Activated Platelets and Monocytes Generate Four Hydroxyphosphatidylethanololamines via Lipoygenase*

Benjamin H. Maskrey1, Alexandra Bermúdez-Fajardo6, Alwena H. Morgan1, Esther Stewart-Jones1, Vincent Dioszeghy1, Graham W. Taylor5, Paul R. S. Baker6, Barbara Coles6, Marcus J. Coffey1, Hartmut Kühn1, and Valerie B. O’Donnell1

From the 1Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, United Kingdom, 2Centre for Amyloidosis and Acute Phase Proteins, Royal Free and University College Medical School, London NW3 2PF, United Kingdom, 3Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, and 4Institute of Biochemistry, Humboldt University, D10117 Berlin, Germany

12/15-Lipoxygenase (LOX) mediates immune-regulatory activities not accounted for by its known free acid eicosanoids, suggesting that additional lipids may be generated by activated cells. To characterize novel LOX-derived lipids, a lipidomic approach was utilized. Ionophore-activated interleukin-4-treated human peripheral monocytes generated up to 10-fold more esterified 15-hydroxyeicosatetraenoic acid (15-HETE) than free in a phosphatidylinositol 3-kinase- and protein kinase C-sensitive manner. Precursor scanning electrospray ionization/tandem spectroscopy for C-sensitive ions at m/z 319 (HETE, [M-H]-) showed 4 ions at m/z 738, 764, 766, and 782 that were identified using tandem spectroscopy and MS3 as specific diacyl and plasmalog-en 15-HETE phosphatidylethanololamines. Using H218O water, the compounds were shown to form by direct oxidation of endogenous phosphatidylethanolamine (PE) by 15-LOX, with PE being the preferred phospholipid pool containing 15-HETE. Similarly, human platelets generated 4 analogous PE lipids that contained 12-HETE and increased significantly in response to ionophore, collagen, or convulxin. These products were retained in the cells, in contrast to free acids, which are primarily secreted. Precursor scanning of platelet extracts for the major platelet-derived prostanoit, thromboxane B2 (m/z 369.2), did not reveal PE esters, indicating that this modification is restricted to the LOX pathway. In summary, we show formation of PE-esterified HETEs in immune cells that may contribute to LOX signaling in inflammation.

Mammalian LOXs2 oxidize arachidonate to form hydro(pero)-xy-eicosatetraenoic acids (H(p)ETE). There are several isoforms expressed in circulating vascular cells, including 12-LOX (platelets), 12/15-LOX (monocytes/macrophages, eosinophils), and 5-LOX (neutrophils). Overall, they play immune modulatory roles in diseases such as asthma, atherosclerosis, diabetes, and hypertension (1–4).

12/15-LOX is expressed by monocytes/macrophages in response to interleukin-4 and -13 and oxidizes arachidonate at either C12 (mouse, rat, pig) or C15 (human, rabbit) (5, 6). In contrast, platelet 12-LOX forms 12-HpETE. After their generation, 12/15-LOX-derived hydroperoxides are either reduced by glutathione peroxidases forming HETEs or further metabolized by LOX to epoxy or tri-hydroxy products, termed hepxolin or lipoxins (7–11). Up to now these compounds have been assumed to be the sole arachidonate-derived products, although the detailed metabolic pathways for H(p)ETEs in immune cells are largely uncharacterized. One modification of endogenously generated H(p)ETEs is their esterification into membrane phospholipids or other complex lipid pools. Several years ago, before the advent of high sensitivity mass spectrometry, this question was addressed by the addition of radiolabeled HETEs to cells followed by examination of their fate by radio-TLC (12–17). However, this is indirect, since exogenous H(p)ETE differs considerably in fate from endogenously generated product (18). Also, this approach is insensitive, and furthermore, does not allow identification of specific molecular species.

Expression of 12/15-LOX in macrophages without release of detectable free H(p)ETEs or lipoxin modulates immune cell function (19–23). This suggests that H(p)ETE metabolites formed within or close to the membrane during LOX turnover may contribute; however, the identities of such lipids are currently unknown. Given the recent studies suggesting an important anti-inflammatory and pro-resolving function for 12/15-LOX, identification of novel products of this pathway is a clinically relevant goal (24, 25).

Herein, we utilized a lipidomic technique, precursor ESI/MS/MS to identify complex pools of endogenous H(p)ETE in immune cells. The studies identify specific esterified eicosanoids that form after immune cell activation and, furthermore, show the powerful nature of precursor scanning as a lipidomic tool for identifying novel endogenously generated lipid adducts.
Phosphatidylethanolamine Products of Lipoxygenase

**FIGURE 1.** 15-HETE generated by IL-4-treated monocytes is predominantly esterified. Panel A, 15-H(p)E TE generation in response to ionophore activation. IL-4-treated human monocytes were activated with A23187 before analysis for 15-HE TE using HPLC-UV as described under “Experimental Procedures.” Panel B, generation of esterified 15-HETE parallels free acid but continues for longer. Shown is the time course of free and esterified 15-H(p)E TE generation by IL-4-treated monocytes activated with 10 μM A23187 for 2–15 min. Panel C, peritoneal macrophages from 12/15-LOX−/− mice do not generate free or esterified 15-HETE. Free and esterified 12-HETE generated by murine peritoneal macrophages from wild-type and 12/15 LOX−/− was determined using LC-UV before and after activation with 10 μM A23187 (n = 3; mean ± S.E.; * p < 0.05 versus unactivated control, Student’s t test). Panel D, involvement of phosphatidylinositol 3-kinase in generation of free and esterified 15-HETE. Generation of 15-HETE was determined after activation of cells with 10 μM A23187 with/without 100 μM wortmannin. Panel E, protein kinase C stimulates generation of free and esterified 15-HETE in monocytes. IL-4-induced human monocytes were activated with 10 μM A23187 with/without 0.8 μM phorbol 12-myristate 13-acetate (PMA) and/or 10 μM bisindolylmaleimide (BIS) (n = 3; mean ± S.E.; * p < 0.05 versus A23187 alone, Student’s t test).

**EXPERIMENTAL PROCEDURES**

Materials—15(S)-Hydroxy-[S-(E,Z,Z,Z)]-5,8,11,13-eicosatetraenoic acid (15(S)-HETE), 12(S)-hydroxy-[S-(E,Z,Z,Z)]-5,8,10,14-Eicosa tetraenoic acid (12(S)-HETE), 15S-hydroxy-11Z,13E-eicosadienoic acid, palmitoyl trifluoromethyl ketone, and oleyxoyethyl phosphocholine were from Alexis Chemicals Ltd., Nottingham, UK. Human recombinant interleukin 4 (IL-4) was from Promega, wortmannin and bisindolylmaleimide were from Calbiochem. Lymphoprep™ was fromAxis- Shield, Oslo, Norway. H218O was from Cambridge Isotope Laboratories, Andover, MA. All other reagents were from Sigma unless otherwise stated.

