New families of bioactive oxidized phospholipids generated by immune cells:
identification and signaling actions

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Running title: New bioactive lipids generated by immune cells.

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Abbreviations: LOX, lipoxygenase, COX, cyclooxygenase, HETE,
hydroxyeicosatetraenoic acid, HpETE, hydroperoxyeicosatetraenoic acid, KETE, keto-
eicosatetraenoic acid, PE, phosphatidylethanolamine, PC, phosphatidylcholine, LC-
MS/MS, high performance liquid chromatography, PLA₂, phospholipase A₂,
Abstract
Phospholipids are of critical importance in mammalian cell biology, both through providing a permeability barrier and acting as substrates for synthesis of lipid mediators. Recently, several new families of bioactive lipids were identified that form through the enzymatic oxidation of membrane phospholipids in circulating innate immune cells and platelets. These comprise eicosanoids attached to phosphatidylethanolamine (PE) and phosphatidylcholine (PC) and form within 2 - 5 min of cell activation by pathophysiological agonists, via the coordinated action of receptors and enzymes. In this review, we summarize what is currently known regarding their structures, mechanisms of formation, cell biology, and signaling actions. We show that phospholipid oxidation by acutely activated immune cells is a controlled event, and propose a central role in regulating membrane biology and innate immune function during health and disease. We also review the mass spectrometry methods used for identification of the lipids, and describe how these approaches can be used for discovery of new lipid mediators in complex biological samples.
1. Introduction

During infection and injury, circulating innate immune cells and platelets respond acutely to a variety of pathophysiological agonists that mediate profound changes in both their function and physical state. Significant alterations in the lipid compartment, including changes to the plasma membrane and generation of potent signaling mediators occur within 2 - 5 min of stimulation\(^1\)-\(^4\). Activation of neutrophils, monocytes and platelets leads to several common events at the plasma membrane, including shape change, flattening, adhesion, phagocytosis, microvilli and microparticle generation\(^5\)-\(^6\). Phospholipids are the building blocks of the cell membrane, forming a permeability barrier and providing substrates for generation of important signaling mediators, including platelet-activating factor (PAF), phosphoinositides, diacylglycerides (DGs), sphingosine-derived phospholipids, phosphatidic acids and eicosanoids. All of these are formed acutely in immune cells through the actions of phospholipases and other enzymes, for example, phospholipase A\(_2\) (PLA\(_2\)), that hydrolyzes phospholipids at sn2, generating fatty acid substrates for oxidation by cyclooxygenases (COX) and lipoxygenases (LOX)\(^7\)-\(^8\). Thus, acute activation of innate immune cells and platelets results in significant remodeling of the lipid compartment. However, how this is organized on a molecular and cellular level, particularly in terms of membrane lipid composition and biophysics is not well understood.

One group of phospholipid signaling mediators that has been extensively studied in recent years is oxidized phospholipids, generated through non-enzymatic redox cycling reactions that occur in chronic inflammation and atherosclerosis. Hundreds of species are known to form, with only a select few analyzed for structure and function to date (reviewed in\(^9\)). Early studies on these lipids focused on characterizing the biological action of air-oxidized phosphatidylcholine (PC) in vitro\(^9\)-\(^11\). Later, investigators began to fractionate and focus on individual species, including 16:0-05:0(ALDO)-PC (POVPC), and 16:0-
05:0(COOH)-PC (PGPC)\textsuperscript{12-14}. Since these lipids are generated through non-enzymatic reactions, their formation is considered to be uncontrolled and associated with later stage disease, as opposed to a regulated event triggered during innate immune cell activation.

The advent of new generation benchtop mass spectrometers, coupled with high performance liquid chromatography (LC-MS/MS) has led to a significant resurgence in the study of lipids in health and disease\textsuperscript{15-17}. Over the last 10 years, structural identification of low abundance phospholipid molecular species in limited amounts of highly complex cellular lipid extracts has become possible. This has revolutionized the field, with recent highlights including the mass spectrometry analysis of phosphoinositides in neutrophils, studies on the role of phosphatidylcholine metabolites in cardiovascular disease and detailed profiling of the plasma lipidome in humans\textsuperscript{18-20}.

