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Metabolomics-Lipidomics of Eicosanoids and Docosanoids Generated By Phagocytes

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Abstract

Lipid mediators derived from essential fatty acids such as arachidonic acid play important and sometimes pivotal roles in physiologic and pathophysiologic processes. Prostaglandins, thromboxane and leukotrienes are well-known eicosanoids that play a role in hemodynamics and inflammation. More recently, new families of mediators were uncovered that constitute a new genus that stimulate resolution of acute inflammation, are organ-protective and reduce the sequelae of ischemia-reperfusion tissue injury. These include the resolvins (E-series and D-series), protectins (neuroprotectin D1/protectin D1) and maresins biosynthesized from omega-3 essential fatty acids. Phagocytes play a major role in tissues and have a high capacity to produce these mediators, which depend on their tissue and state of activation. Since metabolomic profiling of these biosynthetic pathways in phagocytes can also yield inactive metabolites as well as isomers of specific mediators, it is important to select appropriate methods for identifying target mediators and pathway biomarkers. In this chapter, we review state-of-the-art approaches to identify and profile eicosanoid and docosanoid pathways, including specialized pro-resolving mediators such as resolvins, protectins and maresins that are produced by phagocytes in inflammatory exudates. We provide protocols for isolation and criteria for selecting methods and give examples of metabolomics and lipidomic procedures using liquid chromatography-tandem mass spectrometry-based instrumentation. The approaches reviewed here can provide documentation of bioactive mediators from the eicosanoid and docosanoid-metabolomes in relation to their biosynthesis and inactivation by phagocytes, particularly neutrophils and macrophages.

Keywords

Lipid mediators; inflammation; resolution; prostaglandins; leukotrienes; resolvins; maresins

Introduction

Arachidonic acid is precursor to potent local-acting mediators, also known as autoids, that play key roles in physiological processes and pathophysiologic events including reproductive physiology, hemodynamics, renal function and inflammation (Samuelsson, 1983; Samuelsson et al., 1987). Macrophages play key roles in both host defense and in homeostatic tissue functions and thus have a wide range of functional phenotypes (Russell and Gordon, 2009). Macrophages are endowed with the capability to generate diverse arrays of lipid mediators, depending on their state of activation, tissue of origin and functional roles within the tissue (Fig. 1A). For example, it is well appreciated that isolated human

macrophages, when challenged *in vitro*, can generate prostaglandins, leukotrienes or lipoxins, depending on the stimulus and substrate availability (Calorini et al., 2000). The profiles of lipid mediators produced by macrophages are still an important area for cutting-edge research as macrophages, for example involved in resolution and tissue homeostasis, have the ability to produce novel lipid mediators (Serhan et al., 2009). These small molecules are potent and can alter the function of surrounding tissues as well as play autocrine roles.

In addition to the families of arachidonic acid-derived mediators including prostaglandins, thromboxanes, leukotrienes and lipoxins (Samuelsson et al., 1987), human and murine phagocytes such as neutrophils and macrophages produce novel eicosanoids and docosanoids including the new families resolvins, protectins and maresins (Serhan et al., 2000; Serhan et al., 2002; Serhan et al., 2009). The term eicosanoid refers to the carbon-20 backbone and docosanoid refers to the carbon-22 backbone. Both arachidonic acid (AA) and eicosapentaenoic acid (EPA) are C:20 carbon structures (C20:4 and C20:5, respectively). EPA has five double bonds and AA has four; the bonds are not conjugated. Docosaheptaenoic acid (DHA) is denoted C22:6, indicating that it has six unsaturated double bonds.

These essential fatty acids, particularly EPA and DHA, are enriched in marine oils and are not synthesized to any great extent in mammalian tissues; thus, they are required in the diet (Simopoulos et al., 1999). Arachidonic acid is tightly regulated in its release and is evolved in phospholipid receptor-mediated signal transduction processes (Samuelsson, 1983; Samuelsson et al., 1987). These events with EPA- and DHA-derived mediators have only recently become evident (Kasuga et al., 2008). The resolvins, protectins and maresins are derived from EPA and DHA and play protective, anti-inflammatory and pro-resolving roles in many inflammatory disease models (Serhan et al., 2008). In this chapter, we focus on current state-of-the-art approaches to monitor the production of prostaglandins, leukotrienes and lipoxins from arachidonic acid by human and murine phagocytes as well as to monitor the docosanoids including the new genus of specialized pro-resolving mediators (SPM) produced by phagocytes such as resolvins, protectins and maresins. Since the profiling of these biosynthetic pathways in phagocytes can also yield biologically inactive metabolites and related but inactive isomers of the specific mediators (i.e. *trans* isomers of LTB₄, LXA₄, RvE1, RvD1), we focus this chapter on the procedures required to identify the bioactive target mediator as well as their related biosynthetic profile or pathway metabolome in phagocytes. Some of the cell isolation protocols and procedures have been reviewed elsewhere (Chiang and Serhan, 2006). The focus of this chapter is on current methods for state-of-the-art identification of the potent bioactive mediators derived from essential fatty acids (Fig. 1).

Experimental Design for Eicosanoid and Docosanoid Biosynthesis

Whole blood in the peripheral venous circulation of healthy individuals typically has 1.8 to 7.7×10^9 neutrophils (54% total leukocytes)/L and 130 to 400×10^9 platelets/L. The ratio of neutrophils to platelets markedly increases at sites of vascular inflammation, as these cell types physically interact and total neutrophil numbers dramatically rise (Cotran et al., 1999). In the presence of local agonists, such as thrombin and chemotactic peptides, and circulating agents, such as granulocyte/monocyte colony-stimulating factor (GM-CSF) and other cytokines, platelet-neutrophil interactions lead to the elaboration of lipid mediators that modulate the inflammatory host response and mediate the repair of tissue injury. Dissecting the products, their biosynthesis, and the factors that regulate their formation requires an organized experimental approach (Serhan and Sheppard, 1990; Serhan et al., 2000).