Isolation and Activation of Human Monocytes—50 ml ofuffy-coat blood was diluted 1:1 (v/v) with PBS/citrate/dextran (0.8% w/v citrate, 2% w/v dextran 400, pH 7.4). Red cells were allowed to sediment for 1–2 h. The straw-colored supernatant was collected and underlaid with Lymphoprep 2:1 (v/v, supernatant:Lymphoprep) and centrifuged 800 × g for 20 min at 4 °C. The interface was collected and diluted 1:1 (v/v) with ice-cold PBS containing 0.4% (w/v) citrate, pH 7.4, and spun at 400 × g, 10 min at 4 °C. The supernatant was discarded, and the cell pellet was washed 5× with ice-cold PBS/citrate buffer, 400 × g for 5 min at 4 °C. The cell pellet was finally resuspended in a small volume of RPMI 1640 (10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine). Approximately 10⁸ cells were seeded per T75 flask and incubated at 37 °C for 2 h to allow monocytes to adhere. The medium was replaced, and cells were cultured for 72 h with 700 pm IL-4 to induce 15-LOX1. Monocytes were harvested by centrifugation and resuspended in Krebs buffer (50 mM HEPES, 100 mM NaCl, 5 mM KCl, 1 mM NaH2PO4-2H2O, 1 mM CaCl2, 2 mM glucose). 4 × 10⁶ cells in 1 ml were stimulated with A23187 (10 μM) with/without 0.8 μM phorbol 12-myristate 13-acetate at 37 °C for 10 min. In some experiments H218O was used in place of water in Krebs buffer. Experiments using signaling inhibitors included a 10-min preincubation step at 37 °C before the addition of stimulus. In some experiments 15-HETE-d8 (330 ng) was included during activation of cells. Murine-resident peritoneal cells were isolated from wild-type or 12/15-LOX−/− mice by peritoneal lavage into 2 ml of PBS and used without further purification (26).

Isolation and Activation of Washed Human Platelets—Whole blood was collected from healthy volunteers free from nonsteroidal anti-inflammatory drugs for at least 14 days into acid-citrate-dextrose (ACD); 85 mM trisodium citrate, 65 mM citric acid, 100 mM glucose). 4 × 10⁶ cells in 1 ml were stimulated with A23187 (10 μM) with/without 0.8 μM phorbol 12-myristate 13-acetate (PMA) and/or 10 μM bisindolylmaleimide (BIS) (n = 3; mean ± S.E.; * p < 0.05 versus A23187 alone, Student’s t test).
Phosphatidylethanolamine Products of Lipoygenase

FIGURE 2. Precursor scanning of activated monocyte extracts identifies four ions that are elevated with ionophore activation and co-elute with phosphatidylethanolamine on normal phase HPLC. Panel A, precursor scanning of monocyte extract identifies four ions containing m/z 319.2. Non-saponified lipid extract from A23187-activated human monocytes was infused into the source of the Q-Trap. Negative ion precursor scanning for m/z 319.2 (HETE, [M-H]⁻) performed as described under “Experimental Procedures.” Panel C, levels of the four HETE-containing ions are elevated on ionophore activation of monocytes. 10 μl of diluted non-saponified extracts were injected under flow (1 ml/min) in a methanol:water (50:50) mixture into the electrospray source of the MS with specific MRM transitions monitored using m/z 319.2 as daughter ion and comparing ion intensity to the internal standard, di-14:0-PE (HETE, [M-H]⁻) performed as described under “Experimental Procedures.” Panel B, precursor scanning for HETE in lipid extracts of murine peritoneal lavage identifies identical ions parent ions. A non-saponified lipid extract from unactivated peritoneal macrophages was infused at 10 μl/min into the electrospray source (1:20 dilution) with negative ion precursor scanning for m/z 319.2 (HETE, [M-H]⁻) performed as described under “Experimental Procedures.” Panel D, the four HETE-containing ions co-elute with di-14:0-PE standard on normal phase LC. Non-saponified lipid extracts from ionophore-activated monocytes were analyzed by normal phase LC, and fractions were collected during elution and monitored for specific MRM transitions using 319.2 as daughter ion.

with 10 μM A23187, 10 μg/ml collagen, or 60 ng/ml convulxin before lipid extraction as below.

Lipid Extraction—15(S)-Hydroxyeicosadienoic acid (10 μg for HPLC-UV or 10 ng for LC/MS/MS) and/or 10 μg of di-14:0-phosphatidylethanolamine was added to each sample before extraction as internal standards. Hydroperoxides were then reduced to their corresponding stable alcohols by adding 1 mM SnCl₂ (27). Lipids were extracted by adding a solvent mixture (1 mL acetic acid, 2-propanol, hexane (2:20:30, v/v/v)) to the sample at a ratio of 2.5 mL of solvent mixture/1 mL of sample, vortexing, and then adding 2.5 mL of hexane (27). After vortex and centrifugation, lipids were recovered in the upper hexane layer. The samples were then re-extracted by the addition of an equal volume of hexane followed by vortex and centrifugation. The combined hexane layers were dried under N₂ flow, and then fatty acids were released by base hydrolysis with 1.5 mL of 0.2 M NaOH at 60 °C for 30 min under argon atmosphere. Hydrolyzed samples were acidified to pH 3.0 with 0.5 mL HCl, and then fatty acids were extracted twice with 3 mL of hexane. The combined hexane layers were dried under N₂ flow, resuspended in 100 μL of methanol, and stored under argon at 80 °C until analysis by LC-UV or LC/MS/MS.

HETE Quantitation Using LC/UV or LC/MS/MS—Samples were separated on a C₁₈ (ODS2, 5-μm, 150 × 4.6-mm column (Waters Ltd) using a gradient of 50–90% B over 20 min (A, water:acetonitrile:acetic acid, 75:25:0.1; B, methanol:acetonitrile:acetic acid, 60:40:0.1) at 1 mL/min. Products were quantitated either by UV absorbance (235 nm) or by LC/ESI/MS/MS on a Q-Trap (Applied Biosystems 4000 Q-Trap) using specific parent to daughter transitions of m/z 319.2 (HETE, [M-H]⁻) to m/z 219 (15-HETE) or 179 (12-HETE) and 323.2 to 223 for 15-hydroxyeicosadienoic acid with collision energies of ~20 or ~28 V, respectively. Products were identified and quantified using either 15(S)- or 12(S)-HETE, and 15(S)-hydroxyeicosadienoic acid standards run in parallel under the same conditions.