In this review, we focus on how LC-MS/MS has been used to uncover new families of enzymatically-oxidized phospholipids generated by activated human immune cells and platelets. These differ from the non-enzymatically oxidized phospholipids described above, since they comprise a small number of specific molecular species, and they are formed acutely on immune cell activation. We describe the identification and characterization of these new lipid species, followed by what is known regarding their in vivo generation and biological functions. Lastly, we describe how recently-developed high-resolution rapid-scanning instruments offer significant new possibilities in terms of powerful approaches that aim to discover new structures, and have the potential to redefine how we study the biology and biochemistry of lipid mediators in the immune system.

2. Early studies on esterified eicosanoid generation by immune cells.

During immune cell activation, phospholipase A\textsubscript{2} (PLA\textsubscript{2}) hydrolysis of phospholipids releases free arachidonate, which is oxidized by lipoxygenase (LOX), cyclooxygenase
(COX) or cytochrome P450 (CYP) to generate eicosanoids, including prostaglandins E<sub>2</sub> and D<sub>2</sub>, thromboxane A<sub>2</sub>, leukotrienes, hydroxyeicosatetraenoic acids (HETEs) and others<sup>21,22</sup>. Many are important signaling molecules in innate immunity, through regulating hemostasis, pain, fever, cell adhesion, proliferation and tissue regeneration. Traditionally, eicosanoids have been considered free acid mediators, containing a carboxyl group at the site of PLA<sub>2</sub> hydrolysis. In 1998, Kühn and co-workers noted that alkaline hydrolysis of lipids from ionophore-activated human eosinophils released significant amounts of 15-HETE that had been attached to membrane lipids<sup>23</sup>. However, neither the molecular structures of the HETE-containing lipids, nor their mechanisms of generation were investigated. Around this time, several other groups conducted elegant studies on incorporation of exogenously-added HETE standards into immune cells<sup>24-26</sup>. However, it has since become clear that incorporation of exogenous HETEs into membranes is very different to the fate of endogenously-generated eicosanoids formed via oxidation of cellular substrate, and so this will not be further discussed in this review.

In 2005, Marnett and co-workers demonstrated the cellular generation of esterified prostaglandins for the first time, including both PGE<sub>2</sub> and PGD<sub>2</sub> attached to glycerol and ethanolamine<sup>27-29</sup>. These lipids were initially identified in vitro, generated using purified COX-2, but then subsequently in murine macrophages with endogenous substrate. Shortly after, while investigating the cell biology of 15-LOX, we also noted that significant 15-HETE was acutely generated as esterified products by interleukin-4-treated human monocytes. To determine the molecular species to which this was attached, a targeted lipidomic approach was developed that utilized precursor scanning LC-MS/MS to “fish” for lipids that contained a HETE functional group. These studies led to the discovery of several additional families of phospholipid-esterified eicosanoids, and are described in
detail in the following sections. As part of this work, quantitative assays for all these lipids were established, and protocols are provided in 30.

3. Generation of esterified eicosanoids by monocyte/macrophages. The leukocyte 15-LOX is induced in human monocytes by Th2 cytokines, and its murine homolog 12/15-LOX is highly expressed by certain resident macrophage populations. In mice, genetic deletion of 12/15-LOX protects against atherosclerosis, diabetes and hypertension31-35. 12/15-LOX−/− macrophages show defects in PPARγ and TLR4 signaling, as well as altered IL-12 synthesis, and defective phagocytosis 34, 36. The primary product of 15-LOX, 15-HpETE, is rapidly reduced by glutathione peroxidases to 15-HETE, which can then be oxidized to the electrophilic lipid, 15-oxo-ETE, using prostaglandin dehydrogenase 37. How 12/15-LOX signals to control macrophage biology is not well understood, since known free eicosanoid metabolites (such as HETEs) do not effectively restore the phenotype of LOX-deficient cells 38.

