Lipidomics of polyunsaturated-fatty-acid-derived oxygenated metabolites

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Abstract
Nutritionally important PUFAs (polyunsaturated fatty acids) mediate some of their bioactivities through formation of oxygenated metabolites. These bioactive lipids are formed by COX (cyclo-oxygenase), LOX (lipooxygenase) and cytochrome-P450-catalysed reactions, as well as non-enzymatic lipid peroxidation. These reactions produce numerous species, some of which can be formed through more than one pathway. MS-based lipidomics offers the selectivity and sensitivity required for qualitative and quantitative analysis of multiple lipid species, in a variety of biological systems, and can facilitate the study of these mediators.

Introduction
PUFAs (polyunsaturated fatty acids) are important bioactive nutrients that exert a plethora of biological effects through their ability to alter the composition of cellular membranes, activate gene transcription and signalling cascades, and be metabolized to a wide range of potent lipid mediators [1–3]. Nutritionally important n–3 and n–6 PUFAs mediate many of their functions through oxygenated metabolites formed via, primarily, enzymatic but also non-enzymatic oxidations. Eicosanoids are some of the best known and studied examples of such lipids: they are derivatives of the C20, C22 and C20, C22, C24 fatty acids; Rv, resolvin; TX, thromboxane. Liquid chromatography; LG, levuglandin; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; MS/MS, hydroperoxyeicosatetraenoic acid; HX, hepoxilin; isoP, isoprostane; LA, linoleic acid; LC, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; EPA, eicosapentaenoic acid; ESI, electrospray ionization; HDHA, hydroxydocosahexaenoic acid; 15-LO, 15-lipoxygenase; 5-LO, 5-lipoxygenase; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; 5-HEPE, 5-hydroxyeicosapentaenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; 12-LO, 12-lipoxygenase; 12-HPETE, 12-hydroperoxyeicosatetraenoic acid; 12-HEPE, 12-hydroxyeicosapentaenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; P, prostanoid; PG, prostaglandin; LT, leukotriene; LX, lipoxin; E, eosin; COX, cyclo-oxygenase; acCOX-2, aspirin-acetylated COX-2; COX-2, cyclo-oxygenase; COX-1, the constitutive COX isoform; CYP450, cytochrome P450; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; COX, cyclo-oxygenase; acCOX-2, aspirin-acetylated COX-2; COX-2, cyclo-oxygenase; COX-1, the constitutive COX isoform; CYP450, cytochrome P450; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; COX, cyclo-oxygenase;}
Figure 1 | Schematic overview of the enzymatic and non-enzymatic reactions mediating the production of oxygenated lipid mediators from AA

FLAP, 5-lipoxygenase-activating protein.

acid) and DHA (docosahexaenoic acid; C\textsubscript{22,6,\textit{n}−3}) to 13-HDHA (hydroxydocosahexaenoic acid) \cite{14,15}. COX-2 can also metabolize PUFAs like arachidonic acid and amides such as 2-arachidonylglycerol and anandamide, to glyceryl-PG and prostamides respectively \cite{16}. Finally, acCOX-2 (aspirin-acetylated COX-2), although not able to act as a COX, appears to retain its oxygenase activity forming exclusively (15\textit{R})-HETE from AA \cite{17}, while EPA and DHA can be metabolized to (18\textit{R})-HEPE (hydroxyeicosapentaenoic acid) and (17\textit{R})-HDHA respectively \cite{15,18,19}. These oxygenated mediators are biochemical precursors of anti-inflammatory di- and tri-hydroxy-PUFA mediators \cite{12}. Cyclopentanone PGs are a family of COX-derived mediators with anti-inflammatory properties. They are generated through non-enzymatic dehydration of PG such as PGD\textsubscript{2} that gives rise to PGJ\textsubscript{2}, Δ\textsubscript{12}-PGJ\textsubscript{2} and 15d-PGJ\textsubscript{2} \cite{20} (Figure 1). These electrophilic lipids exert their activity through various mechanisms, including binding to nuclear and membrane receptors, covalent modification of thiol groups and addition to protein cysteine residues that, in turn, influence redox signalling pathways \cite{21}. Recent reports show the formation of electrophilic oxo-derivatives from DHA and DPA (docosapentaenoic acid, C\textsubscript{22,5,\textit{n}−3}) by acCOX-2, \textit{in vitro}. These reactive mediators may exert signalling effects through adduction to proteins and, in this way, contribute to the anti-inflammatory profiles of n−3 PUFAs \cite{22}.