Precursor Scanning Mass Spectrometry—Electrospray mass spectra were obtained on a Q-Trap instrument (Applied Biosystems 4000 Q-Trap) operating in the negative mode. Lipid extracts were diluted (1:50–1:100) and introduced at 10 μl/min in methanol using a Hamilton syringe. Instrument settings were determined by tuning on an oxidized phosphatidylethanolamine (PE) standard and run with declustering potential −140 V and collision energy −45 V. Spectra were obtained from 550–1000 atomic mass units over 12 s, with typically 10 scans acquired and averaged. For determination of fold changes in specific 15-HETE-containing lipids, 10 μl of diluted extracts were injected under flow (1 mL/min) in a methanol-water (50:50) mixture with specific multiple reaction monitoring (MRM) transitions monitored using m/z 319.2 as daughter ion and comparing ion intensity to the internal standard, di-14:0-PE (MRM m/z 634.5 → 227.2) (n = 6; mean ± S.E.; *, p < 0.05, versus unactivated control, Student’s t test). Panel D, the four HETE-containing ions co-elute with di-14:0-PE standard on normal phase LC. Non-saponified lipid extracts from ionophore-activated monocytes were analyzed by normal phase LC, and fractions were collected during elution and monitored for specific MRM transitions using 319.2 as daughter ion.

JOURNAL OF BIOLOGICAL CHEMISTRY 20153

JULY 13, 2007•VOLUME 282•NUMBER 28
resuspending in 100 μl of methanol, 10-μl samples of each fraction were injected under flow (1 ml/min) in a methanol:water (50:50) mixture into the electrospray source, with specific MRM transitions monitored using m/z 319.2 as the daughter ion, and areas for each transition were determined in each fraction by integration of the peaks. These were then replotted versus time to obtain normal phase elution profiles for each HETE-containing ion.

Reverse Phase LC/MS/MS of Phospholipids—Online reverse phase separation of phospholipids to separate based on acyl chain was carried out using a Luna 3-μm C18 (2) 150 × 2-mm column (Phenomenex, Ltd) with a gradient of 0–100% B over 30 min (A, acetonitrile:methanol, 35:65; B, acetonitrile:methanol:triethylamine, 35:65:1.5) at a flow rate of 200 μl/min. MS/MS scans using the ion trap mode of the Q-Trap were triggered at the apex of an MRM transition (parent → 219 or 179 for 15- or 12-HETE, respectively). The scan was from 150 to 800 atomic mass units over 0.65 s, with a linear ion trap fill time of 200 ms and Q0 trapping. MS3 was carried out using the electrospray source, with direct infusion of the normal phase PE fraction diluted 1:20 in methanol at 10 μl/min. The linear ion trap fill time was 250 ms with excitation time 200 ms, excitation energy 200 V, with a mass range scanned from 100 to 320 atomic mass units.

HETE Isomer Determination—Lipid extracts were separated by normal phase HPLC, the PE-containing fraction (5.5–8 min) was collected, saponified, then resuspended in 50 μl of methanol, and HETE isomers were analyzed by reverse phase LC/MS/MS. For chiral phase analysis 15-HETE was collected, resuspended in the chiral phase mobile phase (hexane:2-propanol:acetic acid, 100:5:0.1), and injected onto a Chiralcel OD 0.46 × 25-cm column (Chiral Technologies Ltd, Exton, PA) with isocratic separation at 1 ml/min with absorbance monitored at 235 nm.

15-HETE Quantitation in Phospholipid Classes—Lipid extracts from A23187-activated monocytes were separated by normal phase LC-UV, and 1-min fractions were collected from 0 to 20 min. Fractions were dried under N2 and resuspended in methanol. Fractions containing each phospholipid class were identified by head-group precursor scanning (PE = 7–9 min, phosphatidylcholine (PC) = 18–20 min, phosphatidylinositol = 11–13 min, phosphatidylglycerol = 7–9 min, phosphatidylserine = 14–16 min) and hydrolyzed as already described, then analyzed by reverse phase LC/MS/MS monitoring for 15-HETE. Amounts of PE and PC were determined using normal phase LC-UV (205 nm).

Generation of 15-HETE-containing Phospholipids by Soybean LOX Oxidation of Commercial Phospholipid Preparations—5 mg/ml 1-α-phosphatidylethanolamine (egg (Sigma) or brain, porcine plasmalogen (Avanti Polar Lipids, Alabaster, AL)) was incubated for 30 min at 37 °C in PBS, pH 7.4, 4% sodium cholate, with 50 kilounits of soybean lipoygenase type IV (Sigma). Samples were then reduced using SnCl2 before lipid extraction to convert all HpETE into the more stable HETE (27). The majority of 15-HETE is in PE. Panel A, crude lipid extracts from activated monocytes were separated by normal phase LC, and fractions were collected as described under “Experimental Procedures.” Fractions corresponding to each individual phospholipid class were identified, combined, saponified, and analyzed by reverse phase MS/MS monitoring for the specific 15-HETE MRM transition (m/z 319 → 219). Panel B, total amounts of the two major phospholipids PE and PC present in the monocyte sample were determined by normal phase HPLC-UV at 205 nm with egg phospholipid as standard. Levels of 15-HETE were then expressed as ng/μg of phospholipid (n = 3, mean ± S.E.). PS, phosphatidylserine; PI, phosphatidylinositol.

RESULTS

Monocytes Generate Predominantly Esterified 15-H(p)ETE after Ionophore Activation—To define pathways that regulate calcium-dependent activation of 15-LOX1, generation of 15-H(p)ETEs after stimulation of IL-4-treated human monocytes with A23187 was examined. Samples were reduced using SnCl2 before lipid extraction to convert all H(p)ETE into the more stable HETE (27). Most 15-H(p)ETE generated on activation was esterified to complex lipids of unknown structure (Fig. 1A). Also, basal levels of esterified 15-H(p)ETE were detected without ionophore activation. Esterified H(p)ETE generation occurred early and continued after free H(p)ETE had plateaued (Fig. 1B). Murine peritoneal macrophages that express 12/15-LOX, the functional equivalent of human 15-LOX1, generated...
esterified 12-H(p)ETE, and this was absent in macrophages from 12/15-LOX−/− mice (Fig. 1C). These data show that the predominant H(p)ETEs generated by monocytes and macrophages on calcium activation are not free acid products, but complex lipids.

**Regulation of 15-H(p)ETE Release by Intracellular Signaling Pathways**—Calcium facilitates membrane association of 12/15-LOX (29). However, it is unknown whether additional signaling pathways participate in monocyte 12/15-LOX regulation by calcium and whether differences exist between regulation of free versus esterified product formation. Inhibition of both H(p)ETE forms was observed on the addition of wortmannin, implicating phosphatidylinositol 3-kinase (Fig. 1D). The protein kinase C inhibitor bisindolylmaleimide did not significantly affect ionophore stimulation of 12/15-LOX; however, activation of protein kinase C using phorbol 12-myristate 13-acetate significantly promoted generation of both forms in a bisindolylmaleimide-sensitive manner (Fig. 1E). This indicates that stimulation of protein kinase C potentiates calcium-dependent activation of 12/15-LOX.