The capacity of individual cell types to generate eicosanoids and/or docosanoids can be evaluated, and, if cell-cell interactions are likely to occur during physiological or pathophysiological states, conditions can be simulated or modeled *ex vivo* to determine the presence of transcellular pathways for eicosanoid formation (Chiang and Serhan, 2006). When investigating transcellular biosynthesis during cell-cell interactions (*in vitro*), it is critical to examine LM (eicosanoid and docosanoid) profiles during each of the following incubation conditions:

- Basal profile of individual cell type (i.e., without agonist);
- Individual cell type after activation by cell type-specific agonist;
- Stimulation of cells during cocubation using individual cell type-specific agonist;
- Costimulation of both cell types during cocubation using shared Receptor-independent stimuli as well as receptor-mediated stimuli.

A range of agonists have been studied includes phagocytic stimuli (e.g., opsonized zymosan, immune complexes, apoptotic neutrophils and urate crystals) and soluble stimuli (e.g., chemotactic peptides, thrombin, calcium ionophores, complement components, and platelet-activating factor and cytokines) (see Table 1 and references therein). Notably, the biosynthetic profile for LT and LX formation during cocubation of differing cell types is unique to the agonists used to activate the cells. The most physiologically relevant stimuli are those that mediate their effects via activation of cellular receptors (e.g., thrombin or GM-CSF), while stimuli that bypass membrane receptors (e.g., calcium ionophores) are potent cellular agonists yet often give a profile of eicosanoid products that is markedly different, both qualitatively and quantitatively, from those obtained with receptor-mediated stimuli. Hence, ionophore can be used to probe the capacity of isolated cells or tissues to produce eicosanoids and/or docosanoids.

To determine the eicosanoid profile of an individual cell type, it is often useful to expose isolated cells during cocubation to cell type-specific agonists. For example, expose a neutrophil (PMN)-platelet (PLT) incubation to thrombin to determine the enzymatic contribution of the platelet. Incremental information can next be derived from cocubations of cells in the presence of a cell type-specific agonist for PMN, such as the chemotactic peptide formyl-methionylleucyl-phenylalanine (fMLP). Last, cocubation of the two cell types with both relevant agonists often yields both increased amounts of the cell type-specific products and generation of *novel* products beyond the enzymatic capacity of either cell type in isolation (Chiang and Serhan, 2006). In addition, each cell type can be labeled independently with radioactive isotope substrate to determine (1) if polyunsaturated fatty acid or a biosynthetic intermediate is transferred between cells and (2) which enzymes are operative in each cell type in the generation of bioactive products (Marcus et al., 1982).

Lipidomics For Monitoring Eicosanoids And Docosanoids Generated By Phagocytes

Routine lipidomic analyses for eicosanoids and docosanoids include using ELISA-, LC-UV-, GC-MS- and LC-UV-MS-MS-based approaches as outlined in Figure 1 (Lu et al., 2005; Lu et al., 2006; Serhan et al., 2007; Serhan, 2008). In order to fulfill the specific requirements of individual experiments, proper methods will be chosen according to the nature of the investigation as described below. The most reliable and sensitive lipidomic approach currently available is the use of LC-UV-MS-MS (liquid chromatography-ultraviolet spectrometry-tandem mass spectrometry instrumentation) (Kiss et al., 1998; Yu et al., 1998; Deems et al., 2007; Ivanova et al., 2007). LC-UV-MS-MS can provide more spectral characterization than LC-MS-MS. With the aid of LC-UV-MS-MS and other

technologies in this laboratory, we identified the resolvins, protectins, and more recently, maresins, using physical properties include characteristic UV spectra and LC retention times as well as mass spectra (Serhan et al., 2000; Serhan et al., 2002; Hong et al., 2003; Serhan et al., 2009). The correlation of MS-MS fragments to structures of some LM and their isomers have been studied (Murphy et al., 2001; Hong et al., 2007; Lu et al., 2007). These results indicate that MS-MS spectra are readily elucidated for the double bonds and functional groups of fatty acids. ELISA (enzyme-linked immunosorbent assay) has been introduced as an approach for lipid mediator quantitation, and it is designed for quantification of specific LM with relatively high selectivity and sensitivity (Chiang et al., 2004). It allows investigators to assess one specific analyte in a large number of samples in a timely fashion (Chiang et al., 2004). The availabilities and cross-reactivities between analytes are the main concern for choosing this method. LC-UV based lipidomics requires neither expensive instrument nor specific reagent, and is a widely used technique for eicosanoid analysis (Serhan, 1990).

Liquid chromatography coupled with UV spectrometry (LC-UV) relies on retention time and on-line UV spectrum to identify and quantify LM (Figure 1). The limitation of this method is that the peaks might not be well resolved (coelution) and/or the analytes lack useable chromophores. LC-UV is used mostly for convenient screening and/or compound isolation (i.e., isolation of intermediates, purification of substrates, etc.; see below). Gas chromatography coupled with mass spectrometry (GC-MS) has long been the method of choice for routine lipidomics of fatty acids and eicosanoids (Ackman, 1984; Serhan et al., 1984b; a). Multiple analytes can be detected in one sample after derivatization (Serhan, 1990); however GC/MS is not ideally suitable for all intact lipids or lipid mediators because pretreatment as well as derivatization are required for some classes of compounds (Murphy et al., 2005; Ivanova et al., 2007). It is often used together with LC-UV-MS-MS to confirm structure elucidation, quantitation as well as identification (Serhan et al., 1984a; Serhan et al., 2000; Kasuga et al., 2008). The detection limits and advantages of each method for lipidomics method selection are listed in Tables 2 and 3.

ELISA-based lipidomics

ELISA enables detection and quantitation of one specific analyte at a time with high sensitivity. It may be used in certain cases to be cost effective to analyze large numbers of samples, e.g. LXA₄, LTB₄ profiling. When commercial ELISA are used for initial analysis, the results will be validated with LC-UV-MS-MS (Chiang et al., 2004). ELISA are not yet available for all eicosanoids and docosanoids.

