its mechanical proprieties, such as curvature and fluidity, and can thereby affect several membrane-dependent events, including phagocytosis (32), ion channel gating (33), and signal transduction (34). Local distribution of lipids in intracellular organelles also coordinates their morphology and functionality, as has been described for mitochondria in which the ratio of the phospholipids phosphatidic acid (PA) and cardiolipin (CL) directs fusion or fission dynamics (35, 36). Remodeling of the cellular lipidome may be driven by perturbations of the extracellular milieu, as occurs during atherosclerosis progression, wherein diet-derived lipid deposition affects the lipid content of phagocytes and thus the features of their cellular processes (37). Alternatively, the remodeling can be actively governed by the cell that, by activating a specific set of enzymes, reshapes its lipid pool to trigger an optimal response toward a stress factor. This is the case when immune cells (such as macrophages) modify their lipidome configurations in relation to the nature of stimulus they receive (38). In this manner, the activation of different classes of Toll-like receptor (TLR) induces distinct lipidomes in macrophages that are necessary to promote an appropriate inflammatory response (38–41).

A second mechanism utilized by lipids to modify cellular signaling is the co- and post-translational protein modification, referred to as "lipidation". Several lipids are covalently attached to proteins and change the folding of the proteins, their half-life, association to membranes and other proteins, sub-cellular localization, and binding affinity to their co-factors and substrates (42). Palmitoylation (the addition of palmitate to a cysteine residue (43)), is one of the best characterized lipid modifications and controls the stability, trafficking and functionality of the target protein. This has been shown for the nucleotide oligomerization domain (NOD)–like receptors 1 and 2 (NOD1/2), which are responsible for detecting bacterial products in immune cells. NOD1/2 require palmitoylation in order to be recruited to bacteria-containing endosomes and to function therein (44). Lipids are also an important source of acetyl-coenzyme A (acetyl-CoA) (45), which is a central metabolite that drives protein acetylation and thereby controls not only gene expression through histone modification, but also other key cellular processes such as DNA repair of double-strand breaks, cell cycle, cellular signaling, protein conformation, autophagy and metabolism (46). For example, acetylation supports the assembly and activation of the NACHT, LRR and PYD domain-containing protein 3 (NLRP3) inflammasome (47), an innate immune sensor that responds to several exogenous and endogenous stressors (48).

Lastly, lipids can be chemically and structurally modified to impact the signaling process. In this case, specific cellular enzymes catalyze definite modifications to a target lipid. Eicosanoids and steroid hormones are lipids that are produced *via* a spatially and temporally controlled multi-step mechanism, in which arachidonic acid (or other related polyunsaturated fatty acids (PUFAs)) and cholesterol, respectively, are converted into their final biological active forms by a succession of enzymatic reactions (49, 50). G protein-coupled receptors for eicosanoids, and nuclear receptors for steroid hormones then coordinate regulatory responses that control cellular as well as systemic metabolism, development, and tissue homeostasis (49, 50). Production of new lipidic molecules can also occur in a non-enzymatic manner: lipids can spontaneously react with free radical species present in both extracellular and intracellular compartments and give rise to a wide variety of biologically active products. PUFAs can undergo uncontrolled nitration (51), sulfation (52) and oxidation (19) during tissue stress conditions. For example, prostaglandins are eicosanoids produced by the strict guide of cyclooxygenase (COX) enzymes, on the contrary, isoprostanes (53) are prostaglandin-like compounds formed by

non-enzymatic peroxidation of the same COX's substrates during oxidative damage. OxPAPC is another important example of a class of chemically modified lipid moieties that are implicated in the development of inflammatory disorders.

OXIDIZED PHOSPHOLIPIDS BOOST AND SUSTAIN INFLAMMATION IN PHAGOCYTES

oxPLs *per se* are weak inductors of pro-inflammatory cytokine production by phagocytes, and they only slightly upregulate the expression of interleukin-6 (IL-6) and IL-1β (20, 54, 55). Nevertheless, oxPLs potently boost and extend the inflammatory capacity of dendritic cells (DCs) and macrophages (56–60). In particular, prolonged exposure of phagocytes to oxPLs strongly potentiates the production of pro-inflammatory cytokines thanks to the ability of oxPLs to reprogram the mitochondrial metabolism of the phagocytes (60) and to activate the release of IL-1β, while maintaining cell viability (56).

Metabolic Activities of Oxidized Phospholipids in Phagocytes

Depending on the type of signal that is detected, phagocytes reprogram their cellular metabolism differently, in order to support a proper response (61). The Gram-negative bacteria lipopolysaccharide (LPS), one of the best characterized exogenous stressors, induces global rewiring of the major metabolic pathways that dictate microbial killing processes, production of pro-inflammatory mediators and the control of cell viability (62–66). LPS-activated phagocytes increase glycolysis and the pentose phosphate pathway (PPP), which in turn provide ATP and metabolic intermediates that support protein translation and the biosynthesis of several macromolecules, such as the fatty acids, necessary for the expansion of secretory compartments (63, 65, 67–70). In the LPS-activated phagocytes, mitochondrial activity undergoes several alterations: i) the tricarboxylic acid (TCA) cycle is "broken" in two places, due to a reduction in isocitrate dehydrogenase (IDH) expression and a decline in succinate dehydrogenase (SDH) functionality; and ii) the electron transport chain (ETC) is suppressed, mainly due to the production of nitric oxide (NO) (63, 64, 66, 71). These changes shorten the cell's lifespan (66) and allows the accumulation of key metabolites such as citrate, succinate and itaconate, which control the activity of transcription factors and effector molecules such as hypoxia-inducible factor 1-alpha (HIF-1α) (63) and the NLRP3 inflammasome (72).

Recent evidence suggests that oxPLs can modify the metabolism of phagocytes, as reported for adipose tissue macrophages (ATM) in obese animals (73) and for circulating and tissue-resident monocytes/macrophages in atherosclerotic mice (60). Prolonged exposure of LPS-activated macrophages to oxPAPC (referred to hereafter as LPS+oxPAPC) profoundly interferes with the behavior of the mitochondria, and induces a novel metabolic state, termed hypermetabolism, that enhances the production of pro-inflammatory cytokines (60) (**Figure 1**). Mitochondrial activity is potentiated in cells treated with LPS+oxPAPC, sustaining the TCA cycle and respiration. The expression of IDH is selectively increased, and NO production is severely impaired, thus preventing the loss and dysfunction of ETC complexes. In this manner, the intact TCA cycle leads to the export of citrate into the cytosol, where it is converted into acetyl-coA and oxaloacetate (OAA) by the enzyme ATP-citrate lyase (ACLY). In turn, OAA, probably through direct inhibition of prolyl hydroxylases (PDH) (63, 74), stimulates stabilization of HIF-1α, which potently increases the transcription and production of IL-1β. This entire process is fed by glutamine catabolism rather than by glycolysis, even though LPS+oxPAPC cells continue to conserve a high rate of glucose utilization, as occurs in response to LPS only. Notably, glutaminolysis also plays a key role in epigenetic reprogramming, which controls long-term macrophage responses such as their inflammatory polarization and trained immunity (75–77). This mechanism is further reinforced by acetyl-coA, formed by ACLY, which directly supports histone modifications and thereby facilitates the transcription of target genes (78–80). In addition, oxPAPC treatment is sufficient to potently increase the mitochondrial potential ($\Delta\psi_m$) of phagocytes (60), which is the gradient of the electric potential on the inner mitochondrial membrane generated by ETC proton pumps (81). $\Delta\psi_m$ has been implicated in several cellular processes in addition to ATP synthesis: these include production of reactive oxygen species (ROS), cell proliferation, functionality of sirtuin deacetylases, cell renewal, and transcription factor activity (82–85). Thus, the conserved and increased mitochondrial fitness induced by oxPLs, possibly assisted also by production of a redox-balancing response (86), may prolong the lifespan of macrophages, as has been described in atheromas (87) and lung injuries (88) - and sustain their inflammatory signature. We propose that all of the metabolic effects induced by oxPLs work in concert, favoring the persistence of long-lived, detrimental, pro-inflammatory phagocytes and collectively contributing to the development of chronic inflammatory diseases.

Inflammasome Activation by Oxidized Phospholipids

Phagocytes are equipped with receptors that allow them to respond to stress stimuli. In particular, inflammasomes are multiprotein platforms that comprise a sensor protein (i.e. NLRP3), inflammatory caspases (i.e. caspase-1) and an adapter protein (i.e. apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD) – ASC); together, inflammasomes integrate various *non-self* and *self*-signals and induce the secretion of active IL-1β and IL-18 (89). Activation of inflammasomes involves two steps: i) a priming step, generally induced by exogenous molecules *via* TLRs (e.g., LPS and TLR4), that is necessary for the expression of pro-IL-1β (an inactive form of IL-1β) and inflammasome components; and ii) an activation step, whereby a repertoire of intracellular stimuli lead to inflammasome assembly and enzymatic activation of dedicated caspases, resulting in the processing and release of IL-

1β through a lytic cell death program (pyroptosis). Typically, perturbations in homeostasis of the cytosolic compartment, such as organelle dysfunction (90–93), ROS production (94, 95), ion flux (96–98), and metabolic alterations (99), prompt "canonical" activation of the NLRP3 inflammasome, while direct recognition of intracellular LPS by caspase-11/4/5 triggers "noncanonical" activation of the inflammasome (100–102). In this latter pathway, LPS elicits oligomerization of caspase-11/4/5, and its activation by auto-proteolytic cleavage (103); this then induces plasma membrane pore formation *via* gasdermin D (GSDMD) (104, 105) and subsequent potassium efflux (106) that, in turn, causes NLRP3 inflammasome activation, pyroptosis and IL-1β secretion.

Extracellular oxPLs can reach the cytosol *via* plasma membrane receptors such as scavenger receptors (107). As with LPS (108), oxPAPC is also a cargo for CD14 (57), which induces internalization of the oxPAPC, and triggers an endocytic process that is mediated by phospholipase C γ (PLCγ) and spleen tyrosine kinase (SYK). How oxPAPC leaves the endosome and enters the cytosol is a mystery. We suggest that other oxPL-specific receptors, such as Transmembrane Protein 30A (TMEM30A) (109) mediate this relocation, but we cannot rule out the possibility that the oxPAPC itself alters the composition of the endosomal membrane and provokes its own leakage from intracellular organelles into cytosol (110). Additionally, oxPLs can be produced intracellularly in response to cellular stress. For example, a recent report showed in a model of age-related macular degeneration that retinal pigmented epithelium cells produce oxPAPC, which supports their pro-inflammatory activity and their role in the development of pathology (111).

Once in the cytosol, oxPAPC binds caspase-11/4/5 and triggers an atypical inflammasome activation, culminating in active release of IL-1β, in the absence of pyroptosis (56) (**Figure 1**). This process, called "hyperactivation", is critical not only for establishing local long-term inflammation, but also for promoting a strong adaptive immune response (56, 112). The persistence of IL-1β-producing DCs in lymph nodes or in the aortic wall (113), can boost T cell activation, proliferation, and Th1/Th17 polarization, thereby further sustaining local and systemic chronic inflammation.

Inflammasome activation governed by hyperactivation differs from non-canonical inflammasome activation driven by LPS. In fact, LPS and oxPAPC are believed to interact with different domains of caspase-11/4/5, and differentially modulate the downstream effects of this enzyme (56). The highly hydrophobic lipid A moiety of LPS binds the CARD domain of caspase-11/4/5, where basic residues are required for interaction with the phosphate head groups of lipid A (102). Upon engaging LPS, caspase-11/4/5 undergoes oligomerization and activation. However, the exact nature of interactions between oxPAPC and caspase-11/4/5 are still debated (56, 114). The first study on oxPAPC-caspase-11/4/5 of Zanoni

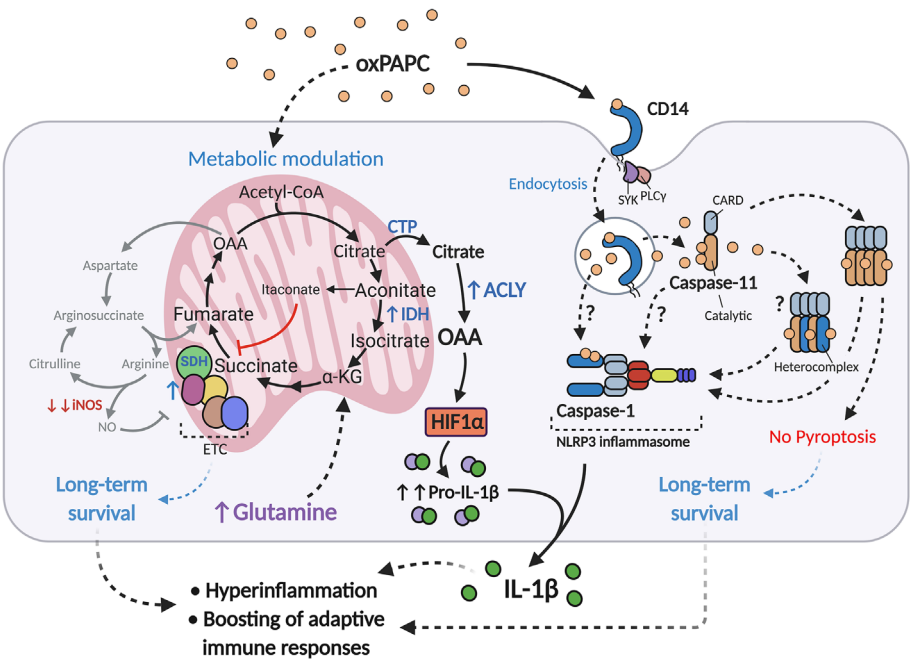

FIGURE 1 | oxPAPC boosts inflammatory responses in LPS-activated macrophages. Upon LPS encounter and/or during atherosclerosis development, oxPAPC induces a metabolic remodeling state in phagocytes, termed hypermetabolism, that is characterized by 1) boosting of mitochondrial activity *via* iNOS inhibition and ETC protection; 2) sustaining the TCA cycle with glutamine and upregulation of IDH; and 3) upregulating ACLY. These events result in the conversion of citrate to OAA, which in turn stabilizes HIF-1α and increases production of pro-IL-1β. OxPAPC is also transported into the cytosol *via* the endocytic module CD14-SYK-PLCγ, where it interacts with caspse-11/4 and induces oligomerization of this enzyme. oxPAPC may also interact with caspase-1, to form caspase-11/4/5-1 hetero-complexes, or to activate the NLRP3 inflammasome. These processes, termed hyperactivation, lead to IL-1β cleavage and release, but not to pyroptosis.