**H(p)ETE Is Esterified to PE in Activated Human Monocytes and Mouse Peritoneal Macrophages**—We sought to determine the specificity of 15-H(p)ETE esterification in monocytes using an MS-based lipidomic approach. Precursor ESI/MS/MS scanning for 319.2 (HETE [M-H]−) of crude lipid extracts from IL-4-treated human monocytes activated with A23187 revealed 4 dominant ions at m/z 738.5, 764.5, 766.5, and 782.5 (Fig. 2A). Also, precursor ESI/MS/MS of murine peritoneal macrophage extracts demonstrated identical ions (Fig. 2B). Next, MRM transitions of the parent-HETE daughter ion were monitored in activated and unactivated human monocytes and compared with an internal standard (di14:0-PE). All four ions increased significantly after calcium mobilization (Fig. 2C). These m/z values are consistent with nitrogen-containing HETE derivatives of two phospholipid species, PE and PC (e.g. m/z 766.5 could be 16:0p/15-HETE-PC or 18:0p/15-HETE-PE). However, the m/z values are not consistent with other phospholipids, including phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, or phosphatidic acids (30). To determine phospholipid class, normal phase LC was undertaken that separates phospholipids according to head group. Fractions were collected, and aliquots of each 1-min fraction were monitored for the parent 3m/z 319.2 transition using MS/MS. Greater than 95% of ion intensity of the transition for all 4 peaks co-eluted with di-14:0-PE standard (5–6-min fraction), with the remainder (<5%) eluting with PC in the 17–19-min fraction (Fig. 2D). These data suggest...
that PE is the predominant phospholipid site of 15-H(p)ETE formation.

**PE Is the Preferred Phospholipid Site for 15-HETE Generation in Monocytes**—In monocytes, precursor scanning MS/MS suggested that PE is the preferred phospholipid site for HETE generation even though SAPC and SAPE are both substrates for purified 15-LOXs (31). The ionization efficiency of equimolar amounts of PC in negative mode is approximately a third that of PE in our system (not shown), and so it is possible that HETE-PC was not readily detected in our experiments. Therefore, to conclusively determine the phospholipid site specificity of HETE formation, the phospholipid fractions from activated monocytes were purified using normal phase HPLC, then hydrolyzed to release free 15-HETE, which could then be directly quantified by LC/MS/MS. Using this approach, >92% of the 15-HETE was found in PE (Fig. 3). The preference of PE over PC as the site of HETE synthesis is observed whether it is expressed as a function of total phospholipid class or per phospholipids, with the level equating to 1.5% of the PE pool in activated monocytes containing 15-HETE (based on detecting 0.7125 nmol of 15-HETE in 48 nmol of purified PE).

**Structural Identification of Individual 15-HETE Ions**—Because of isobaric peaks in the PE fraction, further LC was required to separate different 15-HETE-containing PEs. Reverse phase-LC/MS/MS, which separates based on acyl chain composition, was used on normal phase-purified PE fractions (see “Experimental Procedures”). The parent $m/z$ 219 transition (15-HETE ion) was monitored with an MS/MS spectrum triggered during elution of the MRM transition. The spectrum shown is obtained at the apex of the peak of elution for each compound, which was at the same retention time (4.6 min) for all 4 ions, likely due to the presence of identical sn-2 lipids. Using this approach, spectra were obtained that could be compared with PE oxidized in vitro using soybean 15-LOX.

The product ion spectrum of $m/z$ 782.5 (Fig. 4A) contains major ions at $m/z$ 283 (18:0, stearic acid), $m/z$ 319, 301, and 257 (HETE) along with 219 and 175 (characteristic of the 15-HETE isomer only). Comparison of this with that obtained for $m/z$ 782.5 from soybean 15-LOX-oxidized egg PE shows an identical spectrum (Fig. 4E). Not only are the same ions observed, but the ratios of ions to each other are consistent. This together with an identical retention time on both normal phase and reverse-phase HPLC, supports the identification of $m/z$ 782.5 as 18:0/15-HETE-diacyl-PE (Scheme 1A).

The product ion spectra for $m/z$ 766.5 (Fig. 3E), 764.5 (Fig. 4C) and 738.5 (Fig. 4D) reveal ions characteristic of the HETE carboxylate anion at $m/z$ 319 and its fragments at $m/z$ 301 and 257, with minor 15-HETE ions at $m/z$ 219 and 175 atomic mass units. There was also neutral loss of the free acid [M-H-320] and also the ketene [M-H-302] but no ions characteristic of an sn-1 fatty acid. These spectra suggest that PE possesses either ether or plasmalogen bonds at sn-1. The samples were examined in positive mode, as previously done for plasmalogen PC, but sensitivity was

---

**SCHEME 1.** Proposed structures for the four 15-HETE PE compounds identified in activated human monocytes. A–D show proposed structures, based on comparison with synthetic standards. For platelets, 15-HETE is replaced by 12-HETE.

**FIGURE 5.** MS3 confirms that monocyte ions contain 15-HETE and no other HETE isomer. Diluted samples of normal phase-isolated PE from activated human monocytes was infused at 10 μl/min using the electrospray source. MS3 was carried out on all 4 PE lipids containing HETE, fragmenting the daughter ion $m/z$ 319.2 in the ion trap. Panels A–D show MS3 spectra for $738.5 \rightarrow 319.2$, $764.5 \rightarrow 319.2$, $766.5 \rightarrow 319.2$, and $782.5 \rightarrow 319.2$, respectively. Note: specific 15-HETE ions at $m/z$ 219 and 179, amu, atomic mass units.
lower, and good quality spectra could not be obtained (Ref. 32; data not shown). Plasmalogen phospholipids are sensitive to acidic conditions and can be hydrolyzed by exposure to HCl fumes (32). However, not only did ions at \( m/z \) 738.5, 764.5, and 766.5 degrade relative to the diacyl internal standard (di14:0-PE) after acid exposure but also the \( m/z \) 782.5, which is not a plasmalogen (data not shown), degraded. This suggests that HETE-containing lipids, by virtue of their -OH group on the 20:4, are sensitive to acid hydrolysis. Brain PE, which contains 50% plasmalogen and little ether-linked lipids was, therefore, oxidized using soybean 15-LOX. Spectra obtained during on-line reverse phase LC/MS/MS of normal phase-separated 15-LOX-oxidized brain PE are shown (Figs. 4, F–H). These spectra are identical to those obtained from monocyte extracts, suggesting that the ions at \( m/z \) 738.5, 764.5, and 766.5 are 15-HETE containing plasmalogen PEs, assigned as 16:0p/15-HETE-PE, 18:1p/15-HETE-PE, and 18:0p/15-HETE-PE, respectively (Scheme 1, B–D, calculated from Murphy (30)). However, a variation in the double bond position cannot be conclusively ruled out. Finally, for all four monocyte 15-HETE-PES, MS3 analysis confirmed that daughter ions \( m/z \) 219 and 175 conclusively originate from the 15-HETE fragment at \( m/z \) 319.2 (Fig. 5). No daughter ions that would be expected from other HETE isomers (e.g. 5-HETE, \( m/z \) 115; 8-HETE, \( m/z \) 155; 9-HETE, \( m/z \) 151; 11-HETE, \( m/z \) 167; 12-HETE, \( m/z \) 179) are found. To further confirm the isomer distribution, the PE fraction was hydrolyzed, and each HETE positional isomer was quantified using LC/MS/MS. As shown, only the 15-HETE isomer was detected (Fig. 6A). Furthermore, chiral phase LC-UV analysis of the purified 15-HETE from monocyte PE (collected from reverse phase LC of the hydrolyzed PE fraction) showed that >96% was the \( S \) enantiomer (Fig. 6B). These data collectively indicate that the compounds contain predominantly 15(\( S \))-HETE and can only originate from 15-LOX turnover.