In 2007, precursor scanning of lipid extracts from ionophore-treated IL-4-induced human monocytes revealed four molecular species attached to 15-HETE, subsequently identified as three plasmalogen and one acyl phosphatidylethanolamine (16:0p, 18:1p, 18:0p, and 18:0a/15-HETE-PE) (Figure 1A). Chiral chromatography demonstrated that they contained primarily the 15S-HETE enantiomer, confirming their enzymatic generation, and that they comprised approximately 30% of the total 15-HETE generated 39 (see also erratum for this study). Stable isotope labeling studies revealed that they are generated through direct oxidation of the intact phospholipid by 15-LOX. Analogous 12-HETE-containing PEs are also found in murine peritoneal macrophages, and these are absent in 12/15-LOX−/− (Figure 1B) 40. Unlike free HETE, the lipids are not secreted suggesting an autocrine or local mode of action. More recent studies on human monocytes and murine macrophages
have uncovered several additional related families, including PEs containing either 15- or 12-hydroperoxyeicosatetraenoic acid (HpETE), or 15- or 12-keto-eicosatetraenoic acid (KETE) (Hammond et al, unpublished) (Figure 1C). Up to 1.5% of the total cellular PE pool contains 15-HETE, raising the possibility that local concentrations of esterified eicosanoids could be high enough to regulate biophysical changes to the plasma membrane of activated monocytes/macrophages. Of relevance, 12/15-LOX-deficient macrophages are unable to mount a normal phagocytic response in vitro 41, and we recently observed that they contain larger numbers of cytoplasmic vesicles and abnormal mitochondria, that could represent defective autophagy or exosomal processing (Hammond et al, unpublished).

In vitro, both 18:0a/15-KETE- and 18:0a/15-HETE-PE weakly activate PPARγ transcriptional activity, consistent with the known signaling defect of this transcription factor in murine macrophages deficient in 12/15-LOX (Hammond et al, unpublished and 36). 18:0a/15-HETE-PE also inhibits induction of several cytokines (incl TNFα and GCSF) by lipopolysaccharide (LPS) 40. This most likely results through HETE-PE binding to LPS-binding protein and/or CD14, and is similar to how non-enzymatically oxidized PC analogs were previously shown to act as Toll-like receptor 4 (TLR4) antagonists 42. In these experiments, amounts of HETE-PEs added (< 320 ng/4 x 10^6 cells) were somewhat higher than levels endogenously generated by monocytes (approx. 10 ng or 151 ng/4 x 10^6 for basal and activated cells, respectively) 39. However we note that HETE-PEs are poorly incorporated by leukocytes, with only 5 – 10 % becoming cell-associated over 24 hrs (unpublished data). Thus, added amounts will be much lower than that ultimately present in the cells, and can be considered biologically-relevant. Also, exogenously added lipid will be present in the supernatant primarily, rather than cell associated, as for endogenous HETE-PE. Overall, these signaling actions suggest that HETE-PEs generated by monocyte/macrophage 12/15-LOX display anti-inflammatory bioactivities. This is
consistent with the observation that this LOX isoform is constitutively expressed and basally active in non-inflammatory activated resident macrophage populations, and is cleared during acute peritoneal inflammation 38, 40.

A potential role for esterified eicosanoids in Th2 inflammation is suggested by observations that 12-HETE-PEs are generated during murine lung allergy in vivo, corresponding with the time of greatest IL-4, IL-13 and eosinophil influx 40. Furthermore, two 15-HETE-PEs are generated in response to IL-13 and A23187 by human bronchial epithelial cells in culture 43. 15-HETE-PE was recently shown to bind to PEBP1 in these cells, and induce its dissociation from Raf-1 44. This has downstream effects in activating ERK and elevating IL-4Rα related gene expression, suggesting that the pathway promotes Th2 pathways relevant to asthma pathogenesis. In these experiments, the concentration of HETE-PE added (782 ng/10⁶ cells) is somewhat more than that routinely generated by these cells (typically 10 - 150 ng/10⁶ cells). However, as for monocytes, HETE-PEs are poorly incorporated by bronchial epithelial cells in culture. Lastly, a recent study from Krönke and colleagues showed that resident peritoneal macrophages express binding sites for soluble receptors for apoptotic cells, such as milk fat globule-EGF factor 8, through the presence of oxidized phospholipids on the surface of the cells. This occurs in a 12/15-LOX-dependent manner, regulates clearance of apoptotic cells and maintains immunologic tolerance 45. The identity of the specific oxidized phospholipids involved is not fully clarified. A summary of mechanisms of formation and known signaling actions for 15-LOX-derived esterified eicosanoids in monocytes/macrophages is shown (Figure 2).