et al. using surface plasmon resonance and pull-down approaches, reported that oxPAPC binds the catalytic domain of caspase-11/4/5, and not its CARD domain (56), which enables oxPAPC to promote caspase-11/4/5 oligomerization but does not trigger its enzymatic activity (56). Later on, Chu et al. confirmed the interaction between oxPAPC and caspase-11/4/5, but they found that oxPAPC competes with LPS for the CARD domain of caspase-11/4/5, thus preventing downstream LPS-initiated signaling (114). Although more experiments will be needed to unveil the complex nature of the interactions between oxPAPC, LPS and caspases, a possible explanation for the discrepancies described in the previous studies is that individual oxPAPC constituents bind caspase-11/4/5 in diverse positions, with different affinity and *via* more than one mechanism. In particular, oxPAPC's interaction with proteins occurs *via* at least two mechanisms. Electrophilic oxPAPC components such as 1-palmitoyl-2-(5, 6-epoxyisoprostane E2)-sn-glycero-3-phsphocholine (PEIPC) covalently bind cysteine residues and modulate the activity of their protein targets. This type of interaction has been previously established for H-Ras (115), transient receptor potential cation channel, subfamily A, member 1 (TRPA1) (30), and for Kelch-like ECH-associated protein 1 (Keap-1) (116). Of note, no cysteine residues are present in the CARD domain of murine as well as human caspase-11/4/5 (117), but such residues are relatively abundant in its catalytic subunit. These data support the observation that oxPAPC selectively interacts with the catalytic portion of caspase-11/4/5 rather than competing with LPS for binding to the CARD domain (56). Alternatively, oxPAPC components that incorporate a terminal γ-hydroxy (or oxo)-α,β-unsaturated carbonyl in their *sn-2* chain interact with proteins *via* electrostatic interactions. For example, positively charged residues in the scavenger receptor CD36 are necessary for interactions of the receptor with 1-palmitoyl-2-(5-keto-6-octene-dioyl)-sn-glycero-3-phosphocholine (KOdiAPC) (118, 119). These interactions mirror LPS binding mechanisms identified for LPS binding protein (LPB) (120), caspase-11/4/5 (102), and the newly discovered intracellular LPS receptor guanylate-binding protein 1 (GBP1) (121), which have also been implicated in the interaction of oxPAPC with caspase-11/4/5 (114).

The oligomerization of caspase-11/4/5 induced by oxPAPC is sufficient to stimulate the NLRP3 inflammasome, even in absence of its catalytic activity. Potassium efflux, a downstream effect of caspase-11/4/5 activation, is not required for IL-1β release from oxPAPC-treated DCs (56), which suggests that "silent" caspase-11/4/5 aggregates can also work in other ways to activate NLRP3 inflammasome.

oxPAPC also directly binds caspase-1 (56), as was identified in RAW 264.7 macrophages with use of tandem mass spectrometry (122). We postulate that the hetero-complexes are composed of caspase-11/4/5 and caspase-1, in which the lack of caspase-11/4/5 activity is balanced by the activity of caspase-1. Also, that engagement of caspase-1 by oxPAPC can bypass the requirement for caspase-11/4/5 to start or sustain inflammasome activation. Indeed, after oxPAPC administration, primed DCs that are caspase-11-deficient can decrease - but not abolish - levels of IL-1β, while those that are caspase-1-deficient completely lose the ability to secrete IL-1β (57). Based on this finding, we hypothesize that the oxPAPC-caspase-1 complex can stimulate NLRP3 assembly and activation. However, we cannot exclude the possibility that certain oxPAPC components, depending on their concentration and the responding cell type, can trigger NLRP3 activation also in "canonical mode" (58), through ROS production (58) or metabolic alterations (58, 60).

Once activated by oxPAPC, neither caspase-11/4/5 nor the NLRP3 inflammasome provoke pyroptosis, but the cell nonetheless acquires the ability to secrete IL-1β. How this cytokine is secreted from living cells is unclear, although GSDMD pores are reportedly implicated in this process (59). The pores form small channels for the secretion of cytosolic cytokines, but the lack of a secondary stimulus, such as potassium efflux (see above), may dampen the lytic death program (56, 59). The cell may also activate a repair mechanism that recruits the endosomal sorting complex required for transport (ESCRT) machinery to the site of membrane damage, and eliminate GSDMD pores from the plasma membrane in the form of ectosomes (121). The rapid turnover of the GSDMD pores allows IL-1β secretion but prevents them from causing extensive plasma membrane damage, which thereby protects the cell from pyroptosis. The effects of oxPAPC on mitochondrial activity (see previous paragraph) may also interfere with the mitochondrial damage that is induced by gasdermins (123), and thus may protect the cell from death. Moreover, oxPAPC-potentiated mitochondrial metabolism can lead to accumulation of specific metabolic intermediates that can alter GSDMD functionality. For example, fumarate reacts with GSDMD at critical cysteine residues to form S-(2-succinyl)-cysteine, thwarting its capacity to induce cell death (124). As discussed above for caspase-11/4/5 binding, we speculate that oxPAPC also directly interacts with GS-DMD *via* thiol groups, thus mimicking the effect of cysteine-modifying drugs such as disulfiram, which block GSDMD pore formation (125).

Lastly, fatty acid epoxycyclopentenone, a *sn-2* moiety identified in some oxPAPC components, induces caspase-8 activation and IL-1β secretion (116). Caspase-8 has emerged as a new player in inflammasome induction (89): it participates in an alternative inflammasome activation pathway in human monocytes, wherein TLR engagement is sufficient to trigger inflammasome activation and IL-1β release, without pyroptosis (126). Of note, murine macrophages exposed to oxPAPC for a long time also acquire this capacity after they are stimulated by LPS only - the cells rapidly secrete high amounts of IL-1β, but preserve their viability (60). This phenotype is largely regulated by the metabolism remodeling induced by oxPAPC that boosts mitochondrial activity and favors the accumulation of metabolites; this, in turn, controls transcriptional and epigenetic programs (see previous paragraph). Nevertheless, oxPAPC could also alter the signaling hub mediated by caspase-8, enhance LPS-dependent responses and reshape NLRP3 activity. Thus, although further work is needed to understand whether or not oxPAPC interacts with human and murine caspase-8, and how it does so (directly or indirectly), oxPLs emerge as possible pleiotropic modulators also of

alternative inflammasome pathways in both murine and human phagocytes.

ATHEROSCLEROSIS: ROLES OF OXIDIZED PHOSPHOLIPID-ACTIVATED PHAGOCYTES

Atherosclerosis leads to a chronic and progressive deposition of fatty and fibrous material in arterial walls. This inflammatory condition can lead to a number of serious pathologies known collectively as cardiovascular diseases (CVDs) – these include coronary heart disease, hypertension and stroke (127). Circulating LDLs that accumulate in the intima layer of blood vessels and undergo oxidative modifications are the main initiators of atherosclerosis. However, other stressors may also contribute to this process. For instance, subclinical endotoxemia, which results from gut mucosal leakages induced during chronic infections, obesity, and ageing, may sustain the development of atherosclerosis (128, 129). oxLDLs start an enduring inflammatory reaction that involves multiple cell types, including endothelial cells, smooth muscle cells, resident macrophages and monocytes (127). In particular, activated macrophages proliferate locally (87, 130), and later, monocytes recruited from bloodstream sustain plaque formation (130). These phagocytes produce inflammatory mediators, and favor accumulation of lipid and lipid-laden cells called foam cells. Foam cells originate from macrophages as well as monocytes (130), and by metaplasia of smooth muscle cells (131), gather and progressively form a lipid-rich necrotic core, which increases over time. Non-immune cells also contribute to inflammation and deposition of extracellular material and promote plaque instability and rupture, with severe risk of thrombosis or other complications (132).

Hyperlipidemic humans and animals exhibit high levels of oxPLs, derived from oxLDLs and dead cells in their plasma and atherosclerotic plaques (133–135). These modified molecules control plaque inflammation and progression, and play a key role in the etiology of atherosclerosis (**Figure 2**). Selective oxPL neutralization, mediated by the ectopic expression of E06 antibody (136) single-chain variable fragment (E06-scFv) in high-fat fed mice that are deficient in LDL receptor (LDLR), results in severe reduction and slowing of pathology (21). In this hypercholesterolemic model, E06-scFv binds oxPLs but not unoxidized PLs, impairs pro-inflammatory macrophage activation in the aorta, and diminishes the *in locus* recruitment of monocytes and lymphocytes – this in turn reduces local and systemic inflammation. Thus, E06-scFv decreases the formation of atherosclerotic lesions and prevents valve dysfunction (21). These findings are supported by a report that quenching of reactive dicarbonyls also reduces atherosclerosis in LDLR-deficient mice (137). Indeed, oxidative reactions in the *sn-2* unsaturated chain of PLs may generate highly reactive dicarbonyl moieties such as 4-oxo-nonenal (4-ONE), malondialdehyde (MDA) and isolevuglandins (IsoLGs) (138), which covalently bind proteins and other macromolecules. Thus, use of the dicarbonyl scavenger 2-hydroxybenzylamine (2-HOBA) to block the production of molecular adducts induced by oxPL species reduces systemic inflammation and increases plaque stability (137).

Interfering with the metabolic program induced in phagocytes also indirectly dampens the pro-atherogenic effects of oxPLs. oxPAPC induces glutamine utilization, ACLY-dependent OAA accumulation, and HIF-1α stabilization, and also boosts IL-1β expression. Systemic administration of glutaminolysis or ACLY inhibitors in hypercholesterolemic mice reduces early plaque formation and decreases the production of IL-1β by macrophages in the aorta (60). Additionally, peripheral blood transcriptional signatures from Framingham Heart Study (FHS) (139) participants with pro-atherogenic lipidemia reveal an enrichment of genes that control the same metabolic pathways described for oxPAPC-treated murine macrophages (60) - this indicates that similar metabolic rearrangements are shared between humans and mice, and that metabolic intervention could be a new clinical tool for treating atherosclerosis.

IL-1β produced by myeloid cells is a crucial mediator of atherosclerosis progression (140–142): it acts systemically and in the plaque on bystander cells to augment expression of adhesion molecules and proliferation (143–146). The essential role of this cytokine in atherosclerosis and CVDs has been recently highlighted in the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) trial: treatment with a monoclonal antibody against IL-1β (canakinumab) proved to be protective against cardiovascular dysfunctions in patients with a history of myocardial infarction (MI) and elevated high-sensitivity C-reactive protein (CRP) (147). Single-cell transcriptome analyses of human and murine atherosclerotic lesions have mapped immune populations that participate in plaque inflammation, and underscore the major role of IL-1β (148–151). Of note, lipid-laden macrophages (described as "foamy" $BODIPY^{hi}SSC^{hi}$ or $TREM2^{hi}$ cells) are not pro-inflammatory, while "non-foamy" $CCR2^{+}$ macrophages are strongly enriched in inflammatory transcripts, including for IL-1β (148, 149, 151). Notably, macrophages treated with oxPAPC do not acquire a foamy phenotype and hugely upregulate IL-1β (60). Based on these reports, we speculate that the phenotype of inflammatory lesional non-foamy macrophages is driven by the metabolic program induced by oxPLs. And despite our lack of knowledge about the exact mechanisms that control the cellular and molecular dynamics induced by oxPLs in atheroma, we also propose that IL-1β release from these cells is due either to the direct action of oxPLs on macrophages (hyperactivation) or to canonical inflammasome activation. In the latter case, progressive accumulation of extracellular material such as cholesterol crystals (140) may provide the initiation signals for the activation of the NLRP3 inflammasome. In addition, macrophages and endothelial cells can form a functional circuit controlled by oxPLs (**Figure 2**). Indeed, oxPLs reportedly trigger the production of chemotactic mediators such as CCL2 and CXCL8 from endothelial cells (152–155), and recruit monocytes, thereby increasing the number of

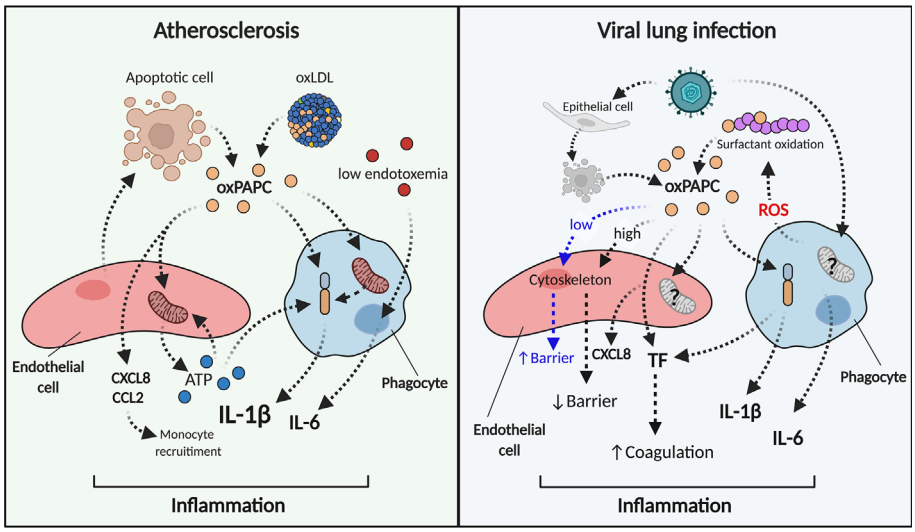

FIGURE 2 | oxPAPC triggers and sustains inflammation in atherosclerosis and viral lung infections. During atherosclerosis (left) oxPAPC released from dying cells or contained in oxLDL induces the release of chemokines and ATP from endothelial cells (red). Phagocytes (blue) become hyperinflammatory, modify their metabolism, and produce pro-inflammatory cytokines such as IL-1β and IL-6. IL-1β can also be induced by extracellular stressors such as ATP. In this manner, the endothelial cell-phagocyte circuit sustains inflammation. During viral infections (right), oxPAPC released from infected-dead cells or from surfactant oxidation interacts with endothelial cells (red) that produce chemokines and TF. Low doses of oxPAPC (early steps of infection) elicit barrier function, while high doses of oxPAPC (late steps of infections) disrupt the endothelial barrier. Phagocytes (blue) activate inflammasome-dependent responses, secrete cytokines and TF and lead to inflammation and coagulation.

oxPL-responsive cells. oxPLs also stimulate purine release from endothelial cells and, *via* a metabolic reprograming that is controlled by mitochondrial methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFD2), also compensate for loss of ATP (156). The extracellular ATP released by endothelial cells can, then, activate the NLRP3 inflammasome in macrophages and trigger IL-1β secretion (89). We also posit that the nature and magnitude of inflammasome activation reflects the progression status of the atherosclerotic plaque: thus, following a dramatic increase of extracellular material in the arterial wall, a prevalence of hyperactivated macrophages is observed at early stages, and then a slow shift toward a pyroptotic phenotype takes place at later stages.

