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Cyclooxygenase-2 Mediated Oxidation of 2-Arachidonoyl-Lysophospholipids Identifies Unknown Lipid Signaling Pathways

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Summary

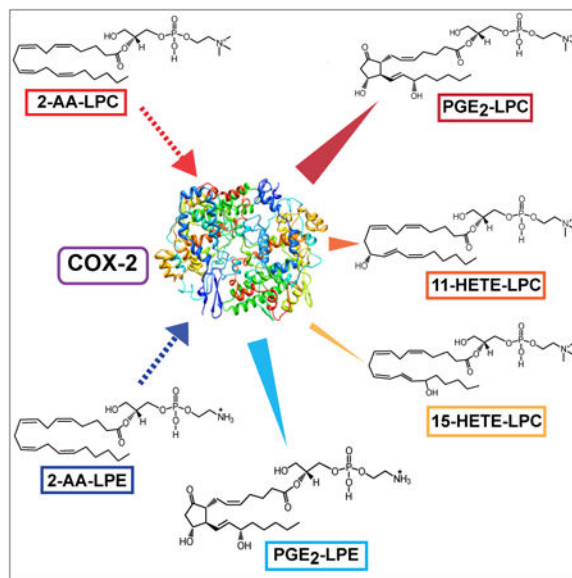
Eicosanoid lipids play important roles in cellular signaling as second messengers in inflammation, immune response, vascular tone, and the central nervous system. Biosynthesis of eicosanoid lipids proceeds via hydrolysis of esterified arachidonic acid from phospholipids followed by the oxidation of the released arachidonic acid by a variety of enzymes including cyclooxygenases (COX). Herein, we demonstrate the remarkable ability of COX-2, but not COX-1, to directly oxidize 2-arachidonoyl-lysolipids resulting in the generation of a previously unknown class of eicosanoid-lysolipids, and provide evidence that intracellular lipases can release eicosanoids from their eicosanoid-lysolipid precursors. Importantly, genetic ablation of a phospholipase, iPLA₂g, significantly reduced the amounts of these eicosanoid-lysolipids in murine hepatic tissue and fibroblasts. Furthermore, calcium-stimulation of wild-type murine lung fibroblasts produced robust increases in these eicosanoid-lysolipids which were markedly attenuated in iPLA₂g^{-/-} fibroblasts. Collectively, these results identify an iPLA₂g-initiated pathway generating a new class of lipid metabolites with potential signaling functions resulting from the direct COX-2 catalyzed oxidation of 2-arachidonoyl-lysolipids.

Graphical abstract

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Introduction

The biosynthesis of prostaglandins from arachidonic acid (AA) is initiated by prostaglandin endoperoxide synthase (PGHS), more commonly referred to as cyclooxygenase (COX) (Funk, 2001; Smith et al., 2000; Marnett et al., 2002; Smith et al., 2011). In humans, two COX isoforms, designated COX-1 and COX-2, are present that are encoded by distinct genes which are differentially regulated (Meade et al., 1993; Herschman et al., 1994). Cyclooxygenases play crucial roles in generating eicosanoid lipid mediators that have multiple diverse effects on cellular signaling, inflammation, vascular tone, and ion channel function as well as numerous other physiologic and pathophysiologic processes (Dubois et al., 1998; Smith et al., 2000; Marnett et al., 2002; Ricciotti and FitzGerald, 2011). Both COX-1 and COX-2 are bifunctional heterodimers that oxidize arachidonic acid to eicosanoid products through two discrete reactions that are catalyzed by functionally interacting active sites within each enzyme (Kurumbail et al., 2001). First, two molecules of diatomic oxygen are added to arachidonic acid in the cyclooxygenase reaction resulting in the generation of the endoperoxide PGG_2 . The second reaction catalyzed by PGHS is a peroxidase reaction, which reduces the 15-hydroperoxy substituent of PGG_2 to the 15-hydroxy endoperoxide PGH_2 . The resultant PGH_2 is a branch point metabolite for the generation of a rich repertoire of signaling eicosanoids (*e.g.*, prostaglandins, thromboxane, and prostacyclin) orchestrated by the integrated actions of multiple downstream enzymes that collectively regulate physiological processes in health, but precipitate pathological alterations in disease (Dogne et al., 2005; Ricciotti and FitzGerald, 2011).

Initially, it was generally believed that COX-1 was constitutively expressed in nearly all cell types while COX-2 was expressed specifically in immune cells promoting inflammation (Dubois et al., 1998; Smith et al., 2000; Marnett et al., 2002). Accordingly, it was assumed that COX-2 specific inhibition would ameliorate the pathologic sequelae of pain and inflammation without the toxic side effects (*e.g.*, gastritis, mucosal ulceration) typically

associated with the use of non-selective COX-1/COX-2 inhibitors. Substantial efforts were devoted to the development of COX-2 specific inhibitors such as Vioxx (Rofecoxib), Celebrex (Celecoxib), and selective NSAIDs. However, treatment of patients with COX-2 specific inhibitors resulted in an unacceptable incidence of cardiovascular events including sudden death (Cannon and Cannon, 2012; Bhalra et al., 2013; Hennekens et al., 2008; Conaghan et al., 2012). The unanticipated mortality associated with use of COX-2 specific inhibitors fueled multiple mechanistic studies that collectively demonstrated that the cardiovascular mortality resulting from COX-2 specific inhibition was due to the combined contributions of the absence of COX-2 reaction products in a variety of cell types (*e.g.*, endothelial cells, platelets, renal cells and cardiac myocytes) (Wang et al., 2009; Streicher et al., 2010; Ricciotti and FitzGerald, 2011; Yu et al., 2012). However, the precise molecular mechanisms leading to the untoward effects of COX-2 specific inhibition remain incompletely understood (Anwar et al., 2015).

Previously, we identified and characterized a novel membrane-associated calcium-independent phospholipase A₂γ (iPLA₂γ, also known as PNPLA8) that is present in myocardium and multiple other tissues (*e.g.*, heart, brain, kidney, lung) (Mancuso et al., 2000). This enzyme catalyzes both the *sn*-1 and the *sn*-2 hydrolysis of phospholipids containing oleic acid at the *sn*-2 position. In sharp contrast, when polyunsaturated aliphatic constituents (*e.g.*, arachidonic acid) are present at the *sn*-2 position, iPLA₂γ catalyzes the highly regioselective cleavage of phospholipids at the *sn*-1 position resulting in the generation of 2-acyl-lysolipids (Yan et al., 2005). Thus, the regiospecificity of iPLA₂γ is dependent upon the nature of the *sn*-2 aliphatic constituent. This unanticipated regiospecificity of iPLA₂γ identified a previously unappreciated signaling pathway resulting in the generation of 2-arachidonoyl-lysophosphatidylcholine (2-AA-LPC) which serves as a central branch point metabolite in multiple signaling cascades (Moon et al., 2012). Since the active site of COX-2 possesses a substantially larger steric volume in comparison to that of COX-1 (Smith et al., 2000; Marnett et al., 2002), we hypothesized that the active site of COX-2 could accommodate 2-arachidonoyl-lysolipids generated from iPLA₂γ resulting in their direct regio- and stereospecific oxidation to generate novel classes of signaling molecules which we have termed “eicosanoid-lysolipids”.

Herein, we report the ability of COX-2 to efficiently and stereospecifically oxidize the arachidonoyl moiety of both 2-AA-LPC and 2-arachidonoyl-lysophosphatidylethanolamine (2-AA-LPE) into a variety of eicosanoid-lysolipids including PGE₂-LPC, PGE₂-LPE, 11(*R*)-HETE-LPC, 15(*R*)-HETE-LPC (major), and 15(*S*)-HETE-LPC (minor). We provide further evidence that these moieties identified *in vitro* are natural products by identifying their presence in fibroblast cell cultures, hepatic tissue and human myocardium. Finally, we demonstrate that acute calcium stimulation of cultured fibroblasts results in the robust production of these metabolites which can be inhibited by genetic ablation of iPLA₂γ. Collectively, these results identify a new class of eicosanoid-lysolipid metabolites that are generated through a novel signaling pathway by the sequential actions of iPLA₂γ mediated hydrolysis of arachidonate-containing phospholipids and direct COX-2 catalyzed oxidation of the arachidonoyl-lysolipid products.