4. Generation of esterified eicosanoids by neutrophils.

Neutrophils express a 5-LOX isoform that is activated by several agonists including bacterial peptides, chemokines and chemical stimuli such phorbol and calcium ionophore.
The primary 5-LOX product, 5-HpETE, is the precursor for leukotriene synthesis, or can be converted by glutathione peroxidases (GPX) to the more stable 5-HETE, which in turn can be oxidized to the potent chemoattractant, 5-oxo-ETE \(^{46}\).

We conducted precursor scanning of lipid extracts from ionophore-activated human neutrophils and detected four molecular species, that were subsequently identified as one HETE-PC (16:0a/5-HETE-PC), and three 5-HETE-PEs (18:0p/, 18:1p/, and 16:0p/5-HETE-PE) \(^{47}\) (Figure 1D). No other positional isomers are present suggesting that they are generated via 5-LOX activity. Similar to 15-HETE-PEs, they retained by the cells, and are localized equally in both nuclear and non-nuclear membrane fractions, consistent with the known translocation of 5-LOX that occurs on neutrophil activation \(^{47}\). The 5-HETE-PC is highly unstable, being metabolized within 5 minutes of its acute generation to unknown products, while 5-HETE-PEs remain cell-associated for up to 3 hrs following their synthesis \(^{47}\). Generation of the lipids in response to fMLP can be significantly enhanced by priming with cytochalasin B, GM-CSF or LPS, and requires calcium mobilization, phospholipase C (PLC), cytosolic phospholipase A\(_2\) (PLA\(_2\)), secretory PLA\(_2\) (sPLA\(_2\)), 5-LOX activating protein (FLAP) and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MAPK/ERK). This indicates a highly coordinated mechanism of formation, involving receptors and intracellular signaling intermediates. Unlike macrophage HETE-PEs, the formation of these lipids requires hydrolysis of arachidonate, and 5-LOX-dependent formation of free 5-HETE, which is then re-esterified back into the phospholipid pool \(^{47}\).

5-HETE-PEs were detected in both human and murine peritonitis, co-inciding with the timepoint of maximum neutrophil influx \(^{47}\). Stimulation of their generation in vitro by live bacteria requires opsonization, indicating that complement or antibody is likely to be
involved. Highest levels were found with gram-positive infection, as expected since neutrophil influx was greatest in these patients 47.

5-HETE-PEs regulate a number of neutrophil activities in vitro, including enhancing superoxide and IL-8 release, and inhibiting neutrophil extracellular trap (NET) release 47. These biological actions were demonstrated at around 7.8 μg added 5-HETE-PE per 10^6 cells, while in contrast neutrophils synthesize around 400 ng/10^6 cells 47. However, as described earlier, exogenously added HETE-PEs are poorly incorporated by primary leukocytes, while almost all that is endogenously-generated is retained 47, and unpublished data. In support of the idea that 5-HETE-PEs mediate these actions when endogenously generated, we found that two pharmacologically-distinct 5-LOX inhibitors (MK886 and zileuton) both enhanced NET generation in vitro 47. Thus, as predicted, inhibition of 5-LOX had the opposing effect to addition of exogenous 5-HETE-PE. NETs are proposed to trap bacteria in the bloodstream where they can be more effectively killed, and limit damage from neutrophil microbicidal proteins. The processes of NET formation are not clear, but have been proposed to involve profound membrane alterations, including dissolving of nuclear and granular membrane, then rupture of plasma membrane 48. Thus, agonist activation of nuclear and plasma membrane phospholipid oxidation by 5-LOX would be consistent with causing cell membrane disruption to facilitate NET release. Overall, whether this is a pro- or anti-inflammatory action in terms of killing bacteria or regulating host damage is not yet clear. A summary of mechanisms of formation and actions of 5-HETE-containing phospholipids is shown in Figure 3.