Sample Preparation and Extraction for LC-UV, LC-UV-MS-MS and GC-MS-Based Lipidomics

Before samples are subject to LC-UV, LC-UV-MS-MS and GC/MS-based analyses, extraction can be used, and in most cases is required, to increase both the specificity and sensitivity (Powell, 1999). Solid-phase extraction is one of the most used extraction techniques (Powell, 1999; Lu et al., 2006; Deems et al., 2007). The following solid-phase extraction procedures are optimized for maximum lipid mediator recovery yield (Lu et al., 2006), which should ideally be >90–95%. Following the addition of two volumes of cold methanol to incubations or tissue samples, they are stored at –20°C for 30 min to 1 h. Samples are extracted with deuterium-containing synthetic or authentic compounds as internal standard (IS) to assess recovery yields and to determine extraction efficiencies (Merched et al., 2008). Ideally, the corresponding deuterated compounds are chosen as IS for an analyte, for example, synthetic d-2 RvD1 is used to quantitate RvD1 in biological samples. If corresponding deuterated compound is not commercially available, deuterium-

labeled docosanoids or eicosanoids with similar polarity to the analyte of interest can be used for estimating the recovery, e.g. d-4 LTB₄ for quantitating dioxygenated products. The commonly used deuterium-labeled internal standards are listed in Figure 5. Samples are centrifuged at 2000rpm, 4°C, for 10 minutes and supernatant collected. Pellets will be suspended in 1 volume of cold ethanol and the centrifuge repeated. The supernatants will be combined and organic solvent evaporated with nitrogen gas or a concentrator (Servant SpeedVac). Samples are then suspended in 10% methanol (e.g. 9 ml H₂O and 1 ml methanol), followed by acidification to pH 3.5.

Before the extraction, C-18 solid phase extraction cartridges should be rinsed with 20 ml methanol and then 20 ml ddH₂O. Acidified samples will be loaded into the cartridges. The cartridges are eluted with 10 ml of hexane, 8 ml of methyl formate and then 10 ml MeOH. Fractions are collected, for example, most of the fatty acids will be enriched in hexane fractions, eicosanoids and docosanoids are enriched in methyl formate fractions and peptido-conjugates (i.e. LTD₄) enriched in methanol fractions (Powell, 1999). Remove the solvent either with nitrogen or SpeedVac and suspend residues in mobile phase immediately prior to the next step of assessment. To minimize potential auto-oxidation and isomerization of analytes, incubation/sample preparations should be subjected to LM lipidomic procedures immediately and not stored for long periods.

HPLC-Based Lipidomics

LC-UV relies on retention time and on-line UV spectrum to identify and quantify LM. We will use LC-UV for rapid and cost effective lipidomic screenings or compound isolations. For example, leukotrienes (LT)s in methanol have a conjugated triene with $\lambda_{\text{max}} \sim 270$ nm for LTB₄ and diHETEs and $\lambda_{\text{max}} \sim 280$ nm for cysteinyl LTs, while both LX and 15-epimer-LX (aspirin-triggered lipoxins) have a conjugated tetraene with $\lambda_{\text{max}} \sim 300$ nm (Figure 4). Although PGB₂ carries a UV chromophore with $\lambda_{\text{max}} \sim 280$ nm, most prostanoids absorb UV at $\lambda_{\text{max}} < 210$ nm, all of which fall in the cut-off wavelength range of LC eluents. Thus, most prostanoids, as well as the LM, without proper chromophores are not suitable for LC-UV-based lipidomics (Masoodi and Nicolaou, 2006). In this lab, HPLC is often used for initial lipidomic screening and compound isolation/purification (Fig. 2).

Polyunsaturated fatty acids (PUFAs) are subject to auto-oxidation and lead to the generation of a series of oxygenated products (Yin et al., 2007). Thus, it is necessary to routinely monitor and isolate/purify PUFAs immediately before use. Figure 2A shows isolation/purification of commercial DHA with RP-HPLC. Insets show online UV spectra of DHA and its auto-oxidation products-monohydroxydocosaheptaenoic acids (HDHA)/monohydroperoxydocosaheptaenoic acids (HpDHA). GC/MS analysis of HPLC isolated DHA is shown in Figure 2B. Only one dominant peak confirms its high purity. The isolation was conducted with 1100 Agilent HPLC equipped with UV detector using a C18 column (4.6 mm $\times$ 150 mm $\times$ 5 μ m; Agilent Eclipse XDB) and a mobile phase consisting of 80%–100% MeOH/H₂O with 0.01% AcOH over 30 min ramp at 1 ml/min (Serhan et al., 2009). This approach can also be conducted for isolating other PUFAs and reagents.

LC-UV-MS-MS-based Lipidomics

LC-UV-MS-MS-based mediator lipidomics includes identification/matching, validation, profiling, structure elucidation, as well as defining novel LM stereochemistry and its biosynthesis as outlined in Figure 3. After positive identification/matching, quantitation is carried out based on the chromatographic area of Qtrap Multiple Reaction Monitoring (MRM) ion pair, and the linear calibration curve of each corresponding synthetic and authentic eicosanoids/docosanoids. We routinely profile up to 52 LM within a single experiment and LC-UV-MS-MS profiling. The representative MRM transitions are shown in

Table 4. Unmatched analytes may be subject to bioactivity assessment, as well as QTOF (Qstar) and GC/MS aided structure elucidation (Lu et al., 2005). For potent bioactive compound(s), isotope trapping and chiral lipidomics of biosynthesis marker(s) are carried out to determine the biosynthesis pathway(s) (Serhan et al., 2009), which will also help to define the stereochemistry of the novel compound. Then total organic synthesis of a series of potential candidates will be undertaken elsewhere for matching. Eicosanoids and docosanoids are potent autotoxins with high stereospecificity; meanwhile, it is impossible to obtain stereochemistry information directly from LC-UV-MS-MS-based lipidomics. It is important to match the physical properties of synthetic candidates, which are then matched with endogenous ones to determine their complete stereochemistry, in addition to matching with bioactivities (Arita et al., 2005; Serhan et al., 2006; Sun et al., 2007; Spite et al., 2009). Matching/identification of physical properties is also necessary for confident profiling and validation of eicosanoids and docosanoids.