**H**\(_2\)\(^{18}\)O Labeling Studies—Monocytes were activated in buffer containing \( H_2\)\(^{18}\)O instead of \( H_2\)\(^{16}\)O. During hydrolysis of membrane lipid, incorporation of the \(^{18}\)O label at the carboxyl group will form arachidonate that is 2 atomic mass units higher than the naturally occurring lipid (33). Oxygenation of this by 15-LOX would form 15-HETE-\(^{18}\)O, with \( m/z \) 321, and subsequent reincorporation of this into PE will retain half the label (e.g. 50% of the PEs will have a mass increase of 2 atomic mass units). On the other hand, direct oxidation of PE by 15-LOX would not generate \(^{18}\)O-labeled species. When monocytes were activated in the presence of \( H_2\)\(^{18}\)O, 94% of 15-HETE formed was labeled with \(^{18}\)O (Fig. 7A and inset). In contrast, monocytes did not incorporate \(^{18}\)O into PE-HETEs, with elevation in HETE-\(^{16}\)O.

### FIGURE 6. Analysis of 15-HETE by HPLC further proves enzymatic generation of 15-HETE-PE. Panel A, LC/MS/MS of hydrolyzed PE fraction from human monocytes. The PE fraction of activated monocytes was isolated using normal phase LC and hydrolyzed as described under “Experimental Procedures.” This was analyzed by reverse phase LC-MS/MS for HETE positional isomers in the MRM mode monitoring from the parent ion of \( m/z \) 319.2 to specific daughter ions for each HETE (15-HETE, 219; 12-HETE, 179; 5-HETE, 115; 8-HETE, 155; 11-HETE, 167). Retention time for the transitions were compared for standards (panels i–v) and hydrolyzed monocyte PE sample (panels vi–x). amu, atomic mass units. Panel B, chiral phase analysis of 15-HETE. PE purified from human monocytes using normal phase LC was hydrolyzed, then separated on reverse phase HPLC, and 15-HETE was collected. This was injected onto a chiral phase column as described under “Experimental Procedures,” monitoring absorbance at 235 nm. Direct comparison was made between authentic standards (panel i) and monocyte PE-derived 15-HETE (panel ii).
Phosphatidylethanolamine Products of Lipoxygenase

FIGURE 7. Monocyte activation in H$_2^{18}$O indicates that PE-HETEs arise from direct oxidation of PE by 15-LOX, and PE-HETEs are retained intracellularly following synthesis. Panel A, free 15-HETE generated by monocyte activation contains predominantly $^{18}$O (15-LOX $^{18}$O). Monocytes were activated using A23187 as described in Fig. 1, in H$_2^{18}$O buffer. 10 µl of diluted samples were analyzed for MRM transitions of 15-HETE-$^{18}$O (m/z 321 $\rightarrow$ 221), 15-HETE-$^{16}$O (m/z 319 $\rightarrow$ 219), and internal standard (di14:0-PE) to reveal -fold changes in species after activation. Inset, product ion spectrum of m/z 321 confirms incorporation of $^{18}$O into carboxyl group of free 15-HETE. Product ion spectra of the $^{18}$O-labeled 15-HETE (M-H $\rightarrow$ 321) shows ions at m/z 303 (loss of water) and m/z 257 (loss of H$_2$O and $^{18}$O-labeled CO$_2$), indicating that $^{18}$O is incorporated into the carboxyl group. There are also characteristic daughter ions arising from cleavage of the carbon backbone at m/z 221 (cleavage at C14-C15) and m/z 175 (C14-C15 + loss of $^{18}$O-labeled CO$_2$), atomic mass units. Panel B, precursor scanning for m/z 319 reveals PE-HETEs containing $^{18}$O. Precursor scanning of activated monocyte extract was conducted with experimental conditions as in Fig. 4. Panel C, precursor scanning for m/z 321 does not show analogous products containing $^{18}$O. Scans were obtained under identical experimental conditions as panel C. Panels D–G, examination of specific PE-HETEs containing either $^{18}$O or $^{16}$O show that only $^{18}$O-containing forms are elevated after ionophore activation of cells. 10 µl of diluted samples were injected into the MS and examined for MRM transitions of the 4 PE-HETEs containing $^{18}$O (m/z 740, 766, 768, 784 $\rightarrow$ 321), PE-HETE-$^{18}$O (m/z 738, 764, 766, 782 $\rightarrow$ 319), and internal standard (di14:0-PE) to reveal -fold changes in each species after activation. Panel H, PE-HETEs are primarily retained in the cells following generation. After ionophore activation, cells were pelleted (800 × g, 5 min, 4 °C). Cells and cell-free supernatant were analyzed for PE-HETE MRM transitions using MS/MS and compared with internal standard (dimyristoylphosphatidylethanolamine).

only after ionophore activation (Fig. 7, B–G). This indicates that formation of PE-HETEs likely does not involve esterification of HETE but direct oxygenation of arachidonate-containing PE by 15-LOX.

To further validate the use of H$_2^{18}$O as a tool to identify direct oxidation of PE by LOX, experiments were conducted using soybean 15-LOX and purified SAPE or SAPC in borate/deoxycholate buffer (32). Both were oxidized at similar rates (1.2 ± 0.15 or 1.4 ± 0.12 nmol/min/8 kilounits of 15-LOX for PE or PC, respectively, mean ± S.E.). PE-HETE was more readily detected as a positive ion but was also observed in negative mode with CID spectra showing diagnostic 15-HETE daughter ions at m/z 319, 219 and 175 (Fig. 8, A and B). In these experiments where LOX directly oxidizes PE, inclusion of H$_2^{18}$O had no effect on the isotope distribution of PE-HETEs formed (Fig. 8, C–F). Specifically, with either H$_2^{18}$O or H$_2^{16}$O-containing buffer, only the $^{16}$O-containing esterified-HETEs are generated. The small amount of esterified-HETE with +2 atomic mass units (m/z 784 for PE or 828 for PC) most likely represents the carbon isotope peak of the $^{16}$O-lipid since it is detected in both H$_2^{16}$O- and H$_2^{18}$O-containing incubations (Fig. 8, C–F). We also confirm in these experiments that SnCl$_2$, included to reduce the primary 15-LOX product, 15-HpETE, to the more stable 15-HETE, does not promote incorporation of $^{18}$O (Fig. 8, C–F).