5. Generation of esterified eicosanoids by human platelets.

Platelets express two eicosanoid generating enzymes, a platelet-specific 12-LOX and cyclooxygenase-1 (COX). The 12-LOX does not appear to play a major role in regulating
direct platelet function since rather high levels of free 12-HETE are required for any effect on the cells. On the other hand, COX-1 generates eicosanoids that are of central importance in thrombosis and hemostasis. The primary COX-1 product, prostaglandin H₂ (PGH₂) is unstable and rapidly converted to thromboxane A₂ (TXA₂) by thromboxane synthase (TXS). TXA₂ is a potent activator of platelet aggregation via the thromboxane receptor (TP), and also can stimulate smooth muscle contraction. Both these enzymes are activated acutely during platelet activation by thrombin or collagen in a calcium-dependent manner.

Precursor scanning of thrombin or ionophore-activated platelet lipid extracts for HETE-containing-lipids identified six species, comprising two PCs (16:0a/18:0/12-HETE-PC) and four PEs (16:0p/18:1p/18:0p/18:0a/12-HETE-PE) (Figure 1B,E). 12S-HETE was the prominent enantiomer, indicating the requirement for 12-LOX. Furthermore, they are absent in platelets from 12-LOX−/− mice (Aldrovandi et al, unpublished). The lipids are generated within 5 min, and levels continue to increase slowly up to 30 min after activation. Esterified HETEs account for up to a quarter of the total 12-HETE generated, and as for other cells, they remain cell associated. In resting cells, PE is predominantly facing the cytosol, but along with phosphatidylinerine (PS), is externalized on cell activation. This is in contrast to PC which predominantly comprises the outer leaflet. We found that a small amount of 12-HETE-PE becomes externalized following its synthesis. This suggests that oxidized phospholipids may regulate extracellular phospholipid-dependent signaling events. One important process that requires the presence of negatively-charged PE and PS on the cell surface is coagulation. In this, several clotting factors become active through proteolytic cleavage in response to injury (release of tissue factor), associate with the plasma membrane, and generate thrombin. Coagulation factor activity can be tested in vitro by activating the cascade in human
plasma using exogenous tissue factor, in the presence of liposomes of varying composition. Using this assay, we found that HETE-PC dose-dependently stimulates tissue factor-dependent thrombin generation at concentrations found in human platelets \(^{52}\). This is consistent with previous reports showing that non-enzymatically oxidized lipid can enhance coagulation, suggests a role for the lipids in promoting hemostasis\(^{53,54}\). As this is currently a preliminary finding, and its biological relevance is not yet known, the mechanisms involved are currently being explored using recombinant coagulation factors, in vitro.

Thrombin-stimulation of 12-HETE-PE/PC synthesis requires src tyrosine kinases, protein kinase C and sPLA\(_2\) and calcium mobilization, and can be triggered by either protease activated receptors (PAR) 1 or 4 \(^{52}\). Similar to neutrophil 5-HETE-PEs, the platelet lipids are generated through PLA\(_2\) hydrolysis of phospholipid, oxidation by LOX and then re-esterification into the plasma membrane. The timescale of generation is fast, similar to that for free 12-HETE generation, suggesting that the proteins involved maybe coupled together in a tight complex. In support, we found that exogenously added 12-HETE-d8 does not become incorporated into PE or PC during the timescale of 12-HETE-PE/PC synthesis \(^{47}\).

