Investigations of Bioactivity, Disposition, and Metabolism of Lipids Through Liquid Chromatography-Mass Spectrometry

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Investigations of Bioactivity, Disposition, and Metabolism of Lipids Through Liquid Chromatography-Mass Spectrometry

Abstract
Inflammatory diseases and multiple human cancers are associated with increased cyclooxygenase-2 (COX-2) expression together with decreased expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH). This has been hypothesized to lead to a milieu of bioactive lipids modulating angiogenesis and cellular proliferation. Further insight into these processes were gained by traditional cell and molecular biology approaches, which were combined with cutting edge liquid chromatography mass spectrometry (LC-MS) methods to elucidate the function, disposition, and metabolism of a major COX-2/15-PGDH derived metabolite, 11-oxo-eicosatetraenoic acid (11-oxo-ETE). 11-oxo-ETE was found to possess significant anti-proliferative activity (IC50 2 microM) in human umbilical endothelial cells (HUVECS), which was 5 times more potent than isomeric 15-oxo-ETE. The corresponding methyl ester derivatives were even more potently anti-proliferative than the free acids. The gold standard method of quantification, stable isotope dilution (SID) LC-MS, was applied to determine the absolute intra- and extra- cellular levels of oxo-ETEs in multiple cell lines. Maximal intracellular concentrations of 11-oxo-ETE were 0.02 ng/4x10^5 cells in human colon adenocarcinoma (LoVo) cells versus 0.58 ng/4x10^5 cells in HUVECs. Its methyl ester derivative increased the intracellular concentration of free 11-oxo-ETE 3-fold. Pharmacological inhibition of relevant transporter proteins increased anti-proliferative activity and the intracellular pool of 11-oxo-ETE. Consistent with an observed structural homology to other bioactive fatty acids, 11-oxo-ETE reduced canonical nuclear factor-kappa B signaling activity. LC-MS revealed a mechanistic explanation of this activity via adduction to critical cysteine or histidine residues of the p50 subunit of the p50/p65 transcription factor. LC- high-resolution MS/MS approaches revealed that the oxo-ETEs underwent substantial intracellular metabolism. Two of the double bonds were reduced and novel coenzyme A (CoA) thioesters were formed followed by reduction of all four of their double bonds. The identification of novel CoA thioester metabolites could have important implications to detection and function of the oxo-ETEs as well as other electrophilic polyunsaturated fatty acids in normal physiology as well as in inflammation and cancer.

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INVESTIGATIONS OF BIOACTIVITY, DISPOSITION, AND METABOLISM OF LIPIDS THROUGH LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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Nathaniel Snyder

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To my family and friends,
my gratitude for years of love and support.

“Measure what is measurable, and make measurable what is not so.”

-Galileo Galilei

“Everything that can be counted does not necessarily count; everything that counts cannot necessarily be counted.”

-Albert Einstein

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In the Amazon rain forest, one of the most important parts of the ecosystem is the taller, older trees that make up the top canopy. These trees provide the shelter and nutrients for a host of other organisms to thrive, and the best view of the whole forest can be seen from the branches of those tall trees. In this metaphor, I would like to thank my thesis advisor, Dr. Ian Blair. I give my grateful acknowledgment of Dr. Xiaojing Liu, Dr. Sanka “Bobby” Basu, Andy Worth, Dr. Stacy Gelhaus, and Dr. Clementina Mesaros for providing invaluable aid throughout every stage of my graduate work and cannot be acknowledged enough for being as unselfish with their time and aid as they were. To Dr. Suhong Zhang, and Jasbir Ahrora, I gratefully acknowledge their help. In addition, I would like to thank Dr. Angela Wehr, Dr. Matthew Macdonald, Dr. Vineet Sangar, and Dr. Sumit Shah for all they provided. I would like to extend special thanks to Christine Shwed for all the work she does for the lab. Lastly, I would also like to thank all former and current members of the Blair Lab.

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Lori, Cara, Julia, Brandon, Bella, and Jill, near and far, for all the support and love they have given me. From Tennyson, I am a part of all that I have met.
ABSTRACT

INVESTIGATIONS OF BIOACTIVITY, DISPOSITION, AND METABOLISM OF LIPIDS
THROUGH LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Nathaniel W. Snyder
Ian A. Blair

Inflammatory diseases and multiple human cancers are associated with increased cyclooxygenase-2 (COX-2) expression together with decreased expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH). This has been hypothesized to lead to a milieu of bioactive lipids modulating angiogenesis and cellular proliferation. Further insight into these processes were gained by traditional cell and molecular biology approaches, which were combined with cutting edge liquid chromatography mass spectrometry (LC-MS) methods to elucidate the function, disposition, and metabolism of a major COX-2/15-PGDH derived metabolite, 11-oxo-eicosatetraenoic acid (11-oxo-ETE). 11-oxo-ETE was found to possess significant anti-proliferative activity (IC\textsubscript{50} 2 μM) in human umbilical endothelial cells (HUVECS), which was 5 times more potent than isomeric 15-oxo-ETE. The corresponding methyl ester derivatives were even more potently anti-proliferative than the free acids. The gold standard method of quantification, stable isotope dilution (SID) LC-MS, was applied to determine the absolute intra- and extra-cellular levels of oxo-ETEs in multiple cell lines. Maximal intracellular concentrations of 11-oxo-ETE were 0.02 ng/4x10\textsuperscript{5} cells in human colon adenocarcinoma (LoVo) cells versus 0.58 ng/4x10\textsuperscript{5} cells in HUVECs. Its methyl ester derivative increased the intracellular concentration of free 11-oxo-ETE 3-fold. Pharmacological inhibition of relevant transporter proteins increased anti-proliferative activity and the intracellular pool of 11-oxo-ETE. Consistent with an observed structural homology to other bioactive fatty acids, 11-oxo-ETE reduced canonical nuclear factor-kappa B signaling activity. LC-MS revealed a mechanistic explanation of this activity via adduction to critical cysteine or histidine residues of the p50 subunit of the p50/p65 transcription factor. LC- high-resolution MS/MS approaches revealed that the oxo-ETEs
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Chapter 1: Introduction

This thesis describes the application of liquid chromatography-mass spectrometry (LC-MS) to study the bioactivity, disposition, and metabolism of lipids. Thus, a brief introduction to lipids with a focus on one of the most abundant endogenous lipids, arachidonic acid (AA), is provided in order to provide a framework for the research presented in this thesis. The relevant nomenclature, structure, metabolism, roles of AA metabolites in disease, and methods for the quantification of AA metabolites are discussed. The history of the scientific study of lipids is storied and important to our understanding of human health and disease. Likewise, a primer on the analytical methods and technologies used in this thesis provides an introduction to the novel studies that have been conducted. As a central methodological theme of this thesis, the theory of LC-MS has also been provided.

1.1 Lipids and Nomenclature

Lipids can be defined as fatty or waxy organic compounds that are readily soluble in nonpolar solvents such as ether but poorly in polar solvents such as water. This definition covers a range of diverse molecules, and can be further refined by a multi-functional nomenclature of lipids proposed by Fahy, et al. The nomenclature classifies lipids into eight categories; fatty acyls (FAs), glycerolipids (GLs), glycerophospholipids (GPs), sphingolipids (SPs), sterol lipids, prenol lipids, saccharolipids, and polyketides (5). This thesis covers mostly investigations into the first group, the FAs (Chapter 1-7), but GLs, GPs and SLs are also discussed (Chapter 6-7).

FAs are structurally simple lipids that form the building blocks of more complex lipids and can be classified as unsaturated or saturated based on the presence or absence, respectively, of double bonds. FAs can be further described by number of carbons, number of double bonds, and by position of the double bonds. Subcategories of FAs can be grouped by distinct physiochemical properties, emergent from chemical moieties present in the structures. For example, polyunsaturated fatty acids (PUFAs) are subject to oxidation by reactive oxygen and
nitrogen species as well as oxygenase enzymes. The resulting oxidized lipids function as a diverse array of signaling molecules (6). When more than one double bond is present in a FA, the connecting group is often a methylene moiety termed a bis-allylic configuration, and the abstraction of a bis-allylic hydrogen is an important initiating step in enzymatic oxidation and lipid peroxidation (7).
As shown in Figure 1.1.A, the application of the above nomenclature can describe many important elements about the FA being described. AA has 20 carbon atoms and 4 double bonds, so it can be abbreviated as 20:4\(\omega\)-6 or 20:4n-6, where the notation \(\omega\)-6 derives from the double bond at C14-C15 being five carbons away from the terminal carbon at C-20 (which is designated

Figure 1.1. Struture of AA, 1-stearoyl-2-arachidonoyl phosphatidylinositol, and arachidonoyl-coenzyme A. (A) The structure of AA. Double bond positions indicated by carbon number. The omega (\(\omega\)) and omega (\(\omega\)-6) carbons are also labeled. Bis-allylic hydrogens are indicated. (B) The structure of a glycerophospholipid with stearic acid at the sn-1 position, AA at the sn-2 position, and a polar phosphoinositol head group at the sn-3 position. (C) The structure of arachidonoyl-Coenzyme A. The arachidonoyl acyl group is linked to the coenzyme A molecule through a thioester bond in the carbonyl of AA.

As shown in Figure 1.1.A, the application of the above nomenclature can describe many important elements about the FA being described. AA has 20 carbon atoms and 4 double bonds, so it can be abbreviated as 20:4\(\omega\)-6 or 20:4n-6, where the notation \(\omega\)-6 derives from the double bond at C14-C15 being five carbons away from the terminal carbon at C-20 (which is designated
by convention as ω). Additionally, the FAs include the subgroup of acyl-coenzyme A (CoA) thioesters (8), a topic covered in Chapter 7 and 8. In the case of the acyl-CoA species, the FA is esterified through a thioester linkage to a bulkier CoA molecule.

Another class of lipids, the phospholipids, are ubiquitous and major components of all cell membranes where they form lipid bilayers (9). Phospholipids can also serve as precursors for bioactive products, or engage in signaling activities directly (Fig. 1.2). Phospholipids are a diverse class of biomolecules, but the structure of a typical GP, 1-stearoyl-2-arachidonoyl-phosphatidylinositol is shown in Figure 1.1.B. In this representative structure, glycerol is esterified with stearic acid (18:0) at the sn-1 position, and it is esterified with AA at sn-2 position with a polar phosphoinositol head group at the sn-3 position. The esterification of AA to yield more complex lipids is an important part of the regulation of AA metabolism and physiology.

1.2 Arachidonic Acid Metabolism and Disease

Metabolism of AA is an active area of study because it involves the generation of diverse biologically active lipid mediators, the eicosanoids (10). The eicosanoids derived from AA act via G-protein coupled receptor (GPCR) agonism or potentially as nuclear transcription factor ligands (6,11,12). They are involved in inflammation (13), cancer (14), and other physiological processes by modulating signaling pathways (15), transcription factor activity (16), and gene expression (17). The diverse roles of various eicosanoids in inflammation, cancer, and other processes are described in more detail in Chapters 2, 3, 4, and 7.

In most animals, AA is a non-essential fatty acid because it can be synthesized from linoleic acid (3). Free AA in mammals is tightly regulated and exists primarily as the esterified form in PLs, GPs, and SLs (Figures 1.1 and 1.2). Release of AA by hydrolysis from esterified lipid stores is an important step in AA metabolism, and critical in the physiological role of AA metabolites. Esterified AA at the sn-2 position on phosphatidylinositol can be directly released through hydrolysis of the PL by phospholipase A2 enzyme (18). Alternatively, phospholipase C-mediated hydrolysis of phospholipids can first occur at the sn-3 position. The polar
phosphoinositol head group is thereby removed, forming a diacylglycerol (DAG) which is acted on by DAG lipase to release free AA (19,20). In the brain, arachidonylethanolamide (AEA) is an endogenous cannabinoid neurotransmitter, and the substrate for fatty acid amide hydrolase, which converts AEA into ethanolamine and AA (21). As shown in Figure 1.2, the tight control of AA directly impacts the generation of eicosanoids in physiologically important pathways.
Figure 1.2: Simplified scheme of AA metabolism. The metabolism of AA starting with release from esterification at the sn-2 position of 1-stearoyl-2-arachidonoyl phosphatidylinositol. The release of AA from esterification allows cyclooxygenase mediated metabolism to downstream products. Not shown: 15-Lipooxygenase can also act on the esterified form of AA. R1: stearoyl (18:0); DAG: diacylglycerol; DAGL: DAG lipase; PI: phosphatidylinositol; PLA2: phospholipase A2; PLC: phospholipase C; AEA: arachidonylethanolamide (anandamide); FAAH: fatty acid amide hydrolase; AA: arachidonic acid; POX: peroxidase; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; PG, prostaglandin; TX, thromboxane.
1.3 Enzymatic and Non-enzymatic Biotransformation of AA

Transformation of AA through the cyclooxygenases (COXs) is one of the primary routes of AA metabolism into potent lipid signaling mediators (Figure 1.2). COX exists as two isoenzymes, COX-1 and COX-2 (22). COX-1 is constitutively expressed in many cells and is considered to function as a house-keeping enzyme where its products help to maintain normal biological processes. COX-2 was identified independently by three groups as a COX isoenzyme that is induced in response to cytokines, growth factors, bacterial endotoxin, and certain other stimuli (23-25).

COXs are membrane bound proteins located on the inner and outer membrane of the nuclear envelope, as well as the surface of the endoplasmic reticulum lumen (26). The enzymes possess dual catalytic functions, containing both COX and peroxidase (POX) active sites. The COX site converts AA into PGG2 and 11(R)- and 15-HPETEs, while the peroxidase site reduces the hydroperoxide groups in PGG2 or HPETEs to the corresponding hydroxyl groups (Figure 1.2). This results in the generation of PGH2 as well as 11(R)- and 15-HETEs (27).

The generally accepted mechanism of COX action involves a tyrosine protein radical (10). The COX catalytic cycle begins when an alkyl hydroperoxide binds to heme in the POX site. The hydroperoxide is reduced to the corresponding alkyl hydroxyl. The heme is thereby oxidized to the oxyferryl Fe$^{4+}=O$ and a protoporphyrin radical cation (Fe$^{4+}=O^+$) is formed. This unstable intermediate abstracts hydrogen from the $^{385}$Tyr side chain, resulting in the formation of $^{385}$Tyr radical and an oxyferryl. The $^{385}$Tyr radical then abstracts the pro-S hydrogen at C13 of AA bound in the COX active site. $^{385}$Tyr is adds a proton, while an allylic radical is formed at C13 of AA. The C11-C12 double bond of AA relocates to C12-C13 and forms a conjugated double bond with C14-C15 and the C13 radical is transferred to C11. Then oxygen is inserted at the C11 position. The radical is transferred to oxygen, which rapidly attacks the C8-C9 double bond. This attack forms a dioxygen (endoperoxide) bridge between C11 and C9, along with a C8 radical. This is followed by a C8 radical attack of the C12-C13 double bond and formation of a C8-C12 single bond, together with a C13 radical. After translocation of the C13 radical and C14-C15 double
bond, a C15 radical is formed and followed by oxygen attachment to C15. Finally, this PGG$_2$ precursor abstracts a hydrogen atom and forms the final PGG$_2$. PGG$_2$ can then function as the initial alkyl hydroperoxide and enter the POX site, where it is reduced to PGH$_2$. After several cycles, COX will usually lose activity via a mechanism that may be related with the quenching of tyrosine radicals or damage to the COX protein by free radicals (28). The mechanism leading to HETE formation is less clear, but notably from the above mechanism, oxygen insertion in the normal catalytic COX cycle occurs at C11 and C15, which would lead to the formation of 11- and 15-H(P)ETE.

COX is a target for the class of therapeutics known as non-steroidal anti-inflammatory drugs (NSAIDs). Aspirin was the first described NSAID and differs mechanistically from other NSAIDs, in that it converts COX-2 into a 15($R$)-lipooxygenase (LOX) via covalent modification, instead of completely inhibiting COX-2 activity. Acetylation of COX-1 via aspirin inactivates the enzyme. Other NSAIDs can be referred to as traditional NSAIDs (tNSAIDs) and include ibuprofen, diclofenac, naproxen, and many others. These tNSAIDs are usually competitive inhibitors of COX and ultimately inhibit prostaglandin (PG) biosynthesis with a variety of effects depending on the drug, dose, timing, co-administration, and selectivity of binding for COX-1/COX-2 (29,30). For example, low dose aspirin will selectively inactivate platelet COX-1 for the life of the platelet, but fail to achieve systemic effects of COX inhibition, resulting in an anti-thrombotic effect while sparing the irritation of gastric mucosa due to inhibition of PG biosynthesis (31). COX-2 specific inhibitors have been developed with the hope of reducing the side effect of gastric irritation, although they have been associated with significant PG/thromboxane dependent adverse cardiovascular events (29,32).

Aside from COXs, AA can also be metabolized by the lipoxygenase (LOX) family. LOXs catalyze a dioxygenase type reaction, as opposed to the PG producing di-oxygen insertion and cyclization of the COXs. The catalytic product of LOXs from AA is a n-6 hydroperoxy fatty acid with a conjugated Z,E-diene system. Similar to the COX reaction, the unstable hydroperoxy moiety is then reduced by cellular POXs to the corresponding hydroxyl group. The nomenclature
for LOXs is based on the specificity of the enzyme for the carbon where molecular oxygen is inserted. Humans possess at least five lipoxygenases. Of these five, two are pure 15-LOXs. The other human LOXs, namely 5-LOX, 8-LOX, and 12-LOXs are reviewed elsewhere (33). The non-human (and non-animal) lipoxygenases are beyond the scope of this thesis.

15-LOX-1 also termed reticulocyte 15-LOX, has been purified from human eosinophils and leukocytes (34,35). 15-LOX-2 has been isolated and described from human skin. Expression of 15-LOX-2 was also observed in prostate, lung, and cornea (36). The specific reduced products of 15-LOX-1 from AA include 15(S)-HETE and 12(S)-HETE because of some 12-LOX-type activity. 15-LOX-2 produces pure 15(S)-HETE. Unlike COXs, LOXs do not have an absolute requirement for acting on free AA. Thus, the possibility of oxidation followed by release by phospholipases must always be considered for lipoxygenase products (33). 15-LOX-1 rapidly inactivates in a turnover dependent mechanism, but 15-LOX-2 is catalytically stable. The role of 15-LOXs in humans is complex and animal models give conflicting results, but an excellent review is available (37). The Brash group at Vanderbilt University describes the possible functions of LOXs as generators of signaling mediators (directly or through intermediates), peroxidation, or as enzymes for lipid mobilization. Ongoing research into these roles is discussed in Chapters 2, 3, 4, and 7.

Besides enzymatic pathways, AA is also subject to autoxidation both in vitro and in vivo. AA has four double bonds and three pairs of bis-allylic hydrogen atoms (Figures 1.1 and 1.2); therefore, it is readily oxidized when it is exposed to air, free radicals, or heat (38). This instability requires careful control of parameters to avoid or control for artifact formation during an experiment. While enzymatically produced AA metabolites are formed with enantiomeric specificity, autooxidation of AA results in the formation of racemic products. The use of chiral chromatography to separate the two enantiomers, discussed below, can differentiate these two pathways of formation in simple systems (39). Thus, both enzymatic and non-enzymatic generation of eicosanoids, can generate a diverse array of products from the parent AA. The
initial composition of this complex mixture of eicosanoids depends upon the enzymatic machinery present and the physiochemical environment.

1.4 Further Metabolism of Eicosanoids

Total eicosanoid levels are controlled not only via COX/LOX dependent generation, but also by eicosanoid catabolism (40). Among the eicosanoid catabolism enzymes, 15-hydroxyprostaglandin dehydrogenase (15-PGDH) has been studied the most intensively. 15-PGDH is strongly down-regulated in numerous cancer cell lines and human cancers resulting in the accumulation of pro-proliferative PGs, such as PGE$_2$ (41). Increasing 15-PGDH levels reduces PGE$_2$ and results in anti-proliferative effects in cell and mouse models (42,43). Additionally, COX-2 inhibitor therapy alone fails in some patients who are 15-PGDH deficient, suggesting that a combination of COX-2 inhibitors and inducers of 15-PGDH provide a better chemoprevention approach (44).

15-PGDH preferentially converts the 15(S)-hydroxyl group on eicosanoids to a keto group (45). For example, PGE$_2$ undergoes 15-PGDH-mediated conversion to 15-keto-PGE$_2$ which can be further metabolized to 13,14-dihydro-15-keto-PGE$_2$ by a specific reductase. The primary cofactor of 15-PGDH is NAD$^+$. A different type II 15-PGDH uses NADP$^+$ as its cofactor, but has a $K_m$ value two orders of magnitude higher than NAD$^+$ dependent 15-PGDH when PGE$_2$ is the substrate (46). Molecules with a $\omega$-6 hydroxyl group, such as the LOX-type product 15(S)-HETE, are also readily oxidized by 15-PGDH (47). This suggests that 15-PGDH could also play an important role in the metabolism of different oxidized fatty acids, which is a topic explored in Chapter 2.

After being reduced, these $\alpha,\beta$-unsaturated ketone containing fatty acids can undergo glutathione S-transferase (GST)-mediated conjugation with glutathione (GSH) (48). The GSH conjugates have higher polarity than their precursors, and they can be excreted out of cells through relevant transporters (49). The interconnected metabolism and function of 15-PGDH products are discussed in more detail in Chapters 2, 3, and 4.
The localization of eicosanoids is also important to their function and metabolism. In the setting of cancer, the generation, signaling, and metabolism of eicosanoids is partly controlled via dysregulation of eicosanoid transporters (50). This is consistent with the intracellular biotransformation of eicosanoids such as PGE\textsubscript{2} and action via GCPRs with binding pockets facing the extracellular space. The transporters thought to be important to eicosanoids include members of the family of Organic Anion Transporters (OATs) and the Multidrug Resistance Proteins (MRPs) of the ATP-Binding Cassette family (ABC). Specifically, the PG transporter (PGT) and OATP-D provide carrier mediated transport for efflux of the eicosanoids (51). MRP4 has also been shown to efflux eicosanoids, notably PGE\textsubscript{2} (52). MRP1 mediates efflux of GSH-conjugates, including LOX products (49). Studies into the effect of eicosanoids may need to take into account the state and relevance of transporters to any bioactivity, potency, or metabolism under investigation as is further discussed in Chapter

<table>
<thead>
<tr>
<th>Term</th>
<th>Co-factor</th>
<th>Variable</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>Eddy Diffusion</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>B/ν</td>
<td>Longitudinal Diffusion</td>
</tr>
<tr>
<td>C</td>
<td>(Cs+Cm)ν</td>
<td>Mass Transfer</td>
</tr>
</tbody>
</table>

Table 1.1: The van Deemter equation for departure from ideal chromatography. Simplified to $H = A + B/ν + C*ν$ from the original $h = 2λ \cdot d_p + (2γ D_m/ν) + (8/π^2) (κ/ (1+κ)^2)(d_f^2/D_1 ν)$ Where $A$= Eddy diffusion term, $B$=coefficient of longitudinal diffusion, $C$=coefficient of mass transfer, $D$= coefficient of molecular diffusion $(m=mobile\ phase, l=liquid\ stationary\ phase), d= diameter\ (p=particle, f=film), k=column\ capacity\ ratio, \ λ=proportionality\ factor, h= plate height, ν=linear velocity. The C term has been expanded to include modern chromatographic instrumentation, and some modern treatments have re-written the fundamental equation. Adapted from (3,4).
1.5 Liquid Chromatography-Mass Spectrometry for Metabolite Measurements

LC uses the differential partition of a molecule of interest to a mobile phase (solvent) and a stationary phase (column) to separate compounds from a complex sample. Thus, LC adds a dimension, termed retention time, to the identity of the molecule being studied (an analyte). Retention time reflects the physical interaction of the liquid chromatography separation (53). The underlying factors of liquid chromatography can be expressed by the Van Deemter equation (Table 1.1). In this equation, the resolving power in height equivalents of a theoretical plate is explained by departure from the ideal chromatography caused by molecular diffusion, eddy diffusion, and mass transfer coefficient (4). This equation explains how the characteristics of a liquid chromatography method can be tuned to maximize the number of theoretical plates by minimizing their theoretical height. This optimization would provide the best separation possible between any set of molecules. Driving improved chromatographic performance is thereby dependent on the ideal packing of the column particle material, low dispersion, fit mass transfer between phases, and optimum velocity of flow. Approaches to chromatographic performance in metabolite analysis are further discussed in Chapter 6.

The chemistry of the column can provide varying selectivity for different compounds based on the characteristics of the compounds being separated (charge, hydrophobicity and chirality) (39). The nature of the solvents can determine the analyte interactions with the column to provide reversed phase chromatography, where a polar solvent is transitioned to an increasing non-polar solvent, or normal chromatography where a non-polar solvent is transitioned to a more polar solvent. Column chemistries and solvent selection can be optimized to provide differing retention times to even closely related compounds, including structural isomers difficult to resolve by other methods (54). However, retention time is often an insufficiently specific dimension for accurate analysis of related molecules or complex mixtures, thus additional terms are required for identification and quantitation of a given analyte. This leads to the necessity of coupling LC to
another instrument in order to provide additional data to describe molecules from a complex mixture.

A powerful instrument called a mass spectrometer can be attached after the LC to provide additional information about an analyte. The fundamental output of a mass spectrometer is the determination of signal intensity for a given mass to charge ratio \((m/z)\). The \(m/z\) is derived from the mass of the molecule as well as the number of charges imparted to the molecule. By definition a mass spectrometer must analyze a charged molecule so; mass spectrometers actually measure the ions (charged species) of a given analyte.

A mass spectrometer operates on the principles of: generation of an ion (at the source), separation of the ion (via a mass analyzer), and detection (at the detector). Each of these segments of the mass spectrometer will be discussed sequentially in the following paragraphs, starting with the ion source. The source of the mass spectrometer determines the type of ions generated, and influences the resulting analysis. Ion polarity can be either positive (+) or negative (-), with the polarity mode of detection imparting some properties to the resulting data based on the specifics of the analyte (55). When coupled with LC, the most widely used source utilizes atmospheric pressure ionization (API) (56). Analytes in the liquid mobile phase are desolvated by charged droplet collapse (electrospray ionization, ESI) (57) or by applying a current to a conducting needle under atmospheric pressure

<table>
<thead>
<tr>
<th>Mass Analyzer</th>
<th>Detection</th>
<th>Separation in Space or Time</th>
<th>Operating Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triple Quadrupole (T5Q/AP4000)</td>
<td>Destructive</td>
<td>Space</td>
<td>Unit</td>
</tr>
<tr>
<td>Linear Ion Trap (LTQ)</td>
<td>Mixed</td>
<td>Time</td>
<td>Unit or better</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Non-destructive</td>
<td>Time</td>
<td>100k - n*10^6 Rxt/T</td>
</tr>
<tr>
<td>LTQ-Orbitrap XL Hybrid</td>
<td>Non-destructive (Mixed)</td>
<td>Time</td>
<td>30k – 120k Rxt/T</td>
</tr>
</tbody>
</table>

*Table 1.2: The mass spectrometers employed in this thesis.* Mode of detection in terms of ion destruction through analysis for each instrument is given. Separation in-time or in-space is given for each instrument for better understanding of the
(atmospheric pressure chemical ionization, APCI) (58). Analytes are charged in solution for ESI by adjusting the pH of the mobile phase, or by other techniques (e.g. derivatization). High temperature, nitrogen gas, a spray/source voltage, and/or vacuum at the source of the instrument may also assist the desolvation process. Flow rates from the LC into the source can drastically affect the nature and output of the source, and the resulting analysis and detection of ions (59). Different analytes are often differentially compatible with ionization by distinct source types; often a chemical knowledge or empirical approach is needed to determine the ideal conditions for ionization, taking into account prior knowledge of matrix effects, solvent effects, thermal stability, propensity for adduction, and stability holding charge (60).