Besides production of IL-1β and other pro-inflammatory mediators, phagocytes carry out numerous functions that are dysregulated in atherosclerosis. For example, removal of dead cells is an essential anti-inflammatory process that slows down the progression of atherosclerotic lesions (157). oxPAPC alters actin polymerization in macrophages, and thereby reduces their phagocytic activity (158). oxPLs may decrease the clearance of dead cells, and thus favor inflammation and plaque widening. Lastly, long-lived inflammatory phagocytes induced by oxPLs promote and sustain the activation and proliferation of $CD4^+$ T cells (56, 113), which in turn maintain chronic inflammation. This effect is further fueled by the capacity of some oxPAPC components, such as 1- palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC), to enhance the ability of antigen presenting cells to migrate to the draining lymph nodes and thus potentiate T cell-dependent responses (112).

In sum, the above findings collectively establish the role of oxPLs in the induction and progression of atherosclerosis, but the proposed cellular and molecular mechanisms that underlie these effects remain to be verified.

LUNG INFECTIONS: PERSPECTIVES ON A NEW ROLE OF OXIDIZED PHOSPHOLIPID IN COVID19

Pulmonary surfactant forms a film at the alveolar air-liquid interface and lowers surface tension, thereby preventing atelectasis during breathing. Surfactant is a complex mixture of lipids and proteins, whose primary components (90-80%) are saturated PLs (such as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)), which are active tension-lowering agents and are unreactive to air oxidation (7–9). Surfactant also contains (4-6%) unsaturated PLs (such as PAPC (7–9)) that can be oxidized (as discussed above). Under physiological conditions, surfactant is protected from atmospheric oxygen by antioxidant processes and by its rapid turnover. The first mechanism is mediated by specific proteins, for example surfactant protein A (SP-A) (159). The second one is carried out by type II pneumocytes and alveolar macrophages, which control the production/recycling and degradation of surfactant respectively (160–162).

Under stress, surfactant/lung homeostasis can be altered, leading to oxidation of PUFA moieties contained in pulmonary PLs. Several infections and treatments, such as acid aspiration,

influenza viruses (H5N1, H1N1 and H3N2), Monkeypox virus, *Yersinia pestis*, *Bacillus anthracis* and severe acute respiratory syndrome coronavirus (SARS-CoV) (20, 163) can induce pulmonary oxPAPC accumulation, which is associated with a detrimental pro-inflammatory response, acute injury, and organ failure (20) (**Figure 2**). These detriment effects are triggered primarily by pathogen-induced generation of ROS from alveolar macrophages. Indeed, the genetic absence of NCF1 (neutrophil cytosolic factor 1) (a key component of the NADPH oxidase complex that is required for ROS production) in mice treated with H5N1 virus reduces generation of oxPL in the lung and alleviates lung dysfunctions (20). Once produced, oxPAPC modulates the inflammatory responses of macrophages, and boosts the production of cytokines such as IL-6 (20). oxPAPC also acts on endothelial cells. Although low doses of oxPAPC enhance the function of the lung endothelial barrier by remodeling the cytoskeleton and tightening cell-cell contacts (164–167), higher doses of oxPAPC, or its fragmented products, have opposite effects, disrupting endothelial barrier integrity (168, 169). This explains how pathogen-induced damage, inflammatory mediators secreted by macrophages and endothelial cell alterations can drive acute lung injury (ALI).

Coronavirus disease 2019 (COVID-19) that is caused by SARS-CoV-2 has become a global pandemic that threatens the lives of hundreds of millions of individuals around the world. SARS-CoV-2 causes mild respiratory symptoms, including fever and cough; but in some subjects it can degenerate to viral pneumonia and acute respiratory distress syndrome (ARDS). Uncontrolled pathology can lead to a cytokine storm, multi-organ failure, septic shock and coagulation abnormalities, which can lead to severe thromboembolic events (170).

SARS-CoV-2 shares 79.6% genomic sequence identity with SARS-CoV, and these two viruses likely share many features of their biology and pathogenesis (170). Notably, quantitative lipidomic and metabolomic profiling of plasma from COVID-19 patients reveals profound metabolic dysregulation, with enhanced oxidative stress and alteration of PUFA-PC homeostasis (171). These data suggest that oxPLs, which accumulate during SARS-CoV infections, also form during SARS-CoV-2 infections, and play a central role in maintaining harmful inflammatory responses. COVID-19 patients show high neutrophilia (172, 173). Since neutrophils are the major producers of ROS (174), we hypothesize that surfactant composition is extremely altered with the massive oxPAPC formation during SARS-CoV-2 infections. Moreover, high levels of IL-1β and IL-6 have been identified in SARS-CoV-2-infected subjects (175), and single-cell transcriptomic analysis of peripheral blood in COVID-19 patients also show increased subsets of IL-1β-producing monocytes (176). In addition, pulmonary arterial thrombosis has been detected in autopsy from SARS-CoV-2 patients (177, 178). In fact, all of these effects can be credited to inflammasome activation (179), which also drives the release of tissue factor (TF) (180, 181), an initiator of the coagulation cascade. Thus, oxPAPC, as an inflammasome modulator, could elicit IL-1β and TF, and coordinate inflammation as well as hemostasis during COVID-19 infection. Indeed, CD14, that regulates inflammasome activation in phagocytes in response to oxPAPC (182), as been proposed as a possible therapeutic target against COVID-19 (183). Lastly, phagocytes infected with SARS-CoV-2 remodel their metabolism and activate HIF-1α to sustain the cytokine storm (182). Accordingly, we propose that the oxPAPC that is produced during viral infections could also act on cellular metabolism, favoring ROS production – in a feed-forward loop. Although not yet validated experimentally, we propose that this detrimental loop feeds PUFA-PC oxidation and controls transcriptional responses *via* regulation of metabolite production.

CONCLUSIONS AND FUTURE DIRECTIONS

Immune cells control tissue homeostasis and respond rapidly to noxious stimuli to maintain physiological conditions. oxPLs are endogenous stressors that reprogram phagocyte metabolism and boost their pro-inflammatory responses, inducing a novel hyperinflammatory phenotype that sustains chronic inflammatory diseases. Several studies focused on oxPAPC have elucidated several molecular events that underlie its effects on phagocytes, but some questions remain unresolved: 1) Given that oxPAPC consists of a mix of biomolecules, and single oxPAPC components can have redundant or even antagonistic effects, what are the metabolic and/or inflammatory responses of unique oxPAPC species? 2) What are the receptors/targets/pathways of oxPAPC that are necessary for inducing its metabolic and/or inflammatory activities? 3) How does oxPAPC modulate the responsivity of phagocytes to other endogenous or exogenous stressors? 4) How does oxPAPC sustain cell viability when the NLR3 inflammasome is activated? 5) Does oxPAPC modulate other processes in phagocytes, such as differentiation, proliferation, motility or migration?

Since oxPLs are virtually always present during inflammation (i.e. through neutrophil-dependent ROS release or tissue damage), we anticipate that identifying their biological targets will be vital for creating new therapies against pathologies initiated by exogenous agents, such as sepsis or cytokine storm, or by endogenous moieties, such as atherosclerosis.

AUTHOR CONTRIBUTIONS

MG conceived and wrote the manuscript, and drew the figures. IZ was involved in discussing the contents of the paper and contributed to the writing. All authors contributed to the article and approved the submitted version.

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REVIEW
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New Insights Into the Pathologic Roles of the Platelet-Activating Factor System

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Described almost 50 years ago, the glycerophosphocholine lipid mediator Platelet-activating factor (PAF) has been implicated in many pathologic processes. Indeed, elevated levels of PAF can be measured in response to almost every type of pathology involving inflammation and cell damage/death. In this review, we provide evidence for PAF involvement in pathologic processes, with focus on cancer, the nervous system, and in photobiology. Importantly, recent insights into how PAF can generate and travel *via* bioactive extracellular vesicles such as microvesicle particles (MVP) are presented. What appears to be emerging from diverse pathologies in different organ systems is a common theme where pro-oxidative stressors generate oxidized glycerophosphocholines with PAF agonistic effects, which then trigger more enzymatic PAF synthesis *via* the PAF receptor. A downstream consequence of PAF receptor activation is the generation and release of MVP which provide a mechanism to transmit PAF as well as other bioactive agents. The knowledge gaps which when addressed could result in novel therapeutic strategies are also discussed. Taken together, an enhanced understanding of the PAF family of lipid mediators is essential in our improved comprehension of the relationship amongst the diverse cutaneous, cancerous, neurologic and systemic pathologic processes.

Keywords: platelet-activating factor (PAF), oxidized glycerophosphocholine, skin, central nervous system, cancer, inflammation, ultraviolet - B, microvesicle particles

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INTRODUCTION

The term "platelet-activating factor" was first given by Benveniste and colleagues in their landmark Journal of Experimental Medicine manuscript in 1972, to a biochemical activity released by activated basophils which caused platelets to aggregate (1). This activity (PAF) was subsequently determined to be a class of glycerophosphocholines (GPC) with 1-hexadecyl-2-acetyl-GPC being amongst the most potent (2, 3). Though PAF has been demonstrated to have multiple biological activities due to a binding at high picomolar-low nanomolar concentrations to single G-protein coupled receptor widely expressed (4, 5), the term Platelet-activating factor has remained. Indeed, the PAF family of lipid mediators have been implicated in pro-inflammatory processes ranging from asthma to sepsis to ultraviolet radiation (UVR) responses. Administration of PAF results in an

acute inflammatory response, yet also can generate immunosuppressive effects *via* upregulation of regulatory T cells (6–9). A recent PubMed search indicates that more than 14,000 publications involve PAF, which attests to the large body of information available on this lipid mediator.

The synthetic pathways for PAF have been extensively reviewed (5, 10). The major pathway associated with cellular stimuli is the remodeling pathway. Cellular activation resulting in increased intracellular calcium levels induces phospholipase A_2 (often group IVA cytosolic $cPLA_2$) activation which releases an unsaturated fatty acid from the *sn-2* position of a GPC, with the released fatty acid often a substrate to form eicosanoids. The lyso GPC species then is acetylated using acetylCoA by an acetyltransferase (10) to form PAF. Of interest, the PAF receptor (PAFR) is a potent stimulus for enzymatic PAF synthesis *via* this pathway, indicating a feed-forward system (11). The limit on enzymatic PAF synthesis appears to be the substrate as well as amounts of acetylCoA available. Once produced, PAF is quickly broken down by cell- and serum-associated PAF acetylhydrolases (PAF-AH) (12). Thus, PAF is a highly potent mediator whose synthesis and degradation are tightly regulated.

In contrast to the highly regulated enzymatic pathways, PAFR agonists can also be formed in response to reactive oxygen species (ROS) *via* the direct attack of free radicals on the *sn-2* long-chained unsaturated fatty acids in the GPC (13–15). Oxidation of the esterified fatty acyl residue can introduce oxy functions, bond rearrangements and can result in the fragmentation of carbon-carbon bonds *via* β-scission. This process can result in a large number of phospholipid reaction products, to include some that exert PAFR agonistic activity. Unlike the tightly regulated enzymatic processes, this non-enzymatic process producing oxidized GPC (ox-GPC) with PAFR activity is dependent upon amounts of GPC substrate, pro-oxidants and antioxidant defenses. It should be noted that the majority of ox-GPC species have not been structurally characterized to allow quantitation using mass spectrometric techniques. Hence, we believe that the most accurate manner to measure PAF is *via* its biochemical effects such as intracellular calcium mobilization responses or release of cytokines such as IL-8 in genetically engineered cell lines with/without the PAFR (14, 16–18).