Assignment of Target Mediator and Related Metabolomic Profiles: Criteria for Matching/Identification of Mediators and Their Metabolites

The matching/identification will usually be undertaken with at least two different instruments/solvent systems. Matching/ identification criteria include LC retention time, maximum absorbance wavelength (λ_{max}) and band shape of UV, and ≥ 6 diagnostic ions of tandem MS-MS spectrum as shown Figure 4 (Lu et al., 2006).

The instrumentation used for LC-UV-MS-MS-based lipidomics includes LCQ, Qtrap 3200 and Qstar XL. The instrument selection will be based on the aspects of each instrument as shown in Table 3. Fast acquisition in MRM mode, high sensitivity and high linear dynamic range of Qtrap 3200 make it optimum for LM profiling (Deems et al., 2007). LCQ can be used as an alternative and a backup for this purpose if Qtrap 3200 is down or requires maintenance service. Its capability of multistage mass spectral analysis makes LCQ ideal for structure elucidation. Qstar is predominantly used for LM identification and structure elucidation when high sensitivity and high mass accuracy are required.

Routine Lipidomics for Eicosanoids and Docosanoids

Typical lipidomic/metabolomic analysis for eicosanoids and docosanoids can use an Agilent 1100 series HPLC coupled with an ABI 3200 Qtrap mass spectrometer equipped with an Agilent Eclipse Plus C18 column (4.6mm $\times$ 50mm $\times$ 1.8 μ m). For ABI Qtrap 3200 mass spectrometer-based lipidomics, instrument control and data acquisition are performed using Analyst 1.4.2 software. The instrument is run in negative ionization mode. For targeted mediator lipidomics, acquisition mode will be Enhanced Product Ion (EPI). The mobile phase consists of ethanol/water/acetic acid (60/40/0.01; v/v/v) and is ramped to 80/20/0.01 (v/v/v) over 7.5 min and to 95/5/0.01 (v/v/v) over the next 4.5 min at a flow rate of 400 μ l/min. The flow rate will be decreased to 200 μ l/min for 3 min, then will be returned to 400 μ l/min and the mobile phase will be ramped up over the next 6 min to ethanol/water/acetic acid 100/0/0.01(v/v/v) before returning to ethanol/water/acetic acid 60/40/0.01 (v/v/v). For lipidomic profiling with Multiple Reaction Monitoring (MRM) coupled with Information Dependent Acquisition (IDA)-Enhanced Production Ions (EPI) data acquisition, the mobile phase consists of methanol/water/acetic acid (60/40/0.01; v/v/v) and will be ramped to 80/20/0.01 (v/v/v) after 5 min, 95/5/0.01 (v/v/v) after 8 min, and 100/0/0.01 after 14 min to wash the column. The diode-array UV detector scans from 200 to 360 nm with step width at 2 nm.

Information Dependent Acquisition criteria: intense peaks from 1 to 1, or 1 to 2; intense peaks exceeds 200 counts; mass tolerance, 250 mDa. Conditions for MS-MS are: electric

spray voltage, 4.2 kV; source temperature, 500°C; Gas1, 50 units, Gas2, 50 units; Curtain Gas, 20 units; Collision Gas, medium. For Enhanced Production Ions mode, Declustering Potential (DP), -35 V; Entrance Potential (EP), -5 V; Collision Cell Entrance Potential (CEP), -12.7 V. For Multiple Reaction Monitor (MRM) mode, each parameter is optimized individually. For Finnigan LCQ based lipidomics, the general conditions will be as follows. The mobile phase will flow at 0.2 ml/min as C (methanol:water:acetic acid 65:35:0.01) from 0 to 8 min, will be ramped to methanol from 8.01 to 30 min, then will flow as methanol for 10 min, and then will run as C again for 10 min. The photodiode-array UV detector will scan from 200 to 360 nm. Conditions for MS-MS will be: electric spray voltage, 4.3 kV; heating capillary, 230°C and -39 V; tube lens offset, 60 V; sheath N₂ gas, 80 units (near 1.2 L/min); auxiliary N₂ gas, 3 units (0.045 L/min).

Next, after positive identification/matching, quantitation is usually carried out. Fast acquisition, high sensitivity and high dynamic range make **Multiple Reaction Monitoring (MRM)** ideal for profiling/quantitating multiple analytes (Deems et al., 2007). Currently, we routinely profile and quantitate up to 52 LM within a single experiment and LC-MS profiling. The representative MRM transitions are shown in Table 4. Individual methods are tailored to fit the specific requirements of individual investigations. Figure 6 shows LC-UV-MS-MS-based lipidomics of 12 synthetic LM and SPM, with demonstration of chromatogram, MS-MS spectrum and online UV of RvE1 and RvD1 (Spite et al., 2009).

Eicosanoid and docosanoid biosynthesis are temporally dissociated in inflammation/resolution. Their productions switch from pro-inflammatory mediators to anti-inflammatory pro-resolving signals, such as lipoxins, resolvins, protectins as well as maresins during resolution phase (Serhan et al., 2008; Serhan et al., 2009). Along with the regulation of these signals, monocytes/macrophages are recruited to the inflamed site for nonphlogistic clearance of apoptotic PMN, pathogens and cell debris, et al. (Lawrence and Gilroy, 2007; Serhan et al., 2008). To elucidate temporal eicosanoid biosynthesis profiles in macrophage phagocytosis of apoptotic PMN, we applied ELISA based lipidomic approach to *in vitro* incubation of elicited macrophages with apoptotic PMNs, and demonstrated the generation of endogenous anti-inflammatory pro-resolving LXA₄ was increased, and peaked at 15 min, but not pro-inflammatory leukotriene B, as shown in Figure 7. ELISA analysis result was validated using LC/UV/MS/MS based lipidomics along with matching/identification criteria shown in figure 4 (Schwab et al., 2007). Tandem mass spectrum of identified biogenic LXA₄ is shown on the right with signature ions shown in red. In presence of aspirin, macrophage phagocytosis of apoptotic PMN also generates anti-inflammatory pro-resolving RvE1. Furthermore, RvE1 level increases when EPA is supplied (Figure 8) (Schwab et al., 2007). Tandem mass spectra of authentic synthetic RvE1 and identified biogenic RvE1 from murine macrophages are shown on the right. Signature ions (shown in red), m/z 291, 195, 153, 129 and 109 confirm the positive identification of biogenic RvE1 (Schwab et al., 2007). These results are consistent with the nonphlogistic nature of the macrophage phagocytosis of apoptotic PMN with implication of amplification of resolution signals in resolution phase.