The mass analyzer is the primary instrument used to distinguish ions with different m/zs, given the resolving power of the mass spectrometer. A functional definition of resolution is the m/z divided by the minimum m/z peak separation of ions with the same nominal mass expressed in Da. (61). A larger value of resolution means better separation of peaks, or an isolated peak with a smaller width as measured from the full width of the peak at half maximum of the peak height (FWHM). Mass spectrometers featuring different analyzers are highlighted in Table 1.2. Low resolution instruments, which typically operate at unit mass resolution, can only distinguish two ions that differ in mass by 1 Da. The TSQ, API4000, and LTQ are the low resolution instruments used in this thesis. High resolution by contrast, could be up to 2,000,000 FWHM (or higher) (62), giving neutron encoded mass defect specific information, but more practically runs a range of 30,000 to 240,000 FWHM. An LTQ-Orbitrap XL and a FT-ICR are high resolution instruments used in this thesis. High resolution instruments confer much higher analytical specificity than the corresponding low resolution instruments.
Figure 1.4: Schematic of tandem MS experiments. (A) Product ion scan for generation of a fragment ion spectra. (B) Precursor ion scan for detection of the set of parent ions giving rise to a particular fragment. (C) Neutral loss scan for synchronized detection of the set of ions that lose a given m/z upon fragmentation. (D) Ion monitoring (Single/Multiple Reaction) for detection of a precursor/fragment pair.

Adapted from (2).

The interplay of the analyzer and detector results in different characteristics of the resulting data acquired, such as scan rate (expressed in Hz), dynamic range, instrument specific strengths and weaknesses, and an increasing requirement for data management strategies as file size increases exponentially. The detector on mass spectrometers also plays an independent role in analysis. Amplification and interpretation of the signal is an important component of detection due to the inherently weak signal generated by a low abundance ion. For induction based detectors, such as utilized in the Orbitrap, Fourier transform is used to convert amplitude to
a useable m/z. Characteristics, utilization, and the future applications of mass analyzers are discussed in greater detail in Chapter 6 and the Conclusion.

Aside from resolution of a mass analyzer, tandem mass analysis (MS/MS or MS\textsuperscript{n}) can be used to improve sensitivity (increased signal/noise) and specificity and increase the information gained from a mass spectrometry experiment (63). Tandem mass spectrometry can be tandem in space where the elements are physically separated across analysis as in a transmission quadrupole (e.g. triple quadrupole (64)) or time-of-flight instrument. Alternatively, tandem in time based analysis involves trapping the ions in a confined physical space, with separations occurring over time, as in an ion trap (65,66). The type of analysis determines the type of experiment which can be performed on the ions of interest. Analysis in space allows true precursor ion and neutral loss scanning, where the ions are scanned through the first mass analyzer and a product ion is scanned through the last mass analyzer. In the case of the neutral loss scan, the first and last mass analyzers are operated at a given offset, so only an ion losing a certain value of m/z is detected. Both analysis in time and analysis in space can perform product ion scans, where a given ion is selected in the first stage, fragmented, and then the resulting product ions are analyzed in the last stage. The final type of tandem MS experiments is selected reaction monitoring, where the first and last stages are set to a given mass range so a distinct precursor and product ion are selected for. This type of analysis can be used to improve performance for a given experiment by tuning sensitivity, specificity, and information gained. This thesis features experiments conducted in all of these analysis modes, where specific instrumentation used for such analysis is indicated in Table 1.2.
Derivatization offers a route to improve liquid chromatography-mass spectrometry performance. Gains in sensitivity, specificity, ionization efficiency, analyte stability, and improved chromatographic characteristics, including retention and peak shape, can be attained through simple derivatization schemes (54,58). The application of specific derivatization techniques to analytical challenges are discussed in chapters 2 and 3. In particular, this thesis takes advantage of liquid chromatography-electron capture atmospheric pressure chemical ionization-mass spectrometry (LC/ECAPCI/MS) via derivatization with pentafluorobenzyl bromide (PFB) (58). This technique can provide increased sensitivity and specificity via increasing the ionization efficiency of a target analyte by tagging an electron capturing group (PFB in this case) to the analyte of interest. The process of derivatization and ECAPCI is shown in Figure 1.5.

Ultimately, LC-MS provides a highly sensitive and specific platform to measure a range of analytes even from complex matrices (67). Advancements in high resolution and accurate mass MS (68,69) coupled with bioinformatics and software platforms (70-72) have made MS the optimal methodology for robust, sensitive, high-throughput metabolomic and proteomic analyses (73,74). Regardless of these advances, a LC-MS system is not truly quantitative, even for relative measurements, without the application of additional analytical methodologies. Appropriate internal standards are especially critical when measuring analytes in complex and/or
biological samples, due to possibly systemic bias introduced by matrix effects on analyte stability, extraction, and ionization efficiency (75-80). Internal standards that faithfully reproduce the properties of the analytes of interest are ideal. Stable isotope analogs containing $[^{15}N_n][^{13}C_n]$, or both isotopes, represent the best internal standards because they have the same chemical properties as the analyte of interest, but can still be uniquely distinguished by a mass spectrometer (81). $[^2H_n]$-containing stable isotope analogs are much easier to synthesize and tend to be used even though there is usually a slight separation between the $[^2H_n]$-analog and the endogenous analyte. Therefore the use of $[^2H_n]$-containing stable isotope analogs is not quite as rigorous as the corresponding $[^{15}N_n]$- or $[^{13}C_n]$-containing stable isotope analogs. Applying stable isotope analogs to a LC-MS experiment allows for quantification based on the observed ratio of analyte to internal standard. This approach is termed stable isotope dilution (SID) LC-MS, and represents the gold standard method of analysis because it corrects for many potential sources of bias and error introduced by the experimenter and instrumentation (82).

The power of LC-MS, especially when coupled to other techniques including but not limited to derivatization and SID-LC-MS, can be applied to quantitatively and qualitatively study metabolism in a rigorous and sensitive manner. This thesis will apply LC-MS as explained in the above paragraphs to the problem of human metabolism, in particular lipid metabolism. The lipid subset of the human metabolic system is particularly interesting because of the incredible diversity of known and potential pathways and products. Lipids are an important element of metabolism because of their involvement in a wide variety of disease states.

Notably, in inflammatory diseases and multiple human cancers, increased COX-2 and decreased 15-PGDH are hypothesized to lead to a milieu of bioactive lipids promoting angiogenesis and cellular proliferation. I hypothesized that this pro-proliferative switch is also dependent on the loss of generation of anti-proliferative homeostatic lipids. In chapter 2, through LC-MS and other analytical techniques, it has been established that 11-oxo-eicosatetraenoic acid (11-oxo-ETE) is a major COX-2/15-PGDH derived metabolite with anti-proliferative function. Based on the observation that a cancer model system rapidly exported 11-oxo-ETE, I sought to
understand the disposition of 11-oxo-ETE and the related 15-oxo-ETE. I hypothesize that the potency of endogenous anti-proliferative lipids is mediated by the decreased availability of those molecules to the intracellular space of cancer cells. The inadequacies of the available chemical tools in this sphere led to a search for new chemical entities to use as probes for future studies. In chapter 3, methyl-ester derivatives of both 11-oxo and 15-oxo-ETE were tested. Using SID-LC-MS, it was possible to show that the methyl esters potently deliver the oxo-ETE to the intracellular space. In chapters 3 and 4, the signaling and disposition of the oxo-ETEs were explored, using the tools that I developed, which are described in chapters 2 and 3.

In my early work, it was observed that the oxo-ETEs were highly transient. This led to the hypothesis that 11-oxo- and 15-oxo-ETE are subject to further metabolic transformation inside the cellular space. To explore the metabolism of the oxo-ETEs untargeted and targeted approaches to biomolecule analysis were developed as described in chapters 5 and 6. Using these untargeted and targeted approaches, limited saturation of the free acids was observed, as well as the formation and saturation up to all four double bonds of a novel coenzyme A thioester of oxo-ETEs. The identification of novel metabolites was supported through LC-MS/MS, time course studies, stable isotope labeling, and treatments with the methyl-esters of the oxo-ETEs as well as isotope labeled 15-oxo-ETE.
Chapter 2: 11-Oxo-eicosatetraenoic Acid is a Cyclooxygenase-2/15-Hydroxyprostaglandin Dehydrogenase-Derived Anti-Proliferative Eicosanoid


2.1 Abstract

Previously, we established that 11(R)-hydroxy-5,8,12,14-(Z,Z,E,Z)-eicosatetraenoic acid (HETE) was a significant cyclooxygenase (COX)-2-derived arachidonic acid (AA) metabolite in epithelial cells. Stable isotope dilution chiral liquid chromatography (LC)-electron capture atmospheric pressure chemical ionization (ECAPCI)/mass spectrometry (MS) was used to quantify COX-2-derived eicosanoids in the human colorectal adenocarcinoma (LoVo) epithelial cell line, which expresses both COX-2 and 15-hydroxy prostaglandin dehydrogenase (15-PGDH). 11(R)-HETE secretion reached peak concentrations within minutes after AA addition before rapidly diminishing, suggesting further metabolism had occurred. Surprisingly, 15-PGDH, which is normally specific for oxidation of eicosanoid 15(S)-hydroxyl groups, was found to convert 11(R)-HETE to 11-oxo-5,8,12,14-(Z,Z,E,Z)-eicosatetraenoic acid (ETE). Furthermore, LoVo cell lysates converted 11(R)-HETE to 11-oxo-ETE and inhibition of 15-PGDH with 5-[[4-
ethoxycarbonyl) phenyl[azo]-2-hydroxy-benzeneacetic acid (CAY 10397) (50 µM) significantly suppressed endogenous 11-oxo-ETE production with a corresponding increase in 11(R)-HETE. These data confirmed COX-2 and 15-PGDH as enzymes responsible for 11-oxo-ETE biosynthesis. Finally, addition of AA to the LoVo cells resulted in rapid secretion of 11-oxo-ETE into the media, reaching peak levels within 20 min of starting the incubation. This was followed by a sharp decrease in 11-oxo-ETE levels. Glutathione (GSH)-S-transferase (GST) was found to metabolize 11-oxo-ETE to the 11-oxo-ETE-GSH (OEG)-adduct in LoVo cells, as confirmed by LC-MS/MS analysis. Bromodeoxyuridine (BrdU)-based cell proliferation assays in human umbilical vein endothelial cells (HUVECs) revealed that the half-maximal inhibitory concentration (IC₅₀) of 11-oxo-ETE for inhibition of HUVEC proliferation was 2.1 µM. These results show that 11-oxo-ETE is a novel COX-2/15-PGDH-derived eicosanoid, which inhibits endothelial cell proliferation with a potency that is similar to that observed for 15d-PGJ₂.

2.2 Introduction

In a previous study, we established that 11(R)-HETE was a significant COX-2-derived AA metabolite in epithelial cells.(83) However, the consequences of this finding were not clear at that time. A number of toxic substances including arsenite,(84) dioxin,(85) benz[a]pyrene-diol-epoxide,(86) and cigarette smoke(87) up-regulate COX-2 expression, which in turn regulates numerous intracellular biochemical pathways. This occurs primarily through the biosynthesis of COX-2-derived AA metabolites, which can exert cell-specific effects on inflammation, cell growth, and proliferation. For example COX-2-derived prostaglandin (PG) E₂ increases tumor proliferation through multiple mechanisms including activation of plasma membrane G-protein-coupled receptors and the nuclear peroxisome proliferator-activated receptor (PPAR)γ.(13)
Steady-state levels of PGE$_2$ are maintained by PGE-synthase-mediated biosynthesis from COX-2-derived PGH$_2$ and catabolism by 15-PGDH-mediated inactivation to 15-oxo-PGE$_2$ (Figure 1). The 15-oxo-PGE$_2$ is then converted to 13,14-dihydro-15-oxo-PGE$_2$ by 15-oxo-prostaglandin-Δ$^{13}$ reductase. (90) Loss of 15-PGDH expression is associated with tumor formation in bladder, breast, colon, intestine, kidney, lung, pancreas, stomach, and skin cancer. (41,91-96) Thus, up-regulation of COX-2(13) and down-regulation of 15-PGDH(41) provides a switch towards endogenous mediators that are significant contributors to cancer progression. (97)

PGD$_2$, another COX-2-derived metabolite is also metabolized by 15-PGDH to form 15-oxo-PGD$_2$, which is then converted to the corresponding inactive 13,14-dihydro-derivative (Figure 1). (98) Alternatively, PGD$_2$ undergoes albumin-mediated dehydration to give PGJ$_2$, followed by a further dehydration to give 15-deoxy-Δ$^{12,14}$-prostaglandin J$_2$; (15d-PGJ$_2$) (Figure 2). (99) Previous studies have shown that 15d-PGJ$_2$ is a PPARγ agonist, (100) which inhibits HUVEC proliferation in culture. (101) In addition, 15d-PGJ$_2$ can induce caspase-mediated endothelial cell apoptosis(102) and inhibit the nuclear factor κB (NFκB) pathway. (103,104) It can also increase levels of p53 in HUVECs, activate p53 phosphorylation, and induce p21. (105)

Studies of purified COX enzymes have shown that 11(R)-HETE, 15(S)-hydroxy-5,8,11,13-(Z,Z,Z,E)-eicosatetraenoic acid (15(S)-HETE), and 15(R)-HETE are the major HETEs that are formed. (106) The HETEs arise from reduction of the corresponding hydroperoxyeicosatetraenoic acids (HPETEs) primarily through the peroxidase (POX) activity of COXs (Figure 1). (107) 11(R)-HETE was a significant eicosanoid secreted by AA-treated rat intestinal epithelial cells that stably express COX-2 (RIES cells) but it was rapidly metabolized. (83) 15(S)-HETE was formed in lower abundance and metabolized to 15-oxo-ETE,(54,108) as expected from its 15(S)-configuration and the substrate specificity of 15-PGDH. 15-Oxo-ETE was also found to inhibit
endothelial cell proliferation, although at concentrations higher than 15d-PGJ$_2$.(108) However, there is little evidence that 15d-PGJ$_2$ can be formed in vivo at concentrations commensurate with an endogenous anti-proliferative role.(109) In our earlier study,(110) the metabolic fate of 11(R)-HETE secreted by the RIES cells was not established. 11-Oxo-ETE has now been synthesized and a LC-selected reaction monitoring (SRM)/MS method for its analysis has been developed. This has made it possible to determine whether 11-oxo-ETE is secreted by human epithelial cell lines with up-regulated COX-2 expression and to identify the dehydrogenase responsible. The ability of 11-oxo-ETE to modulate HUVEC proliferation has also been examined.

2.3 Experimental Procedures

**Chemicals and Reagents.** AA (peroxide-free), 11(R,S)-HETE, 15(R,S)-HETE, [${}^2$H$_6$]-15(S)-HETE, [${}^{13}$C$_{20}$]-15-oxo-ETE, 15-oxo-ETE, PGE$_2$, [${}^2$H$_4$]-PGE$_2$, 13,14-dihydro-15-keto-PGE$_2$, [${}^3$H$_4$]-13,14-dihydro-15-keto-PGE$_2$, 15d-PGJ$_2$, CAY10397, protease inhibitor cocktail and recombinant human 15-PGDH were purchased from Cayman (Ann Harbor, MI). The Dess-Martin reagent [1,1,1-Tris(acetyloxy)-1,1-dihydro-1,2-benziodoxol-3-(1H)-one], 2,3,4,5,6-pentafluorobenzyl (PFB) bromide, Trizma-HCl, lipoxidase from Glycine max (soybean), sodium borohydride, and NAD$^+$ were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was from Gemini Bioproducts (West Sacramento, CA). F12K medium, DMEM medium, Medium 200 (M200), D-glucose, L-glutamine, low-serum growth supplement (LSGS) kit, penicillin, and streptomycin were supplied by Invitrogen (Carlsbad, CA). LC-MS grade water, hexane, methanol, isopropanol, acetonitrile and dichloromethane were obtained from Fisher Scientific (Pittsburgh, PA). Gases were supplied by The Linde Group (Munich, Germany). [${}^{13}$C$_{20}$]-AA was obtained from Spectra Stable Isotopes (Columbia, MD).
**Cell Culture.** Human colorectal adenocarcinoma LoVo cells (ATCC, Manassas, VA) were cultured in F12K medium supplemented with 10% FBS, 2 mM L-glutamine, 100,000 units/l penicillin and 100 mg/l streptomycin. Human colonic adenocarcinoma HCA-7 Colony 29 cells (Sigma-Aldrich, St. Louis, MO) were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 110 mg/L sodium pyruvate, 100,000 units/l penicillin and 100 mg/l streptomycin. For lipidomics analysis, the culture media was replaced with serum-free F12K or DMEM media before the treatment. HUVECs were obtained from Invitrogen (Carlsbad, CA), and cultured on collagen I-coated tissue culture dishes in Medium 200 supplemented with LSGS kit. Cell proliferation assays were performed using HUVECs from passage 4.

**Mass Spectrometry.** A triple stage quadrupole (TSQ Quantum) mass spectrometer (Thermo Electron, San Jose, CA) equipped with an APCI source was used for the quantitative lipidomics analyses. Targeted chiral LC-ECAPCI/SRM/MS analysis was conducted using PFB derivatives of 7 lipids and 4 heavy isotope analog internal standards. For the lipidomics profile, the instrument was operated in the negative ion mode, and unit resolution was maintained for both precursor and fragment ions. Operating conditions for the TSQ Quantum were: vaporizer temperature at 450°C; heated capillary temperature at 250 °C with the corona discharge needle set at 30 µA; nitrogen as sheath (25 psi) and auxiliary (5 arbitrary units) gas. Collision-induced dissociation (CID) was performed using argon as the collision gas at 2.7 mtorr in the RF-only quadrupole. The following SRM transitions were used: 11-oxo-ETE-PFB, m/z 317 → 165 (collision energy (CE), 25 eV), 15-oxo-ETE-PFB, m/z 317 → 113 (CE, 18 eV), [15C20]-15-oxo-ETE-PFB, m/z 337 → 120 (CE, 18 eV), 11(R)-HETE-PFB, m/z 319 → 167 (CE, 16 eV), [14H4]-15(S)-HETE-PFB, m/z 327 → 226 (CE, 13 eV), PGE2-PFB, m/z 351 → 271 (CE, 18 eV), [14H4]-PGE2-PFB, m/z 355 → 275 (CE, 18 eV). 13,14-dihydro-15-keto-PGE2-PFB, m/z 351 → 235 (CE, 22 eV), [14H4]-13,14-dihydro-15-keto-PGE2-PFB, m/z 355 → 239 (CE, 22 eV).
For GSH adduct analysis, the mass spectrometer was operated in the positive ion mode with an electrospray ionization (ESI) source. The operating conditions were: spray voltage at 4 kV; capillary temperature at 350°C; nitrogen as sheath (35 psi) and auxiliary (13 arbitrary units) gas. CID was performed using argon as the collision gas at 2.7 mtorr in the Rf-only quadrupole. The following SRM transition (m/z 626 → 497) was monitored for 11-oxo-ETE-GSH (CE, 18 eV).

**Liquid Chromatography.** LC separations were conducted using a Waters® Alliance 2690 HPLC system. A Chiralpak AD-H column (250 × 4.6 mm inner diameter, 5 µm; Daicel) was employed for normal phase separation (flow-rate 1 ml/min) of PFB-derivatives of eicosanoids. Gradient 1 was used for separating PFB-derivatives of HETEs and PGE₂, whereas gradient 2 was used for PFB derivatives of oxo-ETEs. For gradient 1, solvent A was hexane, and solvent B was methanol/isopropanol (1:1; v/v). Gradient 1 was as follows: 2% B at 0 min, 2% B at 3 min, 3.6% B at 11 min, 8% B at 15 min, 8% B at 27 min, 50% B at 30 min, 50% at 35 min, and 2% B at 37 min. Separations were performed at 30°C using a linear gradient. For gradient 2, solvent A was hexane, and solvent B was isopropanol/hexane (6:4; v/v). Gradient 2 was as follows: 2% B at 0 min, 2% B at 14.5 min, 12% B at 15 min, 23% B at 19 min, 90% B at 19.5 min, 90% B at 23.5 min, and 2% B at 24 min.

A Chiralpak AD-RH column (150 × 4.6 mm inner diameter, 5 µm; Daicel) was used for reverse phase (isocratic method 1, flow-rate 0.5 ml/min) separation of the un-derivatized 11-oxo-ETE. The mobile phase for isocratic separations was methanol/water/formic acid (95:5:0.1; v/v).

Chemically synthesized 11-oxo-ETE was purified by normal-phase (isocratic method 2) preparative LC (Ultrasphere™ 250 × 10 mm, inner diameter, 5 µm; Beckman) using Waters® Alliance 2690 HPLC system by monitoring the UV absorbance at 236 nm. The mobile phase for isocratic method 2 (flow-rate 2.5 ml/min) was hexane/isopropanol/acetic acid (98.5:1.5:0.1 ; v/v).

GSH adducts were separated by reverse phase using gradient 3 on Waters® Alliance 2690 HPLC system. The separation employed a Phenomenex Synergi Hydro-RP column (150 × 4.6
mm inner diameters, 5 µm). Solvent A was 0.1% aqueous formic acid, and solvent B was methanol/acetonitrile (50:50; v/v). Gradient 3 was as follows: 2% B at 0 min, 2% B at 14 min, 30% B at 20 min, 42% B at 21 min, 65% B at 27 min, 80% B at 29 min, 90% B at 33 min, 90% B at 34 min, 2% B at 35 min. The flow rate was 0.4 ml/min. The separation was performed at ambient temperature using a linear gradient.

**PFB Derivatization.** Eicosanoids were dissolved in 100 µl of acetonitrile, and then reacted with 100 µl of PFB bromide in acetonitrile (1:19; v/v) and 100 µl of diisopropylethylamine in acetonitrile (1:9; v/v) at room temperature for 30 min. The derivatives were evaporated to dryness, dissolved in 100 µl of hexane/ethanol (95:5; v/v) and analyzed by chiral LC-ECAPCI/SRM/MS.

**Enzymatic Conversion of 11(R)-HETE by 15-PGDH.** Various concentrations of 11(R)-HETE (0, 2.3 µM, 4.6 µM, 6.9 µM, 9.2 µM and 23 µM) were incubated with 4 nM of recombinant human 15-PGDH (50 ng, 1.8 pmol) and cofactor NAD⁺ (400 µM) in 50 mM Tris-Cl (pH 7.9) for 3.5 min at 37 °C. Each total reaction volume was 200 µl. After a 3.5 min incubation, the enzymatic reaction was quenched with 400 µl of ice cold methanol and [13C20]-15-oxo-ETE (8 ng) added as the internal standard. Eicosanoids were extracted with 1.2 ml of dichloromethane/methanol (8:1; v/v). The lower organic layer was then evaporated to dryness under nitrogen and reconstituted in methanol (100 µl). An aliquot (25 µl) was separated using isocratic method 1 and analyzed by LC-ESI/MS as described above. The retention time for 11-oxo-ETE was 8.7 min. In separate experiments, the formation of 11-oxo-ETE was found to be linear for the first 5 min. Eicosanoids were quantified by interpolation from a standard curve prepared with 11-oxo-ETE using [13C20]-15-oxo-ETE as the internal standard.

**Chemical Synthesis and Purification of 11-Oxo-ETE.** The Dess-Martin reagent (5 mg, 12.0 µmol) was added to a solution of 11(R,S)-HETE (10 mg, 31.0 µmol) in dichloromethane (0.5 ml)
and stirred for 2 h at room temperature. The reaction was monitored by LC-MS using gradient 1 as described above, after PFB derivatization until there was no starting material left. There was only one major product, which corresponded to 11-oxo-ETE. The reaction mixture was centrifuged twice at 3,400 rpm (10 min) and the supernatant was evaporated. The residue was dissolved in the mobile phase (800 µl) and purified by isocratic method 2 as described above. The retention time for 11-oxo-ETE was 13.1 min. High resolution accurate mass measurements were obtained using electrospray ionization on a Thermo LTQ-FT mass spectrometer at a resolution of 100,000 (data not shown). NMR spectra were obtained on a Bruker 500 MHz NMR instrument.

15-PGDH inhibition in LoVo or HCA-7 Cell Lysates by CAY10397. LoVo or HCA-7 cells were grown to 90% confluence, washed with 10 ml of phosphate-buffered saline (PBS) buffer (2 times), and then gently scraped in 600 µl of lysis buffer containing 0.1 M Tris-HCl (pH 7.9) and the protease inhibitor. Cell suspension was transferred to 2 ml Eppendorf tubes, and sonicated for 60 seconds on ice (power 5). Cell lysate was then incubated with or without the selective 15-PGDH inhibitor (CAY10397, 50 µM) and its co-factor (NAD⁺, 500 µM) for 10 min at 37°C. The pH was then adjusted to 4 with 10% aqueous acetic acid (10 µl) followed by addition of the internal standard mix, [¹³C₂₀]-15-oxo-ETE, [³H₃]-15(S)-HETE and [²H₄]-PGE₂ (50 pg/µl, 20 µl). Diethyl ether (600 µl) was added, and samples were vortex-mixed and centrifuged (15000 rpm×2 min). The organic layer was evaporated under nitrogen, and then the eicosanoids were derivatized with PFB bromide as mentioned above. Finally, samples were re-dissolved in hexane/ethanol (95:5; v/v, 100 µl), and analyzed (20 µl) by normal phase LC-ECAPCI/MS. The amounts of eicosanoids were normalized by protein concentrations of each lysate, which were determined by BCA assay.
Metabolism of AA by LoVo or HCA-7 Cells. LoVo or HCA-7 cells were grown to 90% confluence in 6-well plates as described above, and then fed fresh serum-free F-12K or DMEM medium. Cells were then incubated with AA (10 µM) for 0, 5, 10 and 30 min, 1 and 2.5 h at 37°C. At each time point, 0.6 ml medium was taken out, 20 µl 10% aqueous acetic acid was added to adjust pH to 3-4, together with 20 µl internal standards mixture (50 pg/ µl [12C20]-15-oxo-ETE, [3H8]-15(S)-HETE, [3H4]-PGE2 and [3H4]-13,14-dihydro-15-keto-PGE2-PFB). Then diethyl ether (1 ml) was added, and the mixture was vortex-mixed and centrifuged (15000 rpm x 2 min). The upper ether layer was evaporated under nitrogen, and PFB derivatives were synthesized as described above and analyzed by normal phase LC-ECAPCI/MS.

Standard curves for Eicosanoid Quantification. To quantify eicosanoids excreted in the medium, Eppendorf tubes containing 0.6 ml F12K medium were spiked with lipid standards, together with internal standards for ([12C20]-15-oxo-ETE, [3H8]-15(S)-HETE, [3H4]-PGE2 and [3H4]-13,14-dihydro-15-keto-PGE2-PFB 1 ng each). To quantify eicosanoids in the cell lysate, lipids standards and internal standards mixture were spiked into 0.2 ml Tris-HCl buffer. The extraction and PFB derivatization methods are the same as mentioned above.