Platelets also generate several additional phospholipid-esterified hydroxyl-fatty acids. These include four PE-esterified 14-hydroxydocosahexaenoic acids (HDOHE), formed via 12-LOX oxidation of docosahexaneoic acid (DHA)-containing phospholipids \(^{55}\) (Figure 1F). Their levels are lower than 12-HETE-PE/PCs, reflecting the lower amounts of unsaturated fatty acid substrate in the platelet membrane. Similar to 12-HETE-PE/PC, generation of esterified HDOHEs requires calcium and phospholipases. Intriguingly, platelets do not appear to generate esterified thromboxane, although we recently identified COX-1-derived PE esterified PGE\(_2\) and PGD\(_2\), formed on activation of platelets with
thrombin (Aldrovandi et al, unpublished). The mechanism of formation and proposed biological actions of 12-HETE-containing phospholipids generated by human platelets are shown in Figure 4.


The identification of esterified eicosanoids described in this review utilized a mass spectrometry method termed precursor scanning that takes advantage of the facile fragmentation of these species to generate a characteristic eicosanoid carboxylate anion on collision-induced-dissociation. This mode is available on standard triple quadrupole instruments, and when combined with an ion trap (e.g. Q-Trap), enables MS/MS to also be performed during elution, greatly aiding structural identification. Using this mode, lipid extracts were analyzed for families of lipids that contain a common functional group, specifically, an eicosanoid. Since the mass spectrometry fragmentation patterns of both eicosanoids and phospholipids are already well known, the structural characterization of these lipids was quite straightforward. This approach could equally be used for lipids containing other functional groups of interest, e.g. particular fatty acids or short chain modified lipids where the fragment generates a negative ion. Where a charged species is not formed on dissociation, neutral loss scanning can alternatively be used. Our methods coupled precursor scanning to high pressure liquid chromatography. Good separation is essential for analysis of complex lipid extracts, since artifactual adducts of HETEs with other matrix constituents can form in the electrospray source that behave similar to esterified HETEs, when direct infusion of complex lipid extracts is performed. In our studies, an extraction method that extracts most lipid species was utilized, where cells or tissue (homogenized in the presence of antioxidants and metal chelators) is vortexed
vigorously in the presence of hexane:isopropanol:acetic acid (2:20:30) at a ratio of 2.5 ml per ml sample. The method is described in full detail in Morgan et al, 2010\textsuperscript{30}.

Another recently described approach involved derivatization of the PE headgroup using isotope-labeled 4-(dimethyamino)benzoic acid (DMABA)\textsuperscript{56}. Generally, selective detection of PE by MS relies on neutral loss of \textit{m/z} 141. However, this scan does not allow detection of plasmalogen species. Headgroup derivatization by DMABA enables all subclasses of PE to be detected by a common precursor ion scan in positive mode, while also allowing detection in negative ion mode for CID, with identification of radyl groups\textsuperscript{57}. Modification of this approach, where control lipids and 2,2'-azobis-(2-amidinopropane) hydrochloride lipid extracts from RAW264.7 cells were derivatized using d0-DMABA or d6-DMABA respectively, allowed identification of several oxidized PEs including truncated structures\textsuperscript{56}.

Precursor scanning works only for lipid families where part of the structure is already known. More recently, we have begun to utilize high resolution rapid scanning, coupled with peak alignment and integration analysis, that can be performed by hybrid ion trap-Orbitrap instruments. This method catalogs all molecular species present in a lipid mixture that form either positive or negative ions, within a defined mass window. Unbiased methods such as this have been utilized primarily in metabolomic studies, but the increasing scanning speed of newer instruments coupled to ultra-high pressure liquid chromatography (UPLC), offers new exciting approaches that are especially suited to lipid discovery studies. Identification of lipids using databases such as the Human Metabolome Database (HMDB, www.hmdb.ca) can be carried out for known structures. A significant challenge lies in identification of unknowns, especially in complex mixtures of 100’s of lipids that are extracted from activated immune cells. Generation of spectral trees using MS\textsuperscript{n} on high resolution instruments will allow determination of elemental composition, and
will aid identification, while traditional derivatization approaches can determine functional groups. While the MS\textsuperscript{n} approach can be carried out in lipid mixtures, derivatization and analysis requires purification and this can be a significant challenge where lipids of interest may be present in very low amounts compared to other constituents.