A wide range of physiological and pathophysiological events involve cell-cell interactions, during which transcellular biosynthesis plays important roles in eicosanoid and docosanoid generations (Clària and Serhan, 1995; Serhan et al., 2000; Chiang et al., 2004; Morris et al., 2009). Transcellular biosynthesis of aspirin-triggered lipoxins (ATL) was established during endothelial-PMN interaction *in vitro* (Clària and Serhan, 1995), and demonstrated *in vivo* in humans (Chiang et al., 2004; Morris et al., 2009). RvE1 was originally isolated and identified in murine resolving exudates, and the *in vivo* events was recapitulated with human cells *in vitro* (Serhan et al., 2000). Aspirin is well documented to block thromboxane formation via permanently acetylating and inhibiting platelet cyclooxygenase-1 (COX-1), which could account for aspirin's anti-thrombotic properties. In addition, aspirin acetylates

vascular cyclooxygenase-2 (COX-2) and switches its activity from generating PG and TX to producing 15R-H(p)ETE. 15R-H(p)ETE is then transformed by leukocyte 5-lipoxygenase to aspirin-triggered lipoxins (ATL). Along these lines, aspirin-acetylated COX-2 also converts EPA to 18R-HEPE, which is released and then rapidly converted by 5-lipoxygenase in leukocytes to generate RvE1. Therefore, aspirin can jump-start resolution by initiating generation of aspirin-triggered lipoxins and AT-resolvins, which enhance resolution rather than have a negative impact on this essential process (Schwab et al., 2007). The overall regulation of eicosanoid and docosanoid generation by aspirin is shown in Figure 9.

LC-MS-MS-Based Pathway Metabolomics Of Eicosanoids and Docosanoids

Inflammation and resolution are actively regulated processes involving timed generation and inactivation of endogenous signals, where phagocytes, particularly macrophages, are major determinants (Serhan et al., 2008). E-series resolvins are potent anti-inflammatory pro-resolving lipid mediators generated in the resolution phase (Serhan et al., 2008). Like other eicosanoids and docosanoids, they are local-acting autocooids in that they are generated locally, exert their potent biofunctions, and then are rapidly inactivated (Serhan et al., 2000; Tjonahen et al., 2006; Hong et al., 2008). Figure 10 shows E series resolvins biosynthesis and inactivation metabolome. Profiling of RvE1, RvE2 and their biomarker, 18-HEPE, is demonstrated with MRM chromatograms with transition m/z 317>259 for 18-HEPE, m/z 333>115 for RvE2, and m/z 349>195 for RvE1. Instrument setup and parameters for LC-UV-MS-MS based lipidomics are described in section 4.5.2. Major RvE1 metabolism pathways have also been decoded and are shown in Figure 12. Several RvE1 metabolites, namely, 10,11-dihydro-RvE1, 18-oxo-RvE1, 19-hydroxy-RvE1, 20-hydroxy-RvE1 and 20-carboxy-RvE1, have been detected in the incubations of RvE1 with mouse lung, liver, kidney, spleen, human whole blood or isolated PMN (Hong et al., 2008). RvE1 can be converted enzymatically first to an intermediate 12-oxo-RvE1, then rapidly reduced to 10,11-dihydro-12-oxo-RvE1 that can then be further converted to 10,11-dihydro-RvE1. Meanwhile, 20-carboxy-RvE1 is generated via further metabolic oxidation of 20-hydroxy-RvE1. Tandem mass spectra of representative EPA metabolome biosynthesis and inactivation products, 18-HEPE, RvE2, RvE1 and its inactivated metabolite 18-oxo-RvE1, are shown in Figure 11.

During resolution of inflammation, DHA can be utilized by cells, including phagocytes, to enzymatically generate a series of anti-inflammatory pro-resolving docosanoids, including PD1 and RvD1 (for examples, see Serhan et al., 2002; Hong et al., 2003; Serhan et al., 2006; Sun et al., 2007). RvD1 can be further metabolized by 15-prostaglandin dehydrogenase/eicosanoid oxidoreductase to form 17-oxo-RvD1 and 8-oxo-RvD1 (Sun et al., 2007). Figure 12 shows profiling of PD1, RvD1, and their biomarker 17-HDHA with demonstrating MRM chromatograms with transition m/z 343>245 for 17-HDHA, m/z 359>153 for PD1, m/z 375>141 for RvD1. LC-UV-MS-MS setup and parameters are described in section 4.5.2. Figure 13 shows tandem mass spectra of representative DHA metabolome products, 17-HDHA and PD1. See Figure 6 for RvD1 mass spectrum. Metabolomic investigation of eicosanoids and docosanoids will help to elucidate inflammation/resolution circuitry with highly therapeutic relevance. It can also provide the foundation for designing enzymatic stable analogs for clinical application.

LC-UV-MS-MS-Based Reverse Phase Chiral Analysis

Lipids, and in particular polyunsaturated fatty acids, are subject to auto-oxidation, which leads to the formation of racemic mixtures of many compounds, such as isoprostanes (Yin et al., 2007). In contrast, enzymatically produced mediators, especially through the lipoxygenase and cyclooxygenase routes, generate oxygenated products and intermediates

that tend to possess predominant chiralities (Williams et al., 2005). Thus, chiral separations and isomer ratios can be used to distinguish the nonenzymatic and enzymatic pathways of oxygenated lipid-derived products. LC-UV-MS-MS-based reverse phase chiral and lipid analysis enables direct assessment without derivatization; they have been utilized in recent years (Schneider et al., 2007; Serhan et al., 2009). LC-UV-MS-MS based reverse phase chiral analysis is carried out using ABI 3200 Qtrap mass spectrometer coupled with an Agilent 1100 series HPLC with a ChiralPak AD-RH reverse phase chiral column (Chiral Technology, West Chester, PA) (15). The LC mobile phase gradient is set up at acetonitrile:water:acetic acid=70:30:0.05 (v/v/v) to 100:0:0.05 at 0.2mL/min. The mass spectrometer setup, the identification/matching and the quantification are essentially as indicated above. Figure 14 shows MRM chromatograms of chiral separation of racemic HDHA mixture (4-HDHA: m/z 343>101 in blue color, 7-HDHA: m/z 343>141 for 7-HDHA in orange, and 14-HDHA: m/z 343>205 in green). Chiralities were determined by matching with optically pure compounds usually defined by total organic synthesis using criteria as shown in Figure 4.

