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Lipidomics of oxidized polyunsaturated fatty acids

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ABSTRACT

Lipid mediators are produced from the oxidation of polyunsaturated fatty acids through enzymatic and free radical-mediated reactions. When subject to oxygenation via cyclooxygenases, lipoxygenases, and cytochrome P450 monooxygenases, polyunsaturated fatty acids give rise to an array of metabolites including eicosanoids, docosanoids, and octadecanoids. These potent bioactive lipids are involved in many biochemical and signaling pathways, with inflammation being of particular importance. Moreover, because they are produced by more than one pathway and substrate, and are present in a variety of biological milieus, their analysis is not always possible with conventional assays. Liquid chromatography coupled to electrospray mass spectrometry offers a versatile and sensitive approach for the analysis of bioactive lipids, allowing specific and accurate quantitation of multiple species present in the same sample. Here we explain the principles of this approach to mediator lipidomics and present detailed protocols for the assay of enzymatically produced oxygenated metabolites of polyunsaturated fatty acids that can be tailored to answer biological questions or facilitate assessment of nutritional and pharmacological interventions.

Introduction

Polyunsaturated fatty acids (PUFAs) are precursors of numerous metabolites endowed with potent bioactivities and involved in homeostatic and pathophysiological events in the majority of mammalian cells [1–4]. Although these lipid mediators are, primarily, products of enzyme-mediated oxygenations, compounds with similar structures can be produced by reactive oxygen species (ROS)-initiated reactions [5–8]. Enzymatically derived oxygenated PUFAs originate from three main pathways: the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) reactions (Fig. 1). The diversity of PUFA precursors that can be metabolized in this way is quite broad, resulting in a large number of mediators whose range of bioactivities continue to be the focus of current investigations, while new species are constantly being discovered and their activities remain to be explored.

One of the most studied families of bioactive lipids are the eicosanoids. These C20 carboxylic acids are products of arachidonic acid (AA; 20:4$n-6$), eicosapentaenoic acid (EPA; 20:5$n-3$), and dihomo-γ-linolenic acid (DGLA; 20:3$n-6$) (Fig. 1). Best known for their pivotal role in inflammation, AA-derived eicosanoids are considered to be predominantly proinflammatory, whereas EPA- and DGLA-derived mediators are believed to either oppose or dampen down this effect, altering the biological milieu to an anti-inflammatory one [9]. When metabolized by COX isoforms (i.e., the constitutive COX-1 or inducible COX-2) and subject to the prevalence of terminal synthases these C20-PUFA generate prostaglandins (PGs), prostacyclin, and thromboxanes (TXs), collectively termed *prostanoids* [10,11]. Catabolism through dehydrogenation and reduction results in the formation of metabolites with significantly reduced bioactivities (i.e., 15-keto- and 13,14-dihydro-15-keto PGs), representing part of the biological mechanism that controls tissue levels of bioactive lipids [12].

LOX enzymes insert OH groups in a stereoselective manner and on a variety of PUFA substrates, including esterified acyl species [13–15]. However, LOX activities are commonly defined by their positional selectivity when they oxygenate AA: in this way the main mammalian LOX enzymes are defined as 5-LOX, 8-LOX, 12-LOX, and 15-LOX. With the exception of the unique to mammalian skin 12*R*-LOX, all other LOX isoforms give hydroxy fatty acids of the *S* configuration. LOX isozymes initially produce unstable hydroperoxides that are then reduced to hydroxy acids [15]. When subjected to LOX oxygenation PUFAs can generate an array of mono- and polyhydroxy fatty acids: e.g., AA produces hydroxyeicosatetraenoic acids (HETEs), leukotrienes (LTs), and lipoxins (LXs); EPA generates hydroxyeicosapentaenoic acids (HEPEs) and E-series resolvins (RvE's); docosahexaenoic acid (DHA; 22:6$n-3$) produces docosanoids including hydroxydocosahexaenoic acids (HDHAs), maresins, D-series resolvins (RvD's), and protectins (PDs); linoleic acid (LA; 18:2$n-6$) forms octadecanoids such as hydroxyoctadecadienoic acids (HODEs); DGLA forms hydroxyeicosatrienoic acids (HETrE's), etc. (Fig. 1). Interestingly, partially inhibited COX-2 (e.g., acetylated COX-2

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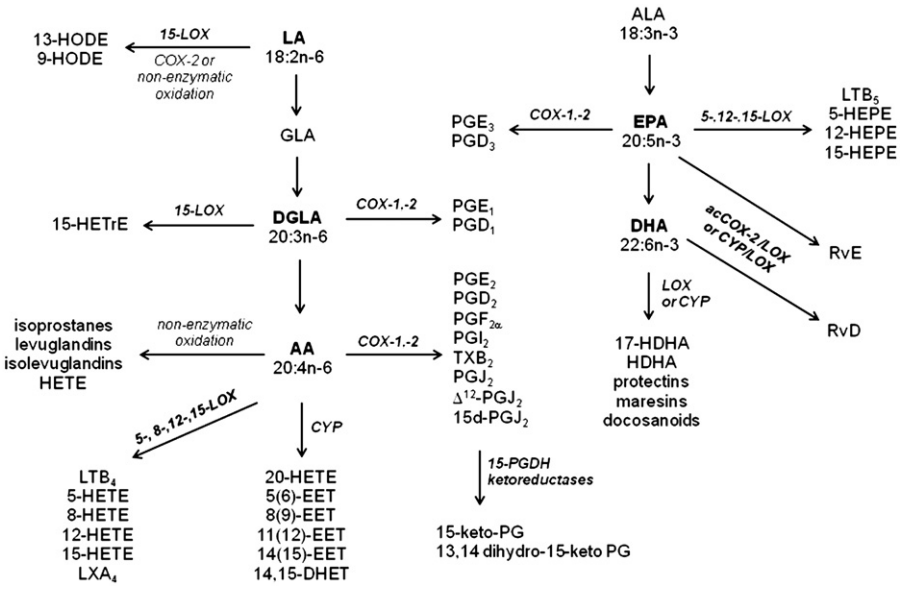