Analysis of 11-OEG adducts in LoVo cell lysate. LoVo cells were grown to 90% confluence and then washed with PBS (10 ml). Cells were gently scraped in 600 µl Tris-HCl buffer (0.1 M, pH=7.9), containing protease inhibitor. Cell lysates were transferred to 2 ml Eppendorf tubes, and sonicated for 60 seconds on ice (power 5). 11-oxo-ETE (20 ng in ethanol) was added to the lysate together with 1 mM GSH. After incubation for 25 min at 37°C, 10 µl of 10% acetic acid was added, and the sample was loaded onto SPE column (oasis HLB, 30 mg) pre-conditioned with methanol and then 0.1% formic acid. The column was washed with 1 ml water and eluted with 250 µl methanol, and then 20 µl was analyzed by reverse phase LC-ESI/MS using gradient 3, as described above.
**Cell proliferation assay.** BrdU incorporation in HUVECs was used to assess the effects of 11-oxo-ETE on cell proliferation. The BrdU assay was performed in a 96-well format using a commercially available colorimetric enzyme-linked immunosorbent assay (ELISA) kit (Roche), and also by immunofluorescence microscopy. Equal numbers of HUVECs in passage 4 were plated in either, collagen-I coated 96-well plates (2000 cells/well), or collagen-I coated 8-chamber tissue culture glass slides (10000 cells/chamber). Cells were allowed to attach overnight in 0.25% DMSO containing media. Eicosanoids were dissolved in DMSO, such that the final concentration of DMSO in cell media was always 0.25% or lower. Cells were then treated for 24 h with either vehicle (0.25% DMSO), 11-oxo-ETE (1 nM-100 μM), or 15d-PGJ₂ (1 nM-100 μM). After 18 h of treatment, BrdU (final concentration, 10 μM in 0.25% DMSO) was added to each treatment group for an additional 6 h.

For the colorimetric ELISA, the manufacturer’s protocol was followed to perform the assay. The absorbance at λ = 370 nm obtained from the assay was transformed to the cell numbers using a standard curve constructed by plating known number of HUVECs in triplicate. The IC₅₀ values for eicosanoid inhibition of HUVEC proliferation were defined as the half maximal inhibitory concentration for endothelial cell proliferation over 24 h when compared with vehicle-treated cells. They were determined from the regression lines of the log inhibitor vs. response curves using a least squares fit.

For the immunofluorescence staining, cells were fixed with neutral buffered formalin for 10 min, permeabilized with methanol for 20 min, and then DNA was denatured by pressure cooking the slides in 10 mM citric acid buffer, pH 6 for 1 h. Cells were then incubated overnight with rat anti-BrdU antibody (1:1000, Accurate Chemical & Scientific Corp.) at 4°C, followed by 30 min incubation at 37 °C with Cy3-conjugated donkey anti-rat secondary antibody (1:600, Jackson Immuno Research). Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI,
Invitrogen) and visualized using a Nikon E600 microscope equipped with differential interference contrast (Nomarski) optics and photographed (x200 magnification) with a Fast 1394 QICam (QImaging). Positive BrdU staining was quantified by image analysis using IVision Analysis Software (Biovision). The percentage of proliferating cells was determined by counting the BrdU-positive cells versus the total number of cells in randomly selected microscopic fields (10/replicate) for each treatment group.

**Statistical Analyses.** All experiments were conducted in triplicate, unless otherwise indicated. Statistical significance (p value ≤ 0.05) was determined using a two-tailed unpaired t-test employing GraphPad® Prism software (v 5.01).

### 2.4 Results

**Biosynthesis of 11-oxo-ETE by 15-PGDH.** Various concentrations of 11(R)-HETE were incubated with recombinant human 15-PGDH and NAD\(^+\) at 37°C. A Michaelis-Menton kinetic analysis of 11-oxo-ETE formation revealed that the \(V_{\text{max}}\) (296 nmol/min/mg), \(K_m\) (3.42 μM), and \(k_{\text{cat}}\) (8.6 min\(^{-1}\)) values for oxidation of 11(R)-HETE (Figure 3) were similar to those for 15(S)-HETE (\(K_m = 1.65 \mu\text{M}, k_{\text{cat}} = 8.6 \text{ min}^{-1}\); Supplementary Figure 1). Therefore, the catalytic efficiency (\(K_{\text{cat}}/K_m\)) for 15-PGDH-mediated oxidation of 11(R)-HETE (2513 min\(^{-1}\)mM\(^{-1}\), Figure 3) was similar to that for 15(S)-HETE (7091 min\(^{-1}\)mM\(^{-1}\); Supplementary Figure 1).

**Chemical Synthesis of Authentic 11-Oxo-ETE.** 11-Oxo-ETE was synthesized chemically by oxidizing racemic 11(R,S)-HETE using the Dess-Martin reagent, and then purified using chromatographic separation. A combined total of 10 mg of the racemic 11(R,S)-HETE resulted in the isolation of 4.9 mg of pure 11-oxo-ETE (overall yield of 49%). High resolution electrospray ionization-MS of 11-oxo-ETE revealed accurate masses of \(m/z\) 319.2263 and \(m/z\) 341.2079 for the protonated and sodiated molecules, respectively (calculated accurate mass for protonated
(C$_{20}$H$_{31}$O$_3$) and sodiated (C$_{20}$H$_{30}$O$_3$Na) molecules, m/z 319.2273 and m/z 341.2093, respectively].

500 MHz $^1$H-NMR ($^\delta$H, CDCl$_3$; Supplementary Figure 2) 7.55 (dd, J$_1$ = 11.5 Hz, J$_2$ =15.5, 1H), 6.21(d, J =15.5Hz, 1H), 6.15-6.11 (m, 1H), 5.97-5.92 (m, 1H), 5.60-5.58 (m, 2H), 5.41-5.38 (m, 2H), 3.35 (d, J =4.5Hz , 2H), 2.83-2.81 (m, 2H), 2.38-2.31 (m, 4H), 2.17-2.12 (m, 2H), 1.74-1.25 (m, 8H), 0.89 (t, J = 7.0 Hz, 3H). Analysis of purified 11-oxo-ETE as its PFB derivative by LC-ECAPCI/MS (Figure 4a) revealed an intense negative ion at m/z 317 corresponding to [M-PFB]-.

CID of [M-PFB] (m/z 317) and MS/MS analysis revealed major product ions at m/z 273, 219, 165, 149, and 123. The UV spectrum was consistent with the presence of a conjugated dienone with a UV $\lambda_{max}$ at 279 nm and molecular extinction coefficient ($\varepsilon$) of 18,985 M$^{-1}$cm$^{-1}$.

Confirmation of 11-oxo-ETE Identity by LC-MS and MS/MS Analyses. The LoVo cell line is known to express both COX-2 and 15-PGDH and only trace amounts of COX-1,(41,111) this was confirmed by Western blot analysis (data not shown). Cell lysates were incubated with 100 nM 11(\textit{R})-HETE in the presence of 500 µM NAD$^+$ for 10 min. LC-MS analysis of PFB derivatives of eicosanoids extracted from the LoVo cell lysate showed that there was a single major metabolite, which eluted at 12.8 min (data not shown). The full scan mass spectrum of this metabolite had only one major ion at m/z 317 corresponding to [M-PFB] (Figure 4b). CID and MS/MS analysis revealed the formation of intense product ions at m/z 123, 149, 165, 219, and 273 (Figure 4b). The product ion spectrum was identical with that obtained from authentic synthetic 11-oxo-ETE-PFB (Fig. 4a), which confirmed the identity of 11-oxo-ETE from LoVo cells. The product ion at m/z 165 in LoVo cell-derived and synthetic 11-oxo-ETE-PFB corresponded to cleavage between C-9 and C-10 and m/z 123 was formed from cleavage between C-11 and C-12 (Fig. 4).

Separation of Eicosanoids by Chiral LC-ECAPCI/SRM/MS. Lysates from LoVo cells were extracted for the eicosanoids, which were then analyzed (after PFB derivatization) by LC-
ECAPCI/SRM/MS. A representative chromatogram (Figure 5) reveals the separation of 11-oxo-ETE (retention time 12.8 min) and 15-oxo-ETE (retention time 12.0 min) that were formed in the LoVo cell lysate. Additional eicosanoids that were observed (Figure 5) include, 13,14-dihydro-15-keto-PGE2 (retention time, 33.6 min), 11(\textit{R})-HETE (retention time, 10.0 min), and PGE2 (retention time, 29.4 min).

**Inhibition of 15-PGDH by CAY10397 in LoVo and HCA-7 cell lysates.** In contrast to LoVo cells, the HCA-7 cell line is known to express COX-2 and only trace amounts of COX-1 and 15-PGDH.\(^{(41,112)}\) This was confirmed by Western blot analysis (data not shown). LoVo (Figure 6a) or HCA-7 (Figure 6b) cell lysates were incubated with 500 mM NAD+, with or without the 15-PGDH-inhibitor, CAY10397 (50 \(\mu\)M) for 10 min. Inhibition of 15-PGDH significantly abolished the formation of endogenous 11-oxo-ETE (Figure 6a) by 92\%, along with the diminished formation of 15-oxo-ETE as well as 13,14-dihydro-15-oxo-PGE2 (Figure 6a) in the LoVo cell lysate. However, since the oxo-ETEs and 13,14-dihydro-15-oxo-PGE2 were undetectable in HCA-7 cell lysate, their precursors were quantified instead, and CAY10397 had no effect on their levels (Figure 6b).

**Secretion of eicosanoids from LoVo and HCA-7 cells following AA addition.** Time-course analyses for the amount of different eicosanoids secreted by LoVo and HCA-7 cells following AA incubation for 0-2.5 h are shown in Figure 7a and Figure 7b, respectively. 11-Oxo-ETE was secreted into the cell media by LoVo cells along with 15-oxo-ETE and 13,14-dihydro-15-oxo-PGE2 (Figure 7a). Interestingly, both 11-oxo-ETE and 15-oxo-ETE reached maximum concentrations 10 min after the addition of AA to the cells. This was followed by a decline of both eicosanoids to steady-state levels after 2.5 h (Figure 7a) in LoVo cells. In contrast, 13,14-dihydro-15-oxo-PGE2 levels did not peak until 1 h after the addition of AA (Figure 7a) and remained constant throughout the remainder of the incubation period. In contrast, none of the
Eicosanoids resulting from 15-PGDH-mediated metabolism were detected in the media of HCA-7 cells incubated with AA (Figure 7b).

**Formation of 11-OEG in LoVo Cell Lysates.** Biosynthesis of 11-OEG was monitored in the cell lysate after the addition of 100 nM 11-oxo-ETE and 1 mM GSH to the LoVo cell lysate. 11-OEG formation was detected and confirmed by LC-MS/MS monitoring of the major metabolite formed after a 30 min incubation. OEG (retention time, 32.2 min, data not shown) was observed as an intense peak in the LC-ESI/MS chromatogram when analyzed by gradient 3 (as described above). CID and MS/MS analysis revealed the formation of intense product ions at m/z 497, 319, 308, and 179 (Figure 8).

**Effects of Eicosanoids on HUVEC Proliferation.** Proliferation by HUVECs was assessed by quantifying the BrdU incorporation into the cells actively synthesizing new DNA. The BrdU assay was performed using a quantitative colorimetric 96-well format ELISA as well as by immunofluorescence microscopy for observing the morphological effects of eicosanoids in HUVECs. For the BrdU ELISA, HUVECs (2000 cells/well) were treated with different doses (0-100 μM) of 11-oxo-ETE and 15d-PGJ₂ for a period of 24h. The cell numbers corresponding to each treatment dose were computed and used to calculate cell proliferation (%) as compared to the vehicle-treated controls. 11-Oxo-ETE (IC₅₀ = 2.1 μM) was equipotent with 15d-PGJ₂ (IC₅₀ = 2.3 μM), a known potent inhibitor of endothelial cell proliferation (Figure 9a). For the immunofluorescence analysis, HUVECs (8000 cells/chamber) were treated with 2 μM of either 11-oxo-ETE or 15d-PGJ₂ for 24 h. BrdU incorporation was assessed by counting the BrdU-positive cells as compared to the total number of cells counted in randomly selected microscopic fields (10 fields/treatment). The photomicrographs clearly show that treatment with 11-oxo-ETE as well as 15d-PGJ₂ remarkably reduced the total number of BrdU-positive cells (stained red) as compared to vehicle-treated controls (Figure 9b). Moreover, there was a distinct change in the
morphological appearance of the DAPI-stained (stained blue) cells in the eicosanoid-treated slides that failed to incorporate BrdU (Figure 9b).

2.5 Discussion

15-PGDH catalyzes NAD$^+$-mediated oxidation of the 15(S)-hydroxyl moiety of PGs and other eicosanoids (Fig 1).(41,98,108) Our previous studies had established that 15-PGDH is also responsible for metabolizing the 15-lipoxygenase-derived 15(S)-HETE in macrophages and monocytes to 15-oxo-ETE.(108) Surprisingly, we have now found that 11(R)-HETE is also metabolized by 15-PGDH to a novel eicosanoid, that was identified as 11-oxo-ETE (Fig 2). Moreover, the catalytic activity of human 15-PGDH for oxidation of 11(R)-HETE (Figure 2) was very similar to that observed for 15(S)-HETE (Supplementary Figure 1). These results were very surprising in view of the lack of a 15(S)-hydroxyl group on 11(R)-HETE as well as the incorrect 11(R)-stereochemistry. The structure of 11-oxo-ETE metabolite was established by chemically synthesizing authentic 11-oxo-ETE from racemic 11-HETE. 11-Oxo-ETE was formed in both LoVo cell lysates (Figure 6a) and secreted from intact cells (Figure 7a); whereas 11-oxo-ETE was not formed by HCA-7 cell lysates (Figure 6b) or secreted from intact cells (Figure 7b). Since LoVo cells express COX-2 as well as 15-PGDH, whereas HCA-7 cells express COX-2 but not 15-PGDH,(41,111,112) these data convincingly prove that 11-oxo-ETE is an endogenous product formed enzymatically by a combination of COX-2 and 15-PGDH.

11-oxo-ETE is isomeric with 15-oxo-ETE (Figure 2) and also has very similar LC properties. Therefore, a reversed phase LC-SRM/MS method was developed to separate the un-derivatized oxo-ETEs, and normal phase was implemented to separate their PFB derivatives. This made it possible to readily analyze the two oxo-ETEs. Endogenous eicosanoids formed by LoVo and HCA-7 epithelial cells and cell lysates as well as those formed after the addition of AA were
analyzed as PFB derivatives by chiral LC-ECAPCI/MS.(110,113) Representative chromatograms of the eicosanoids produced endogenously in the LoVo cell lysates are shown in Figure 5. Inhibition of 15-PGDH enzyme by CAY10397 significantly diminished the formation of endogenous 11-oxo-ETE in LoVo cell lysates (Figure 6a). In addition, the formation of 15-oxo-ETE as well as 13,14-dihydro-15-oxo-PGE₂, the other two 15-PGDH-dependent metabolites, were also significantly reduced in LoVo cell lysates treated with CAY10397 (Figure 6a). In contrast, the three 15-PGDH products, namely, 11-, 15-oxo-ETE and 13,14-dihydro-15-oxo-PGE₂, were undetectable in HCA-7 cells as these cells do not express any 15-PGDH.(41,111) Instead, the corresponding precursors for these three metabolites were observed in the lysates. Furthermore, treatment with CAY10397 had no effect on levels of 11(R)-HETE, 15(S)-HETE or PGE₂ (Figure 6b). Intact LoVo cells also secreted 11-oxo-ETE, 15-oxo-ETE as well as 13,14-dihydro-15-oxo-PGE₂. However, the 11-oxo-ETE was cleared very rapidly (Figure 7a), suggesting that further metabolism was occurring. Subsequently, it was observed that 11-oxo-ETE underwent GST-mediated metabolism to 11-OEG, similar to the formation of 15-OEG in RIES cells (Figure 8).(110)

We realized that 11-oxo-ETE, is very similar in structure to 15d-PGJ₂. It only differs in the lack of a C-8 to C-12 bond, in having a cis-8,9- rather than cis-9,10-double bond, and a cis- rather than trans-14,15-double bond (Figure 2). This suggested that 11-oxo-ETE might be a more effective inhibitor of HUVEC proliferation than 15-oxo-ETE(108) with a potency similar to that observed for 15d-PGJ₂.(101) In fact, 11-oxo-ETE was six times more potent than 15-oxo-ETE (data not shown) and equipotent with 15d-PGJ₂ at inhibition of HUVEC proliferation (Figure 9). Dose-response studies revealed that its IC₅₀ was 2.1 μM compared to an IC₅₀ value of 2.3 μM for 15d-PGJ₂ (Figure 9a). Moreover, immunofluorescence microscopy revealed that 11-oxo-ETE not only inhibited BrdU incorporation into the HUVECs but it also caused a dramatic change in the shape and morphology of these cells (Figure 9b). Although the total number of cells counted in
the eicosanoid-treated groups were quite similar to the vehicle-treated group, the BrdU-negative cells were significantly distorted (Figure 9b). Typically, if the cells are undergoing death by apoptosis, they would appear more compact and round. However, 11-oxo-treated cells were elongated and stretched (Figure 9b), which could be indicative of extensive cytoskeletal remodeling, cell cycle arrest and/or differentiation. Interestingly, 11-oxo-ETE formation could not be detected in HUVEC lysate that was incubated with 11(R)-HETE (data not shown). Finally, immunoblot analysis of HUVEC cell lysate failed to detect COX-2 protein (data not shown). Taken together, these data suggest a paracrine role for 11-oxo-ETE on endothelial cell proliferation.

As noted above, inhibition of 15-PGDH resulted in significant decreases in 11-oxo-ETE formation in LoVo cells (Figure 6). 15-PGDH is down-regulated in numerous cancers types, (41,91,92,94-96) which would cause a decrease in 11-oxo-ETE biosynthesis by preventing the in vivo conversion of 11(R)-HETE to 11-oxo-ETE (Figure 1). This could be particularly devastating as COX-2 becomes up-regulated during tumorigenesis, which would result in elevated PGE2 biosynthesis along with decreased 15-PGDH-mediated inactivation to 15-oxo-PGE2 (Figure 1). Ultimately, this would result in an increase in PGE2-mediated pro-proliferative activity(13,97) without the counter effect of anti-proliferative oxo-ETEs. Increased PGE2 activity can also arise through down-regulation of the influx PG transporter - the organic anion transporter polypeptide (OATP) 2A1 (Figure 1).(50,114)

There is some evidence that 11-oxo-ETE can activate nuclear PPARγ.(16) PPARγ is a ligand-dependent transcription factor responsible for the regulation of a number of cellular events ranging from lipid metabolism to apoptosis.(115) 15d-PGJ2 is a PPARγ agonist, which might account for its ability to inhibit endothelial cell proliferation.(100,101) However, this effect is only observed with pharmacological amounts of 15d-PGJ2 rather than endogenous
concentrations.\(^{(109)}\) Besides PPAR\(\gamma\) agonistic activity, 15d-PGJ\(_2\) is also known to be an inhibitor of NF\(\kappa\)B signaling, a pathway critical to cell proliferation as well as tumorigenesis.\(^{(116,117)}\) In view of the ability of COX-2/15-PGDH to rapidly metabolize AA to nM amounts of oxo-ETEs, together with the structural similarity of oxo-ETEs and 15d-PGJ\(_2\) (Figure 2), it will be important to determine which of these activities are shared by both classes of eicosanoids. It is noteworthy that docosahexaenoic acid and docosapentaenoic acid can be metabolized by the sequential action of COX-2 and dehydrogenises into oxo-eicosanoids that modulate the antioxidant response.\(^{(118)}\) Similarly 5-lipoxygenase-driven 5(S)-HETE is metabolized by 5-hydroxyeicosanoid dehydrogenase into the chemoattractant 5-oxo-ETE.\(^{(39)}\) \(^{(119)}(118)(118)(117)(116)(116)(116)\) Therefore, the oxo-eicosanoids represent a family of oxidized lipids with diverse biological activities.

In summary, our studies have revealed that down-regulation of 15-PGDH inhibits the formation of endogenous anti-proliferative eicosanoid, 11-oxo-ETE. Therefore, 15-PGDH has two quite distinct properties; it can either inactivate PGs or activate HETEs to oxo-ETEs that exert a paracrine effect on endothelial cells (Figure 1). 11-Oxo-ETE, a member of the oxo-ETE family,\(^{(119-121)}\) was observed previously as an endogenously-derived lipid in human atherosclerotic plaques.\(^{(122)}\) However, the biosynthesis of 11-oxo-ETE and its biological activity were not evaluated in that study. Furthermore, there does not appear to be any subsequent report of its formation either, \textit{in vitro} or \textit{in vivo}. We have now shown that in fact 11-oxo-ETE is derived from COX-2/15-PGDH-mediated AA metabolism and that it inhibits endothelial cell proliferation with an IC\(_{50}\) that is very similar to that of 15d-PGJ\(_2\).

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**Figure 2.1. Formation and action of COX-2-derived eicosanoids in epithelial cell models.**

AA is released from membrane phospholipids by calcium-dependent cytosolic phospholipase A2 (cPLA2). The released AA undergoes COX-2-mediated metabolism to PGs or forms the lipid hydroperoxides, 15(S)-hydroperoxyeicosatetraenoic acid; (HPETE), 15(R)-HPETE and 11(R)-HPETE, which are reduced to the corresponding HETEs. PGD₂ and PGE₂ are inactivated by 15-PGDH-mediated conversion to their 15-oxo-metabolites. Both 15-oxo-PGD₂ and 15-oxo-PGE₂ are converted to 13,14-dihydro-5-oxo-PG metabolites. Intact PGD₂ secreted by the epithelial
cells can undergo albumin-mediated dehydration to 15d-PGJ$_2$. PGE$_2$ secreted from the epithelial cells by the ABCC4 transporter is pro-proliferative for tumor cells. Re-uptake of PGE$_2$ by OATP2A1 into the epithelial cells leads to further 15-PGDH-mediated inactivation. In contrast to PGE$_2$ and PGD$_2$, 15(S)-HETE and 11(R)-HETE are activated by 15-PGDH-mediated oxidation to 15-oxo-ETE and 11-oxo-ETE, respectively. The oxo-ETEs are further conjugated to form OEGs. Secreted 15- and 11-oxo-ETE that escape further metabolism can then inhibit endothelial cell proliferation. Therefore, down-regulation of 15-PGDH and OATP2A1 would result in increased PGE$_2$-mediated tumor and endothelial cell proliferation.
Figure 2.2. Chemical structures of COX-2 derived eicosanoids.

Figure 2.3. Kinetic plot of the formation of 11-oxo-ETE by 15-PGDH. Various concentrations of 11(R)-HETE (0-23 µM) were incubated with 4 nM 15-PGDH and cofactor NAD+. Determinations for 11-oxo-ETE were conducted in triplicate (means ± S.E.M.) by stable isotope dilution LC-ESI/MS analyses.
Figure 2.4. Confirmation of 11-oxo-ETE formation by LC-MS/MS analysis.

Specific product ions observed by CID and MS/MS analysis of [M-PFB] (m/z 317), corresponding to 11-oxo-ETE are shown on the relevant chemical structures. (a) Synthetic 11-oxo-ETE-PFB standard; (b) 15-PGDH-derived 11-oxo-ETE-PFB from LoVo cell lysate to which 100 nM 11(R)-HETE had been added.
Figure 2.5. Targeted chiral lipidomics of COX-2 derived eicosanoids from LoVo cells.

LoVo cells were lysed; eicosanoids were extracted, derivatized with PFB bromide, and analyzed by...
LC-ECAPCI/SRM/MS. LoVo cell lysates were pre-treated with 50 µM CAY10397 to inhibit 15-PGDH to be able to detect the 11-, 15-HETEs and PGE₂. Representative chromatograms are shown for (a) 11(R)-HETE-PFB (m/z 319 → 167), (b) [²H₈]-15(S)-HETE-PFB internal standard (m/z 327 → 226), (c) 11-oxo-ETE-PFB (m/z 317 → 165), (c) 15-oxo-ETE-PFB (m/z 317 → 165), (d) [¹³C₂₀]-15-oxo-ETE-PFB internal standard (m/z 337 → 120), (e) PGE₂-PFB (m/z 351 → 271), (f) [²H₄]-PGE₂-PFB (m/z 355 → 275), (g) 13,14-dihydro-15-oxo-PGE₂-PFB (m/z 351 → 235), (h) [²H₄]-13,14-dihydro-15-oxo-PGE₂-PFB (m/z 355 → 239).

**Figure 2.6. Inhibition of 15-PGDH in LoVo and HCA-7 cell lysates by CAY10397.** (a)
LoVo or (b) HCA-7 cell lysate (1x10^6 cells/treatment group) was incubated with or without CAY10397 (50 µM, to inhibit 15-PGDH). Eicosanoids were extracted from the lysates and their levels were determined by chiral LC-ECAPCI/SRM/MS. Analyses were performed in triplicates, and error bars show S.E.M.

(a) LoVo cells

(b) HCA-7 cells

Figure 2.7. Time course for eicosanoids secreted from LoVo and HCA-7 cells.

(a) LoVo or (b) HCA-7 cells were incubated with 10 µM AA for 0-6 h. The different eicosanoids secreted into the media at various time-points were extracted and their levels were determined by stable isotope dilution LC-ECAPCI/SRM/MS analysis of their PFB derivatives. Determinations were conducted in triplicate (means ± S.E.M.), and the quantitation was performed using the standard curves generated for these eicosanoids.
Figure 2.8. LC-MS/MS analysis of 11-OEG adduct. 11-OEG was synthesized by reacting 200 nM 11-oxo-ETE and 1 mM GSH in LoVo cell lysate for 25 min at 37°C. The product was extracted by solid-phase extraction and analyzed by LC-ESI/MS/MS. Specific product ions observed by CID of [MH]+ (m/z 626) are shown with their relevant chemical structures in the MS/MS analysis of 11-OEG.
Figure 2.9. Effect of eicosanoids on cell proliferation of HUVECs. (a) For BrdU ELISA, HUVECs (2000 cells/well) were treated with various doses of 11-oxo-ETE and 15d-PGJ$_2$ for a period of 24 h. Cell proliferation (means ± S.E.M.) was assessed by measuring absorbance at 370 nm and converting it to cell numbers using a standard curve and thereby used to construct the IC$_{50}$ plots. All experiments were conducted three times in triplicate. Representative data from one experiment conducted in triplicate are shown as means ± S.E.M. (b) For immunofluorescence, HUVECs (8000/chamber) were treated with 2 µM of 15d-PGJ$_2$ or 11-oxo-ETE for 24 h and then stained for BrdU and counterstained with DAPI (cells stained blue). Photomicrographs were taken at x200 magnification, and BrdU-positive (stained purple) cells were counted in randomly selected microscopic fields (10/replicate) as compared to the total number of cells in these fields. All experiments were conducted in triplicate. Representative photomicrographs from one set of replicates are shown.
Figure 2.10. Kinetic plot of the formation of 15-oxo-ETE by 15-PGDH. Various concentrations of 15(S)-HETE were incubated with 4 nM 15-PGDH and cofactor NAD⁺. Determinations were conducted in triplicate (means ± S.E.M.) by stable isotope dilution LC-ESI-SRM/MS analyses.