Recently, a novel family of anti-inflammatory and pro-resolving docosanoids -- maresins (macrophage mediators in resolving inflammation) -- has emerged, which was first uncovered as autocoids generated by murine peritoneal macrophages (Serhan et al., 2009). Its biosynthesis has been shown via 12-LOX in human macrophages and 12/15-LOX in murine tissues, which initiate the maresin biosynthetic pathway to produce maresin 1 (MaR1) (Serhan et al., 2009). LC-UV-MS-MS based chiral analysis of incubations of porcine leukocyte type 12-LOX with DHA demonstrated the 14-hydroxydocosaheptaenoic acid (14-HDHA) as a major product. Incubations reduced with NaBH₄ before analysis demonstrated that > 98% *S*-configuration carbon 14 alcohol was generated by the enzyme, as shown in Figure 15A (Serhan et al., 2009). Chirality of 14-HDHA was determined by matching with authentic optical pure compounds using criteria shown in Figure 4. Figure 15B shows the mass spectrum of identified biogenic 14*S*-HDHA from the incubation. Hence, chiral LC-MS-MS can be very powerful in establishing biosynthetic pathways and stereochemistry of specific mediators. This is important because most bioactive eicosanoids and docosanoids are active in a chiral and stereospecific fashion. Hence, some oxidation products are completely devoid of actions and biological functions.

Lipidomic Databases And Searching Algorithms

Databases with appropriate searching algorithms are crucial for the timely identification of these LM via LC-UV-MS-MS analytical runs (Lu et al., 2005). Lipid mediators are identified on the basis of chromatographic features such as retention time, UV and MS-MS spectrum. We modified and refined the available software package Mass Frontier to perform database algorithm searches based on tandem mass spectrum, retention time and UV spectrum (Ivanova et al., 2007). The new program can perform algorithmic search for real databases as well as theoretical databases with UV and MS-MS spectra of a large number of theoretical compounds. Thus far, the database contains over 30 bioactive LM and over 1000 theoretical fatty acid derived compounds and fragments for putative endogenous local mediators.

We are currently building a new database for the newly purchased Qtrap 3200 (work in progress). Lipid mediators are identified on the basis of chromatographic features such as retention time, UV and MS-MS spectra. An example of Qtrap database searching is shown in Figure 17.

GC/MS based lipidomics

GC-MS provides unique information and opportunity for fatty acids and lipid mediator research (Serhan et al., 1984a; Christie, 2003). After derivatization, multiple analytes can be detected in one sample without specialized methods (Serhan et al., 1984a; Christie, 2003). The overview of GC/MS procedure is shown in Figure 16A. After derivatization of lipid mediators (Serhan et al., 2009), the sample is subject to GC/MS analysis. The results are then assessed by data mining through the NIST database. The GC-MS identification is conducted by matching the mass spectra and C-values of unknowns to LM standards. C-values are used instead of retention time to enable comparison of acquisition data obtained from different instruments or different methods (Serhan et al., 2009). Analyte C-value is determined using calibration curve of retention time vs. carbon numbers of a series of saturated fatty acid methyl esters (Lipid standards C14:0 –C24:0, Cat number 18917, Sigma-Aldrich, Milwaukee, WI) as shown in Figure 16 B and C. After the compounds of interest are identified, they will be quantified on the basis of their chromatographic peak areas and linear calibration curves of chromatographic peak areas of authentic and synthetic eicosanoids and docosanoids. Worthy of note, the NIST GC/MS database is commercially available, and can be integrated to GC/MS analysis tools to facilitate data mining (Agilent, Santa Clara, CA). GC/MS is often used together with LC-UV-MS-MS to confirm identification, quantitation and quality control of eicosanoids and docosanoids (Serhan et al., 2002; Kasuga et al., 2008; Serhan et al., 2009). Figure 2B shows the total ion chromatogram (TIC) of derivatized isolated DHA. One dominant peak confirms the integrity of DHA.

Summary

In summation, in this chapter we provide an overview of the current state-of-the-art approaches to identify and quantitate bioactive eicosanoids and docosanoids including prostaglandins, leukotrienes and lipoxins produced from arachidonic acid as well as resolvins, protectins and maresins biosynthesized from omega-3 fatty acids (for example, EPA and DHA). Emphasis is placed on the metabolic profiles of these products produced by phagocytes, in particular peripheral blood neutrophils, inflammatory exudates and macrophages. State-of-the-art techniques reviewed here take advantage of LC-tandem mass spectrometry-based lipidomics for metabolic profiling of the lipid mediators produced by isolated phagocytes. The state of activation of neutrophils and whether they are of peripheral origin or from a site of inflammation dictate the profile of lipid mediators generated. Likewise, macrophages, depending on their functional role and state of activation, have an unusually high capacity to generate a wide range of potent bioactive mediators derived from essential fatty acids. Here, we provide examples of the metabolic profiles obtained from these cells with an emphasis on targeting potent bioactive mediators. In addition, criteria for full identification and matching to synthetic material as well as quantitation using internal standards is reviewed. Metabolomic profiling with phagocytes gives a wealth of information. In addition to bioactive mediators, inactive isomers are products that can be used as biomarkers of pathway activation (i.e., biomarkers include 12-HETE, 14-HDHA and all-*trans*-LTB₄ within these pathways). Since many of these potent mediators are autocoids, they are transiently produced *in vitro* as well as *in vivo* and are further metabolized to biologically inactive products. Profiling of these metabolic inactivation products and their relationship to the target bioactive compound (i.e., LTB₄, RvE1, RvD1, etc.) are also presented, and examples given as illustrations. We trust that with this review the reader will find useful information for identification and profiling of individual bioactive lipid mediators and urge the reader to access the cited literature to provide further detail on the biosynthesis, actions and functional roles of the pathways, bioactive mediators and related compounds produced by phagocytes reviewed here. The approach and methods presented for phagocytes and inflammatory exudates are of wide utility and can be used in other

organs and tissues to identify specific eicosanoids and docosanoids, profiles that can be location (tissue) and/or disease site-specific.