Results

Cyclooxygenase-2, but not cyclooxygenase-1, efficiently catalyzes the oxidation of 2-AA-LPC and 2-AA-LPE to novel eicosanoid-lysolipids

To identify novel eicosanoid-lysolipids produced by COX-2 from 2-AA-LPC by high resolution high mass accuracy (HRAM) mass spectrometry, we first generated a virtual database (VDB) of accurate masses of polyunsaturated lysolipids and their oxidized derivatives. This VDB was comprised of the mass of the starting material (2-AA-LPC or 2-AA-LPE) plus the addition of 1 to 6 oxygen atoms with varying degrees of unsaturation in the acyl chain (Table S1). Next, we synthesized 2-AA-LPC from 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine using *Candida rugosa* lipase as previously described (Creer and Gross, 1985), purified the resultant 2-AA-LPC by HPLC and incubated it with purified recombinant human COX-2. Analysis of the reaction products by LC-MS using HRAM mass spectrometry demonstrated that COX-2 catalyzed the generation of multiple novel metabolites whose accurate mass corresponded to oxidized eicosanoid-lysolipids predicted in the virtual database. Representative extracted ion chromatograms of the observed products from COX-2 mediated oxidation of 2-AA-LPC are shown in Figure 1A. Multistage tandem fragmentation analyses (MSⁿ) (n=2-4) identified multiple informative fragment ions (Figure S1) which in conjunction with the chromatographic elution profiles led to their provisional assignments as PGE₂-LPC, 15-HETE-LPC, and 11-HETE-LPC. Proposed fragmentation pathways of the eicosanoid-lysolipids leading to the observed accurate mass product ions are shown in Figure S2. To substantiate the proposed assignments, we hydrolyzed the *sn*-2 acyl chain of the eicosanoid-lysolipids by treatment with purified recombinant cytosolic phospholipase A₂α (cPLA₂α) (Pete and Exton, 1996; Pete et al., 1996), derivatized the released oxidized non-esterified fatty acid with N-(4-aminomethylphenyl)-pyridinium (AMPP), separated the resultant AMPP-eicosanoids by RPHPLC, and analyzed the products by HRAM mass spectrometry (Liu et al., 2013). The elution times and fragmentation patterns of the AMPP-derivatized eicosanoids released by cPLA₂α from the COX-2 generated eicosanoid-lysolipids were indistinguishable from authentic standards (Figure 2) thereby confirming the proposed structural assignments. In sharp contrast, incubation of 2-AA-LPC with purified recombinant COX-1 (Figure 1A, red line) resulted in the generation of only diminutive amounts of eicosanoid-lysolipids. Control incubations (without COX-1 or COX-2) did not exhibit measurable amounts of the observed eicosanoid-lysolipids (Figure 1A, black line). Thus, the production of PGE₂-LPC and 11-HETE-LPC by COX-2 occurs rapidly, but COX-1 only slowly catalyzes the oxidation of 2-AA-LPC (~5% of that by COX-2).

Next, we sought to determine whether 2-AA-LPE could also serve as substrate for COX-1 and/or COX-2. First, 2-AA-LPE was synthesized by acid hydrolysis of 1-O-1'-(Z)-octadecenyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine and purified by RPHPLC. Incubation of the resultant 2-AA-LPE with purified recombinant COX-2 resulted in the generation of predominantly PGE₂-LPE along with small amounts of 11-HETE-LPE (Figure 1B). Similar to results obtained with 2-AA-LPC, incubation of COX-1 with 2-AA-LPE produced only diminutive amounts of oxidized products. Collectively, these results demonstrate the ability of COX-2 to oxidize 2-AA-lysolipids to novel classes of eicosanoid-

lysolipid products that have the potential to either serve directly as signaling metabolites or indirectly as precursors for a wide variety of downstream lipid 2nd messengers.

The affinity of the COX-2 for 2-AA-lysolipids is a critical determinant of its activity in intact cells. Accordingly, additional experiments were performed to determine the K_m values of COX-2 for the formation of eicosanoid-lysolipid products (Table S2). The K_m values for formation of PGE₂-LPC and 11-HETE-LPC were found to be almost identical (11.7 μ M and 12.3 μ M, respectively) whereas the K_m value for formation of 15-HETE-LPC was 2-3-fold higher (30.0 μ M) in accordance with the lower yield of this minor product at a constant substrate concentration. The difference in K_m values for these COX-2 products of 2-AA-LPC indicate that there are different stereoelectronic relationships leading to its binding at the active site which after removal of the 13-pro S hydrogen, alternatively favoring either cyclization or oxygenation leading to the observed formation of PGE₂-LPC, 11-HETE-LPC, or 15-HETE-LPC, respectively.

Determination of the stereochemistry of COX-2 generated eicosanoid-lysolipids

The absolute stereochemistry of the COX-2 generated eicosanoid-lysolipid products were determined after hydrolysis by recombinant cPLA₂ α , derivatization of the released eicosanoids by AMPP, and separation of enantiomers by chiral chromatography with analysis by HRAM mass spectrometry of both molecular ions and their fragmentation products. Through comparisons with authentic chiral standards, the results demonstrated that the 11-HETE produced by COX-2 was exclusively 11(R)-HETE (Figure 3A-C) while the 15-HETE produced was comprised of ~67% 15(S)-HETE and ~33% 15(R)-HETE (Figure 3D-F). Since PGE₂ contains four chiral centers, the PGE₂ stereoisomers potentially present in the eicosanoid-lysolipids released by treatment with human recombinant cPLA₂ α are diastereomers with differing physical properties that can be separated by RPHPLC. The released PGE₂ from either PGE₂-LPC or PGE₂-LPE was derivatized with AMPP, separated by RPHPLC and analyzed by HRAM mass spectrometry. The resultant product was identified to be exclusively 9-oxo-11 R ,15 S -dihydroxy-5 Z ,13 E -prostadienoic acid by comparisons with authentic PGE₂ diastereomer standards (Figure 3G-I).

Differential effects of aspirin on the production of eicosanoid-lysolipid products

Previous work has demonstrated that aspirin acetylates Ser-516 of COX-2 (Lecomte et al., 1994; Wennogle et al., 1995), thereby completely abolishing the ability of the enzyme to form prostaglandins and concomitantly increasing the production of 11-HETE and 15-HETE (Xiao et al., 1997). To identify the effects of aspirin and resultant Ser-516 acetylation on the formation of eicosanoid-lysolipids, COX-2 was preincubated with aspirin prior to incubation with 2-AA-LPC or 2-AA-LPE. Analysis of reaction products by LC-MS demonstrated that PGE₂-LPC and PGE₂-LPE production were almost completely inhibited by aspirin (Figure S3). Furthermore, the formation of 11-HETE-LPC was also markedly inhibited (>90%) by aspirin pretreatment. In sharp contrast, the formation of 15-HETE-LPC was almost completely unaffected and was the most abundant product after pre-incubation of COX-2 with aspirin utilizing 2-AA-LPC as substrate. Collectively, these results demonstrate the highly specific effects of aspirin on the profile of eicosanoid-lysolipid molecular species produced by COX-2 mediated oxidation of 2-AA-lysolipids.

Increased production of oxidized 2-AA-lysolipids by COX-2 utilizing large unilamellar vesicles (LUVs) in comparison to small unilamellar vesicles (SUVs)

To further examine the activity of COX-2 in the oxidation of 2-AA-LPC and 2-AA-LPE as guests in a host bilayer, COX-2 was incubated with SUVs or LUVs comprised of DOPC/DOPE/2-AA-LPC (molar ratio: 35/10/5) or DOPC/DOPE/2-AA-LPE (molar ratio: 35/10/5). Remarkably, the rates of production of PGE₂-LPC, 11-HETE-LPC, and 15-HETE-LPC by COX-2 mediated oxidation of 2-AA-LPC present in large unilamellar vesicles were three to four-fold higher than in small unilamellar vesicles (Figure S4 A). Similarly, although less pronounced, the rate of oxidation of 2-AA-LPE by COX-2 to form PGE₂-LPE was 50% higher in LUVs than SUVs (Figure S4 B). Collectively, these results demonstrate the ability of COX-2 to preferentially oxidize 2-AA-lysolipids in physiologic membrane bilayers with large radii of curvature (*e.g.*, mimicking the plasma membrane) in comparison to small physically constrained membranes.