Deactivation of prostanoids is mediated by 15-PG dehydrogenases, 13-PG reductases, as well as \(\beta\)- and \(\omega\)-oxidation reactions (reviewed in \cite{12}).

**LOX (lipoxygenase) metabolites**

LOXs are stereospecific dioxygenases catalysing the insertion of molecular oxygen into PUFAs that contain at least one (12,4\textit{Z})-pentadiene system \cite{23,24}. The resulting hydroperoxides are reduced to hydroxy fatty acids by glutathione peroxidases or further metabolized to other bioactive lipids. Historically, animal LOXs have been classified according to their positional selectivity when oxygenating AA, namely 5-LOX, 8-LOX, 12-LOX and 15-LOX. However, this system does not always reflect the increased complexity of the field in terms of substrate specificity and phylogenetic relatedness of the isoenzymes.

5-LOX acts in concert with FLAP (5-lipoxygenase-activating protein) to metabolize AA to (5\textit{S})-HPETE (hydroperoxyeicosatetraenoic acid) \cite{25}. This peroxide is then reduced to (5S)-HETE or dehydrated to LTA\textsubscript{4}, an unstable epoxide containing a conjugated triene system characteristic of all (LTs). LTA\textsubscript{4} is further hydrolysed to LTB\textsubscript{4} or conjugated with GSH to form the cysteinyl-LTs LTC\textsubscript{4}, LTD\textsubscript{4}, LTE\textsubscript{4} or contributes to LX formation. (5\textit{S})-HETE can be also enzymatically reduced to the 5-oxo-ETO (5-oxo-eicosatetraenoic acid), a chemoattractant mediator \cite{26} (Figure 1). While human 5-LOX has preference for non-esterified C\textsubscript{20} fatty acids, it can metabolize EPA to generate (5\textit{S})-HEPE and LTB\textsubscript{5}. Overall, AA-derived LT are potent pro-inflammatory mediators involved in allergic, immune and inflammatory diseases, while EPA metabolites are considered less active \cite{3}.

Mammalian 12- and 15-LOX isoenzymes oxygenate, primarily AA and LA as well as other PUFAs including DGLA, EPA and DHA (Figures 1 and 2). Both
isoenzymes metabolize non-esterified and esterified PUFAs found in membrane phospholipids and lipoproteins [23]. Functionally distinctive mammalian isoforms are classed as leucocyte-type, epidermis-type and platelet-type 12-LOXs, and reticulocyte-type and epidermis-type 15-LOX (15-LOX-1 and 15-LOX-2 respectively). These enzymes have some intriguing features: human 15-LOX-1 can oxidize AA in position C-12 as well as C-15, albeit at lower specificity, forming a mixture of (12S)- and (15S)-HPETE and HETE. With LA the main product of 15-LOX-1 is (15S)-HODE. Furthermore, 15-LOX-1 has high homology with leucocyte-type 12-LOX, and these isoenzymes are frequently classed together as 12/15-LOX. Interestingly, one of the epidermis-type 12-LOX isoforms is a unique mammalian (12R)-LOX producing (12R)-HETE [27] found in human psoriatic tissue. Finally, the human 15-LOX-2 gene shows considerable homology with the mouse 8-LOX gene, although, to date, only the human 8-LOX gene has been cloned [28]. Overall, HETE, HEPE and HODE have been implicated in inflammation and immunity, and can mediate pathways related to cell growth [29].