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Abbreviations

7S,17S-diHpDHA	7S,17S-dihydroperoxydocosa-4Z,8E,10Z,13Z,15E,19Z-hexaenoic acid
7S(8)-epoxy-17S-HDHA	7S(8)-epoxy-17S-hydroxydocosa-4Z,9E,11E,13Z,15E,19Z-hexaenoic acid
LX, lipoxin	lipoxygenase interaction products
LXA₄, lipoxin A₄	5S,6R,15S-trihydroxyeicosa-7E,9Z,11Z,13E-tetraenoic acid
LXB₄, lipoxin B₄	5S,14R,15S-trihydroxyeicosa-6E,8Z,10E,12E-tetraenoic acid
Maresin	macrophage mediators in resolving inflammation
MaR1, maresin 1	7,14S-dihydroxydocosa-4Z,8,10,12,16Z,19-hexaenoic acid
NPD1/PD1, neuroprotectin D1/protectin D1	10R,17S-dihydroxydocosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid
Resolvin	resolution phase interaction products carrying bioactivity
RvD1, resolvin D1	7S,8R,17S-trihydroxydocosa-4Z,9E,11E,13Z,15E,19Z-hexaenoic acid
RvD2, resolvin D2	7S,16R,17S-trihydroxydocosa-4Z,8E,10Z,12E,14E,19Z-hexaenoic acid
RvE1, resolvin E1	5S,12R,18R-trihydroxyeicosa-6Z,8E,10E,14Z,16E-pentaenoic acid
RvE2, resolvin E2	5S,18R-dihydroxyeicosa-6Z,8E,11Z,14Z,16E-pentaenoic acid

Trivial names and Glossaries

ELISA	enzyme-linked immunosorbent assay
EPI	Enhanced Product Ion
GC-MS	gas chromatography coupled with mass spectrometry
IDA	Information Dependent Acquisition
IS	Internal Standards
LC/HPLC	high performance liquid chromatography
LC-UV	liquid chromatography-UV spectrometer
LC-UV	liquid chromatography-UV spectrometer
LC-UV-MS-MS	liquid chromatography-UV coupled with mass spectrometry

LM	lipid mediators
MRM	multiple reaction monitoring
SIM	selected ion monitoring
SPM	specialized pro-resolving mediators
TOF	time-of-flight which is used to determine the mass-to-charge ratio in mass spectrometer

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Figure 1. Overview of A) eicosanoid and docosanoid biosynthesis and B) lipidomic analysis procedures. COX: cyclooxygenase; LOX: lipoxygenase; P-450: cytochrome P-450.

Figure 2. HPLC substrate isolation and GC/MS quality control. A) UV chromatogram (235nm) of HPLC isolation of commercial DHA; B) GC/MS Total Ion Chromatogram (TIC) and spectrum of methylated isolated DHA. See text for details.

Figure 3. Mediator lipidomics flow chart for lipidomic matching/identification, profiling, structure elucidation, and defining novel lipid mediator and its biosynthesis.

Figure 4.
Lipid mediator identification/matching criteria.

Figure 5.
Deuterium labeled compounds for mediator lipidomic profiling.

Figure 6. LC-UV-MS-MS-based lipidomics: authentic and synthetic eicosanoids and docosanoids. LM lipidomics was conducted with an ABI Qtrap using MRM IDA EPI method (See text for details). A) Total ion chromatogram of 12 authentic and synthetic LM; Chromatogram of precursor/product ion pairs m/z 349 > 195 for RvE1 and m/z 375 > 141 for RvD1. B) Demonstration of online UV spectra for RvE1 and RvD1. MS-MS spectra of C) RvD1, and D) RvE1.

Figure 7. Macrophages generate lipoxin A₄ during phagocytosis of apoptotic PMN. A) Lipoxin A₄ production (red circle, quantitated with ELISA) peaked at 15 min during macrophage phagocytosis (green diamond). In contrast, inflammatory LTB₄ production was not increased during this process as shown inset. Quantitation of LXA₄ with ELISA was confirmed by LC-MS-MS. B) Tandem mass spectrum of LXA₄.

Figure 8. Macrophages generate RvE1 during phagocytosis of apoptotic PMN: impact of aspirin and EPA. A) Macrophages, when treated with aspirin prior to coincubation with apoptotic PMN, generated RvE1. This level increased in presence of substrate EPA. B) MS-MS spectra of the authentic synthetic RvE1 and biogenic RvE1 from murine macrophages.

Figure 9. Transcellular biosynthesis: aspirin-dependent biosynthesis regulation of thromboxane, 15-epi-Lipoxin A₄ and resolvin E1 (see text for details).

Figure 10.

E series resolvins pathway metabolome. Profiling of E series resolvins biosynthesis and inactivation in human and murine with LC-UV-MS-MS. Chromatogram of precursor/product ion pairs m/z 317 > 259 for 18-HEPE, m/z 333 > 115 for RvE2, and m/z 349 > 195 for RvE1. See text for more details.

Curr Protoc Immunol. Author manuscript; available in PMC 2012 November 1.

Figure 12. RvD1 pathway metabolome. Profiling of RvD1 biosynthesis and further metabolism with LC-UV-MS-MS. Chromatogram of precursor/product ion pairs m/z 343 > 245 for 17-HDAH, m/z 359 > 153 for PD1, and m/z 375 > 141 for RvD1. See text for more details.

Figure 13. Tandem mass spectra of A) 17-HDHA and B) PD1. Refer to Figure 6 for RvD1 tandem mass spectrum.

Figure 14.