Figure 2.11. 500 MHz ¹H NMR spectrum of 11-oxo-ETE in CDCl₃.
Chapter 3: Cellular Uptake and Anti-proliferative Effects of 11-oxo-eicosatetraenoic Acid


3.1 Abstract

Cyclooxygenases (COXs) metabolize arachidonic acid (AA) to hydroxyeicosatetraenoic acids (HETEs), which can then be oxidized by dehydrogenases such as 15-hydroxyprostaglandin dehydrogenase (15-PGDH) to oxo-eicosatetraenoic acids (ETEs). We have previously established that 11-oxo-eicosatetraenoic acid (oxo-ETE) and 15-oxo-ETE are COX-2/15-PGDH-derived metabolites. Stable isotope dilution chiral liquid chromatography coupled with electron capture atmospheric pressure chemical ionization/mass spectrometry has now been used to quantify uptake of 11-oxo-ETE and 15-oxo-ETE in both and LoVo cells and human umbilical vein endothelial cells (HUVECs). Intracellular 11-oxo- and 15-oxo-ETE concentrations reached maximum levels within 1 h and declined rapidly, with significant quantitative differences in uptake between the LoVo cells and the HUVECs. Maximal intracellular concentrations of 11-oxo-ETE were 0.02 ng/4x10^5 cells in the LoVo cells and 0.58 ng/4x10^5 cells in the HUVECs. Conversely, maximal levels of 15-oxo-ETE were 0.21 ng/4x10^5 in the LoVo cells and 0.01 ng/4x10^5 in the HUVECs. The methyl-esters of both 11-oxo- and 15-oxo-ETE increased the intracellular concentrations of the corresponding free oxo-ETEs by 3-8-fold. 11-oxo-ETE, 15-oxo-ETE, and their methyl esters inhibited proliferation in both
HUVECs and LoVo cells at concentrations of 2-10 μM, with 11-oxo-ETE methyl ester being the most potent inhibitor. Co-treatment with probenecid, an inhibitor of multiple drug resistance transporters (MRPs) 1 and 4, increased anti-proliferative effect of 11-oxo-ETE methyl ester in LoVo cells and increased the intracellular concentrations of 11-oxo-ETE from 0.05 ng/4x10^5 cells to 0.18 ng/4x10^5 cells. Therefore, the present study has established that the COX-2/15-PGDH derived eicosanoids 11-oxo- and 15-oxo-ETE enter target cells, that they inhibit cellular proliferation, and that their inhibitory effects are modulated by MRP exporters.

3.2 Introduction

AA metabolism is implicated in cellular and physiologic regulation, inflammatory diseases, and cancer (123). In colon cancers, COX-2 expression is increased and conversely 15-PGDH is down-regulated (41). There is also evidence for COX-2/15-PGDH counter-regulation in gastric, breast, and lung cancers (88,95,124). The magnitude of the up-regulation/down-regulation may even serve as an independent predictor of progression and survival (125,126). This “proliferative switch” is hypothesized to increase tumorigenesis and angiogenesis via increased prostaglandin (PG) E_2 formation (Fig. 1A) and a feed forward loop for COX-2 (127,128). However, COX-2 mediated AA metabolism also generates other eicosanoids including 11- and 15-hydroperoxyeicosatetraenoic acids (HPETEs) and after reductive metabolism, the more stable 11- and 15-HETEs (27,83) (Figs. 1A, 1B). 15-HPETE is also a major product of the lipoxygenase pathway, through various 15- or 12/15-lipoxygenases (LOXs; Fig. 1B) (33). 15-PGDH then oxidizes 11- or 15-HETE to the α,β-unsaturated ketone-containing oxo-ETEs (108,129). Confirmation of the dehydrogenase pathway has been obtained using numerous experimental paradigms. 15-oxo-ETE was found as a major product of
15-PGDH-mediated oxidation of 15(S)-HETE in rabbit lung, as a major product of AA from mast cells, and as a major product of stenosed canine coronary arteries (130-132). In addition, either COX/15-PGDH or LOX/15-PGDH mediated metabolic pathways are involved in the formation of 15-oxo-ETE (Fig. 1B) (83,108). Furthermore, 11-oxo- and 15-oxo-ETE have been detected in advanced human atherosclerotic lesions, although its route of formation was not examined in that study (122). Finally, we have demonstrated recently that 11-oxo-ETE is generated by COX/15-PGDH mediated metabolism (129).

In spite of significant evidence for the formation of 11- and 15-oxo-ETEs in vivo, the pharmacology of these endogenous metabolites has not been examined in detail. In contrast, the structurally related PG analog, 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2; Fig. 1A) has been very extensively studied as an endogenous PPARγ ligand, NF-κB modulator, and redox signaling mediator (12,133,134). Anti-proliferative and anti-inflammatory properties have also been examined for similar bioactive lipids including: 15-oxo-ETE (108), linoleic acid metabolites (135), a series of long-chain electrophilic fatty acids termed the EFOXs (118), and nitro-fatty acids (136). In the absence of a known G-protein coupled receptor, such as that activated by 5-oxo-ETE,(121) these bioactive lipids are hypothesized to rely on intra-cellular targets. Therefore, understanding the availability of these compounds to the cytoplasmic space is of critical importance. This study was designed to test the cellular uptake, metabolism, and anti-proliferative effects of 11-oxo-ETE in multiple cell models. We examined differential uptake of both 11-oxo- and 15-oxo-ETE between HUVECs and the LoVo colon cancer cell line. Using methyl-ester derivatives of 11-oxo-ETE and 15-oxo-ETE, we studied targeted intracellular delivery to HUVECs and LoVo cells. Finally, we investigated whether there was
potentiation of the anti-proliferative action of oxo-ETEs through targeted delivery or by pharmacological blockade of MRP exporters.

3.3 Materials and Methods

Chemicals and Reagents. LC-MS Optima grade hexanes, methanol, acetonitrile, isopropanol, protease inhibitor, and BCA protein quantification kit were obtained from Fisher Scientific (San Jose, CA). Dichloromethane, N,N-diisopropylethylamine (DIPEA), dimethyl sulfoxide (DMSO), and pentafluorobenzyl bromide (PFB) were from Sigma-Aldrich (St. Louis, MI). Probenecid was obtained from Enzo Life Sciences (Farmingdale, NY). Phosphate buffered saline (PBS) and 3-(N-morpholino) propanesulfonic acid (MOPS) were from Invitrogen (Carlsbad, CA). 11-oxo-ETE, 15-oxo-ETE, $[^{13}C_{20}]$-15-oxo-ETE, as well as the methyl esters of 11-oxo-ETE (11-oxo-ETE-ME) and 15-oxo-ETE (15-oxo-ETE-ME) were prepared in house with standard procedures (129). Western Lightning ECL was obtained from Perkin Elmer (Waltham, MA). Fetal bovine serum (FBS) was obtained from Gemini Bioproducts (West Sacramento, CA). HUVECs, human arterial endothelial cells (HAECs), Medium 200, Low Serum Growth Supplement (LSGS), penicillin, streptomycin, F-12K media, and DMEM media were obtained from Invitrogen (Carlsbad, CA). LoVo, MCF-7, A549, and HCA-7 cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA).

Cell culture. LoVo and adenocarcinoma human alveolar epithelial (A549) cells were maintained in F-12K media supplemented with 2% FBS and 100,000 units/L penicillin and 100mg/L streptomycin. HUVECs and human arterial endothelial cells (HAECs) were maintained in Medium 200 (Invitrogen, Carlsbad, CA) supplemented with the LSGS Kit on Collagen I-coated tissue culture dishes (Becton Dickinson, Bedford, MA). Human colonic adenocarcinoma (HCA-7) cells and the MCF-7 breast cancer cells were
maintained in DMEM supplemented with 2% FBS and 100,000 units/L penicillin and 100mg/L streptomycin. The relevant maintenance media was used for treatment unless otherwise indicated.

**Quantification of cellular uptake.** LoVo cells and HUVECs were plated at 4x10^5 cells / well in a 6-well tissue culture plate (Corning, Corning, NY) or a 6-well collagen coated 6-well plate (Becton Dickinson, Bedford, MA) and allowed to attach for 12 h. 11-oxo-ETE, 15-oxo-ETE, or the respective methyl ester stocks were re-suspended in media containing 0.25% DMSO at indicated concentrations. Either LoVo cells or HUVECs were treated for indicated time-points with indicated compounds. Media was pipetted off; a 3 mL aliquot was spiked with 1 ng [13C_{20}]-15-oxo-ETE internal standard, and taken for analysis. The cells were rinsed 4 times with cold PBS, gently scraped into 3 mL of cold PBS, and spiked with 1 ng [13C_{20}]-15-oxo-ETE internal standard. The final rinse of cold PBS was taken and sampled as a control for residual 11-oxo- or 15-oxo-ETE. Extraction and derivatization has been described in detail elsewhere (137). Briefly, media was extracted with diethyl ether with 0.5% acetic acid with vigorous shaking and the organic phase was separated and evaporated under nitrogen gas. Cells were extracted with dichloromethane/methanol (2:1,v/v, with 0.5% acetic acid) with vigorous shaking and one freeze-thaw cycle, the organic phase was then removed and evaporated under nitrogen gas. After evaporation of the organic phases to dryness, samples were suspended in 100 µL DIPEA in acetonitrile (1:9; v/v) and 200 µL of PFB bromide in acetonitrile (1:19; v/v) and kept at room temperature for 30 min. The derivatized samples were dried down again under nitrogen, and then re-suspended in 100 µL of hexane/ethanol (95:5; v/v) for stable isotope dilution chiral LC-selected reaction monitoring (SRM)/ECAPCI/MS analysis.
Treatment of cells with \[^{13}\text{C}_{20}]-15\text{-oxo-ETE}.\] LoVo cells (5x10^6) were cultured as described above and then treated with 10 mM \[^{13}\text{C}_{20}]-15\text{-oxo-ETE}.\] Cell and media fractions were pooled, and derivatization and analysis were performed as described above except the internal standard was omitted.

Liquid Chromatography. A Water’s Alliance 2690 HPLC system (Waters Ltd, Watford, Hertfordshire, UK) was used for liquid chromatography separations. The PFB derivatives of 11-oxo-ETE and 15-oxo-ETE were separated using normal phase Chiralpak AD-H column (250x4.6mm, 5µm; Daicel Chiral Technologies, Westchester, PA) with a 1 mL/min flow rate. Solvent A was hexanes and solvent B was isopropanol/hexane (6:4; v/v). Gradient composition was: 2% B at 0 min, 2% B at 14.5 min, 12% B at 15 min, 90% B at 17 min, 90% B at 22 min, 2% B at 22.5 min, and 2% B at 29 min.

Mass Spectrometry. A Thermo Triple Stage Quadrupole (TSQ Quantum) mass spectrometer (Thermo Scientific) with an APCI source was operated in negative ion mode. The following transitions corresponding to each compound were monitored; 11-oxo-ETE-PFB, m/z 317→165 [collision energy (CE), 25 eV] 15-oxo-ETE-PFB, m/z 317→113 (CE, 18 eV); \[^{13}\text{C}_{20}]-15\text{-oxo-ETE-PFB, m/z 337→120 (CE, 18 eV).}\] For absolute quantification of 15-oxo-ETE and 11-oxo-ETE, standard curves ranging from 0 to 2 ng and 0 to 4 ng, respectively, were generated in the same matrix under identical extraction conditions with pure compounds. Data analysis was performed using Xcalibur software (Thermo Scientific).

BrdU Incorporation Assays. HUVECs, LoVo, HCA-7, and A549 cells were plated at 2000 cells/well and allowed to attach for 12 h. Treatment media was prepared at indicated concentrations by serial dilution from the most concentrated stock, keeping constant 0.25% DMSO. Cells were treated for 24 h, and then spiked with BrdU for 6 h to
allow incorporation into newly synthesized DNA. The assay was developed using a BrdU cell proliferation kit (Roche Diagnostics) according to the manufacturer’s directions, and a UV-Vis plate reader (BioRad).

**Western Blots.** Cells were collected from pre-confluent cultures and lysed in RIPA buffer containing 1x protease inhibitor cocktail. Protein was quantified with a BCA kit. 30 μg of protein lysate in reducing conditions was loaded into 4-12% gradient gel and ran in MOPS buffer for 50 min at 200V. Proteins were transferred onto a nitrocellulose membrane overnight on ice at 30V. After blocking with 5% BSA in TBS/T, primary antibody was incubated overnight in blocking buffer. Primary antibodies for MRP1, MRP4, and GAPDH were respectively, Abcam (ab32574-100), (ab56675), and (ab8245). Secondary antibody was HRP-conjugated sheep anti-mouse from GE Life Sciences (NA9310). All antibodies were diluted in blocking buffer at 1:1000. Visualization was accomplished with Western Lightning ECL in a digital developer (GE Healthcare).

**MTT Proliferation Assays.** LoVo cells were plated at 2000 cells/well and allowed to attach for 12 h. Treatment media was prepared at indicated concentrations by serial dilution from the most concentrated stock, keeping constant 0.25% DMSO. Probenecid was added from a concentrated stock to 1mM treatment concentration. After indicated time-points, media was replaced with fresh base media containing no FBS or Pen/Strep, and MTT was added to a final concentration of 2 mg/mL and allowed to incubate for 4 h. After incubation, all of the media was removed and the MTT was eluted using pure isopropanol. The resulting absorbance was read at 565 nm in a 96-well plate using a UV-Vis plate reader (BioRad).

**Statistical analysis.** All statistical analyses were carried out using the GraphPad Prism (5) software package.
3.4 Results

**Intracellular 11-oxo-ETE was reduced in LoVo colon cancer cells versus human umbilical vein endothelial cells.** To study the uptake and metabolism of 11-oxo-ETE, LoVo cells or HUVECs were incubated with 10 μM of 11-oxo-ETE, 10 μM of 15-oxo-ETE, or media with 0.25% DMSO vehicle for 4 h. Media and cells were collected at various time-points. Quantification of the free 11-oxo- and 15-oxo-ETE was performed by stable isotope dilution chiral LC-SRM/ECAPI/MS with \[^{13}C_{20}\]-15-oxo-ETE as the internal standard. Cells were carefully normalized to cell count used in the experiments, and only allowed a minimum of time to attach in order to avoid excess growth. Cell volume determination would require lifting of the cells which, especially in the case of the collagen attached, elongated HUVECs, could result in a cell volume change.

Intracellular concentrations of 11-oxo-ETE were reduced in the LoVo cells (Fig. 2A) as when compared with the HUVECs (Fig. 2B) at all time-points examined. 15-oxo-ETE demonstrated the opposite trend, with greater intracellular amounts in LoVo cells (Fig. 2A) versus HUVECs (Fig. 2B). Maximal uptake of 11-oxo-ETE was achieved for LoVo cells and HUVECs at 30 min and 60 min, respectively. 15-oxo-ETE maximal uptake occurred at 30 min for both cell types. Clearance of the free 11-oxo-ETE occurred completely in both cell lines by 4 h, whereas 15-oxo-ETE was still detectable at that time. Treatment of the LoVo cells with \[^{13}C_{20}\]-15-oxo-ETE did not cause the generation of endogenous 11-oxo-ETE or 15-oxo ETE as judged by comparison of the LC-MS chromatogram that was obtained from the cell suspension (Supplementary Fig. 3) with that obtained from the internal standard alone (Supplementary Fig. 1). This conclusively demonstrated that 11-oxo-ETE uptake and metabolism was significantly different between the LoVo cells (Fig. 2A) and HUVECs (Fig. 2B).
11-oxo-ETE inhibited BrdU incorporation across multiple cell lines with varying potency. BrdU incorporation assays were used to measure the anti-proliferative effects of treatments with increasing doses of 11-oxo-ETE. The value obtained for vehicle treatment 0.25% DMSO was arbitrarily set at 100%. Multiple cancer cell lines were used including LoVo, HCA-7 and A549 from colon, colon, and lung cancer respectively. The same assay was conducted with HUVECs to allow comparison to our earlier work on 11-oxo- and 15-oxo-ETE (108,129). Increasing doses showed a dose-dependent reduction in proliferation. HUVECs were the most sensitive to treatment (Fig. 3A) followed by the colon cancer lines LoVo (Fig. 3B) and HCA-7 (Fig. 3C). A549 lung cancer cells showed no significant response to treatment until higher doses of 11-oxo-ETE were used (Fig. 3D).

11-oxo-ETE-ME and 15-oxo-ETE-ME preferentially target the intracellular space. In order to test the targeting of oxo-ETEs to the intracellular environment, 11-oxo-ETE-ME, 15-oxo-ETE-ME, 11-oxo-ETE, 15-oxo-ETE, and a vehicle control were incubated with LoVo cells and HUVECs for 60 min. Media and cells were then extracted and free oxo-ETEs were quantified by LC-MS. The methyl esters significantly increased the levels of free oxo-ETEs in the cell over the amount in the media in both LoVo cells (Fig. 4A) and HUVECs (Fig. 4B). The free 15-oxo-ETE reached a higher intracellular concentration in the LoVo cell (Fig 4A). In contrast the 11-oxo-ETE was higher in the HUVECs (Fig. 4B). Essentially no 11-oxo-ETE or 15-oxo-ETE was detected in the LC-MS chromatogram from the fourth wash of the LoVo cells treated with 11-oxo-ETE, 15-oxo-ETE, 11-oxo-ETE-ME or 15-oxo-ETE-ME (Supplementary Fig. 3) when compared with a control internal standard alone (Supplementary Fig. 2). Similar results were obtained from HUVECs (data not shown). Furthermore, there was no detectable 11-oxo-ETE-ME or 15-oxo-ETE-ME in the fourth wash of the cells (data not shown). This confirmed that
none of the 11-oxo-ETE or 15-oxo-ETE could have arisen from material left on the cell surface and suggested that the methyl ester derivatives could provide a useful delivery strategy to target the intracellular environment for both 11-oxo- and 15-oxo-ETE.

**MTT assays over multiple days demonstrate anti-proliferative effects for 11-oxo-ETE and 11-oxo-ETE-ME.** To observe the anti-proliferative effects of 11-oxo-ETE, 15-oxo-ETE and their methyl esters, MTT assays were carried out over 72 h. Every 24 h, samples were collected, and the media was refreshed. Values obtained for vehicle treatment 0.25% DMSO was arbitrarily set at 100%. 15d-PGJ$_2$ was included as a reference compound. 11-oxo-ETE dose dependently inhibited growth over multiple days (Fig. 5A). Furthermore, 11-oxo-ETE-ME reached significance for inhibition before the free 11-oxo-ETE (Fig. 5A). In all cases, by 72 h, significant anti-proliferative effects were observed versus the vehicle control (Figs. 5A, 5B). Interestingly, 11-oxo-ETE-ME (Fig. 5B) was more potent than 11-oxo-ETE (Fig. 5A), causing a significant antiproliferative effect at all three time points.

The transporter proteins MRP1/MRP4 were expressed in the more resistant cell lines. In order to help understand why there were differences in the intracellular 11-oxo-ETE concentrations the expression of MRP1 and MRP4 membrane transporters was examined. MRP4 expression was robust in the A549 lung cells and significant in the two endothelial lines tested (HUVEC, HAEC); whereas expression of MRP 1 was robust in all cancer lines (LoVo, HCA-7, MCF-7, and A549) when compared with the two endothelial lines (Fig. 6). This suggested that increased MRP1 expression could have been a major determinant of the reduced cellular 11-oxo-ETE levels in LoVo cells (Fig. 2A) when compared with the HUVECs (Fig. 2B). MRP1 has previously been implicated in PG export, especially in the context of cancer cell dependent up-regulation of tumor microenvironment PGE$_2$ (138).
Anti-proliferative effects of 11-oxo-ETE-ME are increased with co-treatment of the drug transport inhibitor probenecid. To test the possibility of blocking the drug transporters to increase anti-proliferative effects, a MTT assay over multiple days using the LoVo cell line was carried out. Treatments with probenecid, 11-oxo-ETE-ME, or the combination of both were compared to vehicle control arbitrarily set at 100%. At both 48 and 72 h, significantly increased anti-proliferative effects were observed for the combination treatment versus either treatment alone (Fig. 7). Pretreatment with probenecid increased the recovery of 11-oxo-ETE from the 11-oxo-ETE-ME treated LoVo cells (Fig. 8). This suggests that increased intracellular 11-oxo-ETE was the mechanism for the synergistic action of probenecid on 11-oxo-ETE-ME anti-proliferative action.

3.5 Discussion

The involvement of COX-2 and 15-PGDH in cancer progression has been well documented (14,40,41,139-142). Pro-proliferative AA metabolites derived from COX-2, such as PGE\(_2\) acting via the G-protein coupled PGE receptors (EP\(_1\), EP\(_2\), and EP\(_4\), induce proliferation and angiogenesis (15,143,144). Autocrine and paracrine signaling of PGE\(_2\) in cancer leads to a feed forward loop modulating local immune responses and increasing angiogenesis and proliferation (139,141). A decrease in catabolic 15-PGDH leads to increased activity of PGE\(_2\) due to its reduced metabolic clearance (41). However, AA metabolism leads to a plethora of metabolites with distinct and sometimes opposing functions (6). Considerable work on the anti-proliferative effects of AA metabolites have focused primarily on 15d-PGJ\(_2\) (17). These studies have been complicated by contradictory results (118,145,146), uncertainty over the actual
physiological relevance of the tested compounds (109), and a lack of dysregulation in disease states(11). Other cyclopentenone PGs such as PGA₂ have been linked to anti-proliferative action via inhibition of the cell cycle through cyclin D1 at 100μM (147).

11-oxo-ETE and 15-oxo-ETE are known endogenous compounds isolated from clinical specimens, and major metabolites of AA via COX/15-PGDH (83,108,122,129,132). In this study, measurable anti-proliferative effects were seen in three of four tested cell lines at 2 μM and all four cell lines at 10 μM (Fig. 4). Although 11-oxo-ETE was clearly more potent than 15-oxo-ETE, the effect of both eicosanoids was significant, and their effects could be modulated by targeted intracellular delivery or pharmacological blockade of transporters (Figs. 5, 6, 7). The amounts of 11-oxo-ETE and 15-oxo-ETE that were detected in the LoVo cells and HUVECs represented only a small fraction of the total amount of each oxo-ETE or oxo-ETE-ME that was added to the cells. From our previous work, we suspect that major amounts of the oxo-ETEs are conjugated to GSH, exported, and cleaved to the cysteinyglycine adduct (83). We are also actively investigating other biotransformation pathways that contribute to metabolic clearance. The finding that intracellular delivery of 11-oxo-ETE through use of the methyl ester derivative increased the anti-proliferative effects in LoVo cells (Figs. 4, 5, 7) lends support to the hypothesis that a plausible mechanism of action may be through intracellular targets. This was particularly evident in the increased anti-proliferative activity of 11-oxo-ETE-ME (Fig. 5B) when compared with the free 11-oxo-ETE (Fig. 5A). The amplification of anti-proliferative effects and increased recovery of 11-oxo-ETE with probenecid co-treatment (Fig. 7) also supports this hypothesis. This is in agreement with an expanding body of work supporting a hypothesis for the mechanism of action for certain bioactive lipids through intracellular signaling mediators (12,118,135,136). These
findings, along with our previous work on the GSH-mediated metabolism of 11-oxo- and
15-oxo-ETE may implicate intracellular uptake as a rate-limiting factor in bioactivity and
metabolism of these compounds (83).

During tumorigenesis, significant up-regulation of COX-2 occurs, which would
increase the production of pro-proliferative PGE$_2$, (13,141) as well as the antiproliferative
oxo-ETEs. However, there is also significant down-regualtion of 15-PGDH,
(41,92,95,96,148) which would result in increased activity of PGE$_2$ due to its decreased
catabolism coupled with a decrease in the formation of the oxo-ETEs (Fig. 1B) (129).
Increased expression of MRP4, (138,149,150) the transporter involved in the efflux of
PGE$_2$ (151,152) also occurs during tumorigenesis. This would further prevent the 15-
PGDH-mediated metabolism of PGE$_2$ in epithelial cells and so further facilitate an
increase in its activity at relevant membrane EPs. In contrast, increased efflux of oxo-
ETEs mediated by MRP4 would result in reduced activity because (as described above)
they have intracellular targets. Finally, the up-regulation of glutathione biosynthesis and
increased glutathione-S-transferase expression (153-155) would result in increased
conversion of oxo-ETEs into their corresponding inactive glutathione adducts (83).
Therefore, tumor progression is associated with substantial activation of pro-proliferative
PGE$_2$ and metabolic inactivation of the the oxo-ETEs.

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**Figure 3.1. Oxo-ETE chemical structures and metabolic pathways.** (A) Chemical structures of compounds used in this study, with PGE$_2$ provided for comparison. (B) The currently elucidated COX-2/15-LOX and 15-PGDH dependent pathway for generation of 11-oxo-ETE and 15-oxo-ETE. AA, Arachidonic Acid, COX, Cyclooxygenase, 15-LOX, 15-Lipoxigenase, cPLA$_2$, cytosolic phospholipase A$_2$, PGH$_2$, prostaglandin H$_2$, HPETE, hydroperoxyeicosatetraenoic acid, HETE, hydroxyeicosatetraenoic acid, 15-PGDH, 15-hydroxyprostaglandin dehydrogenase, POX, peroxidase, DH, dehydrogenase, oxo-ETE, oxo-eicosatetraenoic acid.
Figure 3.2. Disposition of oxo-ETEs in LoVo cells and HUVECs. Stable isotope dilution LC-SRM/ECAPCI/MS quantification of uptake and distribution of 15-oxo-ETE and 11-oxo-ETE by (A) LoVo cells and (B) HUVECs over time. Cells were incubated at 37°C with 15-oxo-ETE (2µM) or 11-oxo-ETE (2µM) for 5, 30, 60, and 240 min. For each time point, cellular fractions were collected, extracted and spiked with \[^{13}\text{C}_{20}\]-15-oxo-ETE, and derivatized with PFB. Each time point is plotted as the mean of triplicates with SEM.
Figure 3.3. Anti-proliferative action of 11-oxo-ETE. BrdU incorporation measured by ELISA in (A) HUVECs, (B) LoVo cells, (C) HCA-7 cells, (D) A549 cells, following 24h treatment with indicated dose of 11-oxo-ETE in 0.25% DMSO compared to vehicle control. 1-way ANOVA with post-hoc Dunnett’s Multiple Comparison test versus vehicle control was used to access statistical significance (* p< 0.05, ** p< 0.01, ***p < 0.001). Data are plotted as the means (n=3-8) with SEM bars, representative of at least two independent experiments.
Figure 3.4. Intracellular oxo-ETEs after methyl ester derivative treatment. Stable isotope dilution chiral LC-SRM/ECAPCI-MS quantification of the ratio of cellular/ media 11-oxo- and 15-oxo-ETE in (A) LoVo cells and (B) HUVECs. Cells were incubated with 15-oxo-ETE (10 µM), 15-oxo-ME (10 µM), 11-oxo-ETE (10 µM), 11-oxo-ME (10 µM), or 0.25% DMSO vehicle control for 60 min. Cellular and media fractions were collected, extracted, and spiked with [13C20]-15-oxo-ETE, and derivatized with PFB. Data are plotted as the means of triplicates with SEMs of the ratio of analytes in the cellular over media fractions. Statistical significance was assessed by 1-way ANOVA with post-hoc Bonferroni Multiple Comparison test (* = p<0.05).
**Figure 3.5. Anti-proliferative action of oxo-ETEs and their methyl esters.** Cell proliferation measured by MTT assay with LoVo cells over multiple days with the indicated compounds in 0.25% DMSO compared to vehicle control. Treatments with the free acids are shown in (A), the methyl-esters are shown in (B). Data are plotted as the means (n=4) with SEMs, representative of at least two independent experiments. Statistical significance was assessed by 1-way ANOVA with post-hoc Bonferroni Multiple Comparison test against the respective vehicle control (* p < 0.05, ** p < 0.01, *** p < 0.001).
Figure 3.6. MRP1 and MRP4 expression. Western blots of HAEC, HUVEC, MCF-7 cells, HCA-7 cells, LoVo cells, and A549 cells for transporters MRP 4 and MRP 1, together with the loading control GAPDH. Increased expression of MRP1 was observed in the cancer cells lines (MCF-7, HCA-7, LoVo, A549) versus the endothelial cells (HAEC, HUVEC). MRP4 expression was detectable in all cell lines, with robust expression in A549 cells.
Figure 3.7. Potentiation of 11-oxo-ETE by probenacid. Cell proliferation of LoVos measured by MTT assay over multiple days with 2 μM 11-oxo-ETE-ME and/or 1 mM probenecid in 0.25% DMSO compared to vehicle control. Data are plotted as the means (n=4) with SEMs, representative of at least two independent experiments. Statistical significance was assessed by 1-way ANOVA with post-hoc Bonferroni multiple comparison test (* p <0.05).