Once PAF is generated, it can either reside in the cellular membranes and potentially act upon the cell itself or to neighbor cells in juxtacrine fashion (19, 20). Some cell types, in particular monocytes, neutrophils and keratinocytes have been demonstrated to release PAF to allow it to exert effects away from the host cell. The exact mechanisms by which cells release PAF is as yet unknown, however, our group has recently demonstrated that a keratinocyte cell line can generate subcellular microvesicle particles (MVP; see ref (21–23) for recent reviews) released from budding from the plasma membrane which contain PAFR agonistic activity (24, 25). As PAFR activation results in both MVP release as well as PAF generation, this results in the potential linkage of the two processes. Presumably, traveling in an MVP might afford protection from degradation by PAF-AH in comparison to being free or protein-bound in tissue fluids. As MVPs merge with target cells, this could place PAF back into a target cell membrane. It should be noted that recent evidence from x-ray crystallography of the PAFR indicates that helix VIII appears to cover the ligand-binding site (26). This novel structural finding could be suggestive that optimal PAFR agonists bind to the receptor while residing in the plasma membrane, rather than accessing the binding site extracellularly. This finding might also provide an explanation for the low affinity of all known PAFR antagonists in comparison to native PAF ligand (27). Of importance, MVP release from many stimuli (including PAF) are dependent upon the lipid enzyme acid sphingomyelinase (aSMase) (25, 28). As aSMase inhibitors including imipramine and other molecules in the tricyclic anti-depressive class of molecules are available (29), this could potentially result in pharmacologic modulation of PAF release. This adds a potential adjunctive pharmaceutical strategy in addition to the use of PAFR antagonists.

PAF AND DISEASE PROCESSES

Because elevated PAF levels can be measured in many diseases, and exogenous PAF can mimic many aspects of disorders, PAF has been implicated in many processes. However, no actual diseases have been demonstrated to be due entirely to the presence or lack of the PAF system. The picture that is emerging is that the PAF system appears to serve as a modulator of pathologic processes. There are three areas that we would like to focus this review upon- the role of PAF in cancers and cancer therapy responses, central nervous system pathologies, and the effects of ultraviolet B radiation. Given that the skin is a complex organ that has epithelial, mesenchymal, immunologic, and neuronal components, all of these areas link to the overall theme of the epilipidome. Moreover, the three areas are connected to what we believe is a common three-part process. First, ROS from various agents generate small levels of PAFR agonists. Second, these PAFR agonists act upon the PAFR resulting in cellular activation and generation of additional PAF enzymatically, potentially allowing a PAFR amplification response. Finally, aSMase activation results in the formation and release of MVP carrying PAFR agonists to other sites. Activation of PAFR in target organs then mediate further pathology. This process provides several therapeutic targets to include antioxidants, PAFR antagonists, aSMase inhibitors as well as agents that block down-stream effects such as cyclooxygenase-2 (COX-2) inhibitors.

PAF AND CANCER AND CANCER THERAPIES

Evidence Linking PAF System to Cancers

The ability of the PAF-PAFR signaling to induce a robust systemic pro-inflammatory, pro-proliferative, and delayed immune suppressive responses, implicated in various pathological

conditions, rationalized its exploration in cancer development as many malignant cells were identified to express PAFR (3–5, 30). Notably, studies by Im and colleagues provided the first report demonstrating that a single systemic administration of PAF can augment IL-1α and TNF-α-induced increased pulmonary metastasis of murine B16F10 melanoma cells in syngeneic C57BL/6 hosts, in a process blocked by the PAFR antagonist BN50739 (31). The critical role of the PAF-PAFR signaling in melanoma tumorigenesis was also supported by the phenotype of the PAFR-overexpressing transgenic mice that exhibited keratinocyte hyperplasia, which was accompanied by hyperpigmentation and increased number of dermal melanocytes in the ear and tail with subsequent development of melanocytic tumors (32, 33). Since the PAFR is expressed in keratinocytes but not melanocytes (34), it is presumed that the melanocytic tumors are in response to inappropriate expression of the PAFR which resulted in a proliferative response. Biancone and colleagues also evaluated the role of the PAF-metabolizing enzyme PAF-AH in melanoma tumor development (35). C57BL/6 mice implanted with PAF-AH-overexpressing B16F10 melanoma cells exhibited significantly decreased tumor vascularization and growth, as well as increased survival compared to the mice harboring PAF-AH-deficient B16F10 tumors (35). These studies provided the rationale to further define the contributions of tumoral versus host PAFR signaling in melanoma development.

Given that ectopic PAF-AH expression in KS-Imm human Kaposi's sarcoma cells, or B16F10 melanoma cells resulted in reduced neoangiogenesis and tumor growth in their respective SCID and C56Bl/6J hosts, and that IFNγ-stimulated PAF synthesis enhanced the invasiveness of F10-M3, a clone of B16F10 melanoma line (35, 36), the direct evidence of tumoral PAFR in melanoma growth remained unclear as unlike many human melanoma cell lines (37–40), murine B16F10 cells do not express PAFR (41, 42). To address this question, our group generated PAFR-expressing B16F10 melanoma cells and demonstrated that regardless of the tumoral-PAFR expression, systemic administration of a PAFR agonist, CPAF resulted in increased growth of melanoma tumors in wild type (WT) mice, but not in PAFR-deficient (PAFR-KO) counterparts (43). Using environmental UVB exposure that generates PAF-agonists (to mirror systemic immunosuppressive model), we observed that similar to systemic CPAF injection, cutaneous UVB radiation also significantly increased the growth of PAFR-deficient, parental B16F10 tumor xenografts in WT hosts, and can be blocked by antioxidants supplementation (43). Importantly, this UVB-mediated increased growth of melanoma tumors was not seen in PAFR-KO hosts (43). Along similar lines, we have also shown that host activation of the host PAFR signaling augments the in-vivo growth and metastatic ability of murine Lewis lung carcinoma cells, yet these effects were not seen in PAFR-KO hosts (44). These studies support an important role of the host PAFR signaling in favoring the development and progression of melanoma and lung tumors.

PAFR Expression in Tumor Proliferation and Clinical Significance

As most malignant cells of murine and human origins such as the Kaposi's sarcoma, breast, prostate, lung, esophageal squamous cell carcinoma, ovarian, and pancreatic cancers express PAFR (40, 45–52), several studies have evaluated its relevance using various experimental *in vitro* models, as well as in clinical settings of cancer patients. Data from *in vitro* cellular models indicate that regardless of the anatomical origins, genetic backgrounds or the mechanisms involved, activation of tumoral-PAFR or PAFR overexpression accelerate the proliferation, aggressiveness, migration and invasion compared to respective control cells in various cancer models (45, 49, 50). Of note, multiple tumor cell lines expressing PAFR have been shown to produce more PAF or undergo increased PAFR expression in response to various stimuli including multiple growth factors and therapeutic agents (39, 40, 42). These lines of evidence also suggest that tumoral-PAFR expression could directly modulate the *in vivo* tumor growth *via* inducing systemic immunosuppressive effects mediated by more enzymatic PAF production by positive feed-forward mechanisms. Of significance, high tumoral-PAFR expression has also been detected in clinical settings of primary as well as lymph node metastatic tumors compared to the matched normal tissue (49, 50). High levels of tumoral PAFR expression was found to be positively correlated with increasing tumor stages, tumor status, tumor invasiveness, and poor prognosis in lung and esophageal squamous cell carcinoma patients (49, 50). Importantly, patients with high PAFR-expressing tumors experienced significantly decreased overall survival compared to the patients with low tumor PAFR expression (49, 50). Moreover, increased PAF concentrations were also detected in tumor samples of esophageal squamous cell carcinoma patients compared to matched adjacent normal tissue (50). These studies suggested the translational significance of tumoral PAFR expression in impacting not only tumor progression but also affecting the prognosis and overall survival of cancer patients.

PAFR Activation Blocking Anti-Tumor Immune Responses

Immune and non-immune factors including inflammatory milieu within the tumor microenvironment play significant roles in fostering tumor growth, angiogenesis, and metastatic progression (53). Given that PAFR activation is critical in both acute inflammatory and delayed systemic immunosuppressive effects, studies including ours have assessed its function in anti-tumor immune responses. Among various immune cell types, macrophages have been recognized for their contributions not only in phagocytosis but also in pathological conditions such as cancer. Macrophages express various receptors including for immunoglobulin (e.g., IgG), endotoxin, phosphatidylserine (PS) etc., which get stimulated upon the engulfment of microbial organisms or their products to then mediate proinflammatory signals. However, when apoptotic bodies are presented, PS interaction with PS-receptor (PSR) on macrophages induce anti-inflammatory signal (54, 55). Importantly, the published reports have also shown that the clearance of apoptotic cells by macrophages induces their differentiation into a regulatory phenotype possessing immune suppressive function. The scavenger receptor CD36 expressed on macrophages, binds to oxidized low density lipoproteins (oxLDL) consisting of phospholipids, which also act as ligands for apoptotic cells.

Thus, CD36 mediates apoptotic cell recognition by macrophages and facilitates its clearance (54, 55). As oxLDL mediated effects were found to be blocked by PAFR antagonists, studies by Oliveira et al., have demonstrated that blockade of PAFR or CD36 inhibited apoptotic cell phagocytosis (i.e., efferocytosis) by bone-marrow derived murine macrophages (56). These studies have also shown that this efferocytosis increased the colocalization of CD36 and PAFR in the plasma membrane of macrophages (56). Overall, the data indicate that apoptotic cell phagocytosis requires the engagement of both CD36 and PAFR in lipid raft, which induces macrophage differentiation into a regulatory phenotype.

Earlier studies have evaluated the role of PAF in macrophage regulation *via* measuring its spreading ability *ex vivo*. Macrophages were isolated from the peritoneal cavity of mice bearing Ehrlich ascites tumor (EAT) tumors and treated with or without PAF antagonist and added over glass coverslips. Increased spreading of macrophages was observed within a shorter period after tumor cell implantation. However, as tumors continued to grow, the spreading of macrophages derived from the normal (vehicle-treated) mice decreased, but macrophages from PAF antagonist-treated mice maintained the elevated levels of spreading (57).

Systemic treatment of PAF antagonists not only reduced the *in vivo* growth of EAT but also restored the spreading capability of macrophages (57). Importantly, PAFR antagonists have also been shown to decrease the growth of B16F10 tumors as well as the number of tumor-associated regulatory immunophenotypes expressing galectin-3 (58). Based upon the stimuli and tumor microenvironment, macrophages can acquire pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes. These findings were supported by a recent report demonstrating that the *in vivo* tumor growth of PAFR-expressing TC-1 carcinoma or PAFR-deficient B16F10 melanoma were significantly reduced in PAFR-KO compared to their WT counterparts. Interestingly, the reduced growth of these tumor types in PAFR KO mice was accompanied by increased infiltration of Gr-1+ neutrophils and CD8+ T cells in B16F10 tumors, and CD4+ T cells in TC-1 tumors (59). In addition, both tumor types from PAFR KO mice exhibited high frequency of CD11c+ M1 and decreased frequency of CD206+ M2 macrophages consistent with higher iNOS, lower arginase activity and IL-10 expression levels as compared to the tumors implanted into WT mice (59). Overall, these findings suggested that endogenous PAF-like molecules bind with macrophages expressing PAFR, which then acquire more M2-like phenotype (TAMs) in the tumor microenvironment to favor tumor promotion. Importantly, a novel macrophage phenotype (i.e., Mox) has also been identified from oxidized phospholipid-treated murine macrophages, which possess distinct genetic profiles characterized by overexpression of Nrf2-mediated redox-regulatory genes, decreased phagocytic and chemotactic capabilities (60). These studies indicated that Mox macrophages could play critical roles in the development of development of atherosclerotic lesion as well as chronic inflammation.

Notably, dendritic cells have been shown to express PAFR, and its stimulation was found to result in increased production of cytokines such as IL-10 and the prostaglandin PGE_2 associated with the regulatory phenotype in a process blocked by PAFR antagonists (61). Our studies have also supported the involvement of immune cells and PAFR in regulating the tumor growth by demonstrating that PAFR activation did not appreciably augment the growth of B16F10 melanoma tumors in immunocompromised SCID mice (43). As other prominent suppressive immunophenotypes which play critical roles in host anti-tumor immune response are regulatory T cells (Tregs), we have shown that increased growth of B16F10 tumors mediated *via* UVB-PAF agonists in syngeneic hosts was correlated with upregulation of tumoral Tregs compared to the sham-treated mice (43). Importantly, depletion of Tregs *via* anti-CD25 Abs or neutralizing Abs against IL-10, a cytokine secreted by immunosuppressive phenotypes including Tregs significantly blocked both UVB- and CPAF-mediated increased growth of B16F10 tumors compared to control groups of mice injected with isotype control Abs (43). Similarly, these effects are blocked by COX-2 inhibitors which appear critical for PAF-mediated Treg generation (18, 42). Myeloid-derived suppressor cells (MDSCs), a heterogeneous group of immature myeloid cells have also been shown to favor tumor development *via* mechanisms including the recruitment of other suppressive immunophenotypes into the tumor microenvironment (62). Given that murine MDSCs express CD11b and Gr-1 surface markers, and that their depletion have been explored as potential therapeutic approaches against solid tumors (reviewed in ref. 62), we evaluated its role in mediating PAFR-dependent tumor growth. Our studies found that UVB- or CPAF-mediated increased growth of B16F10 melanoma tumors was blocked by depleting MDSCs (via systemic injection of anti-Gr-1 Abs) (63). Overall, these studies indicated the relative contributions of several immune cell types in favoring tumor growth.

Multiple Therapies That Kill Tumor Cells Generate PAF

That dying tumor cells could generate PAF ligands provided the premise to explore the significance of PAFR signaling in the therapeutic efficacies of anti-cancer agents with known cytotoxic effects. Studies including ours have shown that multiple tumor lines including melanoma, lung, lymphoma, pancreatic, and nasopharyngeal carcinomas generate oxidized PAF agonists in response to chemotherapeutic agents or radiation therapy, with increased levels were detected in PAFR-expressing tumor cells (41, 42, 52, 64). Importantly, increased tumoral PAFR expression has also been detected upon the treatment of chemotherapy and radiation therapy (39, 64). These findings led to the hypothesis that systemic generation of PAF agonists *via* these therapies could tolerize the tumor bearing mice due to their ability to induce systemic immunosuppression, and thus, can impact their therapeutic efficacies. Notably, one of the major challenges in the medical oncology field is to define the mechanisms involved in inducing tumor resistance to the ongoing therapeutic options for cancer treatment, with the focus of devising novel approaches to improve their effectiveness. To address this clinically relevant question, ours and Sonia Jancar's group have evaluated the potential significance of this "bystander effect" generated by

chemotherapeutic agents and radiation therapy using murine cutaneous melanoma or squamous cell carcinoma (SCC) tumor lines (41, 42, 64).