Eicosanoid-lysolipids are natural products in murine liver and their abundance is decreased by genetic ablation of iPLA₂γ

To determine if the newly identified eicosanoid-lysolipids are natural products, we first examined murine hepatic tissue. Eicosanoid-lysolipids from freshly isolated hepatic tissue were extracted by a modified Bligh and Dyer procedure, enriched by solid phase extraction, and further purified by C18 HPLC column chromatography. The collected eicosanoid-lysolipids were hydrolyzed by purified recombinant cPLA₂α and their resultant eicosanoid products were charge-switch derivatized with AMPP, separated by chiral chromatography and analyzed by LC-MS/MS by comparisons with authentic stereospecific standards. The results clearly identified the presence of 11(*R*)-HETE, 15(*R/S*)-HETE as well as the naturally occurring cyclooxygenase PGE₂ product (*i.e.* 9-oxo-11*R*,15*S*-dihydroxy-5*Z*,13*E*-prostadienoic acid) in mouse hepatic tissues (Figure 4A-C). Next, we quantified the eicosanoid-lysolipids in hepatic tissues from wild-type and germline iPLA₂γ^{-/-} mice that we previously generated and characterized (Mancuso et al., 2007). Genetic ablation of iPLA₂γ resulted in a dramatic reduction of 2-AA-LPC and 2-AA-LPE (Figure 4D). Furthermore, the amounts of PGE₂-LPC, PGE₂-LPE, 11-HETE-LPC and 15-HETE-LPC were also markedly decreased in iPLA₂γ^{-/-} mice in comparison to their WT littermates (Figure 4E). The levels of free eicosanoids PGE₂, 11-HETE and 15-HETE were also decreased in hepatic tissue from the iPLA₂γ^{-/-} mouse in comparison to their WT littermates (Table S3). To exclude the possibility that alterations in COX-2 expression were responsible for the observed decrease in eicosanoid-lysolipids, we measured COX-2 protein content by Western blot analysis. No significant differences in COX-2 protein levels in hepatic tissues from WT *vs.* iPLA₂γ^{-/-} mice were observed (Figure 4F).

The production of eicosanoid-lysolipids is regulated by calcium-mediated activation of iPLA₂γ

To determine whether the generation of eicosanoid-lysolipids was dynamically regulated by increases in intracellular calcium ion, wild-type murine lung fibroblasts (MLF) were grown in culture and treated with either 1.0 μM A23187 or vehicle alone. Calcium influx induced a robust increase in both 2-AA-LPC production and eicosanoid-lysolipid generation (Figure

5). To mechanistically identify whether iPLA₂γ was the enzymatic source of the 2-AA-lysolipids produced, we used cultured lung fibroblasts from the germline iPLA₂γ^{-/-} mice we generated (Mancuso et al., 2007). Ablation of iPLA₂γ markedly decreased the amount of calcium-stimulated eicosanoid-lysolipid production in iPLA₂γ^{-/-} fibroblasts relative to their WT littermate controls (Figure 5). Since calcium activates iPLA₂γ to produce 2-AA-lysolipids (Moon et al., 2012), these results suggest that eicosanoid-lysolipid production is initiated by iPLA₂γ and that the 2-AA-lysolipid products are oxidized by COX-2 to generate eicosanoid-lysolipids in intact cells.

Eicosanoid-lysolipids are present in human myocardium

Since the increased mortality from selective COX-2 inhibition in humans and mice results largely from cardiovascular dysfunction (Hennekens et al., 2008; Conaghan et al., 2012; Wang et al., 2009; Streicher et al., 2010), we sought to identify the presence of eicosanoid-lysolipids in normal human myocardium of cardiac donors that were unmatched for transplantation. Freshly isolated human ventricular myocardium from non-matched deidentified donors was rapidly frozen in liquid nitrogen, pulverized into a fine powder, extracted, and the eicosanoid-lysolipids were isolated following C18 HPLC column separation. The collected eicosanoid-lysolipids were hydrolyzed by purified recombinant cPLA₂α followed by charge-switch derivatization with AMPP, chiral chromatography and high resolution LC-MS/MS. Comparisons with authentic stereospecific standards clearly demonstrated the presence of PGE₂ (*i.e.* 9-oxo-11*R*,15*S*-dihydroxy-5*Z*,13*E*-prostadienoic acid), 15(*R/S*)-HETE, and 11(*R*)-HETE with minor amounts of 11(*S*)-HETE (~5%) (Figure 6A-C). Substantial amounts of 2-AA-LPC and 2-AA-LPE along with their downstream metabolites PGE₂-LPC, PGE₂-LPE, 11-HETE-LPC, and 15-HETE-LPC were detected (Figure 6D and E). The levels of the free eicosanoids PGE₂, 11-HETE and 15-HETE were also quantified and the results are summarized in Table S3. Since cardiac electrophysiologic function has been previously shown to be modulated by lysolipids as well as by multiple eicosanoids (Gross et al., 1982; Gubitosi-Klug et al., 1995; Wang et al., 2009; Streicher et al., 2010; Ricciotti and FitzGerald, 2011; Cedars et al., 2009; Jenkins et al., 2009), these results suggest that selective inhibition of COX-2 may alter the profile of eicosanoid-lysolipids in myocardium thereby contributing to the hemodynamic and electrophysiologic dysfunction observed after selective COX-2 inhibition.

Identification of 15-lipoxygenase-mediated production of 15-hydroxyeicosatetraenoic acid-lysophosphatidylcholine

Since the amount of 15-HETE-LPC produced in calcium-stimulated murine lung fibroblasts greatly exceeded that of other COX-2 oxidation products of 2-AA-LPC and decreased substantially in fibroblasts from germline iPLA₂γ K/O mice, these results suggested that 2-AA-LPC was potentially a 15-lipoxygenase substrate. To address this possibility, we incubated purified human 15-lipoxygenase with 2-AA-LPC for 10 min and the reaction products were analyzed by high resolution LC-MS/MS. Remarkably, human 15-lipoxygenase robustly oxidized 2-AA-LPC generating 15-hydroperoxyeicosatetraenoic acid lysophosphatidylcholine (15-HpETE-LPC) which was subsequently reduced by triphenylphosphine to 15-HETE-LPC (Figure S5). These results demonstrate the suitability of 2-AA-LPC to serve as substrate for both cyclooxygenases and lipoxygenases and further

suggest that 2-AA-lysophospholipids can be oxidized in addition by a multiplicity of oxidases (both known and unknown) to greatly expand the repertoire of lipid 2nd messengers in cells through these novel metabolic and signaling pathways.

Discussion

The present work identifies the previously unrecognized ability of cyclooxygenase-2 to directly oxidize arachidonoyl-lysolipids resulting in the production of new classes of eicosanoid-lysolipids. In this study, we identified the COX-2-dependent oxidation of 2-AA-LPC and 2-AA-LPE, determined the regiochemistry and stereochemistry of the reaction products, and demonstrated the presence of these metabolites in multiple cells and tissues. Moreover, through genetic ablation of iPLA₂γ, we have shown that production of these eicosanoid-lysolipid metabolites is largely dependent upon the calcium-mediated activation of iPLA₂γ to generate 2-arachidonoyl-lysolipid substrates for COX-2. Upon calcium ionophore stimulation, production of PGE₂-LPC, 11-HETE-LPC, and 15-HETE-LPC was dramatically increased in both WT MLF cells and in the extracellular medium of these cells. A substantial portion of the 15-HETE-LPC produced by MLF cells in the current study was likely generated by 15-lipoxygenase using 2-AA-LPC as substrate. Importantly, eicosanoid-lysolipid production was significantly lower after calcium stimulation in MLF cells from iPLA₂γ^{-/-} mice in comparison to their WT counterparts. Collectively, these findings establish a sequential process initiated by calcium-mediated activation of iPLA₂γ to produce 2-arachidonoyl-lysolipids followed by their subsequent COX-2 mediated oxidation to generate eicosanoid-lysolipids. Additionally, these studies demonstrate that the resultant eicosanoid-lysolipids can be directly hydrolyzed by a variety of intracellular phospholipases (*e.g.*, cPLA₂α) resulting in the liberation of non-esterified eicosanoids that have previously been demonstrated to possess potent biological effects in inflammation, vasoregulation, osmoregulation, and hemostasis amongst many other physiologic and pathophysiologic processes (Dubois et al., 1998; Smith et al., 2000; Marnett et al., 2002; Ricciotti and FitzGerald, 2011).