LOX pathways are involved in the formation of various families of bioactive lipids. HXs are epoxyhydroxyicosanoids generated from the isomerization of (12S)-HPETE; HXA2 has been shown to be involved in calcium metabolism [30] (Figure 1). LXs are trihydroxytetraene products of AA formed through sequential reactions mediated by LOX-isosforms that are found in various cell types (transcellular metabolism) [19]: 15-LOX (monocytes, macrophages) and 5-LOX (monocytes) or 5-LOX (leucocytes) and 12-LOX (platelets), generate LXA4, while acCOX-2 or CYP450 (cytochrome P450)-produced (12R)-HETE [discussed in the sections ‘COX (cyclo-oxygenase) metabolites’ and ‘CYP450 metabolites’] is further metabolized by 5-LOX (leucocytes) to generate the epimer 15-epi-LXA4. Similarly, transcellular metabolism of EPA and DHA gives rise to di-and tri-hydroxy PUFAs termed Rvs, PDs and MaRs (maresins) [15,19,31] (Figure 2): EPA is initially transformed to (18R)-HEPE through acCOX-2 or CYP450 [see the sections ‘COX (cyclo-oxygenase) metabolites’ and ‘Metabolites generated through non-enzymatic reactions’], followed by 5-LOX to give RvE1; DHA is metabolized to (17S)- or (17R)-HDHA by 15-LOX or acCOX-2, followed by 5-LOX to give RvD. These mediators are considered anti-inflammatory and protective.

**CYP450 metabolites**

CYP450 mono-oxygenases, better known for their role in detoxification of drugs and xenobiotics, can metabolize PUFAs acting as epoxygenases and hydrolyses [32]: CYP450-catalysed epoxygenation of AA can occur in every double bond resulting in four regio-isomeric cis-EET (epoxyeicosatrienoic acid) (5,6-, 8,9-, 11,12- and 14,15-EET) that can be formed as either R,S or S,R enantiomers. Allylic oxidation of AA can take place between C-5 and C-15 and produces various mid-chain HETE (5-, 8-, 9-, 11-, 12- and 15-HETE) similar to the products of LOX reactions [see section ‘LOX (lipooxygenase) metabolites’]. Furthermore, ω-hydroxylation on Cω–Cω+3, forms 16-, 17-, 18-, 19- and 20-HETE; the presence of an asymmetric carbon in Cω–Cω+1 allows the formation of R- or S-enantiomers [33–35] (Figures 1 and 2). Finally, EET are transformed by epoxide hydrolase to the respective biologically inactive DHET (dihydroeicosatetraenoic acid).

In general, the regioselectivity and stereoselectivity of CYP450-mediated oxygenations are isoform-specific, and various isoenzymes are able to form multiple AA metabolites, as illustrated by the following examples: CYP4A and CYP4F are predominantly ω-hydroxylases producing 20-HETE and, to a lesser extent, 19-HETE; CYP2E1 can form (19R)- and (19S)-HETE at a ratio of 70:30, and optically pure (18R)-HETE; CYPBM-3 (CYP3 from Bacillus megaterium) forms enantiomerically pure (18R)-HETE and (14S,15R)-EET at a ratio 80:20, human CYP2C8 produces (11R,12S) and (14R,15S)-EET with optical purities of approximately 80% (reviewed in [34,35]). EET are involved in vascular relaxation, regulation of cardiac, renal and central nervous system function, angiogenesis and cancer, 20-HETE is a potent vasocostricor, while 18- and 19-HETE appear to mediate vasodilatation [33,36–38].

CYP450s can also metabolize the epoxidation and hydroxylation of a range of n-6 and n-3 PUFAs, such as LA, EPA and DHA [35]. The LA-derived epoxides 9,10- and 12,13-EpOME (epoxyoctadecamonoenoic acid) and HODE are not as well studied as their AA counterparts, although they appear to exhibit some beneficial effects. The main EPA metabolites are various HETEs and five EEQs (epoxyeicosatetraenoic acids), while DHA gives rise to HDHA and six regioisomeric EDPs (epoxydocosapentaenoic acid). CYP450-derived (18R)-HEPE could contribute to RvE formation [discussed in the section ‘LOX (lipoxygenase) metabolites’] [19]. Overall, the ability of CYP450 isoforms to utilize EPA and DHA suggests that the products of these reactions may be responsible for some of the n-3 PUFA bioactivities.

**Metabolites generated through non-enzymatic reactions**

isoPs are a family of regio- and stereo-isomers of prostanoids that are produced by free-radical-induced peroxidation [39]. Contrary to the enzymatically derived PGs, isoPs are formed in situ through the oxidation of phospholipid-esterified AA and are released by PLA2. Furthermore, isoPs differ from the COX-derived PGs in that they are formed as racemic diastereoisomers (Figures 1 and 2). The AA-derived F2-isoPs are chemically stable, have been found in many tissues and biological fluids, appear elevated in many diseases, including atherosclerosis, diabetes and neurodegenerative disease, and are considered reliable markers of lipid peroxidation in humans. Although there is limited work on the bioactivity of isoPs, evidence suggests that 8-iso-PGF2α is a potent vasocostricor. Recently, the formation of IsoP products
from EPA (F₃-isoP) [40] and DHA (F₄-isoP or F₄-neuroprostanes) [41], as well as cyclopentenate isoP [42], has been reported.