Consistent with the systemic immunosuppressive model, we implanted two tumors into the flanks of mice, where one tumor is treated with chemotherapy or radiation therapy and their responses were evaluated on the growth of secondary-untreated tumors. Our studies demonstrated that treatment of one tumor resulted in increased growth of secondary tumor in a PAFR-dependent manner in a process blocked by systemic administration of antioxidants, cyclooxygenase type 2 (COX-2) inhibitors or depleting antibodies against Tregs (41, 42). Consistent with our findings, Jancar's group have shown that co-injection of irradiated TC-1 cells with TC-1 expressing luciferase (TC-1 fluc+) in syngeneic hosts, or human PAFR-expressing KBP cells with irradiated PAFR-deficient KBM cells in immunocompromised mice resulted in increased tumor growth compared to the mice co-injected with unirradiated TC-1 cells with TC-1 fluc+ or KBM cells with irradiated KBM cells (64). In another report, the same group investigated the involvement of PAFR in tumor cell survival following radiation therapy (65). They observed a dose-dependent increased expression of the PAFR, increased generation of PAF agonists, and secretion of PGE_2 after radiation therapy in keratinocytes (HaCaT), cervical cancer (C33, SiHa, HeLa), and squamous carcinoma (SSC78 and SSC90) cell lines compared to their respective unirradiated controls (65). Treatment with PAFR antagonist CV3988 pre-radiation therapy reduced PGE2 secretion and increased tumor cell death compared to untreated controls, indicating the tumor cells generate PAF agonists to protect themselves from cell death (65).

Importantly, our studies have also detected an increased level of PAFR activity in the perfusates collected post-chemotherapy compared to pre-chemotherapy in melanoma patients using the isolated limb chemoperfusion (42). Higher concentrations of tumoral PAF were measured in post-radiation compared to pre-radiation therapy treated basal cell carcinoma (BCC), bladder cancer, or pseudo lymphoma patients (41). Increased tumoral PAFR expression was detected in post-radiation therapy treated compared to untreated cervical invasive carcinoma patients (64). These studies suggest that PAF agonists generated *via* these therapeutic agents impede treatment efficacies in a PAFR dependent manner, and that PAFR serves to mediate pro-survival responses to these agents.

Potential Pharmacologic Strategies

Several groups including ours have proposed PAFR as a promising target to not only inhibit tumor growth but also to increase the efficacy of therapeutic agents. This hypothesis has been tested in multiple experimental models demonstrating that genetic blockade of the PAFR (via studies in PAFR KO mice or PAFR shRNAs) or pharmacologic PAFR antagonists significantly reduced tumor burden, increased murine host survival, as well as augmented the effectiveness of therapeutic agents compared to their respective control groups (39, 41–44, 49, 50, 58, 64, 65). While multiple structurally different but specific PAFR antagonists have been shown to exert promising effects against various experimental tumor models (39, 58, 64, 65), none of these agents have been explored in clinical settings of cancer patients. Importantly, the structural analysis as well as anti-inflammatory effects of several organic compounds (natural and synthetic) and different classes of metal-based inhibitors of PAF have been tested with the focus if these agents could exert anti-cancer properties (66). The authors observed that while these metal-based inorganic compounds possess a very promising class of anti-PAF and anti-inflammatory drugs, the rhodium(III) PAF inhibitor Rh-1 exhibited the moderate cytotoxicity in HEK 293 cell lines, which corroborates with its increased anti-inflammatory action (66). Overall, these studies provided the rationale of designing and exploring a new class of metal-based inhibitors of PAF. Another recent review has summarized the effects and outcomes of major synthetic PAF antagonists tested in clinical trials against several disease pathologies (67). The overall outcomes of these clinical studies are that majority of the synthetic PAF antagonists exerted no significant effects in reducing the clinical symptoms in patients, indicating the exploration of other compounds for their effects as potent inhibitors of PAF or PAFR (67). Thus, other strategies such as the inhibition of ox-GPC formation using antioxidants or the PAFR-mediated immunomodulatory effects using COX-2 inhibitors could be alternative strategies. Finally, the role of MVPs released from tumor cells as the potential source of PAFR agonists could provide an alternative pathway which could be amenable to aSMase inhibitors.

PAF IN CNS FUNCTION AND PATHOLOGY

Evidence for PAF System in CNS Pathologies

At present, it is unclear whether the PAF system plays an important functional role in the nervous system. Though PAF injection into the skin has been reported to be painful (68–71), much of the evidence linking the PAF system and neuropathology is derived from studies of the central nervous system (CNS). Previous work reported that PAFR agonists can increase intracellular calcium concentration (72), inhibit acetylcholine release in hippocampal slices (73), enhance catecholamine release from cultured chromaffin cells (74), inhibit ionotrophic GABA receptor activity in hippocampal neurons (75), and enhance glutamate release from primary hippocampal cultures (76). There is conflicting evidence with regard to PAF's role in long term potentiation (LTP), an example of synaptic plasticity in which a synapse enhances its strength typically resulting from high frequency stimulation. One study reported PAF treatment can induce LTP in hippocampal slices at similar potentiation level as that induced by high frequency stimulation (77). However, these data could not be replicated in a later study, in which they demonstrated that PAF alone could not induce LTP in hippocampal slices (78).

Although the role of PAF in CNS function is unknown, the PAF system has been implicated in multiple CNS pathological states. Elevated PAF levels have been detected in and appear to

correlate with severity of several CNS diseases. The association of elevated PAF with Alzheimer's Disease (AD), multiple sclerosis (MS), cerebral infarction, cerebral ischemia-reperfusion injury, and spinal cord injury has been recently reviewed (79). Moderate increases of 20% in PAF were measured in healthy subjects as they age over a 7.5 year period, whereas a greater than 60% average increase in blood PAF levels was detected in AD patients (80). Similarly, patients suffering from MS have elevated CSF as well as plasma PAF levels as compared to healthy subjects (81). Increase in hippocampal PAF levels was observed after evoked seizures in a mouse model of temporal lobe epilepsy (82). Ischemia and traumatic brain injury (TBI) have also been shown to result in significant production of PAF contributing to the pathophysiological events following ischemia or TBI (83–86).

In addition to increases in PAF levels, a reduction in PAF-AH activity was observed in PD (87) and MS patients (88). PAF-AH is an enzyme that degrades PAF and has been shown to be upregulated in stroke patients likely as a response to elevated blood PAF and PAFR agonist levels (86, 89). There is no clear explanation for the reduction in PAF-AH measured in PD and MS patients that were previously reported. The authors suggest that there may additional lipid oxidation products during PD and MS progression that may act to inhibit the PAF-AH and thus inducing and/or augmenting the elevated PAF levels (87).

PAFR Activation and CNS Toxicity

Relevant to CNS pathologies, PAFR-mediated increases in intracellular calcium levels and glutamate release can result in excitotoxicity and apoptosis (82, 90, 91). On a tissue level, PAFR are expressed in different regions of the brain including hypothalamus, cerebral cortex, olfactory bulb, hippocampus, brainstem and spinal cord (72). At the cellular level, PAFR is expressed predominantly in microglia, the brain resident immune cells (92, 93), but also found in neurons and other glial cells such as astrocytes and oligodendrocyte progenitor cells (72, 94–96). Though high (micromolar) levels of PAF can certainly induce cell toxicity, PAF acts primarily through PAFR. Indeed, its reported effects can be suppressed in the presence of PAFR antagonists or no longer detected in a PAFR knock-out mouse model. PAF-mediated increase in intracellular calcium concentration (72) and neurotransmitter release (73, 74, 76) are no longer detected in the presence of PAFR antagonists. *In vivo* administration of a PAFR antagonist (LAU-0901) in a mouse model of temporal lobe epilepsy attenuated seizure susceptibility and neuronal hyper-excitability as well as reduced hippocampal damage (82, 96). The adverse CNS effects following TBI were alleviated in mice deficient in PAFR expression (97). Ischemic damage can be attenuated by treatment with PAFR antagonist in rabbits (98). Preclinical studies also showed that PAF signaling through PAFR is involved in the dopaminergic neurodegeneration induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as PAFR knockout mice and mice treated with a PAFR antagonist did not suffer neurological deficits from MPTP (99). Furthermore, amyloid beta-induced neurotoxicity can be suppressed by antagonizing PAFR (100, 101).

Although the precise mechanistic steps by which PAF and PAFR play a role in these CNS dysfunction are not yet clearly defined, strong evidence indicates that PAF can induce or enhance neuroinflammation by promoting microglia activation thereby exacerbating disease states and neuronal injury in a PAFR-dependent manner (92, 93). Exogenous application of PAF was shown to induce a microglial chemotactic response that was dependent on PAFR, as chemotaxis was not observed in the presence of PAFR antagonist (WEB2086) or in PAFR deficient mice (102). PAF-mediated activation of microglia is thought to occur through an increase in intracellular calcium concentration (103, 104). The source of the PAF-mediated calcium increase has been reported to be primarily from endoplasmic reticulum stores which in turn induce a more sustained increase in calcium by activation of the store-operated calcium channels (SOCs) (103, 104).

Excessive PAF can also disrupt the blood brain barrier (BBB) *via* a PAFR-dependent mechanism (105–107). Rat brain microvascular endothelial cells, an essential component of the BBB, were shown to have increased PAF production when exposed to hypoxia, consequently resulting in the breakdown of BBB (105). There is evidence that similar to PAF effects on microglia, this PAF-mediated BBB disruption occurs *via* an increase in intracellular calcium (105, 107). However, unlike in microglia, the source of PAF-mediated calcium increase in microvascular endothelial cells is the influx of calcium ions through L-type voltage-gated calcium channels, thereby inducing depolarization, and ultimately resulting in increased BBB permeability (107). Treatment with a PAFR antagonist prevented the PAF-induced increase in calcium concentration and prevented disruption in BBB permeability (105, 107).

MVP and CNS Pathologies

At present, there are no reports as to the identification of PAF-containing MVP in the CNS. However, microglia release MVP containing IL-1β within the CNS upon ATP stimulation (108). Similar to the formation and release of MVP in other systems, this process in microglia was found to be dependent on aSMase (28). In the case of TBI, mice that were genetically deficient in aSMase or pharmacologically treated with aSMase inhibitor experienced significantly less adverse neurological effects at 1 month post injury (109). It is unclear whether these microparticles also contain PAF or PAFR agonists in addition to IL-1β.

Potential Pharmacologic Strategies

In summary, multiple studies have implicated the PAF system in a number of neuroinflammatory and neurodegenerative conditions. As these mechanisms are further elucidated, there is potential for PAF and PAFR targeted therapeutics for various CNS disorders. In particular, one area which has significant promise as a therapeutic target relates to MVPs, which are likely elevated in a broad range of CNS pathologies. It is presumed that an enhanced understanding of the PAF system in CNS disorders would be reflected upon the peripheral nervous system.

PAF AND UVB RADIATION

Evidence Linking PAF System to UVB

There is accumulating data suggestive that the PAF system could play a significant role in cutaneous pathophysiology, in particular how the skin responds to exogenous environmental stressors (110). Cell types in the skin such as the keratinocyte (34), mast cell (111, 112), and multiple monocytic and granulocytic cell types express PAFRs (113, 114). Of interest, melanocytes, skin fibroblasts, and T cells do not, yet B cells express PAFRs (34, 115, 116). Elevated PAFR agonist levels can be detected following multiple pathophysiologic stressors ranging from burn injury to X-radiation (41, 117, 118). Moreover, PAF can be detected in cold urticaria (119), immunobullous diseases such as bullous pemphigoid (120), following sunburn (121), and in the skin disease psoriasis (122). Consistent with the notion that PAF could exert cutaneous effects, intradermal injection or topical application of PAF onto skin results in an almost immediate painful urticarial response (68–71).

Ultraviolet B radiation (290-320 nm; UVB) is a pro-oxidative stressor which exerts profound cutaneous effects. Though only appreciably absorbed in the epidermis, sunlight's "burning rays" are critical for vitamin D metabolism, yet, also generate sunburns as well as both melanoma and non-melanoma skin cancer (8, 9, 123–126). Inasmuch as UVB generates anti-inflammatory effects in skin, phototherapy is used clinically to treat a large number of skin diseases including atopic dermatitis and psoriasis (9)..

Multiple lines of evidence link UVB with the PAF system. UVB generates the production of PAF and ox-GPC with PAFR agonistic activity (14). PAFR signaling is involved in two distinct aspects of photobiology. First, PAFR activation has been implicated in the early acute responses of UVB. In a murine model of photosensitivity from deficiency of the DNA repair enzyme xeroderma pigmentosum type A (XPA), UVB treatment results in dramatically increased levels of ROS, as well as PAF and ox-GPC formation (127). Moreover, UVB-mediated exaggerated pro-inflammatory responses in XPA-deficient mice are blocked by PAFR antagonists (127). Of interest, PAFR-deficient epithelial cells and mice exhibit diminished acute inflammation as well as decreased production of multiple cytokines including tumor necrosis factor in response to UVB (71, 127–129). Second, UVB-mediated systemic immunosuppression involves PAFR signaling *via* ox-GPCs (6–9, 112). Of note, UVB induces both local immunosuppression (where a UVB-treated site is anergic) in addition to systemic immunosuppression (where a non-UVB-treated UVB site is anergic). UVB-mediated local immunosuppression appears to involve dendritic cells, whereas systemic immunosuppression involves mast cells and Tregs. It has been reported by several groups that exogenous PAF is immunosuppressive, and UVB-induced systemic immunosuppression is attenuated by PAFR antagonists and absent in PAFR deficient mice (6–9, 112). However, local immunosuppression is normal in PAFR KO mice (129). As noted elsewhere in this review, exogenous PAF agonists augment experimental melanoma tumor progression (41–43). Finally, systemic PAFR antagonists have been reported to inhibit tumor formation in a murine model of UVB photocarcinogenesis (130).