PE-HETEs Are Retained within the Cells—To determine whether PE-HETEs are primarily secreted similar to free acid eicosanoids or retained intracellularly, monocytes were pelleted after ionophore activation, and levels of free and PE-HETEs were determined separately in supernatant and cell pellets. As expected, free H(p)ETE was primarily secreted (70%, not shown); however, partitioning of PE-HETE was different, with the majority retained within the cells (Fig. 7H). This indicates different
Phosphatidylethanolamine Products of Lipoxygenase

JULY 13, 2007• VOLUME 282 • NUMBER 28

precursor scan for the 15-HETE-d8 daughter ion at 226, which instead detected ions that were not seen in the precursor m/z 327 scan (Fig. 9D). It is, therefore, likely that the precursor 327 scan is detecting esterified docosahexaenoic acid (m/z 327). Furthermore, levels of the ions detected in the precursor 327 scan do not change with cell activation (Fig. 9F). Therefore, it appears that exogenous 15-HETE-d8 is not being incorporated into the phospholipid pool during the timescale of our experiments. Furthermore, our earlier experiments using H$_2$O also showed that endogenously generated 15-HETE is also not incorporated, since m/z 321 (15-HETE-$^{18}$O) was not found attached to monocyte phospholipids after ionophore activation (Fig. 7C).

activated Human Platelets Generate the Same H(p)ETE-PE Products on Activation, however, with Exclusively 12-H(p)ETE—Human platelets contain a different LOX isofrom, 12-LOX, that forms 12-H(p)ETE as its sole product and can be activated by physiological agonists, such as collagen (34, 35). Levels of free and esterified 12-H(p)ETE were determined after activation with either collagen, the glycoprotein VI-selective agonist convulxin or A23187. For all stimuli, esterified H(p)ETE was detected; however, the levels relative to free H(p)ETE were lower than those formed in human monocytes (Fig. 10A). Precursor ESI/MS/MS scanning of ionophore-activated human platelet extracts for m/z 319.2 showed identical ions to human monocytes at m/z 738, 764, 766, and 782 (Fig. 10B). In addition to 12-H(p)ETE, platelets also generate large quantities of a second arachidonate product, thromboxane B$_2$, after activation of prostaglandin H synthase-1. However, precursor scanning for m/z 369.2 revealed no significant thromboxane B$_2$-containing phospholipids (Fig. 10C). This indicates that PE-HETE adduct formation is restricted to LOX metabolites.

As for monocytes, the HETE-containing ions co-eluted with di-14:0-PE on normal-phase LC, indicating that they are PE phospholipids (not shown). By comparison with the internal standard, all 4 peaks increased in intensity after activation with collagen (approx 2–5-fold), convulxin (~5–10 fold), and A23187 (10–20-fold) (Fig. 10D). MS3 of the 4 ions using the 12-HETE daughter ion of m/z 319.2 revealed a characteristic 12-HETE fragmentation pattern with a diagnostic daughter ions.

Cell partitioning of esterified versus free H(p)ETE products and has implications for the potential signaling actions of this class of lipid.

Exogenous or Endogenous 15-HETE Is Not Incorporated into Phospholipids during Activation of 15-LOX—To determine whether 15-HETE becomes esterified during the time course of our studies, monocytes were co-incubated with 15-HETE-d8 at similar concentrations to what is synthesized by the cells with/without ionophore activation. If this was incorporated, it would be observed by precursor scanning for m/z 327. Precursor scanning at m/z 319 or 219 showed that the presence of the deuterated analog did not prevent 15-LOX oxidation of PE, with the characteristic PE-HETE ions being detected (Fig. 9, A, B, and E). Scanning for m/z 327 showed prominent ions 8 atomic mass units higher than the PE-HETEs-d0, suggesting incorporation of the 15-HETE-d8 into PE; however, these were also present when the cells were not supplemented with 15-HETE-d8 (Fig. 9, C with inset, and F) and, furthermore, did not show up in a

FIGURE 8. $^{18}$O from H$_2$$^{18}$O is not incorporated during direct oxidation of SAPE or SAPC by LOX. Panels A and B, soybean LOX generates 15-HETE-PC from arachidonate-containing PC. SAPC was oxidized using 15-LOX and reduced with SnCl$_2$ as described under “Experimental Procedures,” then analyzed by ESI/MS/MS. Product ion spectra for 18:0a/15-HETE-PC were generated at m/z 860.6 [M + H]$^+$ in the positive mode and m/z 806.6 [M + Cl]$^+$ in the negative mode. Panels C–F, SAPC or SAPE, oxidized by soybean LOX in buffer made with H$_2$$^{18}$O (panels C and D) and H$_2$O (panels E and F), were analyzed for 15-HETE-PC or PE by reverse phase LC/MS/MS as described under “Experimental Procedures.” MRM transitions were 782–319 (15-HETE-16O-PE) and 784–321 (15-HETE-18O-PE) in negative mode and 826–184 (15-HETE-18O-PC) and 828–184 (15-HETE-16O-PC) in positive mode. amu, atomic mass units.
ion at 179 (Fig. 11). No ions for 15-HETE or any other isomer are present, indicating that they originate specifically from 12-LOX turnover. Because the four ions have identical $m/z$ as the monocyte products, they are proposed to be the same compounds as in Scheme 1 except for the substitution of 15- with 12-H(p)ETE.

DISCUSSION

The cellular formation of complex LOX products is not characterized. Here we show that the majority of 15-HETE generated in IL-4 treated monocytes after cell activation is esterified and that the preferred phospholipid pool for this eicosanoid is PE (Figs. 1–6). Thus, novel complex LOX products are found in monocytes that may signal in immunity and inflammation resolution.

Comparison of levels of free 15-HETE after hydrolysis of either total lipid extract (Fig. 1) or purified monocyte PE (Fig. 3) show that the PE-esterified HETE does not seem to account for all the esterified HETE formed. Specifically, total esterified HETE varied from 100 to 2000 ng/4 $\times 10^6$ cells (due to donor variation), but only 17.5 ng/4 $\times 10^6$ cells was detected after purification and hydrolysis of PE from one donor. This suggests that 15-HETE is esterified into additional non-phospholipid pools that have not been detected here, though perhaps not being ionized in negative mode. Examples could include diacylglycerides or triglycerides. Further experiments will address this issue and identify additional nonphospholipid sites of HETE esterification in the cells.