Figure 3.8. Increased recovery of 11-oxo-ETE with probenacid treatment. Stable isotope dilution chiral LC-SRM/ECAPCI-MS quantification of the recovery of 11-oxo-ETE
in LoVo cells. Cells were incubated with either 0.25% DMSO or 1mM probenecid for 30 min before incubation with 11-oxo-ME (10 µM) in 0.25% DMSO vehicle control for 60 min. Cellular and media fractions were collected, extracted, and spiked with [^{13}C_{20}]15-oxo-ETE, and derivatized with PFB. Data are plotted as the means of triplicates with SEMs. Statistical significance was assessed by 2-way student’s t-test (** = p<0.01).

Figure 3.9. LC-ECAPCI–MS of 15-oxo-ETE internal standard. 10ng PFB-derivatized \(^{13}\)C\(_{20}\)-15-oxo-ETE internal standard analyzed by normal phase ECAPCI-MS. Noise levels in the 11-oxo-ETE and 15-oxo-ETE channels are shown to establish a baseline.
Figure 3.10. Lack of residual oxo-ETEs after washing of the cells. The 4th wash of the cellular fraction was pooled for a 11-oxo-ETE and a 15-oxo-ETE treatment, derivatized, and analyzed as indicated for the media fractions. 11-oxo-ETE and 15-oxo-ETE channels demonstrate noise levels of signal, indicating that the wash steps were sufficient to remove detectable 11-oxo- or 15-oxo-ETE.

Figure 3.11. Lack of induction of oxo-ETEs by treatment with $^{13}$C$_{20}$-15-oxo-ETE.

LoVo cells were cultured and treated as indicated in the methods section, and then treated with 10μM $^{13}$C$_{20}$-15-oxo-ETE. Cell and media fractions were pooled, and derivitization and analysis were performed as indicated except the internal standard was ommitted. No detectable 11-oxo-ETE of 15-oxo-ETE was found, indicating that the $^{13}$C$_{20}$-15-oxo-ETE did not induce generation of 11-oxo-ETE or 15-oxo-ETE.
Chapter 4. A Covalently Modifying Endogenous Canonical NF-κB Inhibitor from Arachidonic Acid Metabolism

In preparation for submission as: A covalently modifying endogenous canonical NF-κB inhibitor from arachidonic acid metabolism. Nathaniel W. Snyder, Guang Yang, Xiaojing Liu, Suhong Zhang, Phyllis A. Dennery, and Ian A. Blair.

**Background:** Arachidonic acid (AA) metabolism results in the formation of eicosanoids including 11-oxo-eicosatetraenoic acid (11-oxo-ETE).

**Results:** 11-oxo-eicosatetraenoic acid was found to inhibit canonical p50/p65 NF-κB signaling via a covalent modification.

**Conclusion:** 11-oxo-ETE is a feedback mediator for arachidonic acid metabolism and NF-κB signaling.

**Significance:** Inflammatory states involved in normal physiology and disease are regulated by a diverse set of mediators.

**Summary**

α,β-unsaturated ketone containing molecules have been shown to bind to critical cysteine residues of proteins. Metabolism of arachidonic acid (AA) via Cyclooxygenase/15-hydroxyprostaglandin dehydrogenase (COX/15-PGDH) leads to the formation of 11-oxo-eicosatetraenoic acid (oxo-ETE), an α,β-unsaturated ketone containing eicosanoid with structural similarity to other bioactive lipids. 11-oxo-ETE has
no known receptor or function, but has been detected in human samples. Here, we show that pre-treatment of human umbilical vein endothelial cells (HUVECs) by 11-oxo-ETE reduced p50/p65 binding to consensus sequence DNA when activated by TNF-α and Kdo2-Lipid A. Inhibition of canonical NF-κB signaling was confirmed through electrophoretic mobility shift assays, western blot for manganese super oxide dismutase from TNF-α challenged HUVECs, and a stably expressed firefly luciferase NF-κB reporter assay in HEK293 cells. Western blots of phosphorylated inhibitor of kappa B (IκB) α and inhibitor of kappa B kinase (IKK) β revealed a decrease in phosphorylation of IκBα, with no change in phosphorylation of IKKβ. This finding was confirmed by in vitro kinase studies of IKKβ. LC-MS/MS of tryptic peptides from the DNA binding domain of p50/p65 revealed a Michael addition adduct of 11-oxo-ETE to a critical cysteine residue, as well as a histidine adduct. These experiments demonstrate that products of AA through COX/15-PGDH metabolism may serve as a negative feedback loop via a redox sensitive lipid mediator, 11-oxo-ETE. Therefore, it may be of interest to design stable 11-oxo-ETE mimetics or modulators of AA metabolism to induce 15-PGDH.

Introduction

Initiation, control, and resolution of inflammation are all regulated, in part, by arachidonic acid (AA) metabolism, and thus by the enzymes responsible for transforming the AA-derived bioactive lipids (6). However, the roles of several of these major downstream products remain to be elucidated.

AA metabolism by the cyclooxygenase enzymes (COX) begins with abstraction of the 13 pro-S hydrogen (26). Formation of prostaglandin (PG) G₂ occurs through insertion of molecular oxygen, followed by a second insertion and a cyclization reaction. A monoxygenase type reaction may also occur, with insertion of molecular oxygen at either C11 or C15 resulting in 11(R)-
hydroperoxyeicoatetraenoic acid (HPETE) or a mixture of 15(S) and 15(R)-HPETEs (156). The C15 monoxygenase reaction to 15(R)-HPETE is a major pathway after acetylation by aspirin. The HPETEs are then reduced by peroxidases yielding stable hydroxyeicosatetraenoic acids (HETEs) (157). 15-HPETE can also be generated from arachidonic acid by various lipoxygenases (LO) with specificity for the carbon where the oxygen insertion occurs, including a 12/15-LO and a 15-LO (33). 11- or 15-HETEs are then transformed into oxo-ETEs via a second level of metabolism involving 15-hydroxyprostaglandin dehydrogenase (15-PGDH), converting the hydroxyl group to a carbonyl (83,108,129). This creates an electrophilic α,β-unsaturated ketone that could react via a Michael addition with a nucleophilic cysteine or histidine residue.

The metabolic pathway for generation of the 11-oxo- and 15-oxo-ETE has been demonstrated in cell lines expressing both COX-2 and 15-PGDH (83,108,129). 11-oxo-ETE has been detected in advanced human atherosclerotic plaques (122). Despite our understanding of the metabolism and a demonstration of the endogenous presence of 11-oxo-ETE, there is no known G-protein coupled receptor, or any well described signaling pathway for this molecule. However, similar molecules such as the electrophilic oxo-derivatives (EFOXs) (118) and 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) (12), also contain α,β-unsaturated ketones, and have been shown to inhibit NF-κB signaling. Therefore, we hypothesized that 11-oxo-ETE may act via a similar mechanism. This study was aimed at investigating the effects of 11-oxo-ETE on the canonical signaling pathway of NF-κB mediated by the transcription factors p50/p65, and test a plausible mechanism based on covalent adduction to explain the inhibitory effects observed in a cell model system.

**Experimental Procedures**

*Chemicals and Reagents*—Protease inhibitor cocktail (100x), phosphatase inhibitor (100x) Trizma-HCl, ethacrynic acid, pentafluorobenzyl bromide (PFB), and clear flat-
bottom streptavidin coated 96-well plates were purchased from Sigma-Aldrich (St. Louis, MO). Medium 200 and low serum growth supplement (LSGS) were supplied by Invitrogen (Carlsbad, CA). RIPA and MOPS buffers, LC-MS grade (Optima) formic acid, water, and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA). Gases were supplied by The Linde Group (Munich, Germany). Electrophoretic mobility shift assay (EMSA) probes were purchased from Integrated DNA Technologies (Coralville, IA). Unless otherwise noted, antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Recombinant IKKβ, biotin-tagged IκB-α substrate peptide, ATP, and kinase buffer were also from Cell Signaling Technologies.

Cell Culture–Human umbilical vein endothelial cells (HUVECs) were obtained from Invitrogen (Carlsbad, CA) and were cultured in Medium 200 with low serum growth supplement on Collagen IV coated tissue culture plates from Becton Dickinson (Bedford, MA). HEK293 cells stably expressing firefly luciferase driven by a 5x-κB reporter were a kind gift of the Hogenesch lab at University of Pennsylvania and were maintained in DMEM with 2% FBS and 100,000 units/L penicillin and 100 mg/L streptomycin.

Transcription Factor Binding Assays–HUVECs were treated with 2 μM 11-oxo-ETE, 2 μM 15d-PGJ₂, or vehicle control 0.25% DMSO in maintenance media. After 15 minutes the cells were then challenged with 0.5 mg/mL Kdo₂-Lipid A and 40 ng/mL TNF-α or vehicle control for 45 minutes without changing the media. Nuclear fractions were taken from cells using a nuclear extraction kit from Cayman Chemical (Ann Arbor, MI) and p50/p65 binding was assayed using a p50/p65 binding EIA based detection kit from Cayman Chemical.
Electrophoretic Mobility Shift Assay—HUVECs were treated with 0.2 μM 11-oxo-ETE, 2 μM 11-oxo-ETE, the NF-κB inhibitor BAY 11-7085, or vehicle control 0.25% DMSO. After 15 minutes the cells were then challenged with 40 ng/mL TNF-α or vehicle control for 45 minutes without changing the media. Nuclear extracts were taken as described above. Duplexes of oligonucleotide containing the consensus sequence (5'-GCC TGG GAA A CT C GC CTC AAC T-3') or mutated sequence (5'-GCC TGG GAA ACT CGC CTC AAC T-3') of NF-κB were synthesized by Integrated DNA Technologies. EMSA assay was performed as described previously (158) with minor modification. Briefly, a \(^{32}\)P-labeled consensus NF-κB was used as a probe to evaluate NF-κB binding ability. To identify nonspecific binding of the nuclear proteins, competition reactions were performed by addition of either 50-fold excess of non-radiolabeled NF-κB or 50-fold excess of non-radiolabeled mutated NF-κB to the reaction mixtures prior to electrophoresis. In order to identify the NF-κB subunit proteins in the complex, 1 μL of p50 or p65 antibody, ab7971 or ab7970, respectively, from Abcam (Cambridge, MA) was incubated with the nuclear protein prior to addition of the radiolabeled probe to visualize any supershift-retarded bands in the NF-κB complex (11). A loading control oligomer SP-1 (5'-ATT CGA TCG GGG CGG GGC GAG C-3') was obtained from Promega (Madison, WI) (12). An AP-1 oligomer (5'-TTC CGG CTG AGT CAT CAA GCG C-3') from IDT was used as an additional control. Purity of all oligomers was checked by gel electrophoresis prior to use (data not shown).

Superoxide Dismutase Western Blotting—HUVECs were treated with 2 μM 11-oxo-ETE, 2 μM 11-oxo-ETE-methyl ester (11-oxo-ETE-ME), or vehicle control 0.25% DMSO in maintenance media. After 15 minutes the cells were challenged with 40 ng/mL TNF-α or
vehicle control for 6 hours without changing the media. Cells were lysed in RIPA buffer with protease inhibitor cocktail and flash frozen in dry ice/ethanol then stored at -80°C until analysis. Protein was quantified with a micro-BCA kit from Thermo-Pierce, and 30 μg of protein/well was loaded in a 4-12% gradient Bis-Tris gel and ran for 50 minutes at 200 V in MOPS buffer. Proteins were transferred to a nitrocellulose membrane in transfer buffer with 10% methanol at 30 V overnight at 4°C and blocked for an hour in 5% skim milk in TBS/T. The membrane was cut and incubated overnight at 4°C with the primary antibody. After washing, the membrane was incubated for 2 hours at room temperature with the secondary antibody. Visualization was performed in a digital analyzer (GE healthcare) with Western Lightning ECL reagent (Perkin Elmer).

**Phospho-protein Western Blotting**–HUVECs were treated with 2 μM 11-oxo-ETE, 2 μM 11-oxo-ETE-ME, or vehicle control 0.25% DMSO in maintenance media. After either 5 minutes or 6 hours the cells were challenged with 40 ng/mL TNF-α or vehicle control for 45 minutes without changing the media. Cells were lysed in RIPA buffer with 1x protease and 1x phosphatase inhibitor cocktail and flash frozen in dry ice/ethanol then stored at -80°C until analysis. Blotting and visualization were performed as above.

**IKKβ Kinase Assay**–HTScan IKKβ Kinase assay kit (Cell Signaling) was used as directed with minor modification to screen for IKKβ inhibition. Briefly, recombinant IKKβ was incubated with indicated compounds at the indicated concentrations for 15 minutes in 1x kinase buffer prepared separately from the kit using the reducing agent TCEP instead of DTT. Biotin-tagged IκB-α and ATP were added to a final concentration of 1.5 μM and 200 μM respectively, and the mix was incubated for 30 minutes at room temperature. The reaction was quenched with addition of 50 mM EDTA pH 8. The cocktail was loaded into strepavidin coated clear 96-well plates, allowed to bind for 2
hours at room temperature, washed with PBS/T three times. The plate was incubated with 100 mL 1:1000 anti-phospho-κB-α antibody in TBS/T with 5% BSA for 2 hours, then washed three times with 200 mL PBS/T. The plate was incubated with 100 mL 1:1000 secondary anti-rabbit antibody coupled to HRP for an hour at room temperature. The plate was developed and read in a UV-Vis spectrometer (BioRad).

*Adduct Formation and Analysis*—A synthetic peptide with a tryptic overhang of two residues on each terminus (FRYVCEGPSHGGLPGASSEKNK) corresponding to the human and mouse p50 DNA binding domain (Supplemental Fig. 1) was obtained from AnaSpec (Freemont, CA). The peptide was incubated overnight with 10 μM 11-oxo-ETE in 1 mM ammonium bicarbonate buffer pH 7.5 then digested with sequencing grade trypsin (Promega) for 16 hours at 37°C. The sample was then desalted on a C18 spin column from The Nest Group (Southborough, MA), evaporated in a vacuum dessicator, and dissolved in mobile phase A for LC-MS analysis. LC separations were conducted using a Waters nano-ACQUITY UPLC system (Waters Corp., Milford, MA, USA). A Waters BEH130 C18 column (100 μm x 150 mm inner diameter, 1.7 μm pore size; Waters Corp) was employed for reversed phase separation (flow-rate 1.8 μL/min). Solvent A was 98% water/2% acetonitrile with 0.1% formic acid, and solvent B was 99.5% acetonitrile/0.5% water with 0.1% formic acid. The gradient was as follows: 5% B at 0 min, 5% B at 3 min, 60% B at 35 min, 95% B at 37 min, 95% B at 43 min, 5% B at 44 min, and 5% at 60 min. Separations were performed at 30°C. A LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Fisher) was used in positive ion mode with a Michrom captive spray electrospray ionization (ESI) source. The operating conditions were: spray voltage at 4 kV; capillary temperature at 250°C; capillary voltage at 35 V;
collision induced dissociation (CID) was performed using helium as the collision gas in the ion trap (normalized collision energy [CE], 30%).

**NF-κB reporter assay**—HEK293 cells stably expressing firefly luciferase driven by a 5x-κB reporter were incubated with increasing concentrations of 11-oxo-ETE for 5 minutes before treatment with 40 ng/mL TNF-α for 6 hours. Treatment media was the same as maintenance media with 0.25% DMSO.

**LC-MS Quantification of Cellular Uptake**—HEK293s as above were plated at 4x10^5 cells / well in a 6-well tissue culture plate (Corning, Corning, NY) and allowed to attach for 12 hours. 11-oxo-ETE was re-suspended in media containing 0.25% DMSO at indicated concentrations by serial dilution from the most concentrated treatment. After 90 minutes, the cells were gently scraped in the media; an aliquot of 250 μL of the cell suspension was taken and spiked with 1 ng [^{13}C_{20}]-15-oxo-ETE internal standard. Extraction and derivatization has been described in detail elsewhere (159). Briefly, the samples were extracted with dichloromethane/methanol (2:1,v/v) containing 0.5% by volume acetic acid with vigorous shaking, the organic phase was then removed and evaporated under nitrogen gas. After evaporation of the organic phases to dryness, samples were suspended in 100 μL DIPEA in acetonitrile (1:9; v/v) and 200 μL of PFB bromide in acetonitrile (1:19; v/v) and kept at room temperature for 30 minutes. The derivatized samples were dried down again under nitrogen, and then re-suspended in 100 μL of hexane/ethanol (95:5; v/v) for stable isotope dilution chiral LC-selected reaction monitoring (SRM)/electron capture atmospheric pressure chemical ionization (ECAPCI)/MS analysis. A Water’s Alliance 2690 HPLC system (Waters Corp.) was used for liquid chromatography separations. The PFB derivatives of 11-oxo-ETE and 15-oxo-ETE were separated using normal phase Chiralpak AD-H column (250 x 4.6 mm, 5 μm;
Daicel Chiral Technologies, Westchester, PA) with a 1 mL/min flow rate. Solvent A was hexanes and solvent B was isopropanol/hexane (6:4; v/v). Gradient composition was:

- 2% B at 0-14.5 min, 12% B at 15 min, 90% B at 17 min, 90% B at 22 min, 2% B at 22.5 min, and 2% B at 29 min. Separations were performed at 30°C. A Thermo Triple Stage Quadrupole (TSQ Quantum) mass spectrometer (Thermo Scientific) with an APCI source was operated in negative ion mode as previously described (129). The following transitions corresponding to each compound were monitored: 11-oxo-ETE-PFB, m/z 317→165 (CE, 25 eV) and [13C20]-15-oxo-ETE-PFB, m/z 337→120 (CE, 18 eV). For absolute quantification of 11-oxo-ETE, a standard curve ranging from 0 to 2 ng was generated in the same matrix under identical extraction conditions with pure compound. Data analysis was performed using Xcalibur software (Thermo Scientific).

**Results**

11-oxo-ETE inhibits p50/p65 DNA Binding in HUVECs—Pretreatment of the HUVECs with 11-oxo-ETE or 15d-PGJ2 significantly reduced the additional p50 (Figs. 2A, 2C) and p65 (Figs. 2B, 2D) binding induced by treatment with Kdo2/TNF-α. These results were confirmed by EMSA. Either 2 μM 11-oxo-ETE (Fig. 3, lane 6) or the NF-κB inhibitor BAY 11-7085 (Fig. 3, lane 4) abolished the induced NF-κB signaling in the TNF-α treated HUVECs. Competition assays revealed that the induced factor was in fact NF-κB, and predominantly p65 (Fig. 3, lane 9).

11-oxo-ETE Dose-Dependently Inhibits NF-κB Driven Luciferase Activity—HEK293 cells stably expressing firefly luciferase driven by a 5x-κB reporter were incubated with increasing concentrations of 11-oxo-ETE for 5 minutes before treatment of 40 ng/mL
TNF-α for 6 h. 11-oxo-ETE reduced firefly luciferase activity in a dose-dependent manner versus the vehicle control (Fig. 4A). With a 30 minute pretreatment of 100 μM of the GST inhibitor ethacrynic acid the effect of 11-oxo-ETE on NF-κB inhibition was more evident (Fig. 4B), and recovery of 11-oxo-ETE from the treated cells was higher at 90 minutes (Fig 4B, 4C).

11-oxo-ETE Inhibits Downstream of IKKβ in HUVECs—Pretreatment of 11-oxo-ETE for 5 minutes before TNF-α challenge for 45 minutes caused a modest reduction in TNF-α-mediated elevation of phospho-IkB-α levels. The methyl ester of 11-oxo-ETE, which targets the intracellular space, also caused a modest reduction in phospho-IkB-α levels (Fig. 5A). However, a 6 hour pretreatment with 11-oxo-ETE or 11-oxo-ETE-ME, caused no inhibition of TNF-α-mediated phospho-IkB-α elevation (Fig. 5B).

To exclude the possibility of effects on upstream kinases, levels of phospho-IKKβ were surveyed by Western blotting from the same cell extracts. No reduction in phosphor-IKKβ was observed in either time point. In no cases did the 11-oxo-ETE or 11-oxo-ETE-ME alone induce significant phosphorylation of IKKβ or IkB-α (Fig. 5A, 5B).

In vitro screening of IKKβ activity with recombinant IKKβ, a biotin linked substrate, capture on a streptavidin coated plate, and HRP-based visualization was used to study the effects of α,β-unsaturated ketone containing eicosanoids on IKKβ kinase activity. This experiment demonstrated dose dependent inhibition of IKKβ by 15d-PGJ₂ and 11-oxo-ETE (Fig. 5C). 15-oxo-ETE was much less potent (data not shown).

11-oxo-ETE Treatment in HUVECs Inhibits the Induction of Manganese Superoxide Dismutase—Treatment of HUVECs with 11-oxo-ETE and 11-oxo-ETE-ME before TNF-α
challenge strongly inhibited the induction of the NF-κB target manganese superoxide
dismutase after 6 h (Fig. 6).

11-oxo-ETE Forms a Covalent Adduct with Amino Acids within the p50 DNA Binding
Domain—In order to mechanistically explain the reduction in p50/p65 activity, we
investigated the possible formation of a covalent adduct forming between the
electrophilic α,β-unsaturated ketone of 11-oxo-ETE and the nucleophilic residues of
p50/p65. The tryptic peptide corresponding to the cysteine and histidine containing
DNA-binding domain of p50 was chosen because it would offer the most structural
information for complete elucidation of the adduct (Supplemental Fig. 1A).

The synthetic peptide with a tryptic overhang of 2 residues on each terminus was
obtained from AnaSpec, incubated overnight with 10 μM 11-oxo-ETE then digested with
trypsin for 16 h. The sample was then desalted using a C18 spin column (The Nest
Group), evaporated in a vacuum desiccator, and dissolved in mobile phase A for
chromatographic separation and mass spectrometry.

Reversed LC-MS/MS revealed two putative covalent adducts that were isomeric.
Positive ion mode charge state determination and high-resolution accurate mass
determination in the Orbitrap showed that the unmodified and modified peptide was
primarily triply charged (MH₃⁺) (Supplemental Fig. 2A, 2B). The LC retention time of the
unmodified peptide (17.4 min; Fig 7A) was shorter than the two modified peptides (24.8
min, 28.3 min; Fig. 7B). This was consistent with the increased hydrophobicity derived
from the addition of a fatty acid. MS/MS analysis of MH₄⁺ (m/z 698.35) from the later
eluting adduct, suggested that 11-oxo-ETE had added to the peptide residue
corresponding to cysteine-61 (Supplementary Fig. 4) of the p50 protein (Supplementary
Fig. 1). Three relatively high abundance $y_{11}^{+2}, y_{13}^{+2}, y_{14}^{+2}$ ions were observed containing an unmodified histidine residue provided compelling evidence for a covalent modification on cysteine (Fig. 8, Supplemental Fig. 4). The presence of the oxo-ETE moiety was confirmed by the ion at m/z 319.2258 (Calculated m/z for $C_{20}H_{36}O_3 = 319.2268$).

The earlier eluting adduct had a product ion spectrum that was consistent with addition of 11-oxo-ETE to the histidine moiety (Fig. 8, Supplemental Fig. 3). The spectrum was quite different from that observed for the cysteine adduct. In particular, there was a relatively intense $y_{16}$ ion containing the oxo-ETE moiety. Additional minor product ions confirmed that the attachment of oxo-ETE was at the peptide residue corresponding to histidine-64 (Fig. 8) of the p50 DNA binding domain.

**Discussion**

The specific identity and mechanism for lipid mediators of resolution remain controversial. Anti-inflammatory lipids with a mechanism of action through a GPCR, such as the lipoxins, may constitute one family of signaling molecules. Other molecules such as 15d-PGJ$_2$, the EFOXs derived from eicosapentaenoic acid and docosahexaenoic acid, nitro-fatty acids, and now 11-oxo-ETE have non-G protein-coupled receptor mechanisms of action (12,118,135,136,145,160).

$\alpha,\beta$-unsaturated ketone containing fatty acids can form covalent adducts to cysteine and histidine residues of proteins (161). In the case of adduction to signaling proteins, such as Keap1-Nrf2 (135), PPAR$\gamma$ (12), NF-\kappaB (12), or IKK$\beta$ (133) this may mediate a cellular response as a redox sensor. Such a system of redox-mediated feedback has been hypothesized to be important in the control and resolution of inflammatory
responses. Alternatively, other adduct forming entities such as 4-hydroxynonenal have been implicated in pathological oxidative stress (162).

The present study has unequivocally shown that 11-oxo-ETE is directly analogous to (and slightly more potent than) the widely studied PGD$_2$ degradation product 15-d-PGJ$_2$ in its ability to inhibit the activity of NF-κB (Fig. 2). This occurs through a pathway that did not appear to involve diminished phosphorylation of IKKβ (Fig. 5A) or IκB-α (Fig. 5B). In spite of a very modest change in the phosphorylation status of IKKβ there was a clear dose-dependent decrease in the activity of NF-κB (Fig. 5C).

The structure/function relationship of electrophiles to inhibition of NF-κB is likely complex, especially within the wider pathological context. This may be attributable to the structural properties of targets for electrophiles, as has been suggested by studies with PPARγ (16,163). Available structural data on the NF-κB family members examined in our study points to the critical nature of the contact of the cysteine binding domain (Supplementary Fig. 1) with the target consensus sequence DNA (164,165). Interactions may also be a property of the contextually dependent metabolism of the electrophile (166).

Identifying 11-oxo-ETE-mediated covalent modifications to NF-κB components such as p50 or p65 in a cellular context would be extremely challenging because of the trace amounts that are present. Furthermore, the purified proteins require substantial amounts of free thiols to maintain their activity. This would prevent the covalent attachment of any electrophilic Michael acceptors such as 11-oxo-ETE. Therefore, we used a potential tryptic peptide (with two amino acid overhangs at the trypsin sites) derived from the p50 component of NF-κB (Supplementary Figure 1A). After treatment of the peptide with 11-oxo-ETE and trypsin digestion, an adduct was identified with a structure that was consistent with covalent attachment of 11-oxo-ETE to cysteine-61.
(Fig. 8, Supplementary Fig. 4) and an adduct at histidine-66 (Fig. 8, Supplementary Fig. 3). Availability of these covalently modified peptides show that the 11-oxo-ETE modifications were a plausible hypothesis to explain the cellular responses observed earlier. However, current LC-MS methodology does not have sufficient sensitivity to determine whether the 11-oxo-ETE modifications can also occur on the endogenous p50 protein.