Fig. 1. Schematic showing an abridged overview of the main pathways involved in the production of polyunsaturated fatty acid-derived oxygenated mediators. acCOX-2, acetylated COX-2.

after treatment with aspirin) can generate LOX-like products with the OH group at the *R* configuration, e.g., 15*(R)*-HETE from AA and 18(*R*)-HEPE from EPA. These products are important metabolic precursors of 15-*epi*-LXA_4 and RvE_1, respectively, which, in turn, are generated through sequential LOX reactions via transcellular metabolism [16,17].

CYP monooxygenases relevant to PUFA metabolism catalyze epoxygenations and midchain and ω-hydroxylations [18–20]. Substrate specificity and stereoselectivity varies widely between CYP isoforms, e.g., when using AA as substrate CYP can form epoxides on every one of the four double bonds of the acyl chain, forming epoxyeicosatrienoic acid (EET) regioisomers that are further metabolized by epoxide hydrolases to biologically inactive dihydroeicosatetraenoic acids (DHET) (Fig. 1). Midchain hydroxylations produce a range of LOX-like hydroxy fatty acids (e.g., HETE, HEPE) although these mediators are not necessarily of the *S* configuration and may not even be enantiomerically pure but racemic mixtures (reviewed in [5]).

Finally, ROS-mediated reactions can form a range of small lipid molecules with structures resembling these of enzymatically produced mediators (Fig. 1). Examples include the isoprostanes, a family of PG-like regio- and stereoisomeric derivatives formed through the oxidation of phospholipid esterified PUFAs, the highly reactive keto-aldehydes levuglandins and isolevuglandins, as well as a wide range of monohydroxy fatty acids formed as racemic mixtures [6,8,21–23].

Given the immense biological importance, increasing number, and diversity of PUFA-derived oxygenated metabolites, there is a clear need for a sensitive, selective, and accurate assay system suitable for the qualitative and quantitative analysis of these lipid species. Currently, analysis of eicosanoids and other oxygenated PUFA mediators can be performed using various methodologies: enzyme-linked immunosorbent assays and radioimmunoassays are popular but can measure only one metabolite at a time, are not always selective, can be subject to cross-reactivity, and are available only for certain lipids [24,25]. Gas chromatography coupled to mass spectrometry (GC–MS) or tandem mass spectrometry (GC–MS/MS) has been successfully applied to eicosanoid research, although the need to derivatize the lipids to form volatile species causes limitations including the danger of thermal decomposition [26–28]. High-pressure liquid chromatography (HPLC) with fluorescence detection requires derivatization, whereas HPLC–UV is lacking sensitivity and is applicable only to a limited number of UV-active mediators [29–31]. However, the versatility and high separation power of liquid chromatography (LC—as HPLC or UPLC) when coupled to tandem mass spectrometry (LC–MS/MS) have been proven to be an excellent analytical platform for mediator lipidomic assays with detection limits in the picogram range [32–37].

Overall, mass spectrometry-based mediator lipidomics offers a diverse dynamic tool for the simultaneous analysis of multiple mediators formed by various biochemical routes and all present in one single sample and has already made its mark on lipid research: the approach has facilitated the discovery of novel lipid species while being successfully applied to diverse biological matrices including plasma, brain, liver, pancreas, cutaneous blister fluid, myometrial tissue, spinal fluid, breath condensate, cell culture media, solid tumors, urine, etc. [33–35,38–47]. In this article we describe detailed experimental protocols for the study of enzymatically produced oxygenated metabolites of PUFAs including their extraction from various biological materials, quantitation, and elucidation of chirality. These protocols can be tailored to answer targeted or untargeted research questions, investigate the origin of species of interest, and assess effectiveness of nutritional and therapeutic interventions.

Principles

Mass spectrometry measures the mass-to charge (*m*/*z*) ratio of ionized molecules. Further fragmentation of molecular ion species generates product ions that provide structural information for the compound of interest and can inform the development of sensitive quantitative assays. Electrospray ionization (ESI) is a low-energy (soft) ionization technique applicable to the qualitative and quantitative analysis of lipid species [48,49]. Although ESI generates both positively and negatively ionized species, most applications relevant to oxygenated PUFA mediators are in the negative-ion mode (ES^-), in which they form $[M\text{-}H]^-$ carboxylate ion species in high abundance [50] (Fig. 2).