PAF and UVB and MVP

Environmental stressors which produce PAF such as thermal burn injury or UVB also cause the production and release of MVP (24, 52, 131, 132). Indeed, MVP generated in response to UVB or thermal burn injury by keratinocytes *in vitro*, human skin *ex vivo*, and murine skin *in vivo* is dependent upon the PAFR (24, 25). Finally, MVP produced in response to UVB or thermal burn injury contain PAFR agonistic activity (24).

The picture that is emerging (see **Figure 1**) fits with the hypothesis that UVB generates ox-GPC PAFR agonists, which in turn act upon the PAFR-positive keratinocyte resulting in PAFR activation. This PAFR activation then results in additional PAF enzymatically, as well as increased ROS and thus more ox-GPC. PAFR activation also translocates aSMase which then triggers MVP release. The MVP which contain PAF and ox-GPC then travel from the epidermis. One of the critical PAFR cell types these UVB-generated MVP (UVB-MVP) encounter is the dermal mast cell, which contributes to both UVB acute responses as well as delayed systemic immunosuppression.

Potential Pharmacologic Strategies

Knowledge gaps which need to be addressed include the role the PAF system and UVB-MVP in photosensitivity, as well as in photocarcinogenesis. Of note, a human study using the COX-2 inhibitor celecoxib demonstrated decreased numbers of skin cancers (133), which could be in part related to the ability of these agents to block UVB-mediated systemic immunosuppression (6–9). Since MVP generation and release can be modulated pharmacologically by aSMase inhibitors such as imipramine (25, 29), addressing these

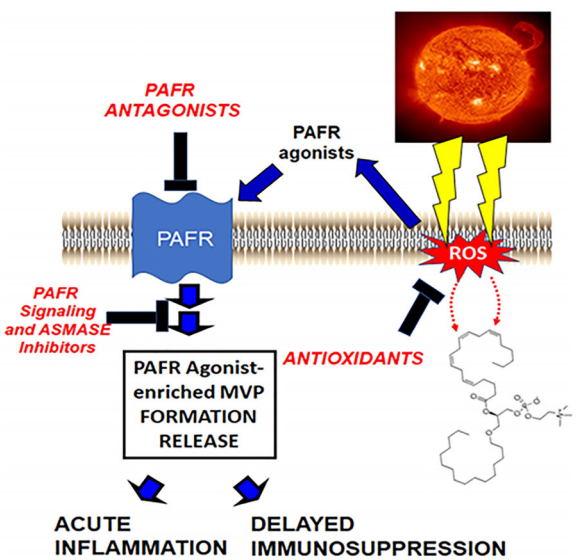

FIGURE 1 | Hypothetical model by which UVB generates PAFR agonists *via* ROS which then result in PAF-laden MVP release. In this model, ROS generated by UVB result in ox-GPC as well as enzymatic PAF synthesis. These PAFR agonists act upon the PAFR resulting in MVP generation release *via* acid sphingomyelinase activation. These MVP contain bioactive agents, especially PAFR agonists which then can mediate UVB effects.

knowledge gaps could result in novel strategies for managing photodermatology disorders and photocarcinogenesis.

DISCUSSION

Though much has been learned about this class of lipid mediators first described almost 50 years ago, significant knowledge gaps remain. Several major hurdles have limited our understanding of the PAF system. First, there is a tremendous heterogeneity of GPC species produced enzymatically and even more *via* non-enzymatic processes which can exert PAFR agonistic effects (5, 15). Moreover, some members of the PAF family of mediators include the 1-acyl species which are in much greater abundance than 1-alkyl GPC, yet have much lower binding affinities and have been suggested to act as antagonists (120, 134, 135). PAF is rapidly metabolized, with a half-life in biological fluids measured in minutes. Hence, it is challenging to be able to accurately measure PAFR activity in biologic systems. A second issue that potentially limits the study of, and ability to use pharmacologic tools is also that PAFR antagonists are of much lower affinity than the most active PAF species, and that inhibitors of the biosynthetic and inactivation enzymatic pathways are not available.

A relatively new area which could result in an enhanced understanding of the PAF system relates to how PAF is released from a host cell. Extracellular vesicles such as MVP appear to be a logical mechanism for the release of PAF and multiple bioactive molecules. Recent findings from our group indicate that PAFR activation is a potent mediator for MVP generation/release, and that these MVP contain PAFR agonistic activity (24). Inasmuch as MVP formation can be blocked by aSMase inhibitors, this allows another potential level of pharmacologic intervention. Future studies should provide new insights into the PAF system which should result in novel targets for diseases of the skin as well as for other systemic disorders.

AUTHOR CONTRIBUTIONS

JT wrote large parts of the manuscript. JR and RS wrote parts of the manuscript and proofread. All authors contributed to the article and approved the submitted version.

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REVIEW
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Oxidized Phospholipids in Control of Endothelial Barrier Function: Mechanisms and Implication in Lung Injury

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Earlier studies investigating the pathogenesis of chronic vascular inflammation associated with atherosclerosis described pro-inflammatory and vascular barrier disruptive effects of lipid oxidation products accumulated in the sites of vascular lesion and atherosclerotic plaque. However, accumulating evidence including studies from our group suggests potent barrier protective and anti-inflammatory properties of certain oxidized phospholipids (OxPLs) in the lung vascular endothelium. Among these OxPLs, oxidized 1-palmitoyl-2-arachdonyl-sn-glycero-3-phosphocholine (OxPAPC) causes sustained enhancement of lung endothelial cell (EC) basal barrier properties and protects against vascular permeability induced by a wide variety of agonists ranging from bacterial pathogens and their cell wall components, endotoxins, thrombin, mechanical insults, and inflammatory cytokines. On the other hand, truncated OxPLs cause acute endothelial barrier disruption and potentiate inflammation. It appears that multiple signaling mechanisms triggering cytoskeletal remodeling are involved in OxPLs-mediated regulation of EC barrier. The promising vascular barrier protective and anti-inflammatory properties exhibited by OxPAPC and its particular components that have been established in the cellular and animal models of sepsis and acute lung injury has prompted consideration of OxPAPC as a prototype therapeutic molecule. In this review, we will summarize signaling and cytoskeletal mechanisms involved in OxPLs-mediated damage, rescue, and restoration of endothelial barrier in various pathophysiological settings and discuss a future potential of OxPAPC in treating lung disorders associated with endothelial barrier dysfunction.

Keywords: oxidized phospholipids, OxPAPC, endothelial barrier, inflammation, lung injury, Rho GTPases, receptor

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INTRODUCTION

In various pathological conditions, especially during inflammation and oxidative stress, circulating and cell membrane phospholipids undergo oxidation to form a diverse group of oxidized phospholipids (OxPLs). These bioactive OxPLs possess profound biological activities and exert both beneficial and harmful effects on human body governed by their biochemical and biophysical

properties. It has been long recognized that OxPLs accumulate in atherosclerotic lesions (1–3), and later studies have shown OxPLs elevation in other cardiopulmonary disorders driven by increased inflammatory responses [Reviewed in (4)]. However, emerging evidence suggests that OxPLs not only induce and propagate inflammatory response but may also contribute to the host response, resolution of inflammation and protection of vascular endothelial barrier properties (5). Endothelial cell (EC) lining of the vascular lumen forms a barrier that controls the passage of fluids, macromolecules, and immune cells between the blood and underlying tissue. The disruption of this selective and semi-permeable barrier results in uncontrolled passage of harmful substances leading to the development of pulmonary edema and acute lung injury (ALI) observed in many disorders: sepsis, severe infection, trauma, toxin inhalation, etc., and culminating in acute respiratory distress syndrome (ARDS) (6, 7). OxPLs play a dual role in regulating endothelial function: some species of OxPLs have been involved in enhancing endothelial barrier integrity while others increased endothelial permeability. The wide structural heterogeneity of OxPLs has been suggested to be responsible for their contrasting biological activities (8, 9). For instance, full length oxidation products of a major membrane phospholipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (OxPAPC, focus of this review) possess potent endothelial barrier protective and anti-inflammatory properties (10). On the other hand, truncated or fragmented products of PAPC oxidation induce acute endothelial barrier disruption and inflammation (11). Multiple signaling pathways including receptor-mediated and cytoskeletal reorganization have been implicated in OxPAPC-induced upregulation of endothelial function which will be discussed in detail in the following sections.

GENERATION OF OxPLs

A diverse spectrum of OxPLs are generated from the oxidation of phospholipids that contain polyunsaturated fatty acids (PUFA) at their *sn*-2 position. PUFAs are highly prone to oxidative modifications by a group of specific enzymes such as cyclooxygenases (COX) and lipoxygenases (LOX) or reactive oxygen species (ROS)-mediated non-enzymatic lipid oxidation (9, 12, 13). Briefly, fatty acids such as arachidonic acid (AA) and linoleic acid (LA) are released from membrane phospholipids by phospholipase A2 (PLA2). In turn, COX-mediated oxidation of AA produces prostaglandins (PGs) and thromboxanes whereas LOX-catalyzed metabolic pathways yield leukotrienes, lipoxins, resolvins, protectins and eoxins. Multiple studies have shown the modulation of endothelial function by PGs but it is out of the scope of this manuscript. Briefly, PGI2, PGE2 and PGA2 exhibited potent, although transient barrier protective activities in pulmonary endothelium in comparison to OxPAPC effects (14, 15). These PGs enhanced endothelial barrier by stimulating cAMP production leading to PKA-dependent activation of Rac and PKA-independent activation of Epac/Rap1/Rac1 signaling cascade (14). PGE2, PGI2, and PGA2 also protect against thrombin-induced hyperpermeability *in vitro* and LPS-induced lung injury *in vivo* (15, 16). Similarly, PGD2 has been shown to enhance endothelial barrier function and protect against ALI (17–19). Furthermore, stable analogs of PGs such as beraprost and iloprost possess protective and anti-inflammatory activities in pulmonary endothelium (20–23). Both COX and LOX are involved in the generation of hydroxyeicosatetraenoic acids (HETEs) from AA oxidation and hydroxyoctadecadienoic acids (HODEs) from LA oxidation. Free radicals-mediated non-enzymatic oxidation of phospholipids produces a heterogenous mixture of bioactive OxPLs species through the classical pathway of lipid peroxidation characterized by initiation, propagation, and termination steps (24).

PAPC, a major membrane phospholipid, undergoes oxidation resulting in generation of wide varieties of full length as well as fragmented oxidized products. The full-length OxPAPC products contain same number of carbon atoms in oxidized arachidonic fatty acid chain as in their precursor. Examples of such products include 1-palmitoyl-2-(5, 6-epoxyisoprstane E2)-sn-glycero-3-phosphatidyl choline (PEIPC) and 1-palmitoyl-2-(5,6 epoxycyclopentenone) *sn* -glycero-3-phsphocholine (5,6-PECPC), among others. Conversely, fragmented OxPAPC products are oxidatively truncated at the *sn*-2 position. 1-Palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-phosphatidylchonine (POVPC), 1-palmitoyl-2glutaroyl *sn*-glycero-phosphocholine (PGPC), 1-(palmitoyl)-2-(5-keto-6-octene-dioyl)phosphatidylcholine (KOdiA-PC) represent examples of such products (25). Besides phosphatidylcholine, other phospholipids containing different polar groups such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylethanolamine (PAPE) and 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylserine (PAPS) are also subjected to oxidative modifications and exhibit similar effects on endothelial barrier regulation and inflammation (15). Furthermore, lipid peroxidation also results in the production of 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) (24).

CONTRASTING EFFECTS OF OxPLs ON ENDOTHELIAL FUNCTION

A continuous monolayer of EC covers vascular lumen and provides a highly selective semi-permeable barrier between the circulation and underlying tissues. EC barrier controls the passage of fluids, solutes, and cells across the vascular endothelium. Various injurious stimuli disrupt the endothelial barrier integrity leading to an increased endothelial permeability for macromolecules and immune cells that is a hallmark of

Abbreviations: AJ, adherens junction; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; COX, cyclooxygenases; EC, endothelial cell; FA, focal adhesion; LOX, lipoxygenases; LPS, bacterial lipopolysaccharide; MLC, myosin light chain; MYPT1, myosin phosphatase 1; OxPLs, oxidized phospholipids; OxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; PGs, prostaglandins; ROS, reactive oxygen species; S1P, sphingosine 1-phosphate; TLR, toll-like receptor; TJ, tight junction; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; VILI, ventilator-induced lung injury.

numerous disorders such as pulmonary edema, ARDS, sepsis, and other pathologies (26–29). Bioactive lipid mediators may have both, positive and negative impact on endothelial barrier function (**Figure 1**). The principal reason behind the contrasting effects of various species of OxPLs is best explained by structure-function analysis. It appears that different molecular species present in OxPAPC govern its function on endothelial barrier. Precisely, full-length OxPLs species such as PEIPC and PECPC mediate barrier protective and barrier-enhancing effects of OxPAPC whereas sn-2-fragmented OxPLs such as PGPC and POVPC induce endothelial permeability (11, 30). Likewise, polar head groups present in OxPAPC also modulate its barrier function. OxPLs with negatively or positively charged polar head groups such as oxidized phosphocholine and phosphoserine exerted potent and sustained barrier-protective effects (31). But oxidized glycerophosphate lacking polar head group had only transient EC barrier protective effects (31).