Interestingly, 15d-PGJ$_2$, 15-oxo-ETE, and 11-oxo-ETE also exhibit anti-proliferative effects (108,129). In inflammatory states and in multiple cancers, COX-2 is up-regulated and 15-PGDH is down-regulated (88,115,127,167,168). This metabolic dysregulation, as well as perturbation of cellular eicosanoid transporters, is hypothesized to contribute to a pro-inflammatory, pro-proliferative, and pro-angiogenic environment. Therefore, the oxo-ETEs could potentially exert both anti-inflammatory and anti-proliferative effects that would be abolished by down regulation of 15-PGDH, which is required for their biosynthesis (83,108,129).

Our findings indicate that therapies designed to modulate, rather than completely inhibit, AA metabolism into downstream products might be a more effective therapy for chronic inflammatory conditions and cancer. Thus, increased COX-2 activity can be compensated by increased 15-PGDH activity through inactivation of pro-inflammatory eicosanoids such as PGE$_2$ and increased formation of eicosanoids that can modulate the activity of NF-$\kappa$B such as 11-oxo-ETE. Research into the metabolism and signaling of other polyunsaturated lipid products, or via other signaling pathways may also provide valuable insight into electrophiles with therapeutic or diagnostic implications.
Figure 4.1. Chemical structures examined in this study. $\alpha,\beta$-unsaturated ketone containing eicosanoids used in this study and the Michael addition adduct of 11-oxo-ETE to a cysteine residue.
Figure 4.2. Inhibition of induced p50/p65 transcription factor binding in activated endothelial cells. (A) NF-κB p50 binding in nuclear extracts from HUVECs pre-treated with 2 μM 11-oxo-ETE. (B) p65 binding in nuclear extracts from HUVECs pre-treated with 2 μM 11-oxo-ETE. (C) p50 binding in nuclear extracts from HUVECs pre-treated with 2 μM 15d-PGJ2. (D) p65 binding in nuclear extracts from HUVECs pre-treated with 2 μM 15d-PGJ2. Cells were first treated with 0.5 mg/mL Kdo2-Lipid A and 40 ng/mL TNF-α for 45 minutes. 0.25 % DMSO was used as control.
Figure 4.3. EMSAs from HUVEC nuclear extracts after various treatments. (1) Free probe for NF-kB. (2) 0.25 % DMSO control. (3) TNF-α. (4) BAY 11-7085 + TNF-α. (5) 0.2 µM 11-oxo-ETE + TNF-α. (6) 2 µM 11-oxo-ETE + TNF-α. (7) 50-fold excess of cold NF-kB (8) Mutated NF-κB cold competition. (9) p50 antibody supershift. (10) p65 antibody supershift. (11) SP-1 cold competition. (12) AP-1 cold competition.
Figure 4.4. NF-κB driven firefly luciferase activity with 11-oxo-ETE treatment. (A)

Firefly luciferase activity versus vehicle control in HEK293 cells stably transfected with a 5x-κB reporter driving firefly luciferase with pretreatment of 11-oxo-ETE for 5 minutes before treatment with 40 ng/mL TNF-α. B) Following an additional pre-treatment of the
cells with 100 μM ethacrylic acid. C) LC-ECAPI/SRM/MS quantification of 11-oxo-ETE from HEK293 cells with or without ethacrylic acid pre-treatment.
Figure 4.5. IKKβ and IκBα phosphorylation in HUVECs. (A) Pretreatment with 11-oxo-ETE or 11-oxo-ETE–ME for 5 minutes before TNF-α challenge for 45 minutes. (B) Pretreatment with 11-oxo-ETE or 11-oxo-ETE–ME for 6 hours before TNF-α challenge for 45 minutes. (C) In vitro IKKβ inhibition with 15d-PGJ₂ (2 or 10 µM) and 11-oxo-ETE (2 or 10 µM).

Figure 4.6. Inhibition of induced manganese superoxide dismutase by 11-oxo-ETE in activated endothelial cells. Western blot of manganese superoxide dismutase from HUVECs pre-treated with 0.25% DMSO vehicle control, 2 µM 11-oxo-ETE, or 2 µM 11-oxo-ETE–ME, and then challenged with 40 ng/mL TNF-α as indicated.
Figure 4.7. LC-MS analysis of tryptic peptides. The LC-MS chromatogram of (A) Unmodified peptide from binding pocket of p50. (B) 11-oxo-ETE adducts of peptides from the DNA binding pocket of p50.
**Figure 4.8.** Peptide fragments detected in MS2 analysis of the 698.3468 m/z precursor ion corresponding to the adduct of the tryptic peptide of the p50 DNA binding domain. (A) Cys-61 adduct (B) His-66 adduct. Ions in bold provide differential

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site localization of the adduct to Cys-61 versus His-66.

Figure 4.9. DNA binding domain of p50 showing cysteine-61 and histidine-66. (A) Sequence homology of mammalian p50s (NFkB1) and p65. (B) Predicted protonated molecules from un-modified and modified tryptic peptides.
Figure 4.10. Protonated molecules with charge states from high resolution LC-MS analysis. (A) 11-oxo-ETE-modified peptide from DNA binding region of p50. (B) Unmodified peptide from DNA binding region of p50.
FIGURE 4.11. MS$^2$ analysis of the 11-oxo-ETE modification on histidine-66 residue of the peptide from the binding pocket of p50.

FIGURE 4.12. MS$^2$ analysis of the 11-oxo-ETE modification on cysteine-61 residue of the peptide from the binding pocket of p50.
FOOTNOTES

*This work was supported by National Institutes of Health grants RO1CA091016 and P30ES013508. NWS was supported by a National Institutes of Health training grant T32-GM-008076.

1To whom correspondence should be addressed: Ian A. Blair, PhD, Center for Cancer Pharmacology, University of Pennsylvania, Philadelphia, PA 19104-6160, Phone: 215-573-9885, Fax: 215-573-9889 Email: ianblair@mail.med.upenn.edu.

2Abbreviations used: 11-oxo-ETE, 11-oxo-eicosatetraenoic acid; 11-oxo-ETE-ME: 11-oxo-ETE-methyl ester; 15-oxo-ETE, 15-oxo-eicosatetraenoic acid; 11-HPETE, 11-hydroperoxy-5,8,12,14-(Z,Z,E,Z)-eicosatetraenoic acid; 15-HPETE, 15-hydroperoxy-5,8,11,13-(Z,Z,Z,E)-eicosatetraenoic acid; AA, arachidonic acid; CID, collision-induced dissociation; ESI, electrospray ionization; PG, prostaglandin; SRM, selected reaction monitoring; ECAPCI, electron capture atmospheric pressure chemical ionization; NF-κB, nuclear factor- kappa B; PPARγ, peroxisome proliferator activated receptor-gamma; Keap1/Nrf2, Kelch-like ECH-associated protein 1/ nuclear factor (erythroid-derived 2)-like 2; iκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IKKβ inhibitor of nuclear factor kappa b kinase subunit beta; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; HUVEC, human umbilical vein endothelial cell; 15d-PGJ2, 15-deoxy-Δ12,14-PGJ2; TCEP, tris(2-carboxyethyl)phosphine; PFB, pentafluorobenzyl bromide; DIPEA, N,N-Diisopropylethylamine
Chapter 5: Stable Isotope Labeling in Cell Culture for Liquid Chromatography-Mass Spectrometry of Mixed Length Acyl-coenzyme A Thioesters.

In preparation for submission as: Stable isotope labeling in cell culture for liquid chromatography mass spectrometry of mixed length acyl-coenzyme A thioesters. Nathaniel W. Snyder, Sankha S. Basu, Zinan Zhou, Andrew J. Worth, Ian A. Blair

Abstract

Rationale: Coenzyme A (CoA) thioesters are a critical component of cellular metabolism. Therefore, the measurement of CoA species is an important analytical task, and can be accomplished with high sensitivity, specificity, and reproducibility by the gold standard method of stable isotope dilution-liquid chromatography-single reaction monitoring-mass spectrometry (SID-LC-SRM/MS).

Methods: Previously, we developed a strategy for producing the required array of short chain acyl-CoA heavy isotopic standards for SID analysis. Here we expand our procedure for producing isotopically labeled CoA thioesters to include lower abundance medium and long chain acyl-CoAs. A fatty acid treatment and solid phase extraction were adapted for acyl-CoA recovery from cell culture. Reversed phase LC-MS/MS with an API4000 mass spectrometer operating in positive ion mode was used for analysis.

Results: We demonstrate induction, recovery, and analysis of low abundance medium and long chain acyl-CoAs. The isotopic purity of our labeling was confirmed to be above
99%. Chromatographic separation of isobaric mixed length chain acyl-CoAs was confirmed against commercial standards. Using SID-LC-SRM/MS, we measure murine liver acyl-CoA levels, and absolutely quantify the induction of arachidonoyl-CoA in cell culture.

Conclusions: This method should further the rigorous quantitative study of acyl-CoA species. This procedure provides a simple cost-effective means of preparing acyl-CoA stable isotope labeled standards.

Introduction

In prokaryotes and eukaryotes, coenzyme A is an essential cofactor for energetic and synthetic metabolic pathways. CoA species are perturbed in a wide variety of diseases with high mortality and morbidity including metabolic dysfunction (169), toxicological insult (170), and a spectrum of genetic disorders (171,172). Fatty acid synthesis, catabolic breakdown of long chain fatty acids, xenobiotic biotransformation, and post-translational modification of proteins rely on acyl-CoA intermediates (8,173-175). However, mammalian cells are incapable of synthesizing the essential CoA precursor pantothenate (vitamin B5) from precursors (176). Previously, we have taken advantage of this incomplete metabolism by replacing pantothenate in cell media with a $[^{13}\text{C}_3\ {^{15}\text{N}}]$-pantothenate heavy isotope labeled analog, which is then incorporated into CoA, and thus, any CoA thioester (177). We termed this methodology stable isotope labeling by essential nutrients in cell culture (SILEC), after the similarity to the stable
isotope labeling by amino acids in cell culture (SILAC) methods employed for proteins (178). For acyl-CoA molecules, this imparts a +4 m/z shift to the internal standard, allowing distinction between the analyte and the internal standard in a mass spectrometer, without significant isotopic overlap or interference from biological matrices (179).

A major limitation of our earlier work was that our extraction and analysis methods only encompassed short chain CoA thioesters, failed to include naturally transient medium chain acyl-CoAs and more hydrophobic longer chain acyl-CoA species. In this study we have adapted a previously reported extraction to encompass a wider range of CoA species (180), and demonstrate recovery of short, medium, and long chain CoA thioesters after an optimized induction of lower abundance species. We further demonstrate a successful application of our SILEC method to create a panel of internal standards. To demonstrate the utility of this method we absolutely quantify induction of arachidonoyl-CoA after treatment with the parent fatty acid in a cell culture system, and measure absolute levels of acyl-CoA species in mouse liver tissue.

Materials and Methods

Reagents

Arachidonoyl-CoA, 5-sulfosalicylic acid (SSA), ammonium formate solution, glacial acetic acid, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Optima LC-MS grade methanol, acetonitrile (ACN), 2-propanol (IPA) and water were purchased from Fisher Scientific (Pittsburgh, PA). 2-(2-pyridyl) ethyl functionalized silica gel 100 mg/1 mL tubes were obtained from Supelco Analytical (Bellefonte, PA). F-12K media, DMEM, medium 200, low serum growth supplement
(LSGS) kit, streptomycin, and penicillin were purchased from Invitrogen (Carlsbad, CA). Charcoal stripped fetal bovine serum (csFBS) was from Gemini Bioproducts (West Sacramento, CA). $[^{13}\text{C}_3^{15}\text{N}_1]$ calcium pantothenate was purchased from Isosciences (King of Prussia, PA). Peroxide free arachidonic acid (AA) was purchased from Cayman Chemical (Ann Arbor, MI). Human colorectal adenocarcinoma cells (LoVo) and human colon adenocarcinoma (HCA7) cells were obtained from ATCC (Manassas, VA).

**Cell Culture**

For SILEC labeling, Hepa1c1c7s were passaged at least 7 times in custom RPMI 1640 media without calcium pantothenate (AthenaES, Baltimore, MD) and containing 10% charcoal stripped-FBS, 100 units/mL penicillin, 100 mg/L streptomycin, and 2 mg/L $[^{13}\text{C}_3^{15}\text{N}_1]$-calcium pantothenate. 24 hours before extraction, “ultra-labeling” with media as above, but omitting csFBS, was carried out for to ensure optimal CoA heavy isotopic labeling. For unlabeled cell culture, HCA7 cells were grown in DMEM media with 2% FBS and 100 units/mL penicillin and 100 mg/L streptomycin. LoVos were grown in F-12K media with 2% FBS and 100 units/mL penicillin and 100 mg/L streptomycin.

**Sample preparation**

For SILEC internal standards, batches were grown as above. Upon completion of labeling method, cells were gently lifted, centrifuged at 500 rcf for 5 minutes and resuspended in 750 µL / 10cm$^2$ plate of 3:1 ACN:IPA. Samples were sonicated with a probe tip sonicator on ice 30 times for 0.5 s. 250 µL of 100 mM KH$_2$PO$_4$ (pH 6.7) were added to the samples, then vortexed and spun down for 10 min at 16,000 rcf at 4°C. The supernatant was pooled and stored in -80°C until use.
For cellular acyl-CoA analysis, cells were gently lifted, centrifuged at 500 rcf for 5 minutes and resuspended in 750 μL / 10 cm² plate of 3:1 ACN:IPA for extraction as previously described (180). Samples were pulse sonicated with a probe tip sonicator on ice 30 times for 0.5 s. 250 μL of 100 mM KH₂PO₄ (pH 6.7) were added to the samples, then vortexed and spun down for 10 min at 16,000 rcf at 4°C. The supernatant was transferred to a glass tube and acidified with 125 μL of glacial acetic acid. SPE columns were equilibrated with 1 mL 9:3:4:4 ACN:IPA:H₂O:acetic acid. The samples were transferred to the column and filtrated. The column was washed two times with 1 mL of the 9:3:4:4 mix. The columns were eluted two times with 500 μL 4:1 methanol:250 mM ammonium formate into glass tubes. Filtrate was evaporated to dryness under nitrogen gas. The filtrate was then re-dissolved in 50 μL of 70:30 H₂O:ACN with 5% SSA (w/v) and transferred to HPLC vials.

**Induction of arachidonoyl-CoA and measurement of mouse liver acyl-CoA**

HCA7 and LoVo cells were grown until 80% confluence as above. 25 μM arachidonic acid with 0.25% DMSO in DMEM media was added and at 30 min, 60 min, 90 min and 4 h, the cells were gently scraped and collected in plastic tubes. Tubes were centrifuged for 5 min at 500 rcf. Media was removed and the cell pellet was dissolved in 550 μL 3:1 ACN:IPA and 200 μL of the internal standard mix was added to each sample. Then the samples were extracted and prepared as above.

For the arachidonoyl-CoA quantification, six standard solutions of arachidonoyl-CoA in methanol were made corresponding to 20 pmol, 2 pmol, 0.2 pmol, 20 fmol, 2 fmol and 0 fmol on column. The standards were dried and then re-dissolved in 800 μL 3:1 ACN:IPA and 200 μL of ISTD were added to each sample. 250 μL of 100 mM KH₂PO₄ (pH 6.7) were added to the samples, then vortexed and spun down for 10 min. at 16,000
The supernatant was transferred to a glass tube and acidified with 125 µL of glacial acetic acid. Then, the samples were extracted using SPE column and the samples were prepared as above. Analysis of the standard curve was performed with Excel.

**LC-MS/MS**

Samples were kept at 4°C in a Leap CTC autosampler (CTC Analytics, Switzerland) with 20 µL injections used for LC-MS analysis. Chromatographic separation was performed using reversed phase Waters XBridge C18 column (2.1 x 150 mm, pore size 3.5 µm) on an Agilent 1100 HPLC system using a three solvent system: (A) 5 mM ammonium acetate in water, (B) 5 mM ammonium acetate in 95/5 acetonitrile/water (v/v), and (C) 80/20/0.1 (v/v/v) acetonitrile/water/formic acid, with a constant flow rate of 0.2 mL/min. Gradient elution was performed as follows: 2% B (isocratic) for 1.5 min, 2% to 20% (linear gradient) over 3.5 min, 20% to 100% B (linear gradient) B over 0.5 min, 100% B (isocratic) for 8 min, 100% C for 5 min, before equilibration at initial conditions for 5 min.

Samples were analyzed using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) in positive electrospray ionization (ESI) mode and data was analyzed using Analyst software as previously described (179). The mass spectrometer operating conditions were as follows: ion spray voltage (5.0 kV), compressed air as curtain gas (15 psi) and nitrogen as nebulizing gas (8 psi), heater (15 psi), and collision-induced dissociation (CID) gas (5 psi). The ESI probe temperature was 450°C, the declustering potential was 105 V, the entrance potential was 10 V, the collision energy was 45 eV, and the collision exit potential was 15 V. CoA thioesters were monitored using the transitions described in Table 2.
Results

Mixed Acyl-CoA Extraction

Extracts of LoVo cells at 80% confluence were analyzed by the LC-MS method described above. The neutral loss scans of 507.1 m/z are shown in Figure 2 for the mixed CoA solid phase extraction (Figure 2A), the short chain CoA solid phase extraction (Figure 2B), and a biphasic liquid-liquid Folch extraction (Figure 2C). In our hands, the mixed extraction recovered the broadest range of acyl-CoA species as evidenced by the peaks corresponding to acyl-CoA species in the short, medium, and long chain range. The short chain extraction we have previously used gave intense CoASH and acetyl-CoA peaks, and overall higher signal intensity, but did not recover significant middle or long chain CoA species. Recovered species appeared less abundant in the Folch extract versus the mixed CoA SPE (Figure 2A, 2C).

Generation of a SILEC standard

Hepa1c1c7 mouse hepatocellular carcinoma cells were grown in RPMI media with pantothenate omitted, 2% charcoal stripped FBS, and 2 mg/L [13C4 15N1] calcium pantothenate added for 7 passages. Cells were “ultra-labeled” for 24 hours in the same media with 0% FBS. The cells were harvested using the mixed CoA solid phase extraction method. Labeling for oleoyl-CoA was determined using integration of the peak area for the 1032 → 525 and 1036 → 529 m/z transitions corresponding respectively to the unlabeled and labeled oleoyl-CoA (Figure 3A). Commercial standards were matched with SILEC internal standards for the following acyl-CoA species; acetyl, butyryl, hexanoyl, octanoyl, decanoyl, lauroyl, tetradecanoyl, palmitoyl, palmitoeloyl, linoleyl, stearoeloyl, oleoyl, and arachidonoyl-CoA (Figure 3B). Importantly, the M2
isotopomers for overlapping species, such as oleoyl-CoA M2 and steareoyl-CoA, are shown to achieve baseline separation with our liquid chromatography method (Figure 3B). Consistent with our previous work on short-chain acyl-CoAs (170,177,179) we find that the distribution of species is unequal, such that certain molecules of interest may be insufficiently synthesized in the internal standard mix for analytical use. To address this problem, we tested induction of acyl-CoAs in Hepa1c1c7 cells with various doses of fatty acid mixtures. A fatty acid mix sodium butyrate, sodium hexanoate, sodium octanoate, sodium decanoate, and sodium dodecanoate at 100 μM was found to be ideal, where above that concentration cellular toxicity was observed (data not shown). A time course study revealed that 3 hour incubation at 100 μM produced the best distribution of acyl-CoA species (Figure 5).

Arachidonoyl-CoA time course

To demonstrate the use of this method for cellular studies, we quantified the levels of arachidonoyl-CoA in a cell system treated with free arachidonic acid. A special SILEC standard was generated by treating the SILEC cells with 25 μM arachidonic acid for one hour before extraction. This provided increased arachidonoyl-CoA internal standard, and shows the applicability of this method for targeted long chain acyl-CoA quantitation. LoVo and HCA-7 colon adenocarcinoma cells were treated with 25 μM arachidonic acid, and the cells were extracted and analyzed by our method at various time points. The resulting standard curve was linear over 4 orders of magnitude from 20 fmol to 20 pmol on column and allowed us to capture the entire concentration range of cellular induction of arachidonoyl-CoA levels in the treated cells with absolute quantification (Figure 4A).

Tissue acyl-CoA quantitation
A set of three mouse liver tissue samples were analyzed by our method, and absolute quantification of select acyl-CoA species is given. We describe pmol/mg of protein levels of long chain CoA thioesters in the mouse liver tissue (Figure 4B). This demonstrates the use of this method for quantitative analysis of acyl-CoA species from tissue samples.

**Conclusion**

Acyl-coenzyme A species are important to cell bioenergetics metabolism, and play critical roles in lipid generation, cell signaling, post-translational modification of proteins, and metabolism of xenobiotics (170,175). However, the variety of individual chemical entities making up the acyl-CoA family poses a challenge to the analytical task of their measurement. LC-UV strategies for analysis of CoA species may be insufficiently specific due to co-eluting peaks (181). Excellent LC-MS/MS strategies for the analysis of acyl-CoAs have been employed by other groups, including validated methods by Magnes, *et al.* (182) and Mauriala *et al.* (183). However, these measurements used no internal standards.

In this study, we expand our previous method to generate stable isotope labeled internal standards for short chain acyl-CoAs to include medium and long chain acyl-CoAs. We were able to generate standards of even the low abundance acyl-CoA species and demonstrate the utility of this method in measuring induction of an acyl-CoA species and measuring absolute levels of CoAs from mouse liver tissue.
Figure 5.1. A) Biosynthesis of long chain acyl-coenzyme A thioesters B)

Coenzyme A MS fragmentation for neutral loss of 507 amu
Figure 5.2. Neutral loss 507 scans of LoVo cells. Cells were extracted by A) mixed acyl-CoA SPE B) Folch extraction C) short chain acyl-CoA SPE.
Figure 5.3. LC-SRM/MS chromatograms of acyl-CoA thioesters. Chromatograms of A) labeling efficiency of unlabeled and $^{13}$C$_3$, $^{15}$N$_1$ labeled oleoyl-CoA from Hepa1c1c7 SILEC extracts, B) labeled SILEC acyl-CoAs and unlabeled authentic standard acyl-CoAs. Where isotopic overlap is observed, chromatographic separation is shown (e.g. the M2 of oleoyl-CoA and M0 stearoyl-CoA).
Figure 5.4. Application of SID-LC-MS/MS of mixed length CoAs to biological systems. A) Absolute quantitation of the induction of arachidonoyl-CoA in two different colon carcinoma cells lines from 0.5 to 4 hours. B) Absolute quantitation of acyl-CoA species from three different mouse liver samples taken from different animals.
Figure 5.5. Neutral loss of 507 amu scans of [13C3, 15N1] Hepa1c1c7 cells extracted at various time points. Spectra from extracted cells of (A) baseline, (B) 30 minutes, (C) 3 hours, (D) 6 hours, (E) 12 hours, (F) 24 hours after treatment with a 100 μM mix of fatty acids as indicated in materials and methods.
Chapter 6: Untargeted Metabolomics from Biological Sources

Using Ultraperformance Liquid Chromatography- High Resolution Mass Spectrometry (UPLC-HRMS)


6.1 Abstract

Here we present a workflow to analyze the metabolic profiles for biological samples of interest including; cells, serum, or tissue. The sample is first separated into polar and non-polar fractions by a liquid-liquid phase extraction, and partially purified to facilitate downstream analysis. Both aqueous (polar metabolites) and organic (non-polar metabolites) phases of the initial extraction are processed to survey a broad range of metabolites. Metabolites are separated by different liquid chromatography methods based upon their partition properties. In this method, we present microflow ultra-performance (UP)LC methods, but the protocol is scalable to higher flows and lower pressures. Introduction into the mass spectrometer can be through either general or compound optimized source conditions. Detection of a broad range of ions is carried out in full scan mode in both positive and negative mode over a broad m/z range using high resolution on a recently calibrated instrument. Label-free differential analysis is
carried out on bioinformatics platforms. Applications of this approach include metabolic pathway screening, biomarker discovery, and drug development.

6.2 Introduction

Due to recent technological advances in the field of HRMS, untargeted, hypothesis-generating metabolomics approaches have become a feasible approach to analysis of complex samples. Mass spectrometers capable of 100,000 resolution facilitating routine low part per million (ppm) mass accuracy have become widely available from multiple vendors. This mass accuracy allows greater specificity and confidence in a preliminary assignment of analyte identity, isotopic pattern recognition, and adduct identification. When coupled with an appropriate extraction procedure and high-performance LC or UPLC, complex mixtures can be analyzed with additional specificity derived from retention time data. UPLC possesses greater chromatographic efficiency and allows greater sensitivity, resolution and analysis time making a greater coverage of the metabolome possible. The resulting large datasets can be integrated into any of multiple differential analysis software and mined for useful patterns or individual analytes of interest. Putative hits can be initially identified using a combination of peak detection algorithms, accurate mass based chemical formula prediction, fragmentation prediction, and chemical database searching. This approach allows prioritization of targets for time-consuming complete structural identification or for development of more sensitive and
more specific stable isotope dilution UPLC/selected or multiple reaction monitoring/MS studies that are the current gold standard methods for quantification.(159)

The varying nature of biological samples has led to optimization of extraction protocols for urine(195), cells(196), serum(197), or tissue(198). This protocol features extractions for cells, serum, and tissue. Where appropriate, comments and additional references have been included for modifications of the procedure to address inclusion of stable isotopes, or for inclusion of especially unstable metabolites.

6.3 Protocol Text

1.) [Sample Extraction from Cells]

1.1) For a 10cm plate of cells: collect 1.5mL of lifted cell suspension in media into a pre-labeled 10mL glass centrifuge tube. For adherent lines, cells should be lifted with gentle scraping in 1.5mL of media kept on ice. Optional: If internal standards are used, add an appropriate aliquot at this step.

Comment: Quenching of cellular metabolism is crucial for certain metabolites. For analysis of time-sensitive metabolites, procedures such as cold methanol extraction should be considered.(199)

1.2) Add 6mL of chloroform (CHCl₃)/methanol (CH₃OH) (2:1, v/v) to each of the 10mL glass tubes containing the samples. Set the samples in a shaker or multi-vortexer on low speed for 30 min. After shaking, the samples are centrifuged with a low acceleration/deceleration setting at 1935 x g for 10 min at 4°C to completely separate
the phases. *Optional:* To avoid using CHCl$_3$, extractions can be conducted with
dichloromethane (CH$_2$Cl$_2$).(159,200)

1.3) Using a long stem Pasteur pipet, transfer the organic (bottom) layer to a new pre-
labeled 10mL glass centrifuge tube. Continue to 4.1.

1.4) Transfer the upper aqueous later into a clean plastic 2mL Eppendorf tube.
Evaporate the sample under nitrogen gas. Continue to 2.6.2 for aqueous phase
desalting or continue to 4.1.

2.) [Sample Extraction from Serum]

2.1) Transfer 20μL of serum into a plastic 2mL Eppendorf tube. *Optional:* If internal
standards are used, add an appropriate aliquot at this step.

2.2) Add 190μL of CH$_3$OH and vortex for 10 sec.

2.3) Add 380μL of CHCl$_3$ and vortex for 10 sec.

2.4) Add 120μL of H$_2$O to induce phase separation. Vortex the samples for 10 sec and
allow to equilibrate at room temperature for 10 min.

2.5) Centrifuge the samples at 8000 x g for 10 min at 4°C to separate the phases.