Recent studies have provided strong evidence that COX-2 inhibition in multiple cell types (*e.g.*, endothelial cells, platelets, kidneys and cardiac myocytes) contributes to the increased risk of cardiovascular events due to disruption of the production of the physiologic repertoire of signaling lipids in a cell-type and context-dependent manner. Previous studies have demonstrated the requirement for COX-2 products for the normal physiologic function of endothelial cells, smooth muscle cells, platelets, and cardiac myocytes (Ricciotti and FitzGerald, 2011). For example, in myocardium, cardiac myocyte specific COX-2 knockout results in electrophysiologic alterations (Wang et al. 2009). Similarly, endothelial specific COX-2 knockout leads to a marked reduction in prostacyclin production in endothelial cells (Tang et al., 2014). These effects may be further exacerbated under conditions of myocardial stress (*e.g.*, ischemia/reperfusion) where activation of phospholipases results in production of excessive amounts of fatty acids and lysolipids which have been demonstrated to compromise ion channel kinetics and electrophysiologic function (Streicher et al., 2010; Ricciotti and FitzGerald, 2011; Yu et al., 2012; Cedars et al., 2009; Jenkins et al., 2009; Liu et al., 2007). Sudden death from specific COX-2 inhibition likely results from lethal changes in cardiac function caused by alterations in the profile of intact eicosanoid-lysolipids or their

hydrolyzed products that regulate ion channel function, responses to stress, and inflammation.

In addition to the well established oxidation of non-esterified arachidonic acid (Funk, 2001; Smith et al., 2000; Marnett et al., 2002; Smith et al., 2011), previous work has shown that COX-2, but not COX-1, can effectively oxidize 2-arachidonoyl glycerol (Kozak et al., 2000) which is consistent with the current results utilizing 2-AA-LPC and 2-AA-LPE as substrates. Recent structural studies determining the position of 1-AA-glycerol within the active site of murine COX-2 have revealed that the glycerol moiety is positioned at the opening of the cyclooxygenase channel near Arg-120 and Tyr-355 (Vecchio and Malkowski, 2011). Interestingly, the 2,3-dihydroxypropyl group of 1-AA-glycerol is not stabilized by either ionic or hydrogen bonding interactions with the polar side chains of Arg-120, Arg-513, or Glu-524 as has been previously proposed (Kozak et al., 2001). Perhaps one or more of these residues may participate in binding of the polar head group of 2-AA-LPC or 2-AA-LPE to the enzyme. In addition, work by Malkowski and coworkers has identified rotamer conformations of Leu-531 in COX-2 which increase the volume of the opening of the cyclooxygenase channel thereby allowing a wider range of substrates to be oxidized by COX-2 relative to COX-1 (Vecchio and Malkowski, 2011). Details at the atomic level of the binding of 2-AA-lysolipids to COX-2 await similar structural crystallographic and solution state NMR studies to determine the stereoelectronic relationships and molecular interactions of 2-AA-lysolipid substrates within the active site.

An interesting aspect of the current results is the functional significance of parallel pathways for the generation of free eicosanoids *vs.* eicosanoid-lysolipids for COX-2 mediated signaling. There are several potential reasons underlying the existence of parallel reaction pathways to generate signaling metabolites. First, eicosanoid-lysolipids are likely endogenous ligands for specific receptors that may have higher affinities than their eicosanoids counterparts due to electrostatic interactions with the quaternary amine polar head group. Second, eicosanoid-lysolipids could serve as latent signaling precursors for the orchestrated spatio-temporal release of free eicosanoids by intracellular lipases. Since the off rate of lysolipids from membrane bilayers greatly exceeds that of non-esterified fatty acids, the ability of oxidized lysophospholipids to readily dissociate from cellular membranes would facilitate inter-organelle communication from their sites of formation to their sites of action or for further metabolism to novel complex signaling moieties (*e.g.*, further oxidation, conjugation to glutathione, *etc.*). Third, since lysolipids are transported by a lysolipid transporter (Tarling et al., 2013; Contreras et al., 2010), it is tempting to speculate that eicosanoid-lysolipids can utilize the same transporter for the delivery of specific eicosanoids to neighboring cells to allow transcellular lipid 2nd messenger signaling. Indeed, it seems likely that the identified metabolites represent only a small fraction of those arising from the oxidation of *sn*-2 polyunsaturated fatty acid-lysolipids (*e.g.*, oxidized linoleic, arachidonic, docosahexaenoic acid-lysolipid adducts). Fourth, we point out that intracellular lysophospholipases C and lysophospholipases D (autotaxin) have previously been shown to hydrolyze arachidonoyl-LPC suggesting that the repertoire of eicosanoid-based lipid signaling can be further diversified by removal of the polar head group in mammalian cells (Di Marzo et al., 1996; Tokumura et al., 2002) (Figure 7). In this regard, polyunsaturated lysophosphatidic acids have been demonstrated to be selectively generated from autotaxin

mediated cleavage of their lysophosphatidylcholine precursors in asthmatic patients following allergen challenge (Ackerman et al., 2016). Thus, eicosanoid-lysolipids represent not only a new class of dynamically regulated signaling molecules, but in addition likely serve as precursors for eicosanoid-glycerols or eicosanoid-lysophosphatidic acids whose relative amounts and biologic effects are likely cell type and context dependent (Di Marzo et al., 1996; Tokumura et al., 2002). Collectively, these results provide a new perspective on lipid 2nd messenger generation identifying eicosanoid-lysolipids as natural products derived from AA-containing lysophospholipids that likely possess receptor-mediated signaling functions that modulate inflammation, cardiovascular effects and ion channel kinetics in myocardium, peripheral vessels and other tissues.

Significance

Calcium-activated iPLA₂γ catalyzes the regiospecific hydrolysis of mitochondrial membrane lipids containing polyunsaturated fatty acids at the *sn*-2 position to generate 2-arachidonoyl-lysolipids which serve as a signaling node in multiple lipid 2nd messenger cascades. The current results indicate that COX-2, but not COX-1, effectively oxidizes 2-arachidonoyl-lysophosphatidylcholine (2-AA-LPC) to generate prostaglandin E₂-LPC (PGE₂-LPC), 11-hydroxyeicosatetraenoic acid-LPC (11-HETE-LPC) and lesser amounts of 15-hydroxyeicosatetraenoic acid-LPC (15-HETE-LPC). In addition, COX-2 oxidizes 2-arachidonoyl-lysophosphatidylethanolamine (2-AA-LPE) to predominantly PGE₂-LPE, but not 11-HETE-LPE or 15-HETE-LPE. Resolution of the PGE₂ diastereomers demonstrated that PGE₂-LPC and PGE₂-LPE were exclusively 9-oxo-11*R*,15*S*-dihydroxy-5*Z*,13*E*-prostadienoic acid-LPC and 9-oxo-11*R*,15*S*-dihydroxy-5*Z*,13*E*-prostadienoic acid-LPE respectively. Furthermore, 11-HETE-LPC was exclusively 11(*R*)-HETE-LPC while 15-HETE-LPC was comprised of ~67% 15(*S*)-HETE-LPC and ~33% 15(*R*)-HETE-LPC. These eicosanoid-lysolipids are natural products since they are present in cultured murine fibroblasts, murine liver and fresh human myocardium in high enantiomeric excess. The pathway for their synthesis was identified through reverse genetics using tissue and cultures of fibroblasts from germline iPLA₂γ knockout mice. Since the amounts of these eicosanoid-lysolipid molecular species in hepatic tissue and fibroblast cultures from iPLA₂γ^{-/-} mice were markedly reduced in comparison to their wild type counterparts, these results demonstrate that iPLA₂γ initiates the production of 2-AA-lysolipids that are subsequently oxidized to generate these novel metabolites. This notion is substantiated by demonstration that calcium-stimulation of wild type murine lung fibroblasts produced robust increases in eicosanoid-lysolipids which are markedly attenuated by genetic ablation of iPLA₂γ. Collectively, these results identify novel signaling pathways employing direct oxidation of arachidonoylated-lysolipids by COX-2 to generate multiple previously unknown lipid 2nd messengers that may either serve directly as novel signaling molecules or be subjected to further metabolism to produce bioactive molecules for a wide variety of lipid signaling cascades.