7. Summary: Common themes of oxidized phospholipid generation by immune cells.

In this review, the identification and characterization of oxidized phospholipids that are generated acutely by human and murine immune cells is described. The lipids represent several families of related structures, containing an oxidized fatty acid moiety attached to a phospholipid. Generation of esterified eicosanoids is restricted to cells that express lipid oxidation enzymes. Thus, under basal or acute inflammatory conditions (e.g. infection), esterified LOX products are generated by resident macrophages or neutrophils \textsuperscript{40, 47}. Induction of 15-LOX by Th2 cytokines leads to generation of 15-HETE-PEs in monocytes and airway epithelium \textsuperscript{43}. Eosinophils are also a likely source of these lipids since they basally express 15-LOX, and are present at elevated amounts in asthmatic airway along with IL-4 and IL-13. Platelets synthesize esterified HETEs, HDOHEs and prostaglandins, thus, these lipids are most likely generated during hemostasis, or in conditions where platelet activation is elevated \textsuperscript{52, 55}.

COX isoforms are either constitutive (COX-1, platelet, renal, gastric) or inducible (COX-2 induced in most cell types by bacterial peptides or pro-inflammatory cytokines). We have observed acute generation of esterified COX-1 lipids in thrombin-activated platelets, but not yet studied COX-2-expressing cells. Since macrophages expressing COX-2 have already been shown to synthesize glyceryl-PGE\textsubscript{2}/D\textsubscript{2} in response to
Lipopolysaccharide, generation of phospholipid-esterified prostaglandins is likely by this isoform 58.

Esterified eicosanoids are closely related to non-enzymatically oxidized phospholipids, that comprise >100’s of molecules originally identified during air or chemical oxidation of unsaturated phospholipids in vitro (for review see 9). Indeed, the molecular structures described herein that originate from LOX or COX turnover, will also be present at low concentrations in non-enzymatically-generated mixtures. The key difference is that enzymes generate a restricted number of specific products, with oxygen insertion effectively dictated by the enzyme pathway involved. Non-enzymatically oxidized phospholipids display pleiotropic biological actions, including some that are common to the lipids described herein, such as modulation of TLR and PPARγ signaling (reviewed in 9).

Non-enzymatically oxidized phospholipids that originate from decomposition of phospholipid hydroperoxides have been measured in inflammation and atheroma (reviewed in 9). However, while the decomposition itself is likely to be uncontrolled, whether the primary hydroperoxides originate from enzymatic or non-enzymatic oxidation in vivo is not clear. An important difference in the processes that generate these two lipid classes is that enzymatically generated oxidized phospholipids are formed through controlled processes involving receptors and intracellular signaling pathways, using enzymes that are conserved among all mammalian species. This indicates that these lipids are of physiological relevance, and likely to be important in innate immune events including hemostasis and bacterial killing. In contrast, non-enzymatic oxidized phospholipids will be generated during chronic inflammation and atherosclerosis, through redox cycling of metals or uncontrolled oxidant toxicity. In both cases, common bioactivities are displayed, although in chronic disease these processes will be occurring in an inappropriate and uncontrolled manner that contributes to the disease, rather than
being required for maintenance of health and homeostasis. Thus, both are of importance in human health and disease, but the relative contribution of signaling will depend on the inflammatory context and the types and amounts of each species generated.

In summary, we describe several new families of enzymatically oxidized phospholipids formed by acutely activated cells. Their generation and retention in the cell occurs in concert with profound changes in plasma membrane function. Thus, future work will determine how the lipids regulate membrane behavior at local level, in particular changes in shape and form such as occurring during phagocytosis, aggregation, microparticle release and chemotaxis.