2.6) Transfer the lower organic phase into a clean plastic 2mL Eppendorf tube. Continue
to 4.1.

2.6.1) Transfer the upper aqueous later into a clean plastic 2mL Eppendorf tube.
Evaporate the sample under nitrogen gas.
2.6.2) Re-suspend the sample in 200μL H₂O. Acid or base reagents (0.1% Acetic acid, formic acid or 0.1% ammonium hydroxide) may be used to preferentially extract pH sensitive metabolites. Sonicate for 20 min on ice to re-suspend the sample completely.

2.6.3) Activate the C18 spin columns with 500μL CH₃OH.

2.6.4) Equilibrate the spin column with two washes of 500μL H₂O and then load the sample. If acid/base reagents are used maintain this concentration in the wash steps.

2.6.5) Wash with 500μL H₂O to desalt the sample. Again, if acid/base reagents are used maintain this concentration in the wash steps.

2.6.6) Elute with two volumes of 200μL 80% CH₃OH in H₂O into a pre-labeled clean 2mL Eppendorf tube. Again, if acid/base reagents are used maintain this concentration in the wash steps. Continue to 4.1.

3.) [Sample Extraction from Tissue]

3.1.) Depending on the tissue, use between 10-100 mg of frozen tissue. Add the whole piece of tissue to a pre-labeled 2mL low retention Eppendorf tube with 1mL of PBS (1M, pH 6.8) in an ice bath. Optional: If internal standards are used, add it into the buffer, before adding the tissue.

3.2.) Homogenize the tissue in ice with an electric tissue grinder for 30 sec in each Eppendorf. Between samples, clean the tissue grinder in two separate tubes containing CH₃OH and H₂O, respectively.
3.3) Transfer the tissue homogenate with a plastic pipette into a pre-labeled 10mL glass centrifuge tube. After the transfer, rinse each Eppendorf tube two times with 1mL of CH$_3$OH and add the washes to the tissue homogenate in the glass tubes.

3.5) Add 4mL of CHCl$_3$ to each of the 10mL glass tubes containing the tissue homogenate and CH$_3$OH washes. Set the samples in a shaker or multi-vortexer on low speed for 30 min.

3.6) After shaking, centrifuge the samples with a low acceleration/deceleration setting at 1935 x g for 10 min to completely separate the phases. Optional: To avoid using chloroform, extractions have been developed with CH$_2$Cl$_2$.

3.7) Using a long stem Pasteur pipette, transfer the organic (bottom) layer to a new pre-labeled 10mL glass centrifuge tube. Continue to 4.1.

3.8) Using a long stem Pasteur pipette, transfer the aqueous layer to a new pre-labeled 10mL glass centrifuge tube. Go to 2.6.2 for desalting or continue to 4.1.

4.) [Re-suspension and filtration of samples for UPLC]

4.1) Evaporate the samples under nitrogen gas.

4.2) Reconstitute dried down samples in an appropriate volume of the starting solution for the desired UPLC method (see Table 1). 50-100µL is usually a desirable re-suspension volume. Gently vortex and/or pipette the sample up and down to aid in dissolving the analytes.
4.3.) Transfer the sample into a 0.22μm nylon tube filter and spin at up to 14000 x g until the sample has completely passed through the filter (~5 min). Make sure that there are no visible precipitates remaining in the sample.

4.4.) Transfer the supernatant of the filtered sample into a pre-labeled UPLC vial with insert. Cap vial, then flick the vial to remove any bubbles from the bottom of the sample vial. Place the sample in the chilled autosampler (4-5°C).

5.) [UPLC Setup]

5.1.) Using the control software for the liquid chromatograph, create a run for the organic sample based off of the conditions listed in Table 1. Then create a method for the aqueous phase off the conditions listed in Table 1. *Optional:* If a known set of target analytes are of high interest, optimize the UPLC conditions using the heavy labeled analog of these compounds, and generate a method using these conditions.

5.3.) Allow the UPLC to adequately equilibrate. This should include a full priming of solvents before attaching a column, and following the manufacturer’s directions for equilibration volume of a new column (usually 3-5 column volumes). Maintain a log of starting back pressures to help diagnose future problems.

6.) [Mass Spectrometer Setup]

6.1.) Using the control software for the high resolution mass spectrometer, create a positive mode method using the conditions listed in Table 2. Then using the same conditions, create a negative mode method. *Optional:* If a known set of target analytes
are of high interest, optimize source and optics conditions using the heavy labeled analog of these compounds, and generate a method using these conditions.

6.2) Clean and calibrate the instrument, establish a stable spray into the spectrometer, and allow time for the calibration solution to adequately dissipate from the source and optics. Acceptable stability of spray should give a 3-5% RSD of intensity over at least 100 scans with injection of calibration solution at the same slow rate as the LC method being used.

6.3) Set up the sequence for all the samples, set appropriate injection volume, and run a blank before the first sample. If carryover or matrix effects between samples are suspected, run adequate blanks/washes. Samples should be setup in a randomized manner using a random number generator and a key to avoid any batch effects introduced by analysis.

Comment: Quality control samples including stable isotope standards or analytical standards of likely targets can be extremely valuable in troubleshooting and assuring reliable, reproducible, and valid results. A technical replicate of a standard only sample followed by a solvent blank can be used to accesses deviation of signal intensity and retention time, as well as carryover into the blank run.

6.4) Begin sequence and monitor periodically for problems including pressure fluctuations, or loss of signal intensity across the run. If severe problems are detected, stop the run, and repeat from 6.2.
7.) **Differential Analysis**

7.1.) Two workflows are presented for this protocol. The first is through SIEVE 2.0, proprietary label-free differential analysis software sold by Thermo Fisher. The second workflow is through XCMS Online, a free platform through Scripps Research Institute. Before beginning differential analysis, manually check the TICs or look at filtered chromatograms for any known compound in the sample to ensure reproducibility of runs and injection/UPLC/spray stability throughout all the samples.

7.2.) **SIEVE**

7.2.1) Transfer the .raw data files from the mass spectrometer to the hard drive of the workstation where SIEVE is installed. (Note: running SIEVE with data stored on a networked or USB port connected drive is not advisable as it can limit the speed of analysis)

7.2.2) Open SIEVE, and begin a new experiment.

7.2.3) Load the appropriate files into SIEVE.

7.2.4) Assign comparison groups and select a single reference file to allow SIEVE to generate the parameters for analysis.

7.2.5) Follow the prompts and adjust any parameters per requirements of any individual experiment. It is often advisable to start with stringent parameters and then go back and loosen the parameters as more generous settings can lengthen analysis time.
7.2.6) Load the correct adduct list for positive or negative mode in the parameters.

7.3.) **XCMS Online**

7.3.1) Convert files into an acceptable format for XCMS Online. In our case, we convert to mzXML format.(201)

7.3.2) Open XCMS Online, and Create Job.

7.3.3) Upload and name datasets. Only two datasets (e.g. Control vs Treatment) can be uploaded as XCMS stand-alone is limited to head-to-head comparisons.

7.3.4) Select desired settings from the drop-down list. Pre-populated parameters are available for different instrumentation setups, and these can be further modified for experimental needs. In our case, we use the HPLC-Orbi2 settings.

7.3.5) Submit and confirm job.

7.4) **Data Analysis**

7.4.1) For SIEVE and XCMS a list of frames and features, respectively, will be populated once the analysis is finished. Ensure that the LC alignment overlays any landmark peaks. If any of the unaligned LC runs show large deviation in landmark peaks this may indicate a problem with the sample.

*Optional:* For SIEVE, if internal standards are used, locate the corresponding peak group and normalize to the selected frame. For XCMS On-line, normalization can be done in a spreadsheet after export.
7.4.2) Plot the data by coefficient of variation (CV) within a like sample population (e.g. control samples). Any large CV values may indicate a problem with one or more sample. Sort the list based on desired traits (e.g. p-value < 0.05, Fold Change > 1.5, low standard deviation within a group, etc.)

7.4.3) Examine each peak for appropriate peak alignment across groups, peak integration, Gaussian peak shape, signal intensity, isotopes, and adduct assignment.

7.4.4) Export data as desired for further analysis or to generate targeted mass lists for confirmation and MSⁿ experiments.

6.4 Representative Results

The results presented show selected data from a 6-h treatment of SH-SY5Y glioblastoma cells with the pesticide and mitochondrial complex I inhibitor rotenone. For brevity, only the organic phase positive mode data is presented. The samples were processed and analyzed as described above (Figure 1, Table 1, Table 2) and loaded onto two differential analysis platforms for label-free quantification, SIEVE and XCMS online. Although a large number of hits (Figure 2, Figure 3) are identified by the two programs used for differential analysis these features include likely artifacts, poorly integrated peaks, and other features of questionable analytical value (Figure 6). This can be judged by screening the hits for appropriate signal intensity, low variation between samples of the same group, background levels, and good chromatographic peak shapes (Figure 3).
To demonstrate the downstream confirmation of initial hits, we isolate a feature with a mass corresponding to our treatment compound rotenone within 3ppm (Figure 3, Figure 4). We confirm the identity of this analyte with UPLC-HR/tandem mass spectrometry (MS/MS) of our sample and the pure compound (Figure 5). We also identify a differentially abundant feature reduced in the rotenone treated group, tentatively identified as D-pantethine by accurate mass database searching (Figure 4).

Tables and Figures:

Figure 6.1. General Experimental Outline for Sample Preparation and Analysis by Microspray UPLC-HRMS.
A general outline of sample preparation, including the optional introduction of internal standards and protein or lipid/protein content analysis. Phase separation, liquid chromatography, and analysis by different ion modes in the mass spectrometer are outlined.
Table 6.1: [UPLC Conditions]

General and compound optimized UPLC conditions. For organic phase 1, constant flow rate of 1.8μL/min through a Waters nanoACQUITY C18 BEH130 column kept in a heater at 35°C was used. Solvent A: 99.5% water/0.5% acetonitrile with 0.1% formic acid, Solvent B: 98% acetonitrile/2% water with 0.1% formic acid. For organic phase 2, constant flow rate of 3.4μL/min through an Acentis Express C8 column kept in a heater at 35°C was used. Solvent A: 40% water/20% acetonitrile with 0.1% formic acid and 10mM ammonium formate, Solvent B: 90% isopropanol/10% acetonitrile with 0.1% formic acid and 10mM ammonium formate. For the aqueous phase, constant flow rate of 1.2μL/min through a Waters nanoACQUITY C18 BEH130 column kept in a heater at

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35°C was used. Solvent A: 95% water/5% methanol with 0.1% ammonium hydroxide, Solvent B: 95% methanol/5% water with 0.1% ammonium hydroxide.

Figure 6.2. Mirror Plot Generated with XCMS.

A mirror plot showing differentially abundant features as detected and quantified through XCMS online from the organic phase positive mode UPLC-MS analysis. Each dot represents a distinct “feature.” Green dots are features more abundant (larger integrated area) in the control, whereas red dots are less abundant in the treatment. Increasing size of the dot indicates increasing magnitude of fold change between the groups, and the intensity of color indicates a decreasing p-value with increasing saturation of color.
Figure 6.3. Analyte Peaks.

Chromatograms showing the organic phase positive ion mode UPLC-MS analysis. A) Corrected (aligned) Total ion current (TIC) from SIEVE 2.0. B) Extracted Ion Chromatogram for the feature tentatively identified as D-pantethine through XCMS. C) Extracted Ion Chromatogram for the feature later identified as rotenone from SIEVE. D) XIC for the feature later identified as rotenone from SIEVE normalized to TIC.
Figure 6.4. Database Search Results.

Database hits for A) Human Metabolome Database (http://www.hmdb.ca) and B) ChemSpider/KEGG (via SIEVE) (ChemSpider alone can also be employed - http://www.chemspider.com) using accurate mass determinations for the features shown in Figures 3B, 3C, and 3D corresponding to the ions of m/z 555.2516 and 395.1481 based on the identification of these ions as [MH]^+ species.

![Figure 6.4](image)

Figure 6.5. HR/MS/MS Structural Confirmation.

Confirmation of identification of the feature corresponding to the ion at 395.1481 m/z with a retention time of 23.22 min as rotenone based on UPLC-MS/MS comparison to a commercial standard with A) similar retention time and B) the same m/z (395.1481, ~0ppm error) with nearly identical fragmentation.

![Figure 6.5](image)
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**Table 6.2: [MS Settings]**

General and compound optimized mass spectrometer settings used for the LTQ XL-Orbitrap with a Michrom Thermo Advance captive spray ESI source.

**6.5 Discussion**

Untargeted metabolomics offers a powerful tool for investigating endogenous or xenobiotic biotransformations, or capturing a metabolic profile from a sample of interest. The output of the technique scales with the resolution and sensitivity of the technology used to separate and analyze the sample, the ability to deal with the large datasets generated, and the ability to mine the dataset for useful information (e.g. accurate mass database searching). Recently, this has been facilitated by advances in high resolution mass spectrometers, and high- or ultra-performance liquid chromatography. Differential analysis software has addressed the analysis bottleneck, and can accomplish peak detection adjustment for retention time shifts, filtering, and statistical analysis with high-throughput. (202-204) Selection of the proper informatics pipeline should include considerations as to data centroiding algorithms, peak detection, peak integration, alignment, ability to integrate MS/MS data, and ability to deal with isotopes or adducts. (205) Selection of appropriate cheminformatics databases
should also be considered. (206-209) The current inadequacy of any particular database
to comprehensively identify compounds and integrate accurate mass data, MS/MS data,
or LC data remains a major problem in the field.

The workflow presented here integrates liquid-liquid extraction, micro-flow
liquid chromatography, and high resolution mass spectrometry with two different
differential analysis software platforms demonstrated in a cell culture treatment model.
Additionally, extraction protocols are listed for serum and tissue, as these may serve as
useful samples for similar analysis.

Although any method used for untargeted metabolomics should be optimized
for repeatability, stable UPLC conditions, and source stability, some variation is
inevitable. Both SIEVE and XCMS allow correction for retention time shift, but special
attention should be paid to ensure that the parameters set in the experiment are
adequate to correct variation. Also, stable isotope labeled internal standard(s) can be
easily integrated into this procedure to reduce artifacts and inter-sample variation
caused by differences in sample extractions or LC-MS analysis. (159) As with all sensitive
LC-MS methodology, it is crucial to use high purity reagents, and ensure that the sample
preparation removes undesirable particulate matter or aggregate. Artifacts can be
generated by the normal variation in contaminants, and it may be desirable to confirm
that putative hits are not in fact ubiquitous contaminants of the extraction or analysis
process.
Lastly, although the method is labeled as “untargeted” or “unbiased” metabolomics, this is a partial misnomer. The nature of the extraction, separation, and analysis will favor metabolites with certain characteristics including stability during extraction, interaction with stationary and mobile phases during separation, and ionization at the source of the mass spectrometer. (210-212) Depending on the available instrumentation, this procedure can be adapted to different extractions, flow rates, LC pressures, ion sources, or mass spectrometers. Therefore, we have included notes in the procedure where certain conditions can be optimized based on general knowledge or internal standards representative of a class of molecules of interest.

Acknowledgments

We acknowledge support of NIH grants P30ES013508 and 5T32GM008076. We also thank Thermo Scientific for access to SIEVE 2.0 and Drs. Eugene Ciccimaro and Mark Sanders of Thermo Scientific for useful discussions.

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Chapter 7. Saturation and Coenzyme A Thioester Formation of
11- and 15-oxo-eicosatetraenoic Acid

In preparation for submission as: Saturation and coenzyme A thioester formation of 11- and 15-oxo-eicosatetraenoic acid. Nathaniel W. Snyder, Alejandro D. Arroyo, Clementina A. Mesaros, Suhong Zhang, Xiaojing Liu, Ian A. Blair

Abstract

Arachidonic acid metabolism by cyclooxygenase-2 (COX-2) and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) results in the formation of 11-oxo- and 15-oxo-eicosatetraenoic acid (oxo-ETE). Both 11-oxo- and 15-oxo-ETE have been found in human specimens; however their further metabolism remains unknown. This lack of understanding limits the biological measurement of these metabolites, as well as hindering elucidation of their function. In this study, we describe metabolic pathways for both 11-oxo- and 15-oxo-ETE including reduction of double bonds and thioester formation. Using an untargeted liquid chromatography mass spectrometry (LC-MS) approach we identify a mono-saturated product of both 11-oxo- and 15-oxo-ETE. We also identify a di-saturated product of 11-oxo-ETE. We confirm these findings using LC-MS/MS and treatment with aracidonic acid, [13C20]-15-oxo-ETE, as well as the methyl-ester of 11-oxo-ETE. Time course experiments show that these metabolites accumulate after treatment with the parent compounds. Examination of the Michael addition adducts of the parent compounds and metabolites showed that the α,β-
unsaturated ketone moiety was absent in the doubly saturated product. With a targeted LC-MS/MS approach we also describe the formation of CoA thioesters of both 11-oxo- and 15-oxo-ETE. Subsequently, we demonstrate that these acyl-CoA species undergo up to 4 double bond reductions. These findings should facilitate the further study of polyunsaturated fatty acid metabolism in inflammatory diseases and cancer.

**Introduction**

Cyclooxygenases are major enzymes responsible for the generation of bioactive lipids from the preferred precursor arachidonic acid (AA) (22). The resulting products are responsible for a vast array of cellular responses through families of G-coupled protein receptors (GPCRs) (6). Evidence for non-GPCR mediated signaling exists (12,16,17,136,145,163), but is still controversial for some mediators (11). The diversity of physiological responses therefore stems from relatively small alterations in the structure of the parent molecule. Oxidation, reduction, cyclization, isomerization, and conjugation can fundamentally alter the function of eicosanoids. Certain transformation result in changing of the affinity for receptors, increasing clearance, or effectively inactivating a functionality of a given molecule (40).

The AA oxidation product 11-oxo-eicosatetraenoic acid (11-oxo-ETE) is generated by the sequential action of cyclooxygenases (COX), peroxidases, and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) on free AA (129). The known intermediates of this pathway are the unstable 11-hydroperoxyeicosatetraenoic acid (11-HPETE) and the stable product 11-hydroxyeicosatetraenoic acid (11-HETE) (27). 15-oxo-ETE can be generated by a COX/15-PGDH pathway from free AA or a 15-LOX/15-
PGDH or 12/15-LOX/15-PGDH pathway from free or esterified AA (83,108). The further metabolism of these molecules however remains poorly described.

In human cancers and inflammatory conditions, COX-2 and 15-PGDH are counter-regulated, whereby COX-2 expression is increased and 15-PGDH levels are reduced (42,88,142). Electrophilic $\alpha,\beta$-unsaturated ketone containing fatty acids derived from $\omega$-6 and $\omega$-3 polyunsaturated fatty acids have anti-proliferative, anti-inflammatory, and redox signaling properties (12,108,118,129,136). The involvement of oxidized phospholipids in atherosclerosis and inflammation also warrant interest in the metabolism of oxidized lipids (213).

A more complete understanding of the metabolism of these molecules is needed to understand the roles such lipids may play in normal and pathological function. Understanding the exact biotransformation will facilitate identification of the responsible enzyme(s). A variety of distinct fatty acid metabolizing enzymes exist with varying specificity for substrate and reaction (40). Furthermore, the lack of an identified GPCR for either 11-oxo- or 15-oxo-ETE complicates the elucidation of their biological function. The possibility that the terminal signaling metabolite generated through 11-oxo- or 15-oxo-ETE is not in fact the oxo-eicosatetraenoic acid cannot yet be discounted.

Due to the involvement of endothelial cells in eicosanoid metabolism, atherosclerosis, tumor angiogenesis, inflammatory responses, and relevance to redox signaling we chose to study the metabolism of 11-oxo- and 15-oxo-ETE in human umbilical vein endothelial cells (HUVECs). We also employ a COX-2/15-PGDH stably expressing human colon adenocarcinoma cell line (LoVo) that was used in previous studies of 11-oxo- and 15-oxo-ETE (129). Through a combination of targeted and untargeted approaches, we examined 11-oxo- and 15-oxo-ETE metabolism.
Specifically, we investigated the interdependent saturation and CoA thioester formation of 11-oxo- and 15-oxo-ETE.

**Materials and Methods**

**Reagents.** 11-oxoeicosatetraenoic acid (11-oxo-ETE), 15-oxoeicosatetraenoic acid (15-oxo-ETE), 11-oxo-eicosatetraenoic acid methyl ester (11-oxo-ETE-ME), 15-oxo-eicosatetraenoic acid methyl ester (15-oxo-ETE-ME) and the $^{13}$C$_{20}$-labeled 15-oxo-ETE internal standard were synthesized in-house as previously reported (129). Peroxide free arachidonic acid (AA) was purchased from Cayman Chemical (Ann Arbor, MI). 5-sulfosalicylic acid (SSA), ammonium formate solution, glacial acetic acid, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade chloroform and ammonium acetate as well as Optima LC-MS grade methanol, acetonitrile, water, isopropanol, ammonium acetate, ammonium formate, and formic acid were purchased from Fisher Scientific (Pittsburgh, PA). 2-(2-pyridyl) ethyl functionalized silica gel (100 mg/mL) tubes were obtained from Supelco Analytical (Bellefonte, PA). F-12K media, medium 200, low serum growth supplement (LSGS) kit, human umbilical vein endothelial cells (HUVECs), streptomycin, and penicillin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Gemini Bioproducts (West Sacramento, CA). Human colorectal adenocarcinoma cells (LoVo and HCA-7) were obtained from ATCC (Manassas, VA).

**Untargeted discovery.** HUVECs were grown until 80% confluence on Collagen IV coated plates from Becton Dickinson (Bedford, MA), and maintained in medium 200 supplemented with low serum growth supplement (LSGS) kit (Invitrogen). Cells were
treated with 10 μM 11-oxo-ETE, 11-oxo-ETE-ME, 15-oxo-ETE, or 15-oxo-ETE-ME in 0.25% DMSO containing media. An initial treatment time of 90 minutes at 37°C was chosen based on previously published uptake data (129,214). Extraction was performed as previously described with minor modifications (137). Cells were gently lifted, mixed, and an aliquot of the resulting suspension was transferred to a glass tube. Folch extractions were performed with 2:1 chloroform:methanol with 0.1% acetic acid. The organic phase was evaporated to dryness under N₂. The samples were dissolved in 70:30 water:acetonitrile with 0.1% formic acid then filtered using 0.22 μm nylon centrifuge filters (Costar) and transferred into HPLC vials for LC-HRMS analysis.

**Metabolic time course.** HUVECs were grown as above. Media containing 0.25% DMSO with 10 μM 11-oxo-ETE, 11-oxo-ETE-ME, 15-oxo-ETE, or 15-oxo-ETE-ME was added. Cells were incubated at 37°C for 5 min, 30 min, 1.5 h, 3 h, 6 h or 9 h. At each time point, 450 μL of the supernatant media was collected and transferred to a glass tube. The cells were gently scraped in the remaining 450 μL of media and transferred to a glass tube. 2.5 ng of [13C₂₀]-15-oxo-ETE was added as an internal standard and, subsequently, Folch extractions were performed as previously described with minor modifications (215). The organic phase was evaporated to dryness under N₂. The samples were dissolved in 100 μM 70:30 water:acetonitrile with 0.1% formic acid then filtered using 0.22 μm nylon centrifuge filters and transferred into HPLC vials for LC-HRMS analysis.

**Acyl-CoA analysis.** Human colorectal adenocarcinoma cells (LoVo) were grown to 80% confluence in F-12K media with 2% FBS and 100,000 units/L penicillin and 100 mg/L streptomycin. HUVECs were grown as above. Extraction and analysis were performed as previously described (177,180). Briefly, cells were gently lifted, and the
resulting suspension was centrifuged at 500 x g at 4°C. The supernatant was removed, and the pellet was suspended in 750 µL of 3:1 acetonitrile:isopropanol (ACN:IPA). The suspension was sonicated with a probe tip sonicator to disrupt the cellular membranes. 250 µL of 100 mM KH₂PO₄ (pH 6.7) was added, the suspension was vortexed, and then centrifuged at 16000 x g at 4°C. The resulting supernatant was transferred to a glass tube and acidified with 125 µL glacial acetic acid for solid phase extraction. 100 mg 2-(2-pyridyl)ethyl-functionalized silica gel solid phase extraction columns (Sigma) were equilibrated with 1 mL 9:3:4:4 ACN:IPA:H₂O:acetic acid. Supernatants were transferred to the column and filtrated. The columns were washed two times with 1 mL of the 9:3:4:4 mixture. The columns were eluted two times with 500 µL 4:1 methanol:250 mM ammonium formate into glass tubes. Filtrate was evaporated to dryness under N₂. The dry samples were dissolved in 50 µL of 70:30 water:acetonitrile with 5% SSA (w/v) and transferred to HPLC vials.

**Liquid chromatography.** For LC-HRMS analysis, LC separations were conducted as previously described (215) using a Waters nano-ACQUITY UPLC system (Waters Corp., Milford, MA, USA). A Waters XBridge BEH130 C18 column (100 µm × 150 mm, 1.7 µm pore size; Waters Corp) was employed for reversed phase separation (flow-rate 1.8 µL/min) with a 3 µL injection. For gradient 1, solvent A was 98/2 water/acetonitrile (v/v) with 0.1% formic acid, and solvent B was 99.5/0.5 acetonitrile/water (v/v) with 0.1% formic acid. The gradient was as follows: 2% B at 0 min, 2% B at 3 min, 3.6% B at 11 min, 8% B at 15 min, 8% B at 27 min, 50% B at 30 min, 50% at 35 min, and 2% B at 37 min. Separations were performed at 30 °C. For gradient 2, Solvent A was 4/6 water/acetonitrile (v/v) with 0.1% formic acid, 10 mM ammonium formate and solvent B was 1/9 acetonitrile/isopropanol (v/v) with 0.1% formic acid, 10 mM ammonium formate.
The gradient was as follows: 30% B at 0 min, 30% B at 6 min, 60% B at 11 min, 60% B at 15 min, 80% B at 27 min, 80% B at 30 min, 85% at 35 min, 85% at 45 min, 30% at 47 min, and 30% B at 55 min. Separations were performed at 50 °C.

For targeted acyl-CoA analysis, samples were maintained at 4°C in a Leap CTC autosampler (CTC Analytics, Switzerland) during sample batch runs. 10 μL injections were used for LC-MS analysis. Chromatographic separation was performed using a reversed phase Waters XBridge BEH130 C18 column (2.1 x 150 mm, 3.5 μm pore size) on an Agilent 1100 HPLC system using a three solvent system: (A) 5 mM ammonium acetate in water, (B) 5 mM ammonium acetate in 95/5 acetonitrile/water (v/v), and (C) 80/20/0.1 (v/v/v) acetonitrile/water/formic acid, with a constant flow rate of 0.2 mL/min. Solvent C was used after analysis to wash the column. The gradient was as follows: 2% B at 0 min, 2% B at 1.5 min, 20% B at 5 min, 100% B at 5.5 min, 100% B at 13.5 min, 100% C at 14 min, 100% C at 19 min, 2% B at 20 min and 2% B at 25 min.

**Mass spectrometry.** For LC-HRMS analysis, an LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Fisher) was used in positive ion mode with a Michrom captive spray ESI source as previously described (215). For gradient 1 from above, the operating conditions were: spray voltage at 4 kV; capillary temperature at 250°C; capillary voltage at 35 V, tube lens 60 V. For gradient 2, the operating conditions were: spray voltage at 1.5 kV; capillary temperature at 200°C; capillary voltage at 0 V, tube lens 80 V.

For targeted acyl-CoA analysis, samples were analyzed using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) in the positive electrospray ionization (ESI) mode and analyzed using Analyst software as previously described (177). The column effluent was diverted to waste before 8 min and after 18
min. The mass spectrometer operating conditions were as follows: ion spray voltage (5.0 kV), compressed air as curtain gas (15 psi) and nitrogen as nebulizing gas (8 psi), heater (15 psi), and collision-induced dissociation (CID) gas (5 psi). The ESI probe temperature was 450°C, the declustering potential was 105 V, the entrance potential was 10 V, the collision energy was 45 eV, and the collision exit potential was 15 V. CoA thioesters were monitored using SRM (selected reaction monitoring mode) as previously published (179).

**Results**

Untargeted discovery reveals distinctive patterns of biotransformation of the oxo-ETEs in HUVECs. To determine possible routes of biotransformation of 11-oxo-ETE and 15-oxo-ETE, HUVECs were treated for 90 minutes with 10 μM of the parent compound in 0.25% DMSO. A biphasic Folch extraction was then performed on the lifted cell suspension to capture a broad range of intra- and extra-cellular metabolites. LC-HRMS was carried out in an untargeted manner on the extracted lipid and aqueous fractions. Analysis of differential abundant features was carried out by SIEVE 2.0, metaboanalyst 2.0, and XCMS (194,216). Metabolic discovery experiments conducted in HUVEC cells revealed a diversity of differentially abundant features from the lipid fraction, with markedly less features in the aqueous fraction as demonstrated by the respective cloud plots generated with XCMS (S Fig. 1). A ranked list by fold change versus control using a t-test (Welch’s unpaired) p-value cut-off of p < 0.01 was generated. Top hits from both programs were manually inspected; chromatograms were extracted from representative raw data samples (Fig. 1).
Two sequential mass shifts of +2 amu and an increased retention time were chosen for follow up targeted analysis for 11-oxo-ETE (Fig. 1A). Only one +2 amu shift was observed for 15-oxo-ETE (Fig. 1B). Interestingly, the free acid of 11-oxo-ETE demonstrated one major saturation and one minor second saturation, however, the methyl-ester of 11-oxo-ETE showed only one +2 amu shift (Fig. 1D). These putative 15-oxo-ETE metabolites were confirmed by treatment with $^{13}C_{20}$-15-oxo-ETE, and a resulting heavy isotope labeled metabolite was found with the same pattern of mass and retention time shift (Fig. 1C). This pattern of retention time and mass shift seems logically indicative of a reduction in double bonds. Accurate mass and isotope patterns matched predicted values for both parent and saturated compounds for 11-oxo-ETE and 15-oxo-ETE in positive and negative ion mode (Supplemental Figure 3 and data not shown).

**Time course studies reveal accumulation of reduced metabolites.** To further describe the dysregulated features, time course studies were carried out using a targeted approach. Cells were treated as above, using 11-oxo-ETE, 15-oxo-ETE, and their methyl esters. Extractions were done over a multi-hour time course to examine the change in levels of targeted metabolites. $^{13}C_{20}$-15-oxo-ETE stable isotope standard was spiked into all extractions to allow quantitation of the metabolites (Fig. 2). The internal standard co-eluted with 15-oxo-ETE (Fig. 2). The area of the target peak was integrated and quantified as a ratio over the area of the internal standard peak. Values were expressed as a fold change log$_{10}$ for each time point in order to cover the range of observed change in signal (Fig. 3). The methyl-ester treated cells showed an order of magnitude amplification in the accumulation of metabolites compared to cells treated without the methyl ester derivatives of 11- and 15-oxo-ETE (Figs. 3B, 3D). This was in
line with our previous work demonstrating that treatment with the methyl esters increased the intracellular pool of the free acid (214).

**MS/MS of parent oxo-ETEs and saturated metabolites.** MS/MS experiments were carried out in both positive and negative mode. (Supplemental Figure 3) Under negative ESI conditions, both mono-saturated oxo-ETEs exhibited an intense parent ion at \( m/z \) 319.2270 (\( \delta = 2.8\text{ppm} \)). (Supplemental Figure 3) The bis-saturated metabolite corresponding to 11-oxo-ETE+4 amu showed \( m/z \) 321.2424 (\( \delta = 3.4\text{ppm} \)). The 11-oxo-ETE MS/MS spectrum exhibited \( m/z \) 165.1283 (\( \delta = 0.6\text{ppm} \)) that is formed by \( \alpha \)-cleavage to the carbonyl at position 11, and thus it retains the original double bonds from C12 and C14. In the MS/MS spectrum of the mono-saturated 11-oxo-ETE, the fragment corresponding to one double bond being reduced in the same fragment was found at \( m/z \) 167.1442 (\( \delta = 0.4\text{ppm} \)) (Supplemental Figure 3). This indicates that the first saturation happens to positions C12 or C14. Consistent with this finding, the molecular fragment \( m/z \) 167.1076 (\( \delta = 0.4\text{ppm} \)) from 11-oxo-ETE, that corresponds to the fragment containing the initial double bonds at C5 and C8, did not have a corresponding ion in the MS/MS spectrum of the 11-oxo-ETE mono-unsaturated (\( m/z \) 169.1234). This further indicated that the first reduction did not occur at the position C5 or C8. The hypothesis that the first reduction happens to positions C12 or C14, was supported by the MS/MS data in positive mode. The \( m/z \) 217.1587 fragment, that contained the two double bonds at position C12 and C14, had a similar ion \( m/z \) 219.1742 in the mono-saturated 11-oxo-ETE, indicating a similar first reduction at one of these positions.

The negative ion MS/MS spectrum of 15-oxoETE was identical with published CID spectrum by Murphy, et. al (217). The MS/MS spectrum of the monosaturated 15-oxoETE contained multiple different fragments that showed a mass increase of 2 amu.
Unfortunately, the best described fragmentation of oxo-ETEs is due to the breaking of the double bond allylic to the carbonyl moiety. This generates the fragments 113.0977, corresponding to the part of the molecule without any double bond, and 219.1394, corresponding to the part of the molecule containing all the double bonds. The MS/MS spectrum of the mono-saturated 15-oxoETE showed the same fragment 113.0977 indicating that the first reduction did not occur at C13. The fragment corresponding to 219.1394 shifted to 221.1549 consistent with the first reduction taking place to one of the initial double bonds at C5, C8 or C11. As discovered by the use of deuterium and oxygen labeled compounds there are simultaneous mechanisms of ion formation in the MS/MS spectra of oxo-ETEs, resulting in isobaric fragments derived from different fragmentation (217). Even if different fragment ions from the MS/MS spectrum of 15-oxo-ETE showed the corresponding ions + 2 amu in the monounsaturated spectrum (Supplemental Figure 3), would be highly speculative to propose the place of the double bond reduction just based on the MS/MS data.

**Michael addition adducts of the parent and saturated products reveal position of double bond reduction.** In order to examine the position of the double bond reductions in the metabolites, the formation of 1,4-Michael adducts was monitored by LC-HRMS. Lipid extracts from treated HUVECs were treated overnight with 5% (v/v) BME at 37°C to adduct any α,β-unsaturated ketone containing compounds (Figs. 4B, 4D). Control treated extracts were treated with 5% (v/v) water and treated identically overnight (Figs. 4A, 4C). Treatment of the 11-oxo-ETE containing extracts reduced the parent and mono-saturated products by over one order of magnitude (Figs. 4A, 4B). In contrast, the doubly saturated product gave a strong peak in both control and BME treated samples.
These findings indicate that the parent and monosaturated product still contain the α,β-unsaturated ketone with the double bond at carbon 12. This is in contrast to the disaturated product of 11-oxo-ETE where one of the two reduced double bonds appears to occur at C12 resulting in the loss of the α,β-unsaturated ketone moiety.

The formation of GSH-adducts from treated HUVECs was also examined by LC-HRMS. GSH-adducts were observed for the parent and monosaturated 11-oxo-ETE (Figs. 5A), as well as the parent and monosaturated 15-oxo-ETE (Figs. 5B). The same pattern was observed for the GSH-adduct of [13C20]-15-oxo-ETE (Figs. 5C). Agreeing with the BME adduction, no GSH-adduct was observed for the doubly saturated 11-oxo-ETE metabolite (Figs. 5A). These data agree with the BME adduction results and indicate a second reduction of a double bond in 11-oxo-ETE abolishes the α,β-unsaturated ketone.

**Targeted acyl-CoA analysis reveals thioester formation and saturation.** The observation of a second reduction in 11-oxo-ETE prompted further experiments to understand the nature of this biotransformation. A cytosolic prostaglandin reductase has been described for prostanoids with a double bond between carbons 13 and 14. However, description of a second saturation is to the author’s knowledge unknown. The possibility of mitochondrial metabolism for the oxo-ETEs was therefore raised. Using a previously described method for analysis of acyl-coenzyme A thioesters, we investigated the possibility of a oxo-ETE-coenzyme A thioester as had been described for similar oxidized fatty acids (166).
A proof-of-principle experiment was conducted using LoVo cells treated with DMSO vehicle control or 25 μM AA (Fig. 6). LC-MS experiments using the CoA stereotypical neutral loss of 507 DA revealed an induction of acyl-CoA species. A strong peak at 1054 m/z corresponding to arachidonoyl-CoA was induced in the treatment versus the control. Interestingly, a peak at 1068 m/z was also observed, which would correspond to a oxo-ETE-CoA thioester (Fig. 6B).

To confirm this initial observation and test if the saturated products previously observed also formed CoA thioesters we treated LoVo cells with 25 μM AA, 10 μM 11-oxo-ETE, 10 μM 11-oxo-ETE-ME, or 10 μM 15-oxo-ETE. Using our previously described extraction methodology, we used LC-SRM/MS with transitions corresponding to the distinct CoA neutral loss of 507 amu fragmentation for the oxo-ETE-thioester with zero to four saturations (Fig. 7). In contrast to the free acid, we observed peaks in more than two channels.

To further investigate the nature of the saturation, we treated two human colon adenocarcinoma lines, LoVo and HCA-7, with 11-oxo-ETE and observed the saturation of the 11-oxo-ETE-CoA thioesters over time. In order to provide a semi-quantitative analysis, heavy labeled arachidonoyl-CoA was generated as previously described (179) and used as an internal standard for normalization and spiked into the sample before extraction. The patterns of saturation observed was different between the two cell lines, where detectable amounts of all four saturations were seen in the LoVo cells, with only 2 saturations at maximum seen in the HCA-7 cells.
Discussion

The study of metabolism of eicosanoids has mostly focused on the generation of bioactive lipids. The transformation of the initial products remains less well described. Existing literature on eicosanoid metabolism for COX/15-PGDH products has focused on PGE$_2$ and its 15-PGDH dependent product 15-keto-PGE$_2$. A second level of transformation via a double bond reduction forms the product 13,14-dihydro-15-keto-PGE$_2$. However, the COX/PGDH pathway also produces 11-oxo- and 15-oxo-ETE. 15-oxo-ETE can also be generated by 15-LOX/PGDH metabolism. Existing work on the metabolism of 15-oxo-ETE has described the generation and signaling of complex lipids integrating 15-oxo-ETE. In this work, we expand the knowledge of these pathways to include additional downstream products.

Our untargeted metabolomics approach revealed a distinct pattern of limited saturation for 11-oxo- and 15-oxo-ETE. The similar metabolism of [$^{13}$C$_{20}$]-15-oxo-ETE, as well as time course studies support the identification of the metabolites are derivatives of the parent oxo-ETEs. By examining the formation of Michael adducts, including BME and GSH adducts, we were able to discern the status of the double bond proximal to the carbonyl in the $\alpha,\beta$-unsaturated ketone moiety of the oxo-ETEs. The parent and predominant first saturation of 11-oxo- and 15-oxo-ETE maintain the $\alpha,\beta$-unsaturated ketone moiety. However, the second saturation of 11-oxo-ETE no longer forms a BME or GSH adduct, indicating the double bond at C12 adjacent to the carbonyl has been reduced. The enzymatic basis of this transformation warrants investigation as the $\alpha,\beta$-unsaturated ketone moiety has been associated with bioactivity in related molecules.
Saturation of mono- or poly-unsaturated fatty acids has been described through mitochondrial metabolism. However, description of eicosanoid thioesters is lacking in the literature. Although we extended our discovery method to specifically look for CoA thioesters of PGE\(_2\) and the proximal metabolites 15-keto-PGE\(_2\) and 13,14-dihydro-15-keto-PGE\(_2\), we could not detect any of these products (Figure 6B and data not shown). We also fail to detect the CoA thioester of the 11-oxo-ETE-methyl ester (data not shown). This further supports the hypothesis that the identified product is in fact a thioester via the carboxylic acid group. This distinction is important since the \(\alpha,\beta\)-unsaturated ketone moiety could result in a 1,4-Michael addition product as identified in our previous work on covalent adduction to a nucleophilic amino acid.

The limited saturation of free 11-oxo-ETE and 15-oxo-ETE and the pattern observed for the CoA thioesters may be relevant to wider metabolism. For the saturation of the CoA thioester we detect up to the full four possible saturation events, which may indicate a difference in mitochondrial versus cytosolic enzyme activity or substrate specificity for only the CoA thioester.

Ultimately, design of therapeutic interventions to modulate or mimic the effects of these molecules could be enabled by a complete elucidation of the pathways of metabolism. This could facilitate the discovery of inhibitors or for the design of stable derivatives. As additional benefit, identification of these novel metabolites may contribute to the ongoing efforts in untargeted metabolomics and an increased appreciation of specificity in lipidomics experiments.
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Figure 7.1. Extracted ion chromatograms of LC-HRMS analysis of lipid extract from oxo-ETE treated HUVECs. Mass spectral features from the discovery experiment were extracted from representative chromatograms with a 5 ppm mass window from the given m/z from positive ion mode LC-HRMS analysis of lipid extracts of HUVECs treated with (A) 11-oxo-ETE, (B) 15-oxo-ETE, (C) [\textsuperscript{13}C\textsubscript{20}]-15-oxo-ETE, or (D) 11-oxo-ETE-ME.
Peaks putatively corresponding to reductions in double bonds (saturations) were apparent for one and two saturations in 11-oxo-ETE. Only one saturation was detectable for 15-oxo-ETE and $[^{13}\text{C}_{20}]$-15-oxo-ETE. 11-oxo-ETE-ME treatment revealed a similar pattern of saturation as observed for 11-oxo-ETE, except the methyl ester itself was only mono-saturated.

**Figure 7.2.** Representative stable isotope dilution-LC-HRMS chromatograms of parent and putative metabolites. Co-elution of 15-oxo-ETE and $[^{13}\text{C}_{20}]$-15-oxo-ETE was observed as well as baseline separation of 11-oxo-ETE from $[^{13}\text{C}_{20}]$-15-oxo-ETE. Extracted ion chromatograms within a 5 ppm window of (A) 11-oxo-ETE and the suspected monosaturated and disaturated products (B) 15-oxo-ETE and the suspected monosaturated product.
Figure 7.3. Time course of accumulation of free oxo-acid metabolites in lipid extracts from HUVECs. Accumulation of putative metabolites after treatment with 10µM (A) 11-oxo-ETE, (B) 11-oxo-ETE-ME, (C) 15-oxo-ETE, or (D) 15-oxo-ETE-ME. Area under the curve was calculated from XICs of metabolites. $[^{13}\text{C}_2\text{O}_4]$-15-oxo-ETE was used as the internal standard and to normalize for extraction and ionization efficiency for relative quantitation. Values are expressed in Log Area under the peak for (Analyte/ISTD) to capture the full range of values over the timecourse.
Figure 7.4. LC-HRMS chromatograms of mock and BME treated oxo-ETE samples for parent oxo-ETEs and metabolites. Lipid extracts of treated HUVECs were treated with or without 5% volume/volume BME overnight at 37°C. (A) 11-oxo-ETE treated samples without BME, (B) 11-oxo-ETE with BME, (C) 15-oxo-ETE without BME (D) 15-oxo-ETE with BME. Panel (E) shows the reaction of 11-oxo-ETE with BME and the resulting adduct.
A. 11-oxo-ETE
GSH-adduct
NL: 1.52E5
m/z=626.3206

Monosaturated
GSH-adduct
NL: 4.21E5
m/z=628.3263

Disaturated
GSH-adduct
NL: 2.41E4
m/z=630.3419

B. 15-oxo-ETE
GSH-adduct
NL: 9.88E6
m/z=626.3206

Monosaturated
GSH-adduct
NL: 6.09E5
m/z=628.3263

C. \(^{13}C_{20}\)-15-oxo-ETE
GSH-adduct
NL: 3.11E6
m/z=646.3777

Monosaturated
GSH-adduct
NL: 1.98E5
m/z=648.3933
Figure 7.5. LC-HRMS chromatograms of GSH-adducts from parent and saturated products of oxo-ETEs. Aqueous phase of Folch extraction from treated HUVECs were analyzed by LC-HRMS after desalting. A 5 ppm mass window around the GSH-adduct is shown for (A) 11-oxo-ETE and saturated products (B) 15-oxo-ETE and its monosaturated product, (C) [13C20]-15-oxo-ETE and its monosaturated product.
Figure 7.6. **Neutral loss of 507 Da scans show inducible acyl-CoA species.** Neutral loss scans of 507 Da of (A) DMSO (B) 25 μM AA treated LoVo cells.
Figure 7.7. LC-MS/MS chromatograms for oxo-ETE-acyl-CoA species and reduced metabolites. Extracted ion chromatograms of acyl-CoA LC-MS/SRM analysis from LoVo Cells treated with (A) 25 µM AA treated LoVo cells (B) 10 µM 11-oxo-ETE (C) 10 µM 15-oxo-ETE (D) 10 µM 11-oxo-ETE-ME. The m/z transitions corresponding to the reduction of 0-4 double bonds were monitored (1068→561, 1070→563, 1072→565, 1074→567, 1076→569). Panel (E) shows the structures of 11-oxo-ETE-CoA and 15-oxo-ETE-CoA.
Figure 7.8. Time course of 11-oxo-ETE-CoA thioesters. Quantification over 4 h of the parent and saturated products of 11-oxo-ETE-CoA in (A) LoVo cells (B) HCA-7 cells. SID-LC-MS/SRM were performed using $[^{13}\text{C}_3\ ^{15}\text{N}_1]$-arachidonoyl-CoA as an internal standard for quantitation. Values are expressed in Log Area under the peak for (Analyte/ISTD) to capture the full range of values over the timecourse.

Supplemental Figures

7.1. Mirror plots from XCMSonline differential analysis of untargeted LC-HRMS of (A) organic phase and (B) aqueous phase from 11-oxo-ETE-ME treated HUVECs.
S Fig 7.2. Background of LC-HRMS analysis of Folch extraction from HUVEC lipid extract for DMSO treated cells. XICs for a 5 ppm mass window corresponding to the parent and reduced oxo-ETEs are shown.
S Fig 7.3. **MS/MS determination of reduced double bond positions.** (A) Negative ion mode ESI fragmentation of 11-oxo-ETE. (B) Chromatograms of diagnostic product ions for 11-oxo-ETE parent and metabolite structural elucidation. (C) Chromatograms of diagnostic product ions for 11-oxo-ETE parent and metabolite structural elucidation confirmed by a second LC gradient. (D) LC-CID-MS/HRMS negative ion product ion scan of 11-oxo-ETE. (E) LC-CID-MS/HRMS negative ion product ion scan of monosaturated 11-oxo-ETE. (F) LC-CID-MS/HRMS negative ion product ion scan of disaturated 11-oxo-ETE. (G) LC-CID-MS/HRMS negative ion and LC-HCD-MS/HRMS product ion scan of 15-oxo-ETE. (H) LC-CID-MS/HRMS negative ion and LC-HCD-MS/HRMS product ion scan of monosaturated 15-oxo-ETE. (I) LC-CID-MS/HRMS negative ion and LC-HCD-MS/HRMS product ion scan of disaturated 15-oxo-ETE.
Chapter 8: Conclusion and future directions

8.1 Conclusions

The work presented above describes the discovery of a new set of lipid metabolites of 11-oxo-ETE as well as description of the bioactivity and possible mechanistic function of the parent 11-oxo-ETE. The methodology used for metabolite characterization and quantification was then applied to the specific public health problem of asbestos exposure. The metabolic pathway under investigation in this work begins when COX-2 converts the substrate arachidonic acid into PGG$_2$, 11($R$)-HPETE, 15($S$)-HPETE and 15($R$)-HPETE. The subsequent action of POXs reduces the unstable hydroperoxide precursors to PGH$_2$, 11($R$)-HETE, 15($S$)-HETE, and 15($R$)-HETE, respectively. From these resulting metabolites, a plethora of other eicosanoids is generated in an enzyme and/or context dependent manner.

In Chapter 2, the ability of 15-PGDH to oxidize 11($R$)-HETE into anti-proliferative 11-oxo-ETE is described. The LoVo colon cancer adenocarcinoma cell model was used to clearly demonstrate that 11-oxo-ETE was rapidly secreted or metabolized by the LoVo cells. Chapter 3 describes an elaboration of the initial findings of rapid export and anti-proliferative action of 11-oxo-ETE by design of potent “pro-drug” methyl-ester forms of 11-oxo-ETE and 15-oxo-ETE that showed increased concentration in the intracellular space and more potent anti-proliferative effects. Furthermore, these effects were increased by pharmacological blockade of MRP exporters, which further implicates these exporters in modulating the biological effects of 11-oxo-ETE. Using the tools described in Chapter 3, the signaling, bioactivity, and mechanistic cellular action of 11-oxo-ETE on the NF-κB pathway were examined. The findings of these studies are described in Chapter 4. Inhibition of NF-κB signaling was demonstrated in HUVECs and with a luciferase reporter assay. A plausible mechanism was elucidated through the characterization of
an 11-oxo-ETE adduct on the cysteine or histidine residue of the DNA binding domain of a NF-κB transcription factor protein.

Chapter 5 describes a targeted approach to acyl-CoA analysis, whereby the novel 11-oxo-ETE-CoA thioester was discovered. Chapter 6 describes the methodology used for untargeted metabolomic analysis through nanoflow-ultraperformance liquid chromatography-high resolution accurate mass-mass spectrometry. The methods described in Chapters 5 and 6 were applied to the study of 11-oxo-ETE and 15-oxo-ETE metabolism to reveal distinct pathways of biotransformation. The results of these metabolic studies are described in Chapter 7.

Overall, this thesis reports the evolution of studies that initially involved the specific problem of understanding how an endogenous metabolite exerted its anti-proliferative effects on cancer cells. When existing methodologies failed to further the understanding of the problem, new methodologies were developed. Untargeted and targeted LC-MS based approaches for sensitive discovery and rigorous quantitation of AA metabolites were developed. The power of these methodologies was then realized in the elucidation of novel metabolism.

Initial studies involved the targeting a specific metabolite of AA, 11-oxo-ETE, together with an investigation of its mechanism of formation. Although 11-oxo-ETE belongs to a physiologically critical and pharmacological important pathway, little previous work had focused on the specific metabolite itself. Through understanding of the exact enzymology of formation, the relevance of 15-PGDH as a pharmacological target was supported. The initial kinetic description of 11-oxo-ETE formation prompted further work to examine its disposition and bioactivity. This required the generation and testing of specific chemical tools, which might be useful to the study of related molecules, or for therapeutic uses. The chemical tools developed in the initial study of 11-oxo-ETE then served as a useful probe in the examination the mechanistic signaling of 11-oxo-ETE in an inflammatory context. This mechanistic understanding expanded a broader scientific understanding of the electrophilic fatty acids, and will further the study of other related molecules for therapeutic and biomarker use. Through use of the most rigorous SID-LC-
MS/MS, the investigation of the formation and disposition of 11-oxo- and 15-oxo-ETE remained internally consistent across studies, and externally consistent with existing literature.

In order to examine the further metabolism of 11-oxo-ETE, specific methodological tools had to be developed. Both untargeted and targeted liquid chromatography-mass spectrometry approaches were created to study pathways of 11-oxo-ETE metabolism. The application of these approaches led to identification of novel metabolites. Pathways of biotransformation discovered for 11-oxo- and 15-oxo-ETE may also play roles in the metabolism of a wide variety of important lipid mediators.

8.2 Future directions

Considering the complexity of human metabolism, a major limitation of current methodology is that it focuses on limited numbers of metabolites within a specific context without consideration of the broader metabolic context (i.e. the other changes within the system). Mass spectrometry platforms, such as LTQ-Orbitrap, Q-exactive, Tribrid, TripleTOF 5600, Synapt, and 12T FT-ICR all provide high resolution capacity that can facilitate good mass accuracy (69,152,185,219-221). Expanded dynamic range, and increased sensitivity afforded by such technological innovation will also drive metabolism research. Supplementing studies with targeted analysis or complementary techniques such as NMR will also provide increased coverage and validity to studies (222). The growth of metabolomics software (e.g. XCMS and SIEVE), bioinformatics platforms with integrated statistical tools (e.g. R and metaboanalyst), and metabolite databases (e.g. METLIN, ChemSpider, LipidMAPS, and HMDB), together with HRMS, will make it possible to begin addressing these issues (190,193,194,207,209,216). Improvements in LC systems, including 2D-LC, and retention time database matching with references standards will also improve metabolomics.

For metabolic studies of lipids, the current small molecule metabolite databases provide incomplete coverage (Chapter 8). Therefore, compared to proteomics studies (with more
sophisticated databases), it is more challenging to assign specific structures to unknown features observed in an LC-MS chromatogram (Chapter 7 and 8). This is an inherent property of the enormous diversity of structures that can be encountered with small molecule metabolites (Chapter 7 and 8), which is also paradoxically what makes global metabolite profiling (metabolomics) such a necessary tool when confronted with such biochemical complexity. HRMS based analysis of small molecule metabolites should not be expected to provide accurate quantitation, complete specificity, or complete structural information in all cases and no single tool that is currently available is applicable to every experimental design. Besides LC-MS, NMR is also crucial for unknown metabolite structure elucidation, but suffers from limitations of sensitivity and capital costs (223). Thus, more basic discovery research into human metabolism (Chapters 1-8) will be required to attain the goal of high confidence assignments from broad coverage metabolomics data sets on a routine basis. Ultimately, validation with targeted analysis using rigorous analytical methodology suited for the task (Chapter 5) will still be required (81).

Importantly, the integration of genomics, transcriptomics, and proteomics/post-translational modifications experiments may provide the complex and enriched data necessary for understanding feedback relationships and the fine tuning of metabolites in physiology and disease. In this thesis research, no specific G-coupled protein receptors were identified for any product along the 11-oxo-ETE or 15-oxo-ETE pathway. Although the first steps in this process were laid in Chapter 1-7, assignment of an unknown receptor/ligand pair is still one of the most difficult problems of pharmacology. The integration of multi-dimensional (gene, RNA, protein, modification, metabolite) –omics data may facilitate this type of identification by virtue of providing a systems biology approach to narrow down candidate receptors. Additionally, increasing power of LC-MS platforms may facilitate better chemical probe approaches (224).

Ultimately, the future directions of the work in this thesis are two-fold: the top-down approach informed from metabolomics driving identification of differentially abundant metabolites from experimental studies and the bottom-up investigation of newly discovered pathways with rigorous validation. Both approaches are complimentary, necessary, and scientifically valid when
conducted with due acknowledgement of the inherent limitations. The historic and ongoing use of metabolites as putative biomarkers, therapeutics in their own right, and receptor or enzyme drug targets makes metabolism an attractive area of study for the betterment of human knowledge and health.

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