Non-enzymatic rearrangement of the unstable endoperoxide PGH₂ can generate LG, while isoP-related pathways form their structural isomers isoLGs (isolevuglandins) [43]. These highly reactive keto-aldehydes can form toxic protein and nucleic acid adducts, and LG are considered to be potent neurotoxic agents.

Lipid peroxidation results in the formation of highly reactive toxic aldehydes. The main metabolite formed upon peroxidation of n–6 PUFAs is HNE (4-hydroxy-2-nonenal) (Figure 2), while n–3 PUFAs generate HHE (4-hydroxy-2-hexanal). HNE and HHE are measured in biological fluids and used as indices of PUFA peroxidation [44]. In addition, during PUFA oxidation, a wide range of regio- and stereoisomers of HODE, HETE, HEPE and HDHA can be formed as racemic mixtures (equal R and S enantiomers) and in a non-selective random manner.

**MS and mediator lipidomics**

MS has played an instrumental role in the discovery and structure elucidation of eicosanoids. Today, MS-based lipidomic protocols offer a versatile, sensitive and accurate means of simultaneously assessing large numbers of PUFA-derived lipid mediators found in a single sample, and in a variety of biological milieux [6,7]. ESI (electrospray ionization) has emerged as the most popular technique for eicosanoids and similar lipid species, since it allows the ionization of these non-volatile compounds without the need for derivatization [45]. These metabolites can form positive ([M + H]⁺) and negative ([M – H]⁻) ion species; however, most applications have been in the negative-ionization mode. Furthermore, ESI is easily coupled with LC (liquid chromatography), allowing for the development of LC–MS assays that combine the immense separation capacity of HPLC or UPLC (ultra-performance LC), and sensitivity of MS. Quantitative analysis is achieved through MS/MS (tandem MS) on an MRM (multiple reaction monitoring) mode, an approach allowing the analysis of species of interest present in complex biological extracts using the formation of compound-specific product ions (Figure 3). Calibration lines using synthetically prepared standards and deuterium-labelled internal standards are then applied for the absolute quantification of the mediators of interest [5,8]. In terms of instrumentation, the most popular spectrometers used in mediator lipidomics are Q³ (triple quadrupoles) or hybrid systems where quadrupoles are coupled to ion trap (Q-Trap) or time-of-flight (Q-TOF) analysers.

These technological developments have now allowed detailed investigations into the role of lipid mediators in complex biological systems and disease states. LC–MS/MS assays permit the simultaneous study of lipids derived from
various PUFA substrates that can be concurrently present and available for metabolism (e.g. $n-3$ and $n-6$ PUFAs competing for the same enzymes), as well as products of specific PUFAs generated by different enzymatic pathways that may be operating in concert or in parallel (e.g. AA metabolized by COX and/or LOX and/or CYP450s) [12]. Targeted lipidomic approaches have already been used to study lipid mediator species involved in CVD (cardiovascular disease), diabetes, neurological disorders, skin inflammation, cancer, pulmonary disease and the reproductive system and have already suggested possible biomarker candidates [35,37,46–49]. This methodology, coupled with transcriptomic and metabolomic approaches, has already shown great potential to become an asset for systems biology approaches [50].

**Concluding remarks**

Oxygenated metabolites of PUFAs play an active role in health and disease, conferring a range of beneficial and toxic effects. Their production through multiple enzymatic and non-enzymatic pathways results in large numbers of bioactive lipids; for instance, AA, one of the most important PUFAs, can generate more than 100 metabolites. The challenges posed by the increased complexity of lipid profiles and the realization that bioactivities conferred by various lipids are varied, and depend on the tissue or biological system in question, have been assisted by MS-based lipidomics. This methodology has the selectivity and sensitivity required to study multiple lipid species, and can be applied to elucidate the bioactivity of PUFAs. Furthermore, mediator lipidomics coupled with transcriptomics and genomics and within a systems biology approach has excellent potential to support the assessment of nutritional interventions and discovery of disease biomarkers.

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