Previous studies examined sites of exogenous 15-HETE esterification into phospholipids and showed this to be mainly phosphatidylinositol or PC (12, 36–40). Herein, we found that PE was the predominant phospholipid pool of 15-HETE after activation of endogenous 15-LOX. The key difference is that endogenously formed products are generated by direct oxidation of PE without prior release of arachidonate to form free 15-HETE (Fig. 7 and Scheme 2). Therefore, this occurs via an entirely different process. Furthermore, during our experiments neither exogenous or endogenous 15-HETE was esterified into any phospholipid pool (Figs. 7C and 9). In human monocytes the predominant phospholipid is PC (40%), with PE only 30%; however, PC is enriched on the outer leaflet, with PE on the inside (41, 42). This suggests that after LOX translocation to the membrane, direct oxidation of complex substrate occurs. Although the ability of purified 15-LOX to oxidize PC in vitro at low rates (compared with arachidonate) is known, PE oxidation by LOX has never been shown to be a source of endogenous products in monocytes or other cells. Phosphatidylinositol 3-kinase and protein kinase C regulated generation of monocyte HETE-PEs, identifying two new signaling pathways that control calcium-dependent activation of 15-LOX (Fig. 1, D and E). Because PE-HETEs are formed independently of phospholipase A$_2$, a direct action of these pathways on activity of the 15-LOX itself is inferred. The mechanisms involved remain to be determined.
PE-HETEs were present in IL-4-treated monocytes, although their levels increase significantly with ionophore activation (Figs. 1, A–C and 2C). This suggests a basal activity of 12/15-LOX that results in continuous generation of esterified but not free HETE. It is known that 12/15-LOX can signal in macrophages without generation of free acid product (19–24). These include IL-4 activation of peroxisome proliferator-activated receptor γ, lipopolysaccharide stimulation of IL-12p40 generation, and phagocytosis/actin polymerization in response to apoptotic thymocytes. Additionally, the requirement for 12/15-LOX cannot be replicated by exogenous 12- and 15-H(p)ETE (24). It is tempting to speculate that complex products such as these are involved, but this remains to be determined. The timescale for formation of esterified 15-HETE in human monocytes was rapid and resulted in 5–10-fold higher levels of esterified product than free being generated. One could speculate that physiological activation of 15-LOX in monocytes might lead to formation of esterified products such as these, which are retained in the cells in vivo without detectable free 15-HETE release. Similar PE species were identified in human platelets (Fig. 10). However, the extent of esterification was lower, as the platelet 12-LOX isoform displays greater preference for free acid substrate. Nonetheless, significant increases in 12-HETE-PE species in response to collagen and convulxin indicate that their formation is regulated via receptor-dependent pathways.

This study utilized a lipidomic technique, termed precursor scanning, which has been used in lipid research primarily for the study of phospholipids based on scanning for their head group ions. To our knowledge, this mode has not before been applied to eicosanoids, although they are especially well suited to this approach. Their carboxyl group is readily ionized in negative ESI, and when

**FIGURE 10.** 12-HETE generation by human platelets reveals generation of analogous HETE-PE compounds after ionophore or agonist activation. Panel A, activated platelets generated esterified 12-HETE. Platelets activated using 10 μg/ml collagen, 60 ng/ml convulxin, or 10 μM A23187 for 15 min were extracted, saponified, and analyzed for 12-HETE using LC/MS/MS as described under "Experimental Procedures." Panel B, precursor scanning of non-saponified platelet extract identifies esterified HETEs. A diluted (1:50 dilution) sample of A23187-activated platelet extract, generated as described under “Experimental Procedures,” was infused into the electrospray source, and a precursor scan was obtained. Panel C, precursor scanning of platelet extract for m/z 369 reveals no esterified thromboxane B2. Samples were infused and scanned as described under “Experimental Procedures.” Panel D, levels of the four HETE-containing ions are elevated on activation of platelets with collagen, convulxin, or ionophore. 10 μl of diluted non-saponified extracts were injected under flow (1 ml/min) in a methanol:water (50:50) mixture into the electrospray source of the MS, with specific MRM transitions monitored, using m/z 319.2 as daughter ion and comparing intensity to the MRM transition of the internal standard, di-14:0-PE (MRM m/z 634.5 → 227.2) (n = 3; mean ± S.E.; *p < 0.05, versus unactivated control, Student’s t test).

**FIGURE 11.** MS3 confirms that platelet ions contain only 12-HETE. Diluted samples of normal phase-isolated PE from activated human platelets was infused at 10 μl/min using the electrospray source. MS3 was carried out on all four PE lipids containing HETE, fragmenting the daughter ion m/z 319.2 in the ion trap. Panels A–D show MS3 spectra for 738.5 → 319.2, 764.5 → 319.2, 766.5 → 319.2, and 782.5 → 319.2, respectively. Note: specific 12-HETE ion at m/z 179. amu, atomic mass units.
exposed to collision-induced dissociation, fatty acid esters preferentially fragment at the carboxyl, eliminating the free fatty acid as a neutral loss and also generating the acid anion which can be seen in negative mode. Additionally, eicosanoids fragment internally under these conditions, generating further ions diagnostic for the specific molecular species attached without the need for MS3. It works especially well with complex lipids that ionize in negative mode but may miss uncharged or positive species. Previous attempts to characterize the esterification of HETEs in cells involved the addition of exogenous radiolabeled eicosanoids followed by radio-TLC or TLC followed by saponification of individual lipids and radio-HPLC (12, 16, 43, 44). However, these approaches suffer from several drawbacks. The behavior of exogenously added HETE is not the same as endogenously produced eicosanoid (18). Also, there are significant sensitivity issues precluding the detection and identification of individual lipid species within a class. It was possible to determine that HETE is contained within a particular phospholipid pool; however, identifying which molecular species contain HETE was never achieved. Precursor scanning MS/MS overcomes all these issues and provides a high sensitivity method with which to (i) identify series of ions that contain eicosanoids in crude lipid extracts without purification (Figs. 2, 10) and (ii) structurally identify each individual compound through subsequent MS/MS analysis of partially purified parent ions (Figs. 4). Additionally, using MS3, the exact HETE positional isomer composition of each lipid can be verified through fragmentation of the HETE (m/z 319.2 [M–H]−) daughter ion generated by collision-induced dissociation of each individual parent (Figs. 5 and 11). In this study MS3 confirmed the enzymatic origin of each PE lipid generated by monocytes or platelets along with the more traditional chromatographic analysis of positional isomers and enantiomers (Fig. 6).

In summary, four specific PE-HETEs that form after activation of human monocytes and platelets have been identified and structurally characterized. The same pattern of products is conserved among two LOX isoforms. Furthermore, the utility of precursor ESI/MS/MS scanning for lipid mediators as a method for identifying families of esterified products that could signal in immune regulation and inflammation is demonstrated.