A role of OxPLs in various cardiopulmonary disorders including ALI (32), ARDS (33), pulmonary hypertension (34), asthma (35), and cystic fibrosis (36) has been suggested by many studies showing the presence of elevated levels of lipid peroxidation products. Furthermore, OxPLs have a direct impact on EC function, as evidenced by OxPLs-driven effects in chronic vascular inflammation associated with atherosclerosis and manifested by enhanced adhesion of monocytes to EC, augmented expression of several inflammatory genes and secretion of inflammatory cytokines and chemokines such as interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) (2, 8, 37). OxPLs are also shown to cause pro-thrombotic phenotype of EC (38, 39) and act as potent oxidative stress inducers in EC (40, 41).

In contrast to these pathological roles of OxPLs, multiple studies have demonstrated potent anti-inflammatory and endothelial barrier protective functions of OxPLs in host defense against bacterial pathogens. Initial studies demonstrated a protective role of OxPAPC against endothelial dysfunction caused by bacterial wall lipopolysaccharide (LPS) in cultured EC as well as in murine models of ALI (30, 38, 39). Later studies have reported the involvement of OxPAPC in rescue and repair of endothelial function disrupted by various injurious factors: live and killed bacteria, components of bacterial wall, edemagenic agonists (thrombin), inflammatory cytokines (TNFα, IL-6), and pathologic mechanical forces: high amplitude cyclic stretch, disturbed flow [Reviewed in (42)]. Several signaling pathways mediating beneficial effects of OxPLs have been described in endothelium (14). Anti-inflammatory roles of OxPAPC have been summarized in our recent reviews (10, 42). This review will solely focus on OxPAPC-mediated endothelial barrier function.

MECHANISMS OF OxPAPC-INDUCED ENHANCEMENT OF ENDOTHELIAL BARRIER FUNCTION

Endothelial barrier is a dynamic structure that constantly undergoes remodeling in response to mechanical forces and various agonists that positively or negatively regulate barrier function. Altered expression of cell junction proteins, the assembly-disassembly dynamics of adherens junction (AJ - VE-cadherin, α, β, γ, and p120-catenins, nectin) and tight junction (TJ - claudin-5, ZO-1, 2, 3, afadin) protein complexes and reorganization of endothelial cytoskeleton in response to chemical and mechanical stimulation are the major determinants of endothelial barrier integrity. While barrier-disruptive insults (edemagenic agonists or high magnitude cyclic stretch) stimulate

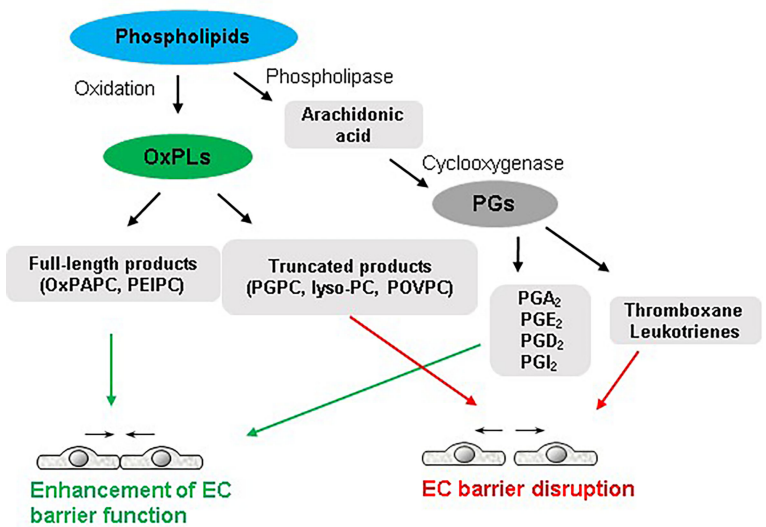

FIGURE 1 | Dual role of phospholipids in endothelial function. Full-length oxidized products of phospholipids and some groups of PGs enhance endothelial barrier integrity. In turn, truncated OxPLs and some products of arachidonic acid such as thromboxane and leukotrienes disrupt endothelial barrier.

stress fiber formation, actomyosin contraction, disassembly of cell junction complexes leading to cell retraction and formation of inter-cellular gaps, barrier-enhancing agonists (OxPAPC, hepatocyte growth factor, sphingosine 1-phosphate) or physiologic mechanical forces (laminar flow, low magnitude cyclic stretch) stimulate enhancement of cortical cytoskeleton, assembly and cooperation of AJ and TJ protein complexes (43, 44). Remodeling of cell junctions and actomyosin cytoskeleton is precisely controlled by signaling protein kinases and small GTPases.

Receptor-Mediated Pathways

A number of receptor-associated signaling pathways mediating OxPAPC actions on lung endothelium has been described. The earlier studies discovered important anti-inflammatory effects of OxPAPC *via* interference with toll-like receptors (TLRs) inflammatory signaling cascade activated by LPS (45, 46). Analysis of OxPAPC-mediated endothelial barrier enhancing mechanisms revealed Akt-dependent transactivation of sphingosine 1-phosphate receptor 1 (S1P1) *via* threonine phosphorylation in caveolin-enriched microdomain that stimulated Rac1- and Rap1-dependent pathways of peripheral F-actin and cell junction enhancement (47). The role of caveolin and S1P1 in mediating the protective effects of OxPAPC was further verified *in vivo* in VILI-induced lung injury where siRNA-mediated knockdown of caveolin or S1P1 abolished the OxPAPC-mediated protection of vascular leak (47). Further analysis elaborated that OxPAPC-induced activation of S1P1 is dependent on the binding of OxPAPC to HTJ-1, a co-factor of cell surface receptor GRP78 (48). siRNA-mediated depletion of HTJ-1 in EC abolished OxPAPC-induced cortical actin formation and knockdown of mouse HTJ-1 homologue suppressed the protective effects of OxPAPC against IL-6 or VILI-induced lung vascular leak (48). Prostaglandin E receptor-4 (EP4) also appears to be involved in endothelial barrier-enhancing responses of OxPAPC as recent study demonstrated that EP4 mediates sustained phase of OxPAPC-induced barrier protective effects (49). A selective role of EP4 in mediating OxPAPC effects was established by the findings that OxPAPC specifically increased EP4 mRNA expression levels in EC and inhibitors targeting EP4 but no other receptors such as EP1-3, PGI2, PGF2, PGD2, and thromboxane had no inhibitory effects on OxPAPC-induced enhancement of EC barrier (49). The role of EP4 in mediating the protective effects of OxPAPC was further established in murine model of ALI as OxPAPC-administered endothelial specific EP4 knockout mice failed to recover from LPS-induced vascular leak and inflammation (49). We recently reported that lipoxin A4 formyl peptide receptor-2(FPR2/ALX) is involved in OxPAPC-induced protection against endothelial permeability caused by TNF-α (50). The endogenous depletion of only FPR2 but no other FPR subtypes inhibited OxPAPC-mediated attenuation of TNF-α-induced increase in endothelial permeability and inflammation suggested the specific involvement of FPR2. Moreover, FPR2 specific inhibitor and FPR2 knockout mice showed reduced inhibition of LPS-induced ALI by OxPAPC further confirmed an essential role of FPR2 in mediating protective effects of OxPAPC (50). Interestingly, FPR2 depletion did not have any inhibitory effects on OxPAPC-induced protection against thrombin-caused EC permeability, suggesting that FPR2 dependent protective pathways also rely on the nature of agonist (e.g., acute vs. chronic effects) that requires further investigations.

Rho GTPases-Mediated Cytoskeletal Remodeling

Ras superfamily of small GTPases specifically Rho GTPases (RhoA, Rac1 and Cdc42) and Rap1 are critical regulators of endothelial barrier in physiological and pathological conditions (51). These GTPases control endothelial permeability by cycling between GTP-bound active and GDP-bound inactive states thereby driving the reorganization of EC junction-associated actin cytoskeleton (52, 53). Among the GTPases, RhoA mediates endothelial barrier disruption caused by various agents while Rac, Cdc42 and Rap1 are involved in maintaining and protecting EC barrier (44, 51, 52, 54). Our initial study showed that cytoskeletal remodeling driven by the combined activation of Rac1 and Cdc42 mediates OxPAPC-induced upregulation of endothelial barrier function (30). The follow-up study revealed that Rac/Cdc42-specific guanine nucleotide exchange factors (GEFs) Tiam1 and betaPIX are involved in Rac-mediated endothelial barrier protective effects of OxPAPC (55). The mechanistic analysis demonstrated that a novel interaction between focal adhesion (FA) and AJ complexes facilitated by the association of paxillin and beta-catenin is essential for Rac/Cdc42-dependent barrier protective responses of OxPAPC (56). Furthermore, Rac effector p21-activated kinase (PAK1)-mediated phosphorylation of paxillin serves as a positive-feedback regulatory pathway contributing to sustained enhancement of endothelial barrier by OxPAPC (57). A further evidence of crucial role of Rac signaling in mediating barrier protective effects of OxPAPC was substantiated by our recent study where knockdown of Rac1/Cdc42 effector IQGAP1 inhibited OxPAPC-induced enhancement of endothelial barrier by suppressing membrane accumulation of AJ proteins VE-cadherin and p120-catenin, and cortactin (58). Consistent with the role of RhoA in endothelial barrier disruption, Rac-mediated barrier protective effects of OxPAPC were achieved by the activation of p190RhoGAP, a negative regulator of RhoA (59). Briefly, OxPAPC-stimulated ECs showed tyrosine phosphorylation and peripheral translocation of p190RhoGAP leading to its association with AJ protein p120-catenin. More importantly, knockdown of p190RhoGAP abolished the protective effects of OxPAPC against the vascular leak induced by pathological cyclic stretch *in vitro* and ventilator-induced lung injury (VILI) *in vivo* (59). Later studies have shown that interaction of p120-catenin with p190RhoGAP was essential for the recruitment of the latter at cell periphery to inhibit Rho signaling (60). This notion was established from the findings that transient expression of p120-catenin mutant lacking 820-843 amino acids residues at C-terminal domain inhibited membrane translocation of p190RhoGAP causing an attenuation of OxPAPC-induced endothelial barrier enhancement resulted from the sustained activation of Rho

signaling and suppression of Rac1 (60). In addition to Rac1/Cdc42, Rap1 is also involved in mediating the barrier protective signals of OxPAPC. Membrane accumulation and increased association of AJ proteins VE-cadherin, p120-catenin, and β-catenin and TJ proteins ZO-1, Occludin, and JAM-A were dependent on OxPAPC-induced Rap1 activation (61). An important role of Rap1 in OxPAPC-induced beneficial effects on vascular endothelium was further substantiated by the findings that Rap1 knockdown suppresses OxPAPC-derived enhancement of EC barrier and represses OxPAPC-induced protection against VILI (61). Our study also demonstrated an essential role of Afadin during Rap1-mediated barrier protective responses of OxPAPC (62). In this regard, OxPAPC treatment of ECs caused Rap1-dependent accumulation of Afadin at cell periphery and its increased association with AJ and TJ proteins- p120-catenin and ZO-1, respectively (62). Consistently, siRNA-mediated knockdown of Afadin or Rap1 binding-defective mutant of Afadin inhibited OxPAPC-induced enhancement of endothelial barrier and Afadin-depleted mice were no longer protected by OxPAPC against VILI-caused vascular leak. Lastly, one of our studies have suggested an existence of Rho GTPase-independent interaction of junction proteins that play a role in OxPAPC-induced positive regulation of endothelial barrier function. In this line, mechanosensitive adaptor protein vinculin seems to be an important regulator of OxPAPC-induced endothelial barrier protection *via* its interaction with AJ protein VE-cadherin (63). The direct activation of Rac1 and Rap1 by OxPAPC leading to enhanced assembly of endothelial cell junction complexes and cytoskeletal remodeling leading to strengthening of endothelial barrier are the best known two critical mechanisms of OxPAPC-induced positive regulation of endothelial barrier (**Figure 2**).