Experimental Procedures

Cyclooxygenase enzymatic assays

(1) *Kinetic analysis of the cyclooxygenase mediated oxidation of 2-AA-LPC or 2-AA-LPE.* Purified COX-1 or COX-2 was preincubated with 1 μ M hematin in 400 μ l of 100 mM Tris-HCl buffer (pH 8.0) containing 10% glycerol for 5 min at room temperature. The substrate 2-AA-LPC or 2-AA-LPE (10 μ M) was then added to initiate the reaction followed by incubation at 30°C for various times. The reaction was then terminated by adding 80 μ l of methanol acidified to pH 4 with glacial acetic acid and applied to a Strata-X solid phase extraction cartridge previously preconditioned with 3 ml of methanol and equilibrated with 3 ml of 20% methanol/80% H₂O. After washing with H₂O, the reaction products were eluted with methanol and analyzed by LC-MSⁿ methods as described below. (2) *Inhibition of COX-2 mediated oxidation of 2-AA-LPC or 2-AA-LPE by aspirin.* Purified human COX-2 was diluted in 400 μ l of 100 mM Tris-HCl buffer pH 8.0 and treated with 2 mM aspirin at 37°C for 30 min. The buffer was then supplemented with hematin (1 μ M) for 5 min at room temperature, and 2-AA-LPC or 2-AA-LPE (10 μ M) was then added. After incubation at various times at 30°C, reactions were terminated with acidified methanol and the products were extracted by solid phase extraction as described above and analyzed by LC-MSⁿ as described below.

15-Lipoxygenase enzymatic assay

Purified 15-LOX (2 μ g) was incubated with 2-AA-LPC (10 μ M) in 400 μ l of 50 mM Tris-HCl buffer (pH 7.2) containing 10% glycerol for 10min in room temperature (23°C). The reaction was then terminated by addition of 80 μ l of methanol acidified to pH 4 with glacial acetic acid and applied to a Strata-X solid phase extraction cartridge previously preconditioned with 3 ml of methanol and equilibrated with 3 ml of 20% methanol/80% H₂O. After washing with H₂O, the reaction products were eluted with methanol and analyzed by LC-MSⁿ methods as described below.

Reverse phase liquid chromatography-tandem mass spectrometry (LC-MSⁿ)

LC-MSⁿ analyses were performed using an LTQ-Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a Surveyor HPLC system (Thermo Scientific, San Jose, CA). Detailed instrumentation settings were provided in Supplemental Information.

Identification of the eicosanoid products generated through hydrolysis of eicosanoid-lysolipids by cPLA₂ α

The eicosanoids-lysolipid products generated by COX-2 were incubated with purified cPLA₂ α (5 μ g) in 200 μ l of HEPES buffer (10 mM HEPES, pH 7.4, 100 mM KCl, 0.5 mM CaCl₂, and 10% glycerol) for 15 min at 37°C. Reactions were stopped by addition of 40 μ l methanol followed by acidification of the reaction mixture to pH 4 with glacial acetic acid. Terminated reactions were immediately applied to a Strata-X solid phase extraction and the products were derivatized with AMPP as previously described (Liu et al., 2013). Samples were then analyzed by LC-MSⁿ methods as described above in the section “Reverse phase liquid chromatography-tandem mass spectrometry” and in the section “Chiral phase

chromatography-tandem mass spectrometry” that was described in details in Supplemental Information.

Human myocardium and murine hepatic tissue sample preparation and analysis of eicosanoids and eicosanoid-lysolipids

Human myocardial tissue was obtained through the Washington University Translational Cardiovascular Tissue Core (WUTCT) in accordance with protocols approved by WUTCT Advisory Committee at Washington University School of Medicine. All animal breeding, care and euthanasia were performed according to protocols approved by the Animal Studies Committee at Washington University School of Medicine. Detailed procedures for tissue preparation and quantitation were provided in Supplemental Information.

Endogenous eicosanoid-lysolipid production in mouse lung fibroblasts (MLF) from wild-type and iPLA₂ γ ^{-/-} mice

Mouse lung fibroblasts were isolated from wild type (WT) or iPLA₂ γ ^{-/-} mice as previously described (Seluanov et al., 2010). Primary cells were cultured in minimum essential media (MEM) containing 15% heat-inactivated fetal bovine serum, 1X MEM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml of penicillin, and 100 μ g/ml of streptomycin on 10 cm tissue culture dishes. Experiments were performed by using MLF cells between the second and fourth passage. At ~90% confluence, MLF cells were placed in serum-free MEM alone for 3 hrs prior to incubation in fresh serum-free MEM containing either DMSO (0.1%) vehicle alone or 1.0 μ M A23187 for 15 min. The medium was then collected and extracted by Strata-X solid phase extraction as described above. After removal of the media, cells were washed with phosphate buffered saline (PBS), harvested in 1 ml ice-cold PBS by using a cell scraper, and then immediately transferred to 2 ml CHCl₃/CH₃OH (1:1, v/v) and vortexed. Following phase separation by centrifugation at 15,000g for 15 min, the CHCl₃ layer was evaporated under a nitrogen stream and resuspended in 100 μ l of 80% CH₃OH in H₂O for LC-MS/MS analysis as described above in the section “Reverse phase liquid chromatography-tandem mass spectrometry”.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlight

- COX-2 directly oxidizes 2-arachidonoyl-lysophospholipids to eicosanoid-lysolipids.
- Lipases (*e.g.*, cPLA₂α) release eicosanoids from eicosanoid-lysolipid precursors.
- Genetic ablation of iPLA₂γ reduces the amounts of eicosanoid-lysolipids.
- Calcium-stimulation robustly increases the production of eicosanoid-lysolipids.

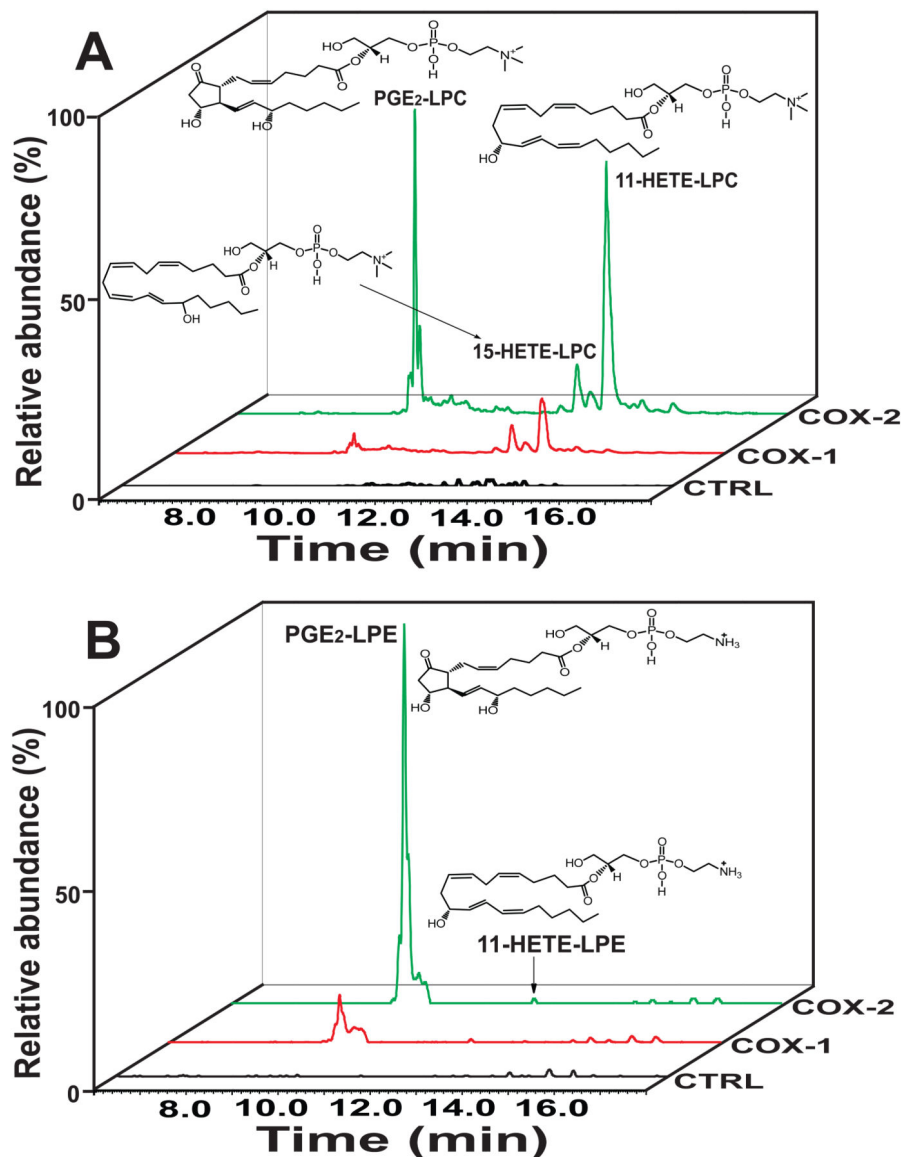


Figure 1. Cyclooxygenases mediate oxidation of 2-arachidonoyl-lysolipids (2-AA-LPC and 2-AA-LPE) to produce novel eicosanoid-lysolipids