REFERENCES

1. Anning, P. B., Coles, B., Bermudez-Fajardo, A., Martin, P. E., Levison, B. S., Hazen, S. L., Funk, C. D., Kuhn, H., and O’Donnell, V. B. (2005) *Am. J. Pathol.* **166**, 653–662
2. Cyrus, T., Witztum, J. L., Rader, D. J., Tangirala, R., Fazio, S., Linton, M. F., and Funk, C. D. (1999) *J. Clin. Invest.* **103**, 1597–1604
3. Bleich, D., Chen, S., Zipser, B., Sun, D., Funk, C. D., and Nadler, J. L. (1999) *J. Clin. Invest.* **103**, 1431–1436
4. Kim, D. C., Hsu, F. I., Barrett, N. A., Friend, D. S., Grenningloh, R., Ho, I. C., Al-Garawi, A., Lora, J. M., Lam, B. K., Austen, K. F., and Kanaoka, Y. (2006) *J. Immunol.* **176**, 4440–4448
5. Schewe, T., Halangk, W., Hiesbsch, C., and Rapoport, S. M. (1979) *FEBS Lett.* **60**, 149–152
6. Kuhn, H., Belkner, J., Suzuki, H., and Yamamoto, S. (1994) *J. Lipid Res.* **35**, 1749–1759
7. Ursini, F., and Bindoli, A. (1987) *Chem. Phys. Lipids* **44**, 255–276
8. Ursini, F., Faiorino, M., and Brigelius-Flohe, R. (1995) *Methods Enzymol.* **252**, 28–53
9. Papayianni, A., Serhan, C. N., Phillips, M. L., Rennke, H. G., and Brady, H. R. (1995) *Kidney Int.* **47**, 1295–1302
10. Kuhn, H., Wiesner, R., Alder, L., Fitzsimmons, B. J., Rokach, J., and Brash, A. R. (1987) *Eur. J. Biochem.* **169**, 593–601
11. Pace-Asciak, C. R., Reynaud, D., Demin, P., and Nigam, S. (1999) *Adv. Exp. Med. Biol.* **447**, 123–132
12. Vernhet, L., Sobo, G., Wang, J., Gueddari, A., Oates, J. A., and Legrand, A. B. (1997) *Life Sci.* **61**, 1667–1678
13. Wallukat, G., Morwinski, R., and Kuhn, H. (1994) *J. Biol. Chem.* **269**, 29055–29060
14. Girtton, R. A., Spector, A. A., and Gordon, J. A. (1994) *Kidney Int.* **45**, 972–980
15. Alpert, S. E., and Walenga, R. W. (1993) *Am. J. Respir. Cell Mol. Biol.* **8**, 273–281
16. Brezinski, M. E., and Serhan, C. N. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 6248–6252
17. Serhan, C. N., and Brezinski, M. E. (1991) *Adv. Prostaglandin Thromboxane Leukotriene Res.* **21**, 105–108
18. Serhan, C. N., and Brezinski, M. E. (1991) *Adv. Prostaglandin Thromboxane Leukotriene Res.* **21**, 105–108
19. Arai, M., Imai, H., Metori, A., and Nakagawa, Y. (1997) *Eur. J. Biochem.* **244**, 513–519
20. Huang, J. T., Welch, J. S., Ricote, M., Binder, C. J., Willson, T. M., Kelly, C., Witztum, J. L., Funk, C. D., Conrad, D., and Glass, C. K. (1999) *Nature* **400**, 378–382
21. Shappell, S. B., Gupta, R. A., Manning, S., Whitehead, R., Boeglin, W. E., Schneider, C., Case, T., Price, J., Jack, G. S., Wheeler, T. M., Matusik, R. J., Brash, A. R., and Dubois, R. N. (2001) *Cancer Res.* **61**, 497–503
22. Shankaranarayan, P., and Nigam, S. (2003) *J. Immunol.* **170**, 887–894
23. Zhao, L., Cuff, C. A., Mose, E., Wille, U., Cyrus, T., Klein, E. A., Pratico, D., Rader, D. J., Hunter, C. A., Pure, E., and Funk, C. D. (2002) *J. Biol. Chem.* **277**, 35350–35356
24. Miller, Y. I., Chang, M. K., Funk, C. D., Fermanisco, J. R., and Witztum, J. L. (2001) *J. Biol. Chem.* **276**, 19431–19439
25. Middleton, M. K., Rubinstein, T., and Pure, E. (2006) *J. Immunol.* **176**, 265–274
26. Gronert, K., Maheshwari, N., Khan, N., Hassan, I. R., Dunn, M., and Laniado Schwartzman, M. (2005) *J. Biol. Chem.* **280**, 15267–15278
27. Sun, D., and Funk, C. D. (1996) *J. Biol. Chem.* **271**, 24055–24062
28. Zhang, R., Brennan, M. L., Shen, Z., MacPherson, J. C., Schmitt, D., Molenda, C. E., and Hazen, S. L. (2002) *J. Biol. Chem.* **277**, 46116–46122
29. Dugan, L. L., Demediuk, P., Pendley, C. E., Jr., and Horrocks, L. A. (1986) *J. Chromatogr.* **378**, 317–327
30. Brinckmann, R., Schnurr, K., Heydeck, D., Rosenbach, T., Kolde, G., and Kuhn, H. (1998) *Blood* **81**, 64–74
31. Murphy, R. (2002) *Mass Spectrometry of Phospholipids: Tables of Molecular and Product Ions*, Illuminati Press, Denver, CO
32. Brash, A. R., Ingram, C. D., and Harris, T. M. (1987) *Biochemistry* **26**, 5465–5471
32. Zemski Berry, K. A., and Murphy, R. C. (2004) *J. Am. Soc. Mass Spectrom.* 15, 1499–1508
33. Brash, A. R., and Ingram, C. D. (1986) *Prostaglandins Leukotrienes Med.* 23, 149–154
34. Coffey, M. J., Coles, B., Locke, M., Bermudez-Fajardo, A., Williams, P. C., Jarvis, G. E., and O’Donnell, V. B. (2004) *FEBS Lett.* 576, 165–168
35. Coffey, M. J., Jarvis, G. E., Gibbins, J. M., Coles, B., Barrett, N. E., Wylie, O. R., and O’Donnell, V. B. (2004) *Circ. Res.* 94, 1598–1605
36. Legrand, A. B., Wang, J., Sobo, G., Blair, I. A., Brash, A. R., and Oates, J. A. (1996) *Biochim. Biophys. Acta* 1301, 150–160
37. Pawlowski, N. A., Scott, W. A., Andreach, M., and Cohn, Z. A. (1982) *J. Exp. Med.* 155, 1653–1664
38. Stenson, W. F., Nickells, M. W., and Atkinson, J. P. (1983) *Prostaglandins* 26, 253–264
39. Shen, X. Y., Figard, P. H., Kaduce, T. L., and Spector, A. A. (1988) *Biochemistry* 27, 996–1004
40. Vernhet, L., Hichami, A., Hamon, L., Cochet, M. F., and Legrand, A. B. (1996) *J. Lipid Med. Cell Signal.* 13, 233–248
41. Kennett, F. F., Schenkein, H. A., Ellis, T. M., and Rutherford, R. B. (1984) *Biochim. Biophys. Acta* 804, 301–307
42. Marinetti, G. V., and Cattieu, K. (1982) *Chem. Phys. Lipids* 31, 169–177
43. Profita, M., Vignola, A. M., Sala, A., Mirabella, A., Siena, L., Pace, E., Folco, G., and Bonsignore, G. (1999) *Am. J. Respir. Cell Mol. Biol.* 20, 61–68
44. Legrand, A. B., Lawson, J. A., Meyrick, B. O., Blair, I. A., and Oates, J. A. (1991) *J. Biol. Chem.* 266, 7570–7577