Intracellular Signaling Pathways

A role of intracellular signaling pathways involving various kinases has also been described to mediate the barrier protective signals of OxPAPC. OxPAPC-stimulated ECs showed time-dependent activation of protein kinase A (PKA), protein kinase C (PKC), Raf-MEK MAP kinase cascade, and JNK MAP kinase (64). Furthermore, OxPAPC treatment also induced a transient increase in global tyrosine phosphorylation and Src kinase-dependent phosphorylation of FA proteins- paxillin and FA kinase in EC (64). By employing pathways-specific pharmacological inhibitors, our study showed that PKA, PKC, Src family kinases, and tyrosine kinases play essential roles during OxPAPC-induced enhancement of endothelial barrier (14). The same study ruled out the involvement of Rho kinase, PI3 kinase, and p38 MAP kinases in mediating Rac-mediated barrier enhancing responses of OxPAPC. OxPAPC has been shown to induce the expression of anti-inflammatory proteins such as heme oxygenase-1 *via* activation of PKA, PKC, and MAP kinase pathways (65), and *via* Nrf2 activation (66) but its impact on endothelial barrier function needs to be investigated. An elevation of cAMP levels *via* PKA-mediated or PKA-independent and Rap-mediated pathways induced by OxPAPC are also suggested to play roles in barrier protective effects of OxPAPC (14). **Figure 2** demonstrates a network of signaling pathways activated by OxPAPC and involved in EC barrier regulation. Of note, EC barrier protective effects of OxPAPC seems to vary among different EC types. In general, EC of microvascular origin tend to be more sensitive towards OxPAPC effects with more pronounced protective effects against agonist-induced permeability (67). It will be interesting to study if a variation of OxPAPC effects exists in the

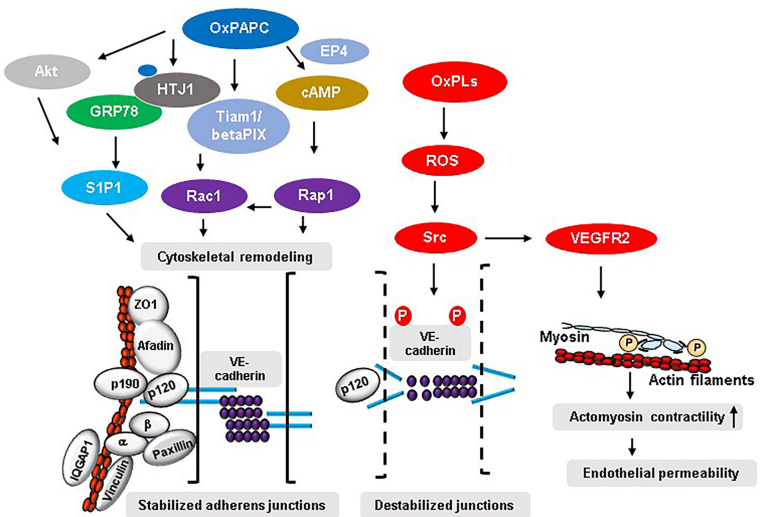

FIGURE 2 | Mechanisms of OxPLs-mediated regulation of endothelial barrier. OxPAPC-induced activation of S1P1 in Akt or GRP78-dependent manner leads to an increased interaction between various AJ, TJ, and FA proteins resulting in enhancement of endothelial barrier integrity. Similarly, OxPAPC-mediated activation of Rac1 and Rap1 induces cytoskeletal reorganization favoring stabilized junctional assembly. On the other hand, truncated OxPLs-stimulated EC produce ROS that activates Src which phosphorylates tyrosine residues in VE-cadherin causing its internalization, degradation, and ultimately leading to disrupted endothelial barrier. Src also activates VEGFR2 that induces endothelial permeability *via* enhanced actomyosin contractility caused by increased myosin light chain phosphorylation.

endothelium from different vascular beds such as lung, heart, and brain.

MECHANISMS OF OxPLs-INDUCED DISRUPTION OF ENDOTHELIAL BARRIER

As discussed above, truncated products of phospholipids oxidation possess endothelial barrier disruptive properties. Moreover, even OxPAPC at higher concentration (≥ 50 μg/ml) causes barrier disruption as opposed to its barrier enhancing function at lower concentrations (5-20 μg/ml) (11, 68). These contrasting biological effects of OxPLs on lung endothelium are largely due to the different signaling pathways activated by them. This notion was best exemplified by one of our studies where the same protein vinculin associated with two distinct proteins- with FA protein talin during thrombin-induced barrier disruption and with VE-cadherin during OxPAPC-induced barrier enhancement (63). The selective activation of various signaling pathways depending on the dose and structural variations of OxPLs appears to be the primary determinants of their biological function.

Role of Vascular Endothelial Growth Factor Receptor 2 (VEGFR2)

The binding of VEGFA to VEGFR2 results in the phosphorylation of the latter leading to the signaling cascades that regulate endothelial survival, proliferation, and migration but VEGFR2 activation is also linked to increased endothelial permeability (69, 70). Interestingly, OxPLs are shown to activate VEGFR2 in EC in Src-dependent manner (71). A role of VEGFR2 in OxPLs-induced endothelial barrier disruption was revealed by our study where only higher concentrations of OxPAPC increased endothelial permeability *via* VEGFR2 activation (72). Mechanistically, higher OxPAPC dose-induced endothelial barrier disruption was accompanied by Rho activation-led increased actomyosin contractility evidenced by increased phosphorylation of myosin phosphatase (MYPT1) and myosin light chain (MLC) that were attenuated by siRNA-mediated knockdown of VEGFR2 (72).

Reactive Oxygen Species (ROS)-Mediated VE-Cadherin Phosphorylation

VE-cadherin is the major adhesive protein present in human EC that is critically essential for maintenance of endothelial barrier integrity (73). Tyrosine phosphorylation of this AJ protein leads to its disassociation from the complex with catenin proteins resulting in increased endothelial permeability (74). ECs treated with higher doses of OxPAPC showed the increased tyrosine phosphorylation of VE-cadherin at tyrosine-658 and tyrosine-731 that was absent in cells treated with low, barrier enhancing doses of OxPAPC (68). The endothelial barrier disruption induced by higher doses of OxPAPC was associated with increased protein tyrosine phosphorylation, activation of Src with phosphorylation at tyrosine 418 and dissociation of VE-cadherin complex with p120- and β-catenin. Furthermore, only high doses of OxPAPC exceeding 50 μg/ml exposure to EC resulted in elevation of ROS levels and anti-oxidant N-acetyl cysteine rescued endothelial barrier disruption caused by OxPAPC (68). An identical pathway of ROS production, Src activation, and VE-cadherin phosphorylation at tyrosine 658 and 731 was observed in ECs treated with PGPC, a truncated OxPLs product (11). Likewise, particulate matter-induced endothelial barrier disruption that is associated with increased production of truncated OxPLs involves similar mechanisms of increased oxidative stress-mediated tyrosine phosphorylation of VE-cadherin (75). Thus, ROS-mediated activation of Src followed by tyrosine phosphorylation of VE-cadherin leading to its internalization and degradation appears to be a unifying mechanism of OxPLs-induced endothelial barrier disruption.

OxPLs IN ENDOTHELIAL DYSFUNCTION-DERIVED LUNG DISEASES

It is now widely accepted that OxPLs play dual roles on regulating the function of vascular endothelium and thus they can be either potentially developed into therapeutics or utilized as targets of therapeutic interventions for various cardiopulmonary diseases that are developed secondary to endothelial dysfunction. In particular, with its proven endothelial barrier-protective and anti-inflammatory activities against a broad spectrum of injurious insults such as bacterial pathogens (76), thrombin (30), LPS (45, 77, 78), and mechanical forces represented by VILI or cyclic stretch (67), OxPAPC holds a strong therapeutic potential against these agonists-induced ALI, ARDS and sepsis. The central role of Rac/Rap1-mediated cytoskeletal reorganization in OxPAPC-induced upregulation of endothelial barrier function makes this molecule attractive therapeutic candidate since the activation of these GTPases act as a common platform for conveying barrier enhancing signals originating from numerous barrier protective agents. The involvement of a number of receptors and kinases in mediating the beneficial actions of OxPAPC on the lung endothelium further provides the opportunity to consider these multiple molecules and associated pathways for the therapeutics.

Some OxPLs products are detected at the site of tissue injury, inflammation and their deleterious effects on vascular endothelium has been well documented (2, 4). More recent studies suggest that these groups of OxPLs can also act as secondary injurious insults and exacerbate endothelial dysfunction. For instance, EC or mice lungs exposed to particulate matter from polluted air resulted in the generation of truncated OxPLs species such as POVPC, PGPC, and lyso-PC that caused acute endothelial barrier disruption (75). Furthermore, when combined with suboptimal dose of particulate matter that does not cause endothelial barrier dysfunction on its own, OxPLs augmented endothelial permeability, indicating their additive role in exacerbation of endothelial function in pre-existing disease conditions. This phenomenon was further evident with the presence of higher basal level of truncated OxPLs in aged mice and corresponding

enhanced increase and delayed clearance of these phospholipids in LPS-treated aged mice compared to their younger counterparts (79). Aged mice were more susceptible to TNF-α-induced lung injury and non-toxic dose of POVPC when combined with TNF-α caused similar levels of lung inflammation. These observations suggest that generation of truncated OxPLs and their additive harmful effects with inflammatory agents further exacerbate lung injury/inflammation in aged population who may already have impaired anti-oxidant system. The definite role of truncated OxPLs as secondary injurious agonist was established by the selective removal of these lipids with platelet-activating factor acetyl hydrolase 2 (PAFAH2) which specifically hydrolyzes truncated OxPLs. The overexpression of PAFAH2 in EC rescued particulate matter- or inflammatory cytokines-induced endothelial barrier disruption, while pharmacological inhibition of PAFAH2 worsened lung injury in TNF-α-challenged mice (75, 79). A few other studies have also reported the presence of higher levels of truncated OxPLs in aged mice (80, 81), and increased production of fragmented phosphatidylcholine in human blood plasma as well as higher levels of ester-linked but lower level of ether-linked phosphatidylcholine in aged human individuals (82, 83). The changes in the levels of lipid profile of ester- vs ether-linked with aging has been attributed as a potential risk factor for the development of various diseases in elderly population. Specifically, ether-linked phospholipids are known to possess anti-oxidant activity and decreased serum levels of these PLs is linked to type 2 diabetes and hypertension (84, 85). Likewise, the oxidation of mitochondrial phospholipid cardiolipin has been shown to contribute to EC necrotic death and increased permeability (86), and hyperoxic lung injury (87). The profile of cardiolipin also undergoes changes with ageing as evidenced by the decrease of total cardiolipin content in mitochondria accompanied by an increase in oxidized forms in heart and brain from aged rats (88, 89). All of these findings suggest that altered lipid profile caused by excessive accumulation of bioactive truncated OxPLs due to exaggerated oxidative stress during aging may play a critical role in propagating associated adverse pathologies. On a positive note, targeting such lipid program switch to selectively inhibit the production or remove pre-formed harmful OxPLs products may provide a potential therapeutic avenue.

FUTURE DIRECTIONS OF OxPL RESEARCH AND CLINICAL APPLICATIONS

Although OxPLs oxidized *in vitro* are increasingly recognized for their anti-inflammatory and lung barrier-protecting activities *in vitro* and *in vivo*, they have a number of serious limitations precluding their therapeutic use. PAPC oxidation *in vitro* yields a complex, structurally diverse mixture of OxPLs. Such natural OxPLs are ineffective for *in vivo* therapy due to their various shortcomings. First, natural OxPLs with either barrier-protective or barrier-disruptive properties are rapidly degraded by phospholipases A that are very abundant in blood plasma, tissues, and cells. Second, natural OxPL mixtures contain a large proportion of oxidatively fragmented molecular species which demonstrate low anti-inflammatory activity in combination with increased toxicity and unwanted effects such as disruption of lung endothelial barrier. Therefore, engineering and synthesis of novel class of phospholipase-resistant phospholipids that have enhanced stability and better biological activities could lead to the discovery of effective drugs that preserve and protect endothelial function and ultimately can be used in the prevention and treatment of endothelial dysfunction-driven diseases. To overcome these limitations, we designed, synthesized, and experimentally tested biological properties of a phospholipase resistant phospholipid incorporating a prostaglandin I_2 stable analog iloprost (ILO) as a prototype synthetic phospholipid compound with barrier-protective and anti-inflammatory properties (90). Such incorporation of ILO into synthetic phospholipid backbone led to more prolonged EC barrier enhancement and more pronounced anti-inflammatory effect, credited to its ability to cause a prolonged activation of Rap1 and Rac. In our continued efforts to synthesize such optimal phospholipids derivatives, recently an alkyl-amide OxPLs was synthesized that retained endothelial barrier enhancement and anti-inflammatory effects (91). As demonstrated by these examples, chemically modified phospholipase-resistant phospholipids may exhibit simultaneously two types of activities. First, these compounds inhibit signaling induced by TLRs (92). Second, these synthetic compounds enhance vascular endothelial barrier, reverse action of edemagenic mediators and prevent formation of lung edema *in vivo* (90, 91). Such poly-pharmacological mode of action can make these compounds especially effective for treatment of severe infections leading to the development of lung edema.

The generation of chemically diverse species of OxPLs and corresponding contrasting biological effects requires precise structure-function analysis of these lipid mediators to define their accurate pathophysiological roles. The advancements in liquid chromatography-mass spectrometry (LC-MS) techniques along with the use of sophisticated bioinformatics analysis has now made possible to detect minor modifications in OxPLs structure and its possible impact on biological activities. These advanced analysis and detection techniques combined with omics analysis approaches including lipidomics, metabolomic, transcriptomic, and proteomics will reveal the signaling cascades associated with OxPLs that will assist in identifying potential therapeutic targets for OxPLs-derived diseases. Furthermore, oxidative phospholipidomics analysis may serve as a valuable tool for identification of biomarkers as suggested by a recent study characterizing oxygenated cardiolipins and phosphatidylethanolamines as predictive biomarkers of apoptotic and ferroptotic cell death, respectively (93). Future projects also should consider exploring the role of OxPLs in the pathogenesis of other diseases besides its established role in cardiopulmonary disorders since recent findings suggest their

involvement in other diseases including aging (79) and traumatic brain injury (94). The translation of potent beneficial effects of OxPAPC into clinics is the most exciting challenge ahead and other additional molecular targets such as receptors and Rho GTPases that mediate OxPAPC actions on lung endothelium could also be considered for therapeutic targets. Moreover, enzymes such as PAFAH2 represent another category of potential therapeutic candidates to prevent excessive accumulation of deleterious OxPL products.

CONCLUSION

It is now widely appreciated that the complexity of contrasting biological effects of OxPLs largely relies on their structural heterogeneity with full-length oxidized products exerting beneficial effects and truncated species acting as pathogenic factor in various cardiopulmonary disorders. The studies so far have established that OxPAPC itself or signaling intermediates that mediate its functions on endothelium, barrier-disruptive OxPLs targeting enzymes such as PAFAH2, and structure-based synthetic analogues of barrier-protective OxPLs could be developed into therapeutics against acute endothelial dysfunction associated with injury, infection, or sepsis. The utilization of LC-MS advanced detection techniques in combination with next generation omics analytical tools has enabled to precisely monitor the lipid modifications and possibly use these modifications as biomarkers for various pathologies. Thus, the dual biological nature of OxPLs presents the opportunity to consider both "good" and "bad" oxidized products of circulating or membrane phospholipids for therapeutic interventions.

AUTHOR CONTRIBUTIONS

PK and KB outlined the review. PK drafted the manuscript and KB finalized. All authors contributed to the article and approved the submitted version.

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