Extracted ion chromatograms of COX-1 and COX-2 oxidized eicosanoid-lysolipid products utilizing either 2-AA-LPC (**A**) or 2-AA-LPE (**B**) as substrate. Purified recombinant COX-1 (2 μ g) or COX-2 (2 μ g) was incubated with 2-AA-LPC (10 μ M) or 2-AA-LPE (10 μ M) in 100 mM Tris-HCl buffer (pH 8.0) at 30°C for 10 min. The reaction was terminated by addition of methanol and acidified to pH 4 with glacial acetic acid. The reaction products were then purified by solid phase extraction following separation on a C18 HPLC column prior to analysis utilizing an LTQ-Orbitrap mass spectrometer with a mass resolution of 30,000 at $m/z=400$ in the positive ion mode. The extracted ion chromatograms (with 3 ppm mass window) for the identified metabolic products of PGE₂-LPC (m/z 592.3245), 11-HETE-LPC (m/z 560.3347), and 15-HETE-LPC (m/z 560.3347) for 2-AA-LPC as well as PGE₂-LPE (m/z 550.2726) and 11-HETE-LPE (m/z 518.2877) for 2-AA-LPE are shown.

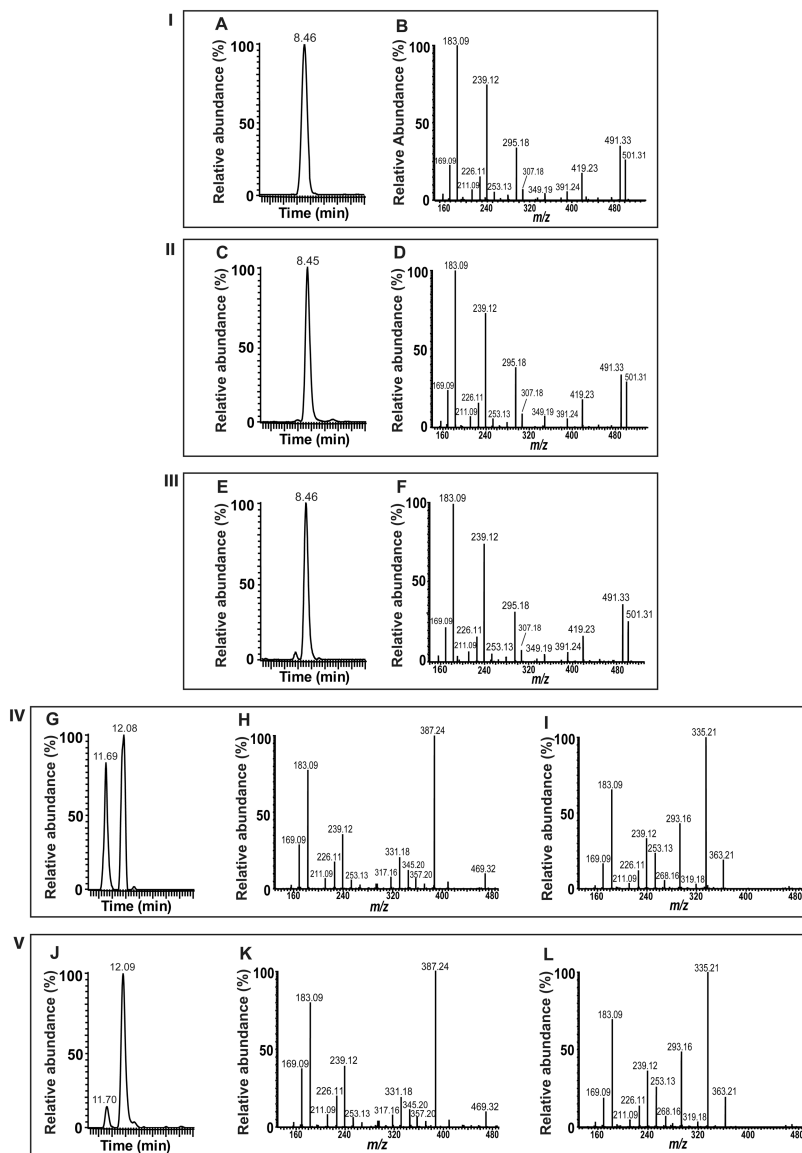


Figure 2. Identification of eicosanoid-lysolipids generated by COX-2 catalyzed oxidation of 2-AA-LPC and 2-AA-LPE
 COX-2 generated eicosanoid-lysolipids were treated with cPLA₂ α and the resultant released eicosanoid products were chemically derivatized with AMPP, separated on a C18 HPLC column and analyzed by mass spectrometry. **Panel-I**, standard PGE₂ elution time (A) and its MS/MS product ion spectrum (B); **Panel-II**, elution time (C) and MS/MS product ion spectrum (D) for the product generated by cPLA₂ α mediated hydrolysis of the COX-2 oxidation of 2-AA-LPC; **Panel-III**, elution time (E) and MS/MS product ion spectrum (F) for the product generated by cPLA₂ α mediated hydrolysis of the COX-2 oxidation of 2-AA-LPE; **Panel-IV**, elution times (G) for 15-HETE (11.69 min) and 11-HETE (12.08 min) standards along with their MS/MS product ion spectra H for 15-HETE and I for 11-HETE, respectively; **Panel-V**, elution times (J) and MS/MS product ion spectra (K and L) for the products generated by cPLA₂ α mediated hydrolysis of the COX-2 oxidized products of 2-AA-LPC.

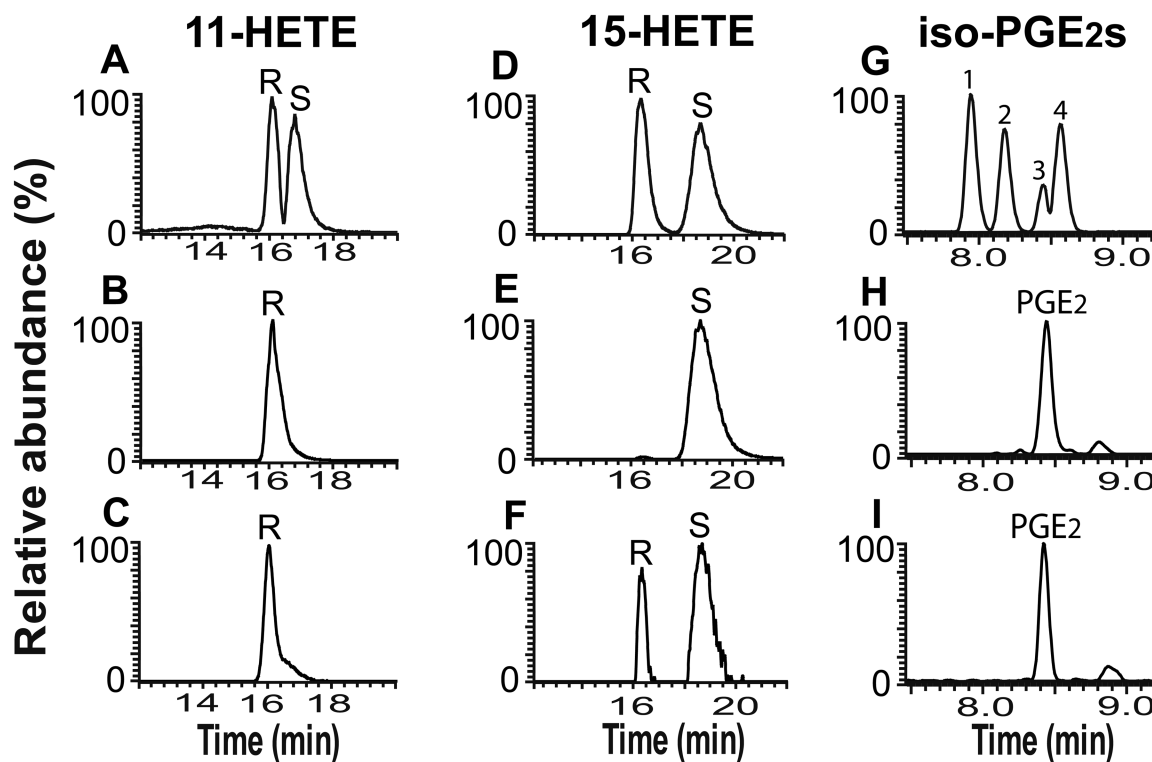


Figure 3. Chiral-phase and reverse-phase HPLC-MS analyses of eicosanoids produced following cPLA₂α mediated hydrolysis of PGE₂-LPC, PGE₂-LPE, 11-HETE-LPC and 15-HETE-LPC generated by COX-2 catalyzed oxidation of 2-AA-LPC and 2-AA-LPE