LC-UV-MS-MS based chiral analysis of racemic 4-HDHA, 7-HDHA, and 14-HDHA mixture. Chiralities are determined by marching retention time with optical pure compounds. Total Ion Chromatogram (TIC) of racemic 4-HDHA, 7-HDHA, and 14-HDHA mixture is shown in brown color. Chromatogram of precursor/product ion pair m/z 343>101 for racemic 4-HDHA is shown in blue, m/z 343 >141 for 7-HDHA in orange, and m/z 343 > 205 for 14-HDHA in green.

Figure 15.

LC-UV-MS-MS based chiral analysis shows incubation of porcine 12-LOX with DHA generated product with > 98% *S*-configuration. The incubation was reduced with NaBH₄ before analysis. The assignment was confirmed by co-eluting (retention time), online UV and diagnostic ions of ms/ms spectrum with authentic synthetic standards. The spectrum of 14-HDHA is shown on the right.

Figure 16. GC/MS based LM lipidomics. A) Overview of GC/MS procedure. B) GC-MS C-value determination.

Figure 17.
Examples of database search of RvD1 spectrum on Qtrap 3200 with Analyst 1.41.

Table 1

Stimuli of biosynthetic enzymes for eicosanoid and docosanoid generation

Enzyme	Phagocytic stimuli	Soluble stimuli (Representative)
15-LO	Apoptotic cells (Freire-de-Lima et al., 2006)	IL-4 (Conrad et al., 1992) IL-13 (Nassar et al., 1994)
5-LO	Zymosan (Birmelin and Decker, 1984) IgE (Patterson and Harris, 1981) Monosodium urate crystals (Reibman et al., 1986)	LPS (Aderem and Cohn, 1988) TNF-alpha (Palmantier et al., 1994) GM-CSF (Palmantier et al., 1994)
COX-2	Zymosan (Pouliot et al., 1998a) Monosodium urate crystals (Serhan et al., 1984b; Pouliot et al., 1998b)	IL-1-beta (Raz et al., 1988; Maier et al., 1990) LPS (Fu et al., 1990; Hla and Neilson, 1992) TNF-alpha (Pouliot et al., 1998a) fMLP (Pouliot et al., 1998a) TGF-beta (Gilbert et al., 1994) GM-CSF (Herrmann et al., 1990)

* Pharmacological inhibitors have been developed for each of these enzymes

Table 2

Lipidomic method selection

Method	LM detection limit	Advantage	Limitation	Proposed/demonstrated uses
LC-UV-MS-MS	10pg to 1ng	The most reliable and sensitive method. No need for derivatization.	Specialized (targeted) methods are desired.	Most used methods for lipidomics (See Table 3 for instrument selection) (Powell, 1999; Deems et al., 2007; Ivanova et al., 2007; Schwab et al., 2007; Adler et al., 2009)
GC-MS	100ng	Detection of multiple analytes in one acquisition without specialized (targeted) methods.	Derivatization is required. Not suitable for all LM.	Fatty acids. Together with LC-UV-MS-MS for identification, profiling & structure elucidation (Serhan et al., 1984a; Christie, 2003)
LC-UV	1ng	Online UV and retention time.	Coelution of analytes with similar retention times. Analyte UV chromophore is required.	Convenient assessments and purifications of compounds with UV chromophores (Wei et al., 2006; Schneider et al., 2007; Serhan et al., 2009)
ELISA	1pg to 5pg	High sensitivity and selectivity. Time efficient assessment of large numbers of samples.	Availability for only certain LM. Cross-reactivity between analytes.	One specific LM (e.g. PGE ₂ , PGD ₂ , LTB ₄ , LXA ₄) (Chiang et al., 2004)

Table 3

LC-UV-mass spectrometers instrument selection.

	Qtrap 3200	Qstar XL
Typical detection limit for LM	50pg for EPI, 10pg for MRM	25pg
Multistage (tandem) mass spectral analysis (n ≥ 3)	Not suitable	Not suitable
Mass accuracy	low	high
Linear detection dynamic range	high	low
Applicable areas	High sensitivity, high speed, high dynamic range, ideal for LM identification & profiling	High sensitivity, high mass accuracy for LM identification & structure elucidation

Table 4

MRM transitions for LC-UV-MS-MS based lipidomics

Compound	MRM	Q3	Compound	Q1	Q3
AA	303	259	20-hydroxy-RvE1	365	195
12-oxo-ETE	317	179	20-carboxy-RvE1	379	195
5-oxo-ETE	317	203			
15-HETE	319	219	DHA	327	283
12-HETE	319	179	17-HDHA	343	245
5-HETE	319	115	14-HDHA	343	205
11-HETE	319	167	13-HDHA	343	193
8-HETE	319	155	7-HDHA	343	141
PGA ₂	333	189	4-HDHA	343	101
PGB ₂	333	235	PD1	359	153
5,15-DiHETE	335	201	D ₅ transPD1	359	153
LTB ₄	335	195	MaR1	359	250
PGE ₂	351	189	RvD5	359	137
20-OH LTB ₄	351	195	RvD6	359	257
LXA ₄	351	115	RvD1	375	215
LXB ₄	351	221	RvD2	375	233
15-epi LXA ₄	351	115	Ω-22-hydroxy-PD1	375	195
PGD ₂	351	233			
PGF _{2a}	353	193	d ₈ -AA	331	267
20-COOH LTB ₄	365	195	d ₅ -DHA	332	288
TXB ₂	369	169	d ₅ -EPA	306	262
			d ₈ -15S-HETE	327	226
EPA	301	257	d ₈ -5S-HETE	327	116
18-HEPE	317	259	d ₄ -PGB ₂	337	193
15-HEPE	317	219	d ₄ -PGD ₂	355	193
12-HETE	317	179	d ₄ -PGE ₂	355	193

Compound	MRM	Q3	Compound	Q1	Q3
11-HETE	317	195	d ₄ -LTB ₄	339	197
5-HETE	317	115	d ₅ -LXA ₄	356	115
RvE2	333	115	d ₄ -TXB ₂	373	173
18-oxo-RvE1	347	291	d ₅ -17-HDHA	348	245
RvE1	349	195	d ₂ -PD1	361	153
10,11-dihydro-RvE1	351	197	d ₄ -RvE1	353	195
19-hydroxy-RvE1	365	195			