Acknowledgements

This was supported in part by a grant from the National Institutes of Health (RCM) HL34303, and support from Wellcome Trust, European Union and British Heart Foundation to VBO.

Authorship

This review was co-written and edited by VOD and RCM. The authors declare no conflicts of interest.

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Figure Legends.


Figure 2. Summary of mechanism of formation and action of 12- and 15-HETE-PEs generated by human monocytes and murine peritoneal macrophages. Panel A. HETE- and KETE-PEs are already present in the membranes of IL-4-cultured monocytes and peritoneal macrophages, but their levels are elevated approx. 2-fold on ionophore activation. Generation involves direct oxidation of membrane phospholipids. Panel B. In vitro, HETE-PEs inhibit TLR4 signaling, activate PPAR_γ transcriptional activity, and stimulate dissociation of PEBP1 from Raf. PHGPx, phospholipid hydroperoxide glutathione peroxidase, PGDH, prostaglandin dehydrogenase, PEBP1, PE-binding protein-1, TNFα, tumor necrosis factor-α, GCSF, granulocyte colony stimulating factor, AA, arachidonic acid.

Figure 3. Summary of mechanism of formation and action of 5-HETE-PLs by human neutrophils. Panel A. Generation of the lipids is stimulated via receptor-dependent stimuli, including bacterial peptides, and intracellular signaling mediators. Hydrolysis of arachidonate by cPLA_2 is required, then oxidation by 5-LOX, reduction by GPX, and re-
esterification into the phospholipid membrane. **Panel B.** HETE-PEs enhance superoxide generation and IL-8 release, while inhibiting NET formation. fMLP, N-formyl-methionine-leucine-phenylalanine, PLC, phospholipase C, cPLA$_2$, cytosolic phospholipase A$_2$, FACL, fatty acyl CoA ligase, GPX, glutathione peroxidase.

**Figure 4. Summary of mechanism of formation and action of 12-HETE-PLs in human platelets.** **Panel A.** 12-HETE-PLs are generated in response to thrombin activation of PAR1 and PAR4, via several signaling intermediates. Hydrolysis of arachidonate by cPLA2 is required. **Panel B.** Some HETE-PEs translocate to the outside of the plasma membrane and can enhance tissue factor-dependent thrombin generation. sPLA$_2$, secretory phospholipase A$_2$, FACL, fatty acyl CoA ligase, PLC, phospholipase C.
Figure 2

A

12/15-LOX → Ca\(^{2+}\) → 12/15-LOX → PHGPx → PGDH

Ca\(^{2+}\)

HETE-PE

KETE-PE

B

Translocate to the outside of the cell but are not secreted.

PEBP1

Activation of PPARγ

Inhibit LPS induction of cytokines.

PPARγ

Dissociation of PEBP1 from Raf

TNFα, GCSF

Key

- PC
- PE
- PS
- HpETE
- HETE
- KETE
- AA

Figure 2
Ca^{2+} Enhance IL-8 generation. Inhibit neutrophil extracellular trap release

A

PLC → Ca^{2+} → sPLA_{2}, MAPK/ERK → FLAP/5-LOX/GPX → HETE-PC, HETE-PE

FACL

cPLA_{2}

B

IL-8

Enhance IL-8 generation. Inhibit neutrophil extracellular trap release

O_{2} O_{2}^{•-}

NADPH oxidase

Enhance superoxide generation.

Key

- PC
- PE
- PS
- HETE
- AA

Figure 3
Figure 4

Key
- PC
- PE
- PS
- HETE
- HDOHE
- AA
- PGE2/D2

A

PAR1/PAR4

Thrombin

Src tyrosine kinases,
PLC, sPLA2, Ca^{2+}

cPLA2

12-LOX/GPX

FACL

HETE-PC

HETE-PE

B

Prothrombin

Enhancing tissue factor-
dependent thrombin
generation

Xa/Va

Ca^{2+} Ca^{2+}
New families of bioactive oxidized phospholipids generated by immune cells: identification and signaling actions.

Valerie B. O'Donnell and Robert C. Murphy