COX-2 generated eicosanoid-lysolipids were treated with cPLA₂α and the resultant eicosanoid products were derivatized with AMPP, purified by SPE and analyzed by chiral-phase (for 11-HETE and 15-HETE stereoisomers) HPLC-MS and reverse-phase (for PGE₂ stereoisomers) HPLC-MS analyses. **A**, Standards of racemic 11(*R/S*)-HETE; **B**, Standard of 11(*R*)-HETE; **C**, 11-HETE produced by cPLA₂α mediated hydrolysis of the COX-2 oxidized products of 2-AA-LPC; **D**, Standards of racemic 15(*R/S*)-HETE; **E**, Standard of 15(*S*)-HETE; **F**, 15-HETE produced by cPLA₂α mediated hydrolysis of the COX-2 oxidized products of 2-AA-LPC; **G**, Standards of PGE₂ stereoisomers indicated as follows: 15(*R*)-PGE₂ (**1**), 8-iso-PGE₂ (**2**), PGE₂ (9-oxo-11*R*,15*S*-dihydroxy-5*Z*,13*E*-prostadienoic acid) (**3**), and 11β-PGE₂ (**4**); **H**, PGE₂ produced by cPLA₂α mediated hydrolysis of the COX-2 oxidized products of 2-AA-LPC; **I**, PGE₂ produced by cPLA₂α mediated hydrolysis of the COX-2 oxidized products of 2-AA-LPE.

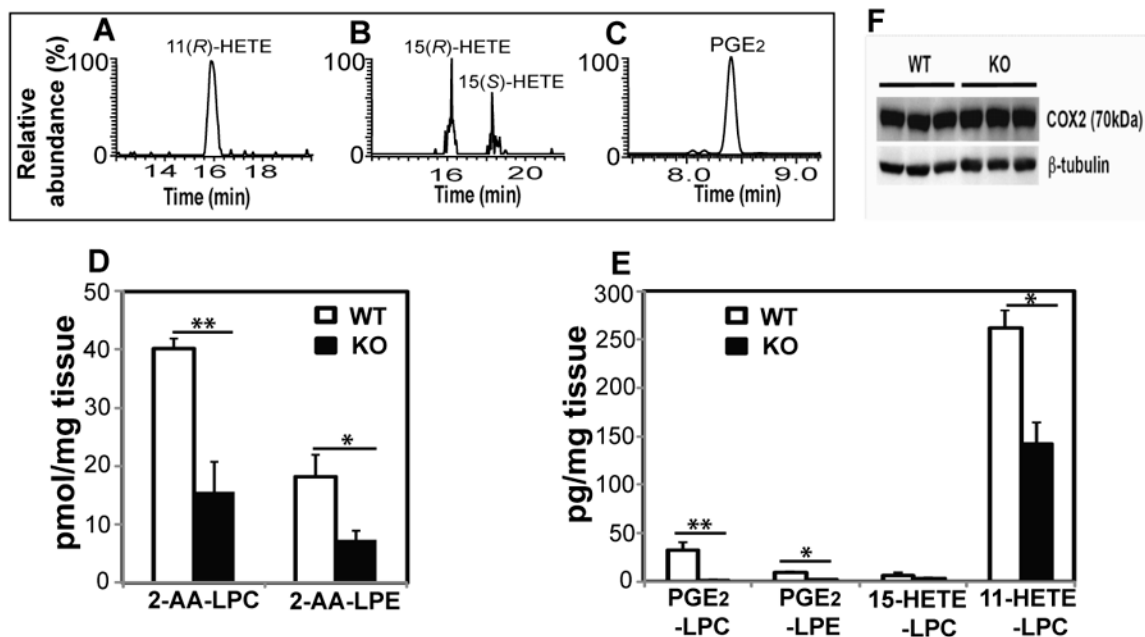


Figure 4. Eicosanoid-lysolipids present in wild-type (WT) and $iPLA_2\gamma^{-/-}$ (KO) murine hepatic tissue

Hepatic tissue was isolated from wild-type (WT) and $iPLA_2\gamma$ knock-out (KO) mice and flash frozen in liquid nitrogen. The extracts were purified by solid-phase extraction and the isolated eicosanoid-lysolipids were hydrolyzed by purified recombinant $cPLA_2\alpha$ followed by charge-switch derivatization with AMPP, chiral chromatographic separation, and compared with authentic stereospecific standards. **A-C**, Stereochemical determination of eicosanoid-lysolipid products present in WT hepatic tissue; **D**, Content of 2-AA-LPC and 2-AA-LPE in hepatic tissue from WT and KO mice; **E**, Content of PGE₂-LPC, PGE₂-LPE, 11-HETE-LPC, and 15-HETE-LPC in hepatic tissue from WT and KO mice. Data are expressed as mean values \pm SEM of six replicates. Statistical analyses were performed using an unpaired two-tailed Student *t* test; $P < 0.05$ was considered significant. * $P < 0.05$ and ** $P < 0.01$; **F**, Western analysis of COX-2 expression in WT and KO mouse liver using β -tubulin as a loading control.

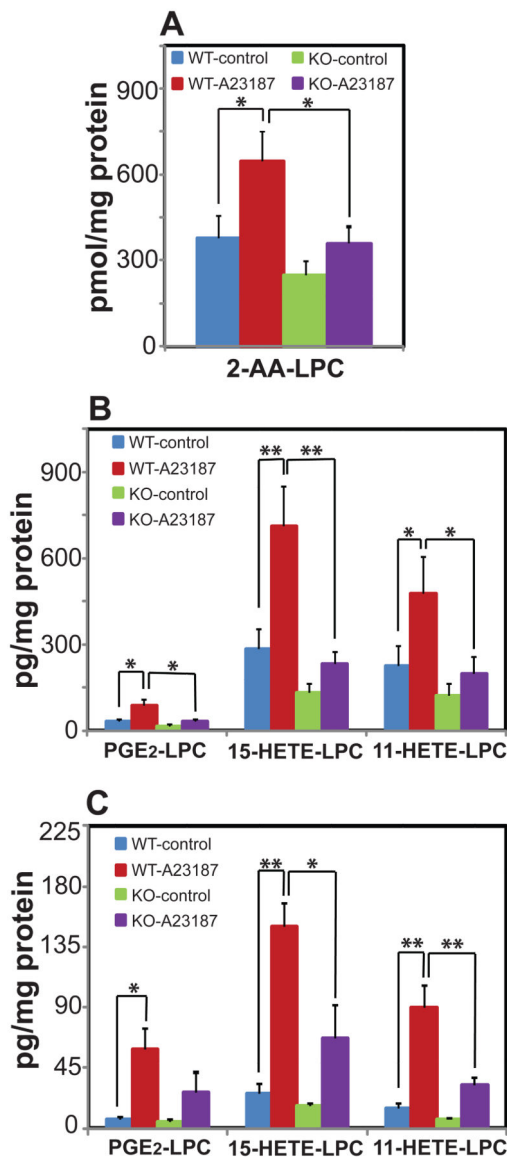


Figure 5. Biosynthesis of 2-AA-LPC, PGE₂-LPC, 11-HETE-LPC and 15-HETE-LPC in murine lung fibroblasts (MLF) from wild-type (WT) and iPLA₂γ^{-/-} (KO) mice stimulated with calcium ionophore

A, Endogenous 2-AA-LPC production from MLF cells from WT and KO mice stimulated with A23187 (1μM) or vehicle alone at 37°C for 15 min.; **B**, Endogenous production of PGE₂-LPC, 11-HETE-LPC and 15-HETE-LPC in MLF cells from WT and KO mice stimulated with or without (vehicle alone) A23187 calcium ionophore; **C**, Quantitation of PGE₂-LPC, 11-HETE-LPC and 15-HETE-LPC released into the extracellular media of MLF cells from WT and KO mice stimulated with or without (vehicle alone) A23187 calcium ionophore. Data are expressed as mean values ± SEM of six replicates. Statistical analyses were performed using unpaired two-tailed Student *t* test; *P*<0.05 was considered significant. **P*<0.05 and ***P*<0.01.

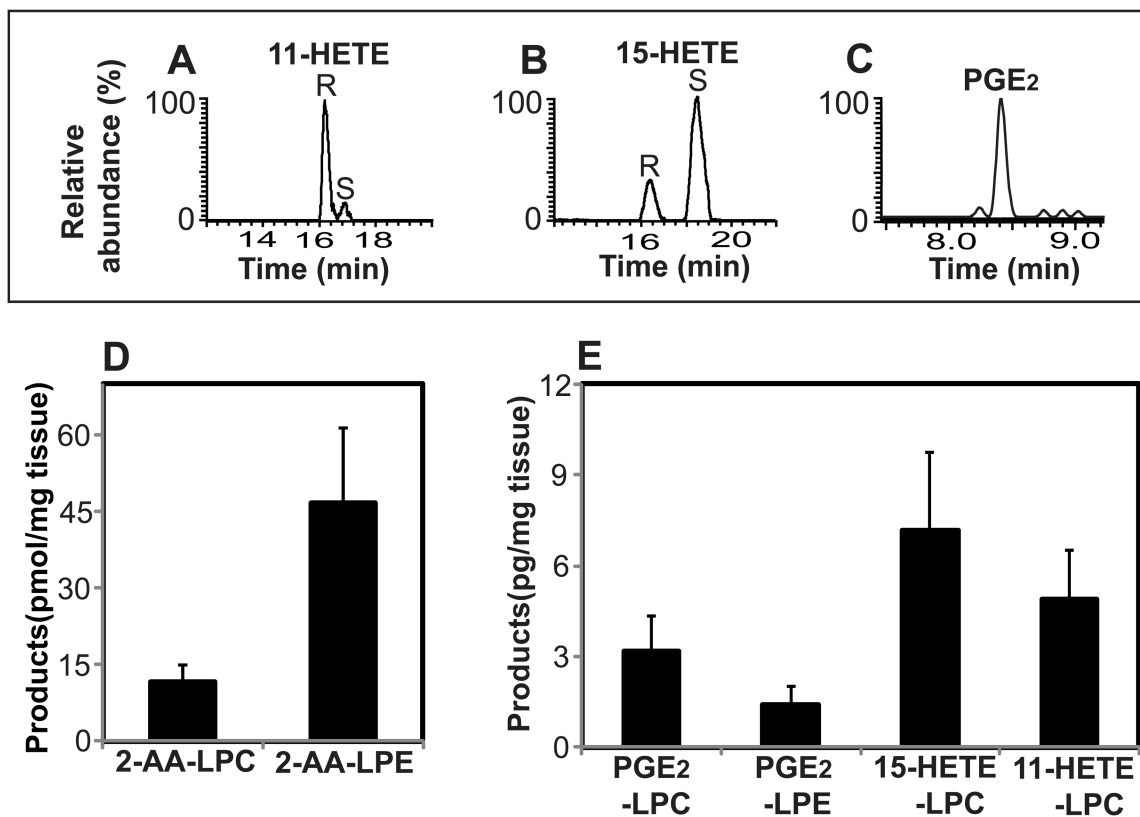


Figure 6. Eicosanoid-lysolipid molecular species present in human myocardium

Freshly isolated human ventricular myocardium from non-matched donors was rapidly frozen in liquid nitrogen, pulverized into a fine powder and eicosanoid-lysolipids were extracted, purified by solid-phase extraction, and the isolated eicosanoid-lysolipids were hydrolyzed by purified recombinant cPLA₂ α followed by charge-switch derivatization of the released eicosanoids with AMPP. The derivatized eicosanoids were separated by chiral HPLC and analyzed by mass spectrometry through comparisons with authentic stereospecific standards. **A-C**, Stereochemical determination of eicosanoid-lysolipid products present in human myocardium; **D**, Content of 2-AA-LPC and 2-AA-LPE present in human myocardium; **E**, Content of PGE₂-LPC, PGE₂-LPE, 11-HETE-LPC, and 15-HETE-LPC present in human myocardium. Data are expressed as mean values \pm SEM of six replicates.

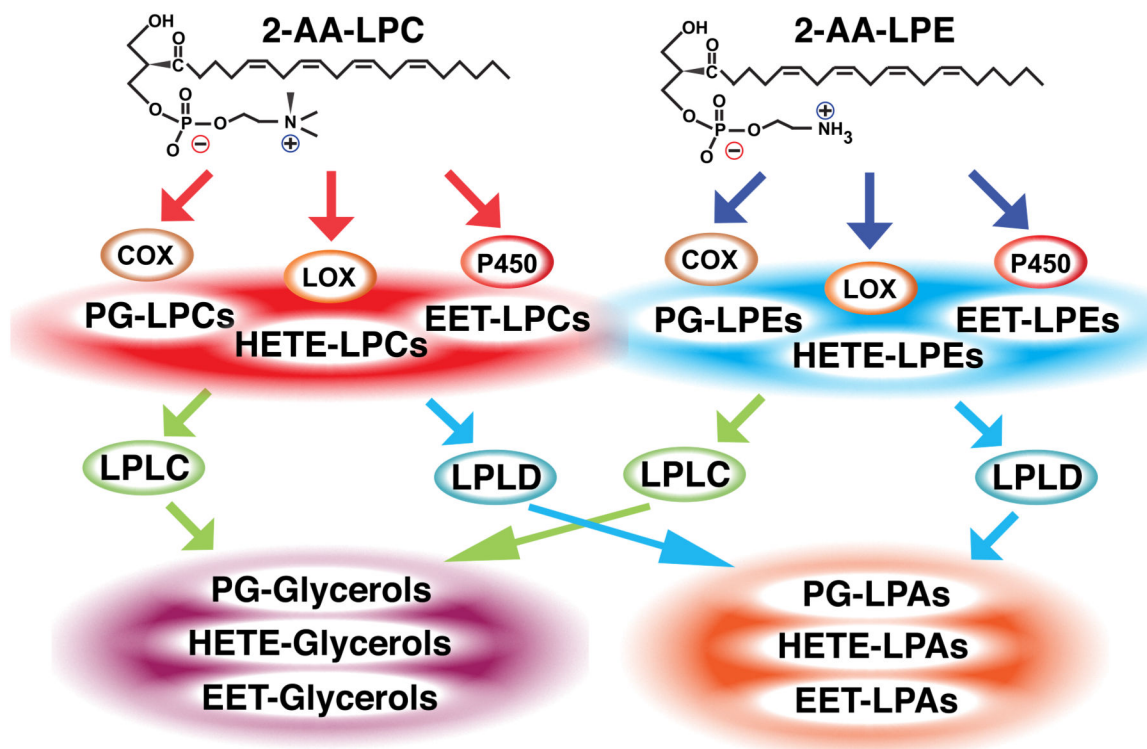


Figure 7. Oxidation of 2-arachidonoyl-lysolipids (2-AA-LPC and 2-AA-LPE) to eicosanoid-lysolipids and subsequent metabolism to eicosanoid-glycerols and eicosanoid-lysophosphatidic acids

Eicosanoid-lysolipids generated from 2-AA-LPC and 2-AA-LPE by multiple oxygenases (*e.g.*, COX, LOX and P450) can be hydrolyzed by intracellular lysophospholipase C (LPLC) and lysophospholipase D (LPLD) to produce eicosanoid-glycerols and eicosanoid-lysophosphatidic acids (LPA), respectively. Thus, eicosanoid-lysolipids represent not only a new class of dynamically regulated signaling molecules, but in addition likely serve as precursors for further metabolism to mediate a wide variety of lipid signaling cascades. PG: prostaglandin; HETE: hydroxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid.