Lipoxin & Aspirin-triggered 15-epi-Lipoxin Related Methods & Physical Analyses

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1. Introduction

The development of new and improved analytical methods for the study of eicosanoid biosynthesis has enabled us to identify and profile products that are generated by individual cell types in vitro. In vivo scenarios encountered in inflammation, thrombosis, atherosclerosis, asthma, and other multicellular events encompass an array of cell-cell interactions as well as a complex interplay of factors that include adhesion molecules, cytokines, lipid mediators, nitric oxide (Parky’s favorite), and chemoattractants. It has thus become apparent that transcellular eicosanoid biosynthesis during these interactions is an important means to amplify production of and/or results in the formation of novel mediators that neither cell type can generate alone (1-3).

The profound impact of cell-cell interactions on product profiles is exemplified by results from studies on interactions between isolated platelets and neutrophils in vitro (4). The lipooxygenase (LO) activity observed in platelets is that of 12-LO, while in human leukocytes it is of 5- and 15-LO. Isotopic labeling of the arachidonic acid (C20:4) stores within isolated cell types enabled the demonstration of the bidirectional transfer of labeled substrates from donor to recipient cell types and the subsequent generation of novel products that neither cell type generated alone. It was thus demonstrated that platelets transfer their native C20:4 and 12-hydroxy-eicosatetraenoic acid (HETE) to surrounding neutrophils to generate leukotriene A₄ (LTA₄), LTB₄ and 5S,12S-diHETE (4). On the other hand, neutrophils upon activation release greater than 45% of their LTA₄ to neighboring cells (5), which is rapidly converted by platelets to both LTC₄ and lipoxins (LXA₄ and LXB₄) (6, 7). The release of LTA₄ by leukocytes is now well established. During interactions with platelets, endothelial, and epithelial cells this release appears to be an important means to generate novel products and amplify eicosanoid levels in a local milieu (8).

The discovery of a novel aspirin-triggered biosynthetic route during neutrophil interactions with either endothelial or epithelial cells also emphasizes the importance of transcellular biosynthesis with other cell types as well as the impact and complexity of the in vivo milieu on this process. In this model, hypoxia or cytokine induction of cyclooxygenase II (COX-II) in
endothelia, enterocytes, or epithelia in the presence of aspirin sets the stage for 15-epi-LX biosynthesis. The acetylated COX-II generates 15(R)-HETE that is rapidly converted to 15-epi-LX by adjacent leukocytes (9, 10) and these aspirin-triggered lipoxins exert potent anti-inflammatory actions both in vitro and in vivo (11, 12). To simulate in vivo inflammatory scenarios, several experimental approaches have been developed involving in vitro models that emphasize the role of receptor-mediated cell activation, the impact of cytokines on eicosanoid biosynthesis, and the profiling of individual products generated during cell-cell interactions.

This compendium outlines and reviews the salient features in these in vitro models and highlight crucial cellular and analytical components in experimental designs and analyses that should enable the in-house investigators at Berlex and Schering to expand this approach to other cell-cell interaction systems as well as to the analyses of materials prepared by total organic synthesis for matching experiments and the development of specific Lipoxin and ATL related analogs and compounds of interest.

2. Materials

1. Most of the major eicosanoids (prostaglandins and leukotrienes) are commercially available from a variety of vendors such as Cayman Chemical Co., Inc. (Ann Arbor, MI), Biomol (Plymouth Meeting, PA), Oxford Biomedical Research (Oxford, MI), and [3H]- or [14C]-labeled eicosanoids are available from DuPont NEN (Boston, MA). However, recent analysis of commercial lipoxin (see insert) revealed that other trihydroxytetraene containing of unknown stereochemistry are being sold with uncharted biological actions. It is essential that the reader refer at this point to the original matching experiments and stereochemistry assignments for each of the lipoxins and 15-epi-lipoxins. The principals used in these matching experiments (for matching biologically isolated LX with synthetic materials using bioassay, HPLC, and mass spectral analysis) form the basis of many of these currently used methods.
Since these very labile eicosanoids are used as standards for quantitation, retention time, or as substrates for biosynthesis, it is essential to determine the concentration and structural integrity of all stocks initially upon receipt and -- as a standard laboratory protocol -- throughout the use of the compound of interest (see Note 1).

2. Buffers and Reagents: Dulbecco's phosphate-buffered saline plus Ca\(^{2+}\) and Mg\(^{2+}\) (DPBS') or minus Ca\(^{2+}\) and Mg\(^{2+}\) (DPBS'), RPMI 1640, Hanks' balanced salt solution (1 or 10 times) without Ca\(^{2+}\) and Mg\(^{2+}\) (HBSS'), and trypan blue (0.9%) for determining cell viability are commercially available. It is essential that all buffers are kept sterile to avoid LPS and bacterial contamination, which are potent stimuli of PMN. Acidic citrate dextrose (anticoagulant): 38 mM of citric acid, 75 mM of trisodium citrate, 136 mM of dextrose. This solution is prepared in water and sterilized by filtration. Prepare a concentrated EDTA (500 mM in water) stock by adjusting the pH of the solution to 8.0 (otherwise EDTA will not go in solution). Hepes-Tyrode buffer (500 ml): NaCl (3.75 g), NaHCO\(_3\) (0.375 g), KCl (0.1 g), KH\(_2\)PO\(_4\) (0.055 g), dextrose (0.5 g), and Heps (1.15 g). Adjust the pH to 7.4, then add 0.102 g MgCl\(_2\), and sterilize buffer by filtration. Lymphocyte separation media (LSM\(^{b}\)), a Ficoll/diatrizoate gradient, is commercially available. Additional reagents required are HPLC-grade methanol and ethanol.

3. Cytokines (see Table 1 and Note 2) that induce lipoxygenases or cyclooxygenase II or enhance the capacity for eicosanoid biosynthesis are available from a variety of vendors including R&D Systems (Minneapolis, MN), Endogen (Woburn, MA), and Boehringer Mannheim (Indianapolis, IN).

4. Agonists and standard enzyme inhibitors (see Notes 3, 4 and Table 2) such as fMLP, calcium ionophore (A23187), PMA, platelet-derived growth factor, and thrombin are commercially available from Sigma Chemical Co (St. Louis, MO). Inhibitors such as aspirin, indomethacin, and esculetin are commercially available from vendors that supply eicosanoids (see 2.1). Prepare the following stocks: A23187 (1.0 -2.0 mM), arachidonic acid (20-100 mM), aspirin
(300-500 mM), and esculetin (100 mM) in ethanol. Note that at higher concentrations both calcium ionophore A23187 (5-10 mM) and indomethacin (100 mM) are more soluble in DMSO as stock solutions. Thrombin stocks are prepared in water.

5. Cell culture for in vitro incubations:

A. Endothelial cells: Human umbilical vein endothelial cells (HUVEC) are available from American Type Culture Collection (Rockville, MD) or can be isolated from two to five normal term human umbilical cord segments by collagenase digestion (0.1% collagenase) (13). Cells are propagated on gelatin-coated (1%) tissue culture plates in RPMI 1640 containing 15% BCS (HyClone Laboratories, Logan, UT), 15% NU-serum (Collaborative Research Inc., Lexington, MA), 50 μg/ml endothelial mitogen (Biomedical Technologies Inc., Stoughton, MA), 8 U/ml heparin, 50 U/ml penicillin, and 50 μg/ml streptomycin. It is preferable to use HUVEC at passages 1-3 to ensure that these non-transformed cells maintain their phenotype. Confluency of endothelial monolayers is confirmed by microscopy (14).

B. Epithelial cells: Human type II epithelial A549 cells from human lung carcinoma and normal human skin fibroblast (breast) are available from the American Type Culture collection (Rockville, MD). The A549 cell line originates from a human alveolar cell carcinoma and has proven to be a useful cell line because they are easily assessable and are maintained in culture without contaminating tissue macrophages. Seed A549 cells into T-75 cm² tissue culture flasks and maintain these adherent epithelial cells in F-12K medium supplemented with 10% heat-inactivated FBS, penicillin (50 U/mL), and streptomycin (50 μg/ml) (10, 15). These type II alveolar-like epithelial cells do not form functional monolayers. Note that other mucosal epithelial and leukocyte interactions have been studied with cell lines such as human intestinal epithelia (16).

C. Monocytes: It is a considerable challenge to isolate large numbers of highly purified human peripheral monocytes (for a detailed method on isolating human peripheral monocytes see 17, 18); therefore, the human acute monocytic leukemia cell line THP-1 (ATCC, Rockville,
MD) is a useful alternative in many cases. These cells produce lysozymes, are phagocytotic, and can be differentiated into macrophage-like cells. THP-1 are maintained in RPMI supplemented with 10% fetal bovine serum and 2-mercaptoethanol (50 mM). Note that these monocytes are non-adherent unless activated and therefore are cultured in suspension.

6. Eicosanoid Analysis:

A. Radioimmunoassays (RIA) or enzyme immunoassays (EIA, ELISA) are available for prostaglandins, leukotrienes, and lipoxins from a few vendors (see 2.1). This is a convenient method that allows for the simultaneous analysis of a large number of samples with assay sensitivity in the low picogram range (vide infra). However, appropriate controls have to be designed to ensure that observed quantities of immuno-reactive eicosanoids are not due to nonspecific cross reactivity with the antibody. Therefore, it is highly recommended that the sample be extracted before immunoassay analysis (see Note 5). In addition, analysis is limited to simple quantitation of a single eicosanoid per assay without offering any structural detail on the compound. Here, I point out that many of the LX stable analogs at higher levels cross react with these assays and therefore maybe helpful in some experiments (call Charlie for unpublished results on file).

B. Reversed Phase High Pressure Liquid Chromatography (RP-HPLC) coupled with either ultraviolet, refractive index, electrochemical detection, or mass spectrometry (MS, see below) is a powerful technique to analyze lipoxygenase-derived products. Many of these eicosanoids carry specific UV chromophores [i.e., leukotrienes carry a conjugated triene chromophore with a \( \lambda_{\text{max}} \) of \( \sim 270 \) nm (diHETEs) or \( \lambda_{\text{max}} \) of \( \sim 280 \) nm (peptide-containing leukotrienes) and lipoxins carry a conjugated tetrane chromophore with a \( \lambda_{\text{max}} \) of \( \sim 300 \) nm; see Fig. 6] that permits their identification and quantitation by spectrophotometric analysis (see Chapter 5 and Reference 19). The development of new HPLC columns and stationary phases as well as diode array detectors has allowed for the on-line analysis of complete lipoxygenase product profiles from cell-cell interactions well within the picogram range. Most prostanoids absorb UV at a
wavelength of ~ 200 nm or less. Because interfering substances in biological samples obscure the detection of compounds in this range, sensitive monitoring of prostanoids requires the coupling of HPLC to: 1) prostanoid specific immunoassay, 2) electrochemical detection, 3) electrospray tandem mass spectrometry (see Fig. 7).

C. Gas chromatography coupled with mass-spectrometry (GC/MS) is a sensitive method (low picogram range) that combines column separation with compound fragmentation for analysis and structural elucidation. Since this method is not dependent on specific UV chromophores, it is suitable for prostaglandin, lipoxin, and leukotriene analyses. However, this level of analysis is dependent upon the ability to volatilize compounds and therefore requires the derivatization (i.e., methyl ester and/or OTMS derivatives) of some eicosanoids. Detection limits and the analytical capabilities of this technique are potentially restricted by compound loss during derivatization work-up.

D. Electrospray mass spectrometry coupled with liquid chromatography (LC/MS) utilizes HPLC separation and spectrophotometric analysis, as well as individual compound fragmentation, as analytical parameters for compound identification and structure elucidation (see Fig. 7). This method represents the most powerful tool for eicosanoid analysis available at this point. With detection limits of about ~1 pg or lower, which is instrument- and operator-dependent, it is as sensitive as immunoassays but offers much greater analytical capabilities. Moreover, this method has the distinct advantage that LX or related compounds do not have to be volatilized as in GC/MS. Hence, derivatization is not required. This reduces losses and saves from isolation-induced changes in the LX and ATLs; also important in the analyses of analogs (see accompanying comparison of saponification methods used in Berlin and Boston).
3. Methods

3.1 Transcellular Biosynthesis of Lipoxins and Leukotrienes

During Platelet and PMN Interactions

Objective: Evaluation of transcellular eicosanoid biosynthesis with peripheral blood cells.

Transcellular biosynthesis is an emerging area of study that has documented only a few potential *in vivo* scenarios. In view of *in vivo* milieus that are defined by continuous cell-cell interactions, it is apparent that the available experimental models are only just beginning to assess this pivotal component of eicosanoid and lipid mediator biosynthesis. *This section outlines specific examples of incubation scenarios, providing a framework (see Fig. 1) for the evaluation other cell-cell interactions that enable the discovery of novel outcomes or pathways.*

The most extensively studied model of transcellular biosynthesis is the interaction of platelets and neutrophils (see Fig. 2). This model of transcellular biosynthesis with isolated peripheral blood cells involves multiple pathways that lead to the generation of novel leukotrienes, lipoxins and diHETES. Enzymes involved in transcellular biosynthesis are the PMN 5-LO and LTA\(_4\) hydrolase, as well as the platelet 12-LO and LTC\(_4\) synthase. Transcellular products generated by the platelet include LXA\(_4\), LXB\(_4\), LTC\(_4\) and 5S-,12S-diHETE, whereas PMN LTB\(_4\) biosynthesis is amplified by platelet-derived C20:4 (see Figs. 6, 7).

To ensure clear evaluation of eicosanoid profiles by criteria outlined in Figure 1, it is essential to obtain highly enriched isolated cell populations. This is a key point, and therefore important steps in the cell isolation are outlined.

3.1.1. Neutrophil Isolation (method modified from Böyum (20))

1. All subsequent steps are performed with sterile techniques to prevent cell activation.
Obtain peripheral blood by venipuncture from healthy donors who have not taken aspirin or other medication in the last 2 weeks. Collect blood in 60 ml syringes containing acid citrate dextrose (7.0 ml) as an anticoagulant.

2. Transfer 30-40 ml of blood to 50 ml Falcon tubes and spin at 800 rpm (134 x g; do not use brake on centrifuge to prevent cell activation).

3. Carefully remove platelet rich plasma (upper layer) without disturbing the interface (For platelet isolation proceed to 3.1.2).

4. Replace the removed plasma with an equal volume of DPBS+ (6-10 ml) and add 8.0 ml of 6% dextran to each Falcon tube. Cap the tube and mix by inverting (mix well) and allow the erythrocytes to sediment for 20-30 min.

5. After sedimentation transfer the upper layer (leukocytes) to a new tube and discard the lower layer (sedimented erythrocytes). Slowly add 10 ml of LSM (Ficoll/Diatrizoate gradient) to the bottom of the tube that contains the leukocyte suspension (DO NOT VIGOROUSLY SHAKE OR MIX). Centrifuge for 30 min at 1500 rpm (514 x g; do not use brake on centrifuge).

6. If desired collect the buffy coat (upper opaque band) that contains mononuclear cells (lymphocytes and monocytes), then remove the remaining supernatant carefully. The pellet at the bottom of the tube contains the enriched neutrophil population.

7. To lyse the contaminating erythrocytes, resuspend the neutrophil pellet in 45 ml of deionized water. After 15-30 s add 5.0 ml of Hanks (10 times) and immediately mix suspension by inverting the tube (mix well). Spin the tubes at 1200 rpm (329 x g) for 15 min.

8. Pour off the supernatant and invert the tubes to drain residual buffer and resuspend the neutrophil pellet in DPBS+.

9. Enumerate neutrophils using a hemocytometer and adjust cell number to ~70 x 10^6 PMN/ml (or desired cell number). To determine integrity of cells before and during incubation conditions monitor the ability of cells to exclude trypan blue. Furthermore, cells should be
stained with Wright/Giemsa (follow manufacturer’s instructions) to confirm neutrophil purity, which should be approximately 98 ± 1%.

3.1.2. Platelet Isolation (21)

1. Collect platelet rich plasma (neutrophil isolation step 3) in 50 ml Falcon tubes and add EDTA to obtain a final concentration of 7.0 mM; this will chelate calcium to prevent platelet activation.

2. Centrifuge at 2000 rpm (914 x g) for 10 min and carefully remove the supernatant using a transfer pipet.

3. Wash the pellet with approximately 2.0 ml HEPES-Tyrode buffer using a transfer pipette (do not dislodge pellet or resuspend erythrocytes).

4. After washing, slowly add buffer and now resuspend the platelets (avoiding erythrocytes). Carefully transfer the platelets to a clean Falcon tube. Bring volume to 25 ml with HEPES-Tyrode buffer.

5. Repeat steps 2-4 and centrifuge at 2000 rpm (914 x g) for 10 min.

6. Remove the supernatant with a transfer pipet and resuspend the pellet in 3.0 ml HEPES-Tyrode buffer containing 0.1% fatty acid free human albumin (see Note 6) and CaCl₂ (1 mM).

7. Enumerate platelets, preferably with an automated counter such as a Coulter counter (Coulter Electronics Inc., Hialeah, FL.). Adjust the cell number to ∼700 x 10⁶ platelets/ml (or desired cell number).

3.1.3. Incubation Conditions

A. Treatment of cells prior to coincubation (optional)

I. If the objective is to determine the cell origin of transferred substrates or intermediates, then C20:4 pools in selected donor cell populations should be labeled (i.e., radiolabeled or deuterium labels if MS is used). This method has been described in detail for both PMN and platelets but can be applied to other cell types. It is essential to establish labeling for
each particular cell to be investigated. As a specific example, the protocol for radiolabeling platelet C20:4 pools is given below. Also cf. (4, 22).

1. Preparation of [³H] sodium arachidonate (see Note 7)
   A. Convert [³H]-C20:4 (≈50 µCi, specific activity 60-100 Ci/mmol) to the sodium salt by taking the suspension to dryness with a stream of nitrogen, making sure that C20:4 stays at the bottom of the tube (note that isolated free acid C20:4 is an oil). Add an equal volume of hexane to the tube and wash the sides of the tube by gentle vortexing. Evaporate the hexane with a stream of nitrogen, ensuring that C20:4 concentrates at the bottom of the tube.
   B. Add 50 µl of 0.01 M Na₂CO₃ and 150 µl of deionized water (bubble both with nitrogen before addition to remove oxygen) to the tube and manually agitate the solution for 15 min at room temperature.
   C. Add 3.0 ml of buffer containing: 15 mM of Tris, 134 mM of glucose, and 0.01% of delipidated bovine serum albumin, pH 7.4. Determine the total activity of the [³H] C20:4 (total counts) sodium salt by removing 3 µl for scintillation counting.

2. Labeling of platelet C20:4 phospholipid stores.
   A. Use 7.0-9.0 x 10⁹ platelets for the labeling protocol and dilute the platelet suspension to a final concentration of 0.2 x 10⁹ cells/ml. Transfer the platelet suspension to a disposable screw-capped plastic container and add the 3 ml [³H]-sodium C20:4.
   B. Incubate platelets in a covered H₂O bath with gentle shaking for 45 min at 37 °C.
   C. After the incubation, cool the container on ice for 10 min, transfer the platelet suspension to a plastic tube, and pellet the cells at 1450 x g for 15 min at 4°C.
   D. After centrifugation, collect the supernatant, which contains the unincorporated C20:4, and wash the platelets once with Hepes-Tyrode buffer (minus albumin and calcium). Collect the supernatant from the wash (unincorporated C20:4). Determine
the total counts of the unincorporated C20:4 and total counts of the \[^{1}H\]-C20:4 (step 1C) by scintillation counting and calculate the percent of incorporated C20:4.

E. Resuspend the platelets in Hepes-Ryode buffer (minus calcium) at the desired number and keep the cells at 4°C until use. The percent of incorporated \(^3H\)-C20:4 should be approximately 50-80% (a value that is both donor-dependent and varies with the state of activation of the cell suspension).

II. If the objective is to eliminate platelet-derived COX products (prostaglandin E\(_2\) and/or thromboxane A\(_2\)) from the eicosanoid profile during platelet/PMN interactions (see Fig. 2), then expose platelets to aspirin (100-500 µM) or indomethacin (100 µM) for 20 min before the incubations (23).

III. If the objective is to eliminate or evaluate the impact of platelet-derived 12-LO products in the coinubcation product profiles, then expose platelets to 12-LO inhibitors such as esculetin (100 µM) for 10-20 min before coinubcations (23).

IV. If the objective is to enhance 5-LO activity, then expose PMN to cytokines such as LPS (1 µg/ml, 30 min) (24) or GM-CSF (200 pM, 90 min) (25) (The full range of cytokine impact in the system remains to be determined since many new cytokines have recently been discovered; see Table 1).

V. Receptor activated cell suspensions (see Table 2, Note 4) release adenosine, an endogenous inhibitor of PMN C20:4 and subsequent 5-LO activity as monitored by LTB\(_4\) production (26). In these cell suspensions, adenosine is rapidly taken up and inactivated by erythrocyes (see Fig. 2A). Thus, if an objective is to eliminate adenosine from the cell incubation to enhance 5-LO product generation, then PMN should be exposed to adenosine deaminase (0.1 U) for 10 min. (This will convert adenosine to inosine and inactivate it, therefore releasing its inhibitory action on the receptor-activated 5-LO pathway).

B. Platelet/Neutrophil: Example of a Coincubation System & Analysis.
1. Combine aliquots of platelets (0.5 ml, 35 x 10^7 cells) and PMN (0.5 ml, 35 x 10^6 cells) into 12 ml Falcon tubes (see Note 8). To compare the coinoculation product profile to products derived from platelets or PMN alone, add 0.5 ml of the appropriate cells to separate tubes containing 0.5 ml of DPBS*.

2. Equilibrate platelets and PMN (5 min, 37°C) before exposure to soluble stimuli.

3. Activate cells by sequentially adding fMLP (100nM), a potent PMN agonist, and thrombin (1 U/ml), a potent platelet agonist (see Notes 3, 4 and Table 2). Gently mix cells after the addition of each agonist and incubate cells for 20 min at 37°C without mixing (see Note 9).

4. Terminate incubations with the addition of 2 volumes of cold methanol (for example, if the total incubation volume is 1 ml, then add 2 ml of methanol, -20°C) containing an appropriate internal standard for extraction recovery such as PGB_2 (see Note 10).

5. Store samples (-20 to -80°C) for extraction and workup (see Note 5).

6. Samples in general should be extracted within 24-48 h, even though LX and ATL can be stored for a longer period of time in the methanol stop solution.

**3.2 Impact of Aspirin on Transcellular Eicosanoid Biosynthesis**

Nonsteroidal antiinflammatory drugs have an important impact on eicosanoid profiles *in vivo* since in general they can be classified as inhibitors of prostaglandin biosynthesis. Of special interest is aspirin, which covalently modifies COX II and triggers a novel biosynthetic pathway (see Fig. 3 point 1, Fig. 5). The impact of aspirin or other NSAIDS on vascular transcellular biosynthesis in a cytokine-enriched inflammatory scenario can be evaluated by analyzing vascular endothelia and PMN interactions (see Fig. 3). A specific example of cytokine-primed endothelial interaction with PMN in the presence of aspirin is given below. In this experimental model, endothelia COX II and LTC_4 synthase interact with the PMN 5-LO. Transcellular-derived products
generated during these cell-cell interactions include both lipoxins and aspirin-triggered lipoxins by PMN and LTC4 by endothelial cells (see Figs. 3, 5).

3.2.1. Endothelia/PMN Interactions (9)

**Objective:** Evaluation of vascular transcellular biosynthesis during endothelia and leukocyte interactions with aspirin treated vascular endothelial cells.

1. Day 1

Confluent monolayers of HUVEC (see 2.5A) are exposed to either IL-1β (1 ng/ml) to induce cyclooxygenase II (see Table 2, Note 11) or, for non-aspirin scenarios, to media alone for 24 hr.

2. Day 2

A. Isolate PMN (see 3.1.1). Adjust the cell suspension to 100 x 10^6 PMN/ml (or desired cell number). The desired ratio of PMN to endothelial cells is 8:1 for biosynthesis of aspirin-triggered lipoxins (see Note 12, 3.1.3A and Table 1 for optional treatment, i.e., cytokines or inhibitors, of cells).

B. After 24 hr, wash endothelial cells that are adherent to the culture flask twice with DPBS* and suspend the adherent endothelial cells in a final volume of 4 ml DPBS* (Note that endothelial cells can also be lifted from the culture flask. The subsequent steps can be carried out with endothelial cells in suspension to increase the available membrane surface area for interaction with PMN). Expose the cells to either aspirin (100-500 μM; see Note 13) or vehicle (ethanol) alone for 20 min prior to coincubation with PMN. After 20 min remove the buffer and suspend the cells in 3.5 ml of DPBS*.

C. Let each cell population or suspension stand for 5 min at 37°C.

D. Expose endothelial cells to thrombin (1 U/ml) for ~2 min (This potent endothelial agonist upregulates adhesion molecules such as P-selectin and stimulates C20:4 release.).
E. Add PMN (50 x 10^6 cells, 0.5 ml) to each monolayer of endothelial cells (approximately 6x10^6 HUVEC/75 cm^2 flask). After adding cells for coincubation, add agonists (eg., 100 nM of fMLP) and coincubate the cells without mixing for 30 min (see Note 9).

F. Terminate coincubation with the addition of 2 volumes of cold methanol (-20°C) containing appropriate standards such as PGB₂ (100 ng) (see Note 10). To optimize recovery of LXs use disposable tissue culture cell scrapers to remove the endothelial cells from the culture plates. Collect the cells and solution in a Falcon tube.

G. Store samples (-20 to -80°C) for extraction and workup (see Note 5). Samples in general should be extracted within 24-48 hr, even though eicosanoids can be stored for a longer period of time in the methanol stop solution.

3.2.2. Epithelium/PMN Interactions (10)

Objective: Evaluation of transcellular biosynthesis during mucosal epithelia and leukocyte interactions with, for example, aspirin-treated cells.

PMN recruitment to sites of inflammation can result in the emigration of PMN into adjacent tissues (see Fig. 3). Interaction of these emigrated PMN with cytokine-exposed tissues in the presence of aspirin can be evaluated in vitro. Models have been developed to examine transcellular eicosanoid biosynthesis during interaction of cytokine-primed mucosal epithelia (airway A549 as well as intestinal HT29 epithelial cells) with PMN (see Figs. 4, 5). A specific example of airway epithelia and PMN transcellular biosynthesis and the impact of aspirin is given below. Enzymes involved in this transcellular pathway are epithelial 15-LO, LTA₄ hydrolase, COX II, and PMN 5-LO (see schematic outline in Fig. 5). Transcellular products generated during these cell-cell interactions are aspirin triggered lipoxins by PMN (Fig. 5), and LXA₄ as well as LTB₄ by epithelia (Fig. 4).
Day 1

To induce COX II, expose A549 cells to IL-1β (1 ng/ml) or, for a non-aspirin scenario, expose cells to media alone for 24 h prior to the experiment. Note that IL-13 and IL-4 are both cytokines that induce 15-LO (see Fig. 4) and may be considered in an experimental protocol if an objective will be to study the contribution of 15-LO-derived products in PMN/epithelial interactions (25, 27).

Day 2

A. Isolate PMN (see 3.1.1). Adjust the cell numbers in suspension to 100 PMN/ml (or desired cell levels). The ratio of PMN to epithelial cells of 8:1 was found to be optimal for the biosynthesis of aspirin-triggered lipoxins (for optional treatment of PMN, see 3.1.3A, Table 1).

B. After 24 hr, wash epithelial cells that are adherent to the culture flasks twice with DPBS* and suspend the cells in a final volume of 4.0 ml DPBS* (Note that epithelial cells can also be lifted from the culture flask. The subsequent steps can be carried out with epithelial cells in suspension to increase the available membrane surface area for interaction with PMN). Expose the cells to either aspirin (300-500 μM) (see Note 13) or vehicle (ethanol calculated as vol/vol) alone for 20 min prior to coincubation with PMN.

Optional Treatments: These epithelial cells (A549) can also generate 15R-HETE via the P450 pathways. Thus, if the objective is to evaluate the relative contribution of COX II and cytochrome P450 in the eicosanoid profile, and especially 15R-HETE and 15-epi-lipoxin biosynthesis, then expose the epithelial cells to the following inhibitors for 20 min: Indomethacin (100 μM), a nonspecific inhibitor of COX (see Note 13); and/or 17-octadecynoic acid (500 μM), a cytochrome P450 inhibitor.

After 20 min remove the buffer and suspend the cells in 3.2 ml of DPBS* (Do not replace buffer in flasks that were exposed to either indomethacin or 17-octadecynoic acid).

C. Let each cell population stand for ~ 3-5 min at 37°C.
D. Expose epithelial cells to C20:4 (20 μM, 1 min) (see Note 14).

E. Add PMN (~8x10^6 cells, 0.8 ml) to monolayers of epithelial cells (approximately 10 x 10^6 cells/75cm^2 flask). After addition, stimulate cells with A23187 (5 μM; activates both cell types) and co-incubate the cells without mixing for 30 min (see Note 9).

F. Terminate coincubations by the addition of 2 volumes of cold methanol (-20°C) containing an appropriate internal standard (see Note 10). Again, to optimize the recovery of eicosanoids, use disposable tissue culture cell scrapers to remove the endothelial cells from the culture plates. Collect the cells and solution in a Falcon tube.

G. Store samples (-20 to -80°C) for extraction and workup (see Note 5). Samples in general should be extracted within 24 to 48 h, even though eicosanoids can be stored for a longer period of time in the methanol stop solution.

3.2.3. Monocytes/PMN Interactions (28)

Objective: Transcellular biosynthesis during leukocyte-leukocyte interactions: Evaluation of the impact of aspirin.

Wound healing and resolution of inflammation involves interactions of PMN and monocytes/macrophages. Experimental models were developed to evaluate the impact of aspirin with these cell types. A specific example that illustrates this impact is a protocol that evaluates the interaction of cytokine-primed monocytes with PMN. Enzymes involved in this route of transcellular biosynthesis are monocyte COX II and PMN 5-LO. Products generated during these cell-cell interactions include the aspirin-triggered lipoxins (illustrated in Fig. 5).

Day 1

Expose monocytes to LPS (1 μg/ml) to induce COX II or, for non-aspirin scenarios, expose cells to media alone (see Note 11) for 16 hr before the experiment. Note that IL-13 and IL-4 are both cytokines that induce 15-LO and should be considered in an experimental design if the
objective is to study the contribution of 15-LO-derived products in PMN/monocyte interactions cf. (25, 27).

Day 2

A. Isolate fresh PMN (typically 3 hours post venipuncture taking cell isolation into consideration; see 3.1.1). Adjust the cell number to 120 x 10^6 PMN/ml (or desired cell number). The ratio of PMN to monocytes of 6:1 was determined to be optimal for 15-epi-LX generation (Chiang et al., JPET 98 see enclosed reprint) (see 3.13A and Table 1 for optional treatment of PMN, cytokines and inhibitors).

B. After 16 h, pellet the non-adherent monocytes. Remove the media and resuspend the monocytes at 20 x 10^6 cells/ml in DPBS*.

C. Expose monocytes to either aspirin (300 μM)(see Note 13) or vehicle (ethanol) alone for 20 min.

D. Pellet monocytes at 1000 rpm (228 x g, 10 min), remove buffer, and resuspend cells at 20 x 10^6 monocytes/ml.

E. Coincubate the cells with freshly isolated PMN by combining 1 ml of the PMN (120 x 10^6 cells) and 1.0 ml of the monocyte (20x10^6 cells) suspension.

F. Equilibrate the cells for 5 min at 37°C.

G. Addition of agonist: Coactivate the cell suspension by adding 20 μM of C20:4 and 5 μM of calcium ionophore (activates both cell types) (see Notes 4, 14). Mix the cells immediately after the addition of agonist. Incubate the cells without mixing for 30 min (see Note 9).

H. Terminate coincubations with the addition of 2 volumes of cold MeOH (-20°C) containing appropriate standards (see Note 10).

I. Store samples (-20 to -80°C) for extraction and workup (for extraction, see Note 5). Samples in general should be extracted within 24 to 48 h, even though eicosanoids can be stored for a longer period of time in the methanol stop solution.
4. Notes

1. Most Prostaglandin and Leukotriene stocks should be stored considering manufacturer’s instructions (usually -20 to -80 °C). A good rule also for LX users.

2. Many of bioactive eicosanoids (including LX) are sensitive to light, oxygen, and heat. This is especially critical for native arachidonic acid, which upon exposure to oxidative conditions will degrade to a variety of compounds including mono- and diHETES and isoprostanes. In this context, C20:4 can be used in the lab as an indicator in parallel to monitor procedures related to LX handling and store procedures --- like a canary in a coal mine. This is especially relevant when evaluating LX analog bioactions. Thus, prepare a concentrated stock and a “working or daily stock” that you will use as reference materials for the experiments. As a general rule each time an LX, ATL or analog stock is used, pass a gentle stream of nitrogen over the vial before closing. This will slow the degradation of LX.

3. Determine the concentration of lipoxin, leukotriene, some and prostaglandin stocks by measuring the UV absorbance and using the specific extinction coefficient (these are available in the Merck Index and from some vendors). This is especially important if stocks are used on a regular basis. Note that the leukotriene and lipoxin platelet intermediate LTA₄ (see Fig. 2B point 1) is a relatively unstable epoxide that is available as a methyl ester and therefore has to be saponified before it can be used as a substrate in the experiment (see ref. 21). If possible, HPLC analysis of your reference eicosanoid stocks is an invaluable tool to determine the integrity and, for example, percent isomerization.

4. It is usually advisable to aliquot cytokines, since they are in general dissolved in water and kept at -20°C. Keep and dissolve cytokine stocks according to the manufacturer’s instructions. Repeated freezing and thawing can lead to degradation of these protein mediators.

5. The total amount of vehicle (ethanol, v/v) added to your cell incubation or coincubations should not exceed 0.1%. Higher concentrations of alcohol or dimethyl sulfoxide can affect cell and LX integrity (i.e., enzyme activity etc). It is therefore desirable to prepare stocks of stimuli
or inhibitors at 1000-5000 times the final concentration so that only small aliquots are added directly to cell suspensions.

6. The calcium ionophore (A23187) is a widely used agonist and is a useful tool to evaluate the full enzymatic potential of individual cells or cell-cell interactions. However, this non-receptor-mediated cell activation is not physiological and induces eicosanoid products that may not reflect in vivo product profiles. Therefore, it is preferable to simulate individual cells and cell coincubations with specific ligands that bear functional surface membrane receptors in these cells (see Table 2).

7. Extraction protocol optimized for lipoxins and aspirin-triggered lipoxins (23, 28): Place the incubation suspension at -20°C for at least 20 min (protein precipitation). Centrifuge the incubation suspension at 2000 rpm (800 x g) for 20 min at 4°C. Collect the supernatant in a 50 ml tube and dilute the sample with at least 5 volumes of pure water. Rapidly adjust the pH of this aqueous suspension to pH 3.5 with HCL (1N). Load the acidified samples into solid phase extraction cartridges (C$_{18}$ Sep-Pak), wash the cartridge with 10 ml of water and elute compounds with 8.0 ml of hexane, followed by 8.0 ml of methyl formate, and a final elution with 8.0 ml of methanol. Lipoxins, HETEs and most leukotrienes elute in the methyl formate fraction, while peptide leukotrienes (LTC$_4$, LTD$_4$, LTE$_4$, etc.) elute in the methanol fraction. Take the methyl formate fraction to dryness under a gentle stream of nitrogen and resuspend eicosanoids in a small volume of methanol (ie., 100 µl). For further consideration of eicosanoid extraction, see Chapter 4.

8. The pivotal leukotriene and lipoxin intermediate in platelets (ie., LTA$_4$) is an unstable epoxide in aqueous environments. Once released by the donor cell, LTA$_4$ is hydrolyzed non-enzymatically within seconds to 6-trans-LTB$_4$ and 6-trans-12-epi-LTB$_4$ (see Fig. 6A). Stability of LTA$_4$ and therefore transcellular biosynthesis are greatly enhanced by albumin or other stabilizing agents such as liposomes, which serve as a carrier for this lipid as other eicosanoids.
9. If the objective is to determine the relative contribution of each cell type in the eicosanoid profile, radiolabel the C20:4 pool from each cell type. For example the PMN C20:4 pool can be labeled with[^14]C-C20:4 while platelet pools would be labeled with[^3H]-C20:4. This dual labeling permits the identification of the cellular source of eicosanoids in a cell-cell interaction profile.

10. The ratio and amounts of platelet-derived LTC₄ and lipoxin are highly dependent on the number of platelets that are coincubated with PMN (6). At a ratio of 1:1-1:100 (PMN:platelets), lipoxins are the predominant LTA₄-derived transcellular product, and at a ratio of 1:100 approach PMN-generated LTB₄ levels. To obtain significant amounts of LTC₄, ratios of 1:100 (PMN:platelets) are indicated.

11. Cell adherence greatly enhances transcellular biosynthesis. Levy et al. (25) have demonstrated that continuous mixing of platelet and PMN coincubations greatly attenuates the amount of lipoxins generated as compared to coincubations that were not disturbed. In addition, Brady and Serhan (29) have demonstrated that inhibition of P-selectin and integrin (CD11/CD18)-mediated adhesion during PMN and endothelia interactions greatly inhibits transcellular eicosanoid biosynthesis.

12. It is essential to terminate incubations with solutions containing an internal standard since this will serve as an invaluable tool for correction of product profiles (e.g., HPLC retention times) and for calculating recoveries of eicosanoids after extraction and sample workup. The internal standard should be a stable measurable compound that is not generated during the cell incubations and does not interfere with the product analysis. PGB₂ usually meets those criteria for analysis of lipoxins and leukotrienes, while 13-HODE is used when analyzing mono-HETEs, and 19-OH-PGB₂ is suitable for analysis of omega or beta oxidation products. Depending on the sensitivity of your analytical assay, 50-200 ng (see Fig. 6B) are added to the incubations.
12. Expression of COX II should be verified in the current experimental conditions. COX II specific antibodies as well as COX II nucleotide primers are available from most vendors that supply eicosanoids. Thus, verification of cytokine COX II induction by either Western blot or RT-PCR is recommended cf. (10, 16).

13. PMN should be used immediately after isolation (i.e., ~3 hr after venipuncture, accounting for isolation) or placed in an ice bath (4°C). This will slow the decrease of enzymatic activity that starts once the cells are removed from blood. Note that careful records should be kept on the blood donors. Unlike cultured cells, peripheral blood cells are exposed to a diverse environment that can include cytokines and monoHETEs that “prime” leukocytes. Such diversity results in noticeable donor variations in the data. For example, in endogenously “primed” PMN such as from asthmatic patients (30), 5S,15S-diHETE and lipoxins, which are normally associated with transcellular biosynthesis, are generated endogenously from a single cell type (PMN).

14. Aspirin hydrolyses rapidly in an aqueous environment. Therefore, prepare aspirin stocks in ethanol just immediately before each experiment and store solid aspirin in a dessicator to prevent decomposition. Note that aspirin is the only known NSAID that induces COX II to generate 15R-HETE by covalently modifying (acetylating) this isozyme.

15. Cyclooxygenase and lipooxygenase activity can be greatly amplified (depending on the cell type) by the addition of exogenous C20:4. However, adding exogenous C20:4 to certain cell types can also eliminate cell regulation of biosynthesis and therefore impacts the eicosanoidand LX profile.

Thus, adding exogenous C20:4 should be avoided, unless the enzymatic potential of a given cell type is investigated and/or a specific product must be amplified for analytical detection.
EXTRAC TIONS

LX and ATL must first be extracted from the reaction materials before they can be concentrated and analyzed. Eicosanoid extraction is complicated by obligate loss of compound. To control for the loss of unknown total amounts of LT and LX, a closely related compound that would not be an anticipated product of the co-incubation should be used as an internal control. It should be added to the reaction materials at the time the co-incubation is terminated. Two major extraction protocols have been optimized for the recovery of LT and LX: liquid-liquid extraction and solid phase extraction.

1. Liquid-liquid extraction -

   1. Terminate co-incubation with \( \geq 2 \) volumes of methanol. After protein precipitation at \(-20^\circ\text{C}\) for at least 60 min, centrifuge (800g, 15 min, 0°C).
   2. Remove supernatant (S\(_{800}\)) to a separatory funnel. Resuspend pellet in \( \geq 2 \) vol ethanol and repeat centrifugation. Pool S\(_{800}\) into the separatory funnel. Repeat wash with MeOH and pool S\(_{800}\).
   3. Add an equal volume of ddH\(_2\)O to the pooled S\(_{800}\). Next add a large amount of ether (\textasciitilde200 ml).

   (Be certain to work in an approved chemical fume hood)

   4. Acidify the lower (i.e., aqueous) phase to pH 1 to 2. Add HCl drop-by-drop and check the lower phase’s pH by pH paper after vigorous mixing.
   5. After pH adjustment, shake and ventilate sep funnel for 1 min. Remove cap and allow interface to form.
6. Remove lower phase (ddH₂O and ethanol layer) into a second sep funnel. Add another ~200 ml ether. Check and adjust pH. Save first funnel with ether.

7. Shake second sep funnel for 1 minute. Remove and discard lower phase. Combine ether phases.

8. Wash ether phases with ~10 ml ddH₂O, shake well, and remove lower phase after interface has formed. Repeat until the wash ddH₂O being removed reaches a pH = 7.0 (usually 2 - 3 washes).

9. Once all the water has been removed, transfer ether phase into a round bottom glass flask. Add a small amount of ethanol.

10. Evaporate under vacuum until sample is completely dry. Add 1 ml MeOH.

11. The sample is now ready for analysis. Store at -20°C in a light impermeable glass test tube.

2. Solid phase extraction

1. Activate a C18 cartridge (Sep-Pak) by pushing 20 ml MeOH followed by 20 ml ddH₂O (drop-wise) through the column with a glass syringe.

2. After sample has been collected in the round bottom flask, rotoevaporate to dryness.

3. Release vacuum and immediately add 200 µl MeOH. Vortex well. Add 5 ml ddH₂O and vortex. Remove sample to the glass syringe.

4. Repeat step #3.
5. Acidify sample in the glass syringe with a few drops of HCl (0.1 N) to a pH ~3.5. Load sample onto activated column.

6. Slowly push 10 ml ddH₂O through the column to neutralize pH (~2 drops/sec). Check the pH of the eluate using pH paper.

7. Push 10 ml hexane through the column. Collect this fraction with a clean borosilicate glass test tube. Phospholipids elute here.

8. Push 8 ml methyl formate through the column. Collect this fraction. Mono-HETE's, most leukotrienes, prostaglandins and lipoxins elute here.


b. Physical methods

Reverse phase - high pressure liquid chromatography (RP-HPLC) separates compounds on the basis of size, charge and hydrophobicity that are present in an extract of materials from cellular incubations. High pressure is used to lessen the time for sample analysis. Eluting materials can be detected in the picogram range by coupling the separation achieved by HPLC to a diode array detector (DAD), electrochemical detector, refractive index measurement or mass spectrometer. Spectrophotometric analysis is most commonly employed because most eicosanoids give distinctive UV chromophores. For example, LT in methanol have a conjugated triene with λmax ~270 nm for LTB₄ and DiHETE's and λmax ~280 nm for cysteinyll LT's, while both LX and epimer-LX have a conjugated tetraene with λmax ~300 nm (see accompanying Figures).
Although PGB₂ carries a UV chromophore with \( \lambda_{\text{max}} \approx 270 \text{ nm} \), most prostanoids absorb UV at \( \lambda_{\text{max}} < 210 \text{ nm} \) (Note: PGB₂ is not a product of PLT-PMN co-incubation and therefore is ideally suited for use as an internal standard in these co-incubations). Because numerous interfering substances absorb UV at <210 nm, the detection of most prostanoids solely on the basis of UV criteria is limited. To avoid this problem, couple RP-HPLC (to separate PG’s) with (1) immunoassay for specific prostaglandin measurement, or (2) with electrochemical detection or electrospray MS to profile PG reaction products and thereby achieve sensitive PG identification and quantitation.

The length of time after injection for a compound to reach the detector is defined as its retention time. The retention time combined with a compound’s other physical properties can be used to identify individual eicosanoids when synthetic standards are available. Retention time and spectrophotometric analysis via diode array detectors permits on-line identification and quantitation of LT and LX. When available, coelution with authentic standards by matching studies (i.e., by injecting 50% sample mixed with 50% standard to determine if the retention times and UV spectra of the sample match the standard) can confirm the identification of unknown compound(s) present in a biological matrix or reaction set.

In our HPLC examples presented here, RP-HPLC analyses were performed using a Hewlett Packard 1100 Series Diode Array Detector equipped with a binary pump and eluted on a LUNA C18-2 microbore column (150 x 1 mm, 5 \( \mu \text{m} \)) (Phenomenex, Torrance, CA) using a mobile phase composed of methanol/ water/ acetate (58/42/0.01, v/v/v) as phase 1 (0-25 min) and a linear gradient with methanol/acetate (99.99/0.01, v/v) as phase 2 (25-37 min) at a flow rate of 0.12 ml/min. Collected UV data was recalled at
270 nm (Panel A) to detect conjugated trienes (e.g., leukotrienes) and 301 nm (Panel B) to detect conjugated tetraenes (e.g., lipoxins).

Also of great utility in the sensitive detection of LT and LX biosynthesis is mass spectrometry (MS) which consists of three basic components: an ion source, a mass analyzer and detector. Several different techniques for generating ions from compounds in solid, liquid or gaseous phases are available with current mass spectrometers, however discussion of this area is beyond the scope of this chapter. Mass analyzers utilize electrical fields to separate molecular ions on the basis of their mass to charge ratio (m/z) which enables detection of unique molecular fragments. Separation of materials entering the MS is required when analyzing individual compounds from a biological matrix and can be carried out by gas chromatography (GC) using an inert carrier gas (e.g., helium) or by liquid chromatography (LC) via either capillary flow, HPLC or direct injection. MS has the advantage of detection in the same low picogram range as UV yet does not require the presence of a chromophore. In addition, structural elucidation of unknown compounds is possible by MS via analysis of its molecular ion, fragmentation pattern (i.e., presence of prominent ions) and changes in the sizes of these fragments after derivitization (e.g., by silylation, ozonolysis or catalytic hydrogenation). Drawbacks of GC/MS include a requirement for compounds to be volatile in order to be suitable for GC and significant losses of material during derivitization reactions.

Newer methods utilize electrospary MS coupled to HPLC to enable analysis of individual compounds directly after elution from the HPLC column. LC/MS/MS is the most powerful technique currently available for LX and ALT analysis with detection limits in the low picogram range.
Several examples of LC/MS/MS analysis of lipoxin are given see accompanying tracings and analysis. Samples were extracted from the incubation mixture by solid phase extraction and materials in the methyl formate elution were injected into a SpectraSYSTEM HPLC (Thermo Separation Products, San Jose, CA) coupled via an electrospray ionization source to an LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA). A LUNA C18-2 (150 x 2 mm, 5 µm) column was eluted isocratically with methanol/water/acetic acid (58:42:0.009) at 0.2 ml/min into the electrospray probe. The LCQ spray voltage was set to 6 kV and the heated capillary to -4 V and 250°C. Full-scan mass spectra (MS) were acquired by scanning between m/z 340-360 in the negative ion mode, followed by the acquisition of product ion mass spectra (MS/MS) for m/z 351.5 ([M-H]- of lipoxin A₄ and lipoxin B₄). See examples of LX and ATL analogs.

**Immunoassay**

Enzyme-based and radioactive immunoassays are available for a wide variety of eicosanoids, including specific LT and LX. This method also detects LT and LX in the picogram range and is an even more convenient and translatable method for analyzing large numbers of samples. *Several caveats should be noted:*
(1) despite using an Ab with high affinity for the compound of interest, non-specific Ab crossreactivity remains a frequent problem,

(2) the presence of interfering substances that inhibit antigen-Ab interaction are common in biological matrices,

(3) analysis is limited to a specific compound, and

(4) consistent and accurate pipetting is essential to acquiring reproducible results.

These common problems plus the lack of structural information necessitate initial validation of the selected immunoassay with physical chemical techniques, running appropriate controls and performing at least 2-3 determinations for each sample. Here we describe a LXA₄ ELISA to exemplify a protocol that is, in general, similar to those used for other eicosanoid ELISA's of interest (e.g., TxB₂, LTB₄ and LTC₄).

LXA₄ ELISA

LXA₄ antisera was prepared by conjugation to keyhole limpets hemocyanin through the succinimide ester of LXA₄. Rabbits were immunized initially with 100 μg of the conjugate per rabbit. Monthly booster injections with 50 μg of the initial conjugate were performed and rabbits were bled through ear veins. Antisera was collected from the blood by centrifugation (900g), stored at -20°C and diluted 1:200 in ELISA buffer (optimal dilutions for the assay conditions). Note: horseradish peroxidase-labeled LXA₄ was also prepared through its succinimide ester.¹²

1. Pre-coat a 96-well microtiter plate with 1 μg affinity-purified goat anti-rabbit IgG per well (or purchase pre-coated plate).

3. In sequence, add 50 μl LXA₄ antiserum (diluted 1:200 in ELISA buffer), then 50 μl LXA₄ standard or sample in duplicate, and finally 50 μl horseradish peroxidase-labeled LXA₄. *(Use a repeating pipettor whenever possible to minimize systematic pipetting error.)*

4. Gently shake plates for 1 hour at 25°C.

5. Wash (x3) with 200 μl ELISA wash buffer per well. *(Use multichannel repeating pipettor.)*

6. Tap plate on absorbent diaper until no visible liquid remains in the wells.

7. Add 150 μl K-Blue® substrate and incubate up to 15 min at 25°C.

8. Stop reaction with 100 μl of 1 N H₂SO₄.

9. Read absorbance at 450 nm. Quantitate amount of LXA₄ in samples by comparison with the LXA₄ serial dilutions. Note: A standard curve must be included in each 96-well microtiter plate for reference.

Figure 6 demonstrates the utility of ELISA’s when measuring LT and LX in biological samples. Levels of immunoreactive LTC₄ and LXA₄ were determined in nasal lavage fluid from aspirin-sensitive asthmatics both before and after a placebo or threshold dose of oral aspirin that elicited an *in vivo* inflammatory response in patient airways (as in 12).

**ELISA Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride</td>
<td>0.9%</td>
</tr>
<tr>
<td>Potassium Phosphate, monobasic</td>
<td>100 mM</td>
</tr>
<tr>
<td>Potassium Phosphate, dibasic (Sigma P-5504)</td>
<td>100 mM</td>
</tr>
<tr>
<td>Bovine Serum Albumin (Sigma A-2153)</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

*Adjust pH to 7.4.

**ELISA Wash Buffer**
Potassium Phosphate, monobasic 10 mM  
Potassium Phosphate, dibasic 10 mM  
Tween 20 0.05%  
*Adjust pH to 7.4.

*K-Blue Substrate* - Purchase with ELISA kit or from Neogen #300175.

**BUFFERS AND SOLUTIONS**

*Acidic citrate dextrose (ACD)*
Citric Acid (Sigma C-0759) 38 mM  
Trisodium Citrate (Sigma C-7254) 75 mM  
Dextrose (Sigma G-8870) 136 mM

*Hepes-Tyrode*
Sodium Chloride (Sigma S-7653) 128 mM  
Sodium Bicarbonate (Sigma S-6297) 8.9 mM  
Potassium Chloride (Sigma P-9333) 2.7 mM  
Potassium Phosphate, monobasic (Sigma P-5379) 0.8 mM  
Dextrose 5.5 mM  
Hepes (Sigma H-7523) 9.7 mM  
*Titrate pH to 7.4, then add:  
Magnesium Chloride (Sigma M-2670) 1.0 mM

*Dulbecco’s Phosphate Buffered Saline (PBS)* - BIOWHITAKER #17-512F
Potassium Chloride 2.7 mM  
Potassium Phosphate, monobasic 1.5 mM  
Sodium Phosphate, dibasic (Sigma S-7907) 8.1 mM  
Sodium Phosphate, heptahydrate (Sigma S-9390) 8.1 mM  
Sodium Chloride 137 mM  
* Adjust pH to 7.4

*Hanks’ Balanced Salt Solution (HBSS) (10x)* - Gibco #14180-012
Potassium Chloride 53.7 mM  
Potassium Phosphate, monobasic 4.4 mM  
Sodium Chloride 1.37 M  
Sodium Phosphate, heptahydrate 3.4 mM  
Dextrose 55.5 mM

*DEPC-H$_2$O* Diethyl pyrocarbonate (DEPC) (Sigma D-5758) 1 ml/l dH$_2$O  
*Shake, let stand overnight. Autoclave for 30 min.

*TBE* Trizma Base (Sigma T-6791) 100 mM  
Boric acid (Sigma B-7660) 90 mM
Ethylenediaminetetraacetic acid (EDTA) (Sigma E-9884)  1 mM
*Adjust pH to 8.4. Autoclave for 30 min.
Models for \textit{in vivo} scenarios of transcellular biosynthesis provide invaluable information about the regulation of eicosanoid biosynthesis that is likely to occur during multicellular events \textit{in vivo}. The experimental approach of studying eicosanoid generation during cell-cell interactions and receptor-mediated cell activation represents a significant advancement beyond initial observations of eicosanoid formation and bioaction in isolated cell types that were activated under less physiologically relevant conditions. The experimental models reviewed in this chapter should be viewed as specific examples or as approaches to the study of cell-cell interactions. These examples may serve as guidelines to investigate novel cell-cell scenarios (see Fig. 1) and advance the emerging area of transcellular biosynthesis of bioactive lipid mediators.
<table>
<thead>
<tr>
<th>Cytokine/Agonist</th>
<th>enzyme/protein</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>cytosolic phospholipase A₂</td>
<td>Neutrophil</td>
</tr>
<tr>
<td>TGFβ</td>
<td>5-lipoxygenase</td>
<td>Monocyte</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5-lipoxygenase</td>
<td>Mast Cell</td>
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<tr>
<td>GM-CSF</td>
<td>5-lipoxygenase</td>
<td>Monocytes</td>
</tr>
<tr>
<td>IL-3</td>
<td>15-lipoxygenase</td>
<td>Neutrophil</td>
</tr>
<tr>
<td>IL-4</td>
<td>12-lipoxygenase</td>
<td>Tracheal Epithelia</td>
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<td>IL-13</td>
<td>12-lipoxygenase</td>
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<tr>
<td>growth factors</td>
<td>12-lipoxygenase</td>
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<td>IL-1β</td>
<td>Cyclooxygenase II</td>
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<tr>
<td>TNF</td>
<td>Cyclooxygenase II</td>
<td>GI Epithelia</td>
</tr>
<tr>
<td>Endothelia growth factors</td>
<td>Cyclooxygenase II</td>
<td>Neutrophil</td>
</tr>
<tr>
<td>tumor promoters</td>
<td>Cyclooxygenase II</td>
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<td>LPS</td>
<td>Cyclooxygenase II</td>
<td>Vascular endothelia</td>
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<td>c-kit</td>
<td>LTC₄ synthase</td>
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<td>GM-CSF c-kit</td>
<td>five lipoxygenase activating protein</td>
<td>Neutrophil</td>
</tr>
<tr>
<td></td>
<td>(FLAP)</td>
<td>Monocyte</td>
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Table 2. Agonists that stimulate formation of novel lipoxygenase derived products during cell-cell interactions

<table>
<thead>
<tr>
<th>Cell-Cell Interactions</th>
<th>Receptor Bypass</th>
<th>Product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(From Healthy donors)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil-Platelet</td>
<td>Ca(^{2+}) Ionophore</td>
<td>LTC(_4)</td>
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<tr>
<td>Neutrophil-Platelet</td>
<td>Urate Crystals</td>
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<tr>
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<td>LTC(_4), LX</td>
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<td>Leukocyte-Endothelial Cell</td>
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<td>Alveolar Macrophage-Epithelia</td>
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<td>Neutrophil-Epithelial Cell</td>
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<table>
<thead>
<tr>
<th>Cell-Cell Interactions</th>
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<th>Products</th>
<th>Reference</th>
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<tr>
<td>Neutrophil-Platelet</td>
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<tr>
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<td>Neutrophil-Platelet</td>
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<td>LTC(_4)</td>
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<tr>
<td>Neutrophil-Endothelial Cells</td>
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<td>15-epi-LX</td>
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<tr>
<td>Neutrophil-Epithelial Cell</td>
<td>II-1 (+ASA), Ca(^{2+}) Ionophore</td>
<td>15-epi-LX</td>
<td>9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell type or cell-cell interaction</th>
<th>Disease</th>
<th>Products</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>(Diseased donors or disease model)</td>
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<td>Neutrophils</td>
<td>Asthma</td>
<td>LX, LTB(_4)</td>
<td>31</td>
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<td>Rheumatoid Arthritis</td>
<td>LX, LTB(_4)</td>
<td>42</td>
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<tr>
<td>macrophage-astrocytes</td>
<td>HIV</td>
<td>LX, LTB(_4), LTD(_4)</td>
<td>43</td>
</tr>
</tbody>
</table>
Figure 1. **General outline for investigating transcellular biosynthesis.**

General scheme illustrating the experimental approach to investigate transcellular biosynthesis. Pivotal experimental components are identified and include the initial characterization of cell type specific product profiles with receptor-triggered activation. In coinubcation scenarios, the bidirectional transfer of intermediates from both cell types A and B needs to be considered. The intermediates from the donor cell are potentially transformed enzymatically by the recipient cell to a novel product that neither cell type A nor B generates alone. This general experimental approach can be applied to investigate product profiles of all cell-cell interactions including scenarios that involve more than two cell types.

Figure 2. **Transcellular biosynthesis with peripheral blood cells.**

A) Predominant products generated by isolated neutrophils (PMN) and platelets that are activated via specific serpine receptors (i.e., thrombin and fMLP) are indicated. Isolated erythrocytes (RBC) do not generate significant amounts of eicosanoids but take up adenosine, an endogenous product that is released by PMN and inhibits 5-LO product generation. B) Scenarios of transcellular biosynthesis during platelet and PMN interaction: 1) PMN activation stimulates the generation of the intermediate LTA₄ that is transformed by adhering platelets to the novel products lipoxins and LTC₄ by 12-lipoxygenase and LTC₄ synthase, respectively. 2) Platelet activation stimulates the generation of 12-HETE that is transformed by PMN to the novel product 5S,12S-diHETE. 3) Activated platelets release the eicosanoid precursor arachidonic acid that amplifies PMN LTB₄ and LTA₄ generation.
Figure 3. **Vascular endothelia and PMN interactions in the presence of aspirin.**

1) Cytokine exposure induces COX II expression in vascular endothelial cells. This isozyme is acetylated by aspirin, and cell activation via serpentine receptors (i.e., thrombin) generates 15R-HETE that is transformed by the 5-LO of adhering PMN to the novel products 15-epi-lipoxins. 2) Receptor-triggered activation (i.e., fMLP) of adhering PMN stimulates the generation of LTA₄ that is transformed by endothelial LTC₄ synthase to the novel product LTC₄. This transcellular biosynthesis is diminished by antibodies specific for the adhesion molecules L-selectin and beta-2 integrins (CD 11/CD18).

Figure 4. **Bidirectional transfer of eicosanoid intermediates during PMN and epithelia interactions.**

Epithelia exposure to cytokines (i.e., IL-13, IL-4) induces the expression of 15-LO. In subsequent interactions with adjacent leukocytes, the 5-LO-derived intermediate LTA₄ is transformed by LTA₄ hydrolase and 15-LO of epithelial cells to LTB₄ and the novel product LXA₄, respectively. Another component of this cell-cell interaction is the biosynthesis of 15-HETE by epithelial cells and its transformation by the 5-LO of adjacent leukocytes to the novel products LXA₄ and LXB₄.

Figure 5. **Novel aspirin-triggered pathway in PMN interactions with epithelia and endothelia.**

Endothelia, airway epithelia, or enterocyte exposure to cytokines in an inflammatory milieu induces the expression of cyclooxygenase II. In the presence of aspirin, this isozyme is acetylated. This covalent modification results in the inhibition of prostaglandin biosynthesis and the generation of 15R-HETE in activated cells. This COX II-derived intermediate is transformed by the 5-LO of adjacent leukocytes to novel 15-epi-LXA₄. In addition, cytokine exposure (i.e., IL-13, IL-4) induces 15-LO in epithelial cells. Subsequent cell activation generates 15S-HETE that is
transformed by adjacent PMN to lipoxins. Transcellular biosynthesis of lipoxins and aspirin triggered lipoxins attenuates leukotriene biosynthesis and therefore alters the eicosanoid profile in an inflammatory microenvironment.

Figure 6. **Transcellular biosynthesis of lipoxins during platelet-PMN interactions**.

(A) Representative RP-HPLC chromatogram of endogenous products obtained from human PMN exposed to LPS (1 μg/ml) and stimulated with platelet-activating factor (PAF, 300 nM) and fMLP (100 nM). Products were analyzed by RP-HPLC with a Hewlett Packard 1100 Series diode array detector equipped with a binary pump and eluted on a Phenomenex LUNA C18-2 microbore column (150 x 1 mm, 5 μm) using a mobile phase composed of methanol/water/acetate (58/42/0.01, v/v/v) as phase 1 (0-25 min) and a linear gradient with methanol/acetate (99.99/0.01, v/v) as phase 2 (25-37 min), at a flow rate of 0.12 ml/min. Collected UV data were recalled at 270 nm to detect conjugated trienes (5-lipoxygenase products, i.e. leukotrienes). Arrows indicate the retention times of synthetic standards. UV chromophores of endogenous LTB₄ and the non-enzymatic hydrolysis products of the epoxide LTA₄, denoted as compound I (6-trans-LTB₄) and compound II (6-trans-12-epi-LTB₄), are shown in the insets. (B) RP-HPLC chromatogram of products obtained from human platelets incubated with LTA₄. Platelets (1.6 x 10⁸ cells) suspended in PBS containing 0.1% BSA were incubated with 20 μM LTA₄ and 5 μM A23187 for 20 min at 37°C. Trans-A denotes retention times of 11-trans-LXA₄ and 6S-11-trans-LXA₄, and trans-B denotes 8-trans-LXB₄ and 14S-8-trans-LXB₄. The specific UV chromophores of LTA₄-derived lipoxins and the internal PGB₂ standard are shown in the insets.

Figure 7. **LC/MS/MS analysis of platelet and LTA₄ derived lipoxins**

LC/MS/MS (liquid chromatography-tandem mass spectrometry) was performed on a Finnigan LCQ quadrupole ion trap mass spectrometer system equipped with an electrospray atmospheric
pressure ionization probe. A SpectraSYSTEM AS3000 autosampler was used to inject methanol samples into the HPLC component, which consisted of a SpectraSYSTEM P4000 quaternary gradient pump, a Phenomenex LUNA C18-2 column (150 x 2 mm, 5 μm), and a SpectraSYSTEM UV2000 scanning UV/VIS absorbance detector. The column was eluted isocratically with methanol/water/acetic acid (58:42:0.009) at 0.2 ml/min into the electrospray probe. The LCQ spray voltage was set to 6 kV and the heated capillary to -4 V and 250 °C. Over a 1.2 s scan cycle, full-scan mass spectra (MS) were acquired by scanning between m/z 340-360 in the negative ion mode, followed by the acquisition of product ion mass spectra (MS/MS) for m/z 351.5 ([M-H]⁻ of lipoxins A₄ and B₄). (A) Representative LC/MS/MS m/z 351 SIM (selected ion monitoring) profile of products obtained from human platelets incubated with LTA₄. Products were obtained and isolated as in Figure 6B. (B) Representative MS/MS spectrum of LXB₄ peak from profile shown in A. MS/MS spectrum of LXA₄ peak from profile shown in A.

**LXB₄ Fragments:** The MS/MS spectrum of LXB₄ shows prominent ions of diagnostic value at m/z 333 [351 - H₂O], 315 [351 - 2H₂O], 307 [351 - CO₂], 289 [351 - H₂O, - CO₂], 271 [351 - 2H₂O, - CO₂], 251 [351 - CHO(CH₂)₄CH₃], 233 [351 - H₂O, - CHO(CH₂)₄CH₃], 221 [351 - CHOCHOH(CH₂)₄CH₃], 207 [351 - CO₂, - CHO(CH₂)₄CH₃], 189 [351 - H₂O, - CO₂, - CHO(CH₂)₄CH₃], 163 [351 - CO₂, - CH₂COHCHOH(CH₂)₄CH₃], 129 [CH₃CO(CH₂)₃COO⁻], and 115 [CHO(CH₂)₃COO⁻].

**LXA₄ Fragments:** The MS/MS spectrum of LXA₄ shows prominent ions of diagnostic value at m/z 333 [351 - H₂O], 315 [351 - 2H₂O], 307 [351 - CO₂], 289 [351 - H₂O, - CO₂], 271 [351 - 2H₂O, - CO₂], 251 [351 - CHO(CH₂)₄CH₃], 235 [351 - CHO(CH₂)₃COOH], 233 [351 - H₂O, - CHO(CH₂)₄CH₃], 207 [351 - CO₂, - CHO(CH₂)₄CH₃], 189 [351 - H₂O, - CO₂, - CHO(CH₂)₄CH₃], 135 [351 - CHO(CH₂)₃COOH, - CHO(CH₂)₄CH₃], and 115 [CHO(CH₂)₃COO⁻] (cf. (31, 32)
• Eicosanoid profiles for each cell type with and without stimuli
  • Cell type A specific profile with cell type specific stimuli
  • Cell type B specific profile with cell type specific stimuli
• Cell A-Cell B interaction profile with co-stimulation
• Cell A-Cell B interaction profile with specific receptor stimulation
A) Adenosine → RBC
   5-LO
   \[ \text{fMLP} \] → LTA₄ → 12-LO → COX I → TXA₂
   PMN
   LTB₄

B) Thrombin
   12-LO
   5S, 12S diHETE
   1
   LTA₄
   Lipoxins
   COX II
   \[ \text{fMLP} \] → 5-LO
   LTC₄
   12-HETE
   2
   C20:4
   3
1) 15-epi-Lipoxins

PMN

fMLP

5-LO

IL-1β, TNF-α

ASA--COX II

15R-HETE

Endothelia

2) LTC₄

Blocked by anti-CD11/CD18 and L-selectin AB

LTA₄

LTC₄ synthase

Thrombin
Microenvironment cytokines (TNF-α, IL-1β, LPS) & inflammatory mediators

Endothelia
Epithelia (airway, intestine)
Monocytes

Cyclooxygenase-2 ---- Aspirin

Prostaglandins

Transcellular Biosynthesis

15 R-HETE

Leukocytes

15-epi-Lipoxins

Leukotrienes

5-Lipoxygenase

15 R

15 S

15 S-HETE

15-Lipoxygenase (IL-4, IL-13)

Epithelia

15 S-HETE

Lipoxins
Fig. 7

A

trans-B

LXA₄

trans-A

LXB₄

m/z 351 SW Relative Intensity

Time (min)

B

LXB₄

Relative Abundance

m/z

115 129 163 189 207 221 233 251 271 289 307 315 333 351

C

LXA₄

Relative Abundance

m/z

115 135 189 207 233 251 289 307 315 333 351
LX analog General Design Chart

LX Natural Products & Metabolites → Bioactions in vitro Cellular Assays

Leukocyte assays
endothelial cells
GI epithelial cells
Enzyme conversion

Analog Design

Synthesis

Selection criteria

In vivo Models

GI inflammation
Nephritis (rat)
Reperfusion injury (mouse)

Therapeutic Indications
15-Deoxy-LXA₄ methyl ester

15-Cyclohexyl LXA₄ methyl ester

16-Phenoxy LXA₄ methyl ester

16-(p-Fluorophenoxy) LXA₄ methyl ester

15 (R/S)-Methyl LXA₄ methyl ester

15 (R/S)-Methyl LXA₄ N,N-dimethylamide

5 (R/S)-Methyl LXB₄ methyl ester

15-epi LXA₄ methyl ester

15-epi-15-Cyclohexyl LXA₄ methyl ester

15-epi-16-Phenoxy LXA₄ methyl ester

15-epi-16-(p-Fluorophenoxy) LXA₄ methyl ester

15-Cyclooctyl LXA₄ methyl ester

16-Phenoxy LXA₄ N,N-dimethylamide

15-epi LXB₄ methyl ester
LC/MS/MS Method

LC/MS/MS analyses. LC/MS/MS was performed employing an LCQ (Finnigan MAT, San Jose, CA) quadrupole ion trap mass spectrometer system equipped with an electrospray atmospheric pressure ionization probe. Samples were suspended in methanol and injected into the HPLC component, which consisted of a SpectraSYSTEM P4000 (Thermo Separation Products, San Jose, CA) quaternary gradient pump, a Prodigy octadecylsilane-3 (100 x 2 mm, 5 μm) column (Phenomenex, Torrance, CA) or a LUNA C18-2 (150 x 2 mm, 5 μm) column, and a rapid spectra scanning SpectraSYSTEM UV2000 (Thermo Separation Products, San Jose, CA) UV/VIS absorbance detector. The column was eluted isocratically with methanol/water/acetic acid (65:35:0.01, v/v/v) at 0.2 ml/min into the electrospray probe. The spray voltage was set to 5-6 kV and the heated capillary to 250 °C. LXA₄ and the ATLα were quantitated by selected ion monitoring (SIM) for analyte molecular anions (e.g. [M-H]⁻ = m/z 351.5 for LXA₄, m/z 365.5 for ATLα₁, and m/z 405.5 for ATLα₂ free acid) or by UV absorbance at 300 nm. Product ion mass spectra (MS/MS) were also acquired for definitive identification of the compounds.

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Saponification Procedure

Saponification of Methyl Esters with Lithium Hydroxide

Materials

glass penny-head vial with stopper
magnetic stir bar
stir plate with clamp
dry ice/isopropanol bath
aluminum foil
Teflon tape
1 M LiOH solution
tetrahydrofuran (THF)
dH₂O
methyl ester eicosanoid

Procedure

Clean the glass vial and stir bar by rinsing with: (i) 1 M LiOH, (ii) water, (iii) methanol, and (iv) THF
Measure the UV spectrum of the starting material
In a cold room, set up the stir plate and dry ice/isopropanol bath
For saponification of ~ 150 µg of material, use the following amounts of solvent:

- transfer the solution containing the methyl ester to the glass vial
- dry down the solvent under a gentle stream of N₂(g)
- add 500 µl of THF in which to resuspend the material
- carefully put the stir bar in the vial
- place the vial in the dry ice/isopropanol bath and turn on the stir plate
- slowly add 50 µl of 1 M LiOH directly to the THF solution (it should freeze on contact)
- add 15 µl of H₂O
- place the stopper in the vial and seal with Teflon tape
- cover the apparatus with aluminum foil and allow the reaction to go overnight
- the following day, remove the stir bar form the vial (with a larger, external magnet)
- place on ice and allow the lithium salts to settle
- with a glass Pasteur pipette, collect the THF solution, being careful not to take up the precipitate, and transfer the solution to a clean vial.
- before using (including the measurement of the UV spectrum) be sure to dry off the THF and resuspend in either MeOH or EtOH.

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LXA<sub>4</sub>

\[ m/z \ 351: [M-\text{H}]^- \]
\[ m/z \ 333: [M-\text{H}^-] - \text{H}_2\text{O} \]
\[ m/z \ 315: [M-\text{H}^-] - 2\text{H}_2\text{O} \]
\[ m/z \ 307: [M-\text{H}^-] - \text{CO}_2 \]
\[ m/z \ 289: [M-\text{H}^-] - \text{H}_2\text{O}, - \text{CO}_2 \]
\[ m/z \ 271: [M-\text{H}^-] - 2\text{H}_2\text{O}, - \text{CO}_2 \]
\[ m/z \ 251: [M-\text{H}^-] - \text{CHO}((\text{CH}_2)_4\text{CH}_3) \]
\[ m/z \ 235: [M-\text{H}^-] - \text{CHOH}((\text{CH}_2)_3\text{CO}_2^-) \]
\[ m/z \ 233: m/z \ 251 - \text{H}_2\text{O} \]
\[ m/z \ 215: m/z \ 251 - 2\text{H}_2\text{O} \]
\[ m/z \ 207: m/z \ 251 - \text{CO}_2 \]
\[ m/z \ 189: m/z \ 251 - \text{H}_2\text{O}, - \text{CO}_2 \]
\[ m/z \ 135: m/z \ 251 - \text{CHOH}((\text{CH}_2)_3\text{CO}_2^-) \]
\[ m/z \ 115: \text{CHO}((\text{CH}_2)_3\text{CO}_2^-) \]

LXB<sub>4</sub>

\[ m/z \ 351: [M-\text{H}]^- \]
\[ m/z \ 333: [M-\text{H}^-] - \text{H}_2\text{O} \]
\[ m/z \ 315: [M-\text{H}^-] - 2\text{H}_2\text{O} \]
\[ m/z \ 307: [M-\text{H}^-] - \text{CO}_2 \]
\[ m/z \ 289: [M-\text{H}^-] - \text{H}_2\text{O}, - \text{CO}_2 \]
\[ m/z \ 271: [M-\text{H}^-] - 2\text{H}_2\text{O}, - \text{CO}_2 \]
\[ m/z \ 251: [M-\text{H}^-] - \text{CHO}((\text{CH}_2)_4\text{CH}_3) \]
\[ m/z \ 233: m/z \ 251 - \text{H}_2\text{O} \]
\[ m/z \ 221: [M-\text{H}^-] - \text{CHOCHOH}((\text{CH}_2)_4\text{CH}_3) \]
\[ m/z \ 215: m/z \ 251 - 2\text{H}_2\text{O} \]
\[ m/z \ 207: m/z \ 251 - \text{CO}_2 \]
\[ m/z \ 189: m/z \ 251 - \text{H}_2\text{O}, - \text{CO}_2 \]
\[ m/z \ 163: [M-\text{H}^-] - \text{CO}_2, - \text{CH}_2\text{CHOHCHOH}((\text{CH}_2)_4\text{CH}_3) \]
\[ m/z \ 129: \text{CH}_3\text{CO}(\text{CH}_2)_3\text{CO}_2^- \]
\[ m/z \ 115: \text{CHO}(\text{CH}_2)_3\text{CO}_2^- \]

PGE<sub>2</sub>

\[ m/z \ 351: [M-\text{H}]^- \]
\[ m/z \ 333: [M-\text{H}^-] - \text{H}_2\text{O} \]
\[ m/z \ 315: [M-\text{H}^-] - 2\text{H}_2\text{O} \]
\[ m/z \ 271: [M-\text{H}^-] - 2\text{H}_2\text{O}, - \text{CO}_2 \]
\[ m/z \ 233: [M-\text{H}^-] - \text{H}_2\text{O}, - \text{CHO}((\text{CH}_2)_4\text{CH}_3) \]
**LTB$_4$**

$m/z$ 335: [M-H]$^-$
$m/z$ 317: [M-H]$^-$ - H$_2$O
$m/z$ 299: [M-H]$^-$ - 2H$_2$O
$m/z$ 291: [M-H]$^-$ - CO$_2$
$m/z$ 273: [M-H]$^-$ - H$_2$O, - CO$_2$

$m/z$ 195 =

**15(S)-HETE**

$m/z$ 319: [M-H]$^-$
$m/z$ 301: [M-H]$^-$ - H$_2$O
$m/z$ 275: [M-H]$^-$ - CO$_2$
$m/z$ 257: [M-H]$^-$ - H$_2$O, - CO$_2$
$m/z$ 219: [M-H]$^-$ - CHO(CH$_2$)$_4$CH$_3$

**12(S)-HETE**

$m/z$ 319: [M-H]$^-$
$m/z$ 301: [M-H]$^-$ - H$_2$O
$m/z$ 275: [M-H]$^-$ - CO$_2$
$m/z$ 257: [M-H]$^-$ - H$_2$O, - CO$_2$

$m/z$ 195 =

**5(S)-HETE**

$m/z$ 319: [M-H]$^-$
$m/z$ 301: [M-H]$^-$ - H$_2$O
$m/z$ 275: [M-H]$^-$ - CO$_2$
$m/z$ 257: [M-H]$^-$ - H$_2$O, - CO$_2$
$m/z$ 115: [M-H]$^-$ - CHO(CH$_2$)$_3$COO$^-$
15-epi-16-(p-fluoro)-phenoxy-LXA₄: MS and MS/MS

15-epi-16-(para-fluoro)-phenoxy-LXA₄ free acid:
LiOH/THF saponification, overnight, 4 °C

**Preferred Saponification Method**

15-epi-16-(p-F)-phenoxy-LXA₄ free acid eluted isocratically with methanol/water/acetic acid (65:35:0.01, v/v/v) from a LUNA 150 x 2 mm C18-2 column (Phenomenex, Torrance, CA) at 0.2 ml/min.

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15-epi-16-(p-fluoro)-phenoxy-LXA₄: MS and MS/MS

15-epi-16-(para-fluoro)-phenoxy-LXA₄ free acid:
LiOH/THF saponification, overnight, 4 °C

15-epi-16-(p-F)-phenoxy-LXA₄ free acid eluted isocratically with methanol/water/acetic acid (65:35:0.01, v/v/v) from a LUNA 150 x 2 mm C18-2 column (Phenomenex, Torrance, CA) at 0.2 mL/min.

15-epi-16-(para-fluoro)-phenoxy-LXA₄ free acid:
LiOH/EtOH saponification, 30 min, 25 °C

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15-epi-16-(p-fluoro)-phenoxy-LXA₄ MS/MS Fragmentation

$[\text{M-H}]^- = m/z$ 405.5

$m/z$ 387: $[\text{M-H}]^- - \text{H}_2\text{O}$

$m/z$ 369: $[\text{M-H}]^- - 2\text{H}_2\text{O}$

$m/z$ 361: $[\text{M-H}]^- - \text{CO}_2$

$m/z$ 293: $[\text{M-H}]^- - \text{HO-C}_{\text{phenyl}} - \text{F}$

$m/z$ 275: $m/z$ 293 - $\text{H}_2\text{O}$

$m/z$ 289: $[\text{M-H}]^- - \text{CHOH(CH}_2)_3\text{CO}_2^-$

$m/z$ 251: $[\text{M-H}]^- - \text{COOCH}_{\text{phenyl}} - \text{F}$

$m/z$ 233: $m/z$ 251 - $\text{H}_2\text{O}$

$m/z$ 369.4, 387.5

$m/z$ 231: $m/z$ 293 - $\text{H}_2\text{O, - CO}_2$

$m/z$ 215: $m/z$ 251 - 2$\text{H}_2\text{O}$

$m/z$ 213: $m/z$ 293 - 2$\text{H}_2\text{O, - CO}_2$

$m/z$ 207: $m/z$ 251 - $\text{CO}_2$

$m/z$ 189: $m/z$ 251 - $\text{H}_2\text{O, - CO}_2$

$m/z$ 177: $m/z$ 289 - $\text{HO-C}_{\text{phenyl}} - \text{F}$

$m/z$ 135: $m/z$ 251 - $\text{CHOH(CH}_2)_3\text{CO}_2^-$

$m/z$ 115: $\text{CHO(CH}_2)_3\text{CO}_2^-$
15(R/S)-methyl-LXA₄: MS and MS/MS

15(R/S)-methyl-LXA₄ free acid eluted isocratically with methanol/water/acetic acid (65:35:0.01, v/v/v) from a Prodigy 100 x 2 mm ODS-3 column (Phenomenex, Torrance, CA) at 0.2 ml/min.

MS: Base Peak

MS/MS

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15(R/S)-methyl-LXA₄ MS/MS Fragmentation

\[ [\text{M-H}]^- = m/z \ 365.5 \]

\[
\begin{align*}
\text{m/z} \ 347: & \quad [\text{M-H}]^- - \text{H}_2\text{O} \\
\text{m/z} \ 329: & \quad [\text{M-H}]^- - 2\text{H}_2\text{O} \\
\text{m/z} \ 321: & \quad [\text{M-H}]^- - \text{CO}_2 \\
\text{m/z} \ 303: & \quad [\text{M-H}]^- - \text{H}_2\text{O}, - \text{CO}_2 \\
\text{m/z} \ 285: & \quad [\text{M-H}]^- - 2\text{H}_2\text{O}, - \text{CO}_2 \\
\text{m/z} \ 251: & \quad [\text{M-H}]^- - \text{CHO (CH}_2)_4\text{CH}_3 \\
\text{m/z} \ 249: & \quad [\text{M-H}]^- - \text{CHOH(CH}_2)_3\text{CO}_2^- \\
\text{m/z} \ 233: & \quad m/z \ 251 - \text{H}_2\text{O} \\
\text{m/z} \ 207: & \quad m/z \ 251 - \text{CO}_2 \\
\text{m/z} \ 189: & \quad m/z \ 251 - \text{H}_2\text{O}, - \text{CO}_2 \\
\text{m/z} \ 135: & \quad m/z \ 251 - \text{CHOH(CH}_2)_3\text{CO}_2^- \\
\text{m/z} \ 115: & \quad \text{CHO(CH}_2)_3\text{CO}_2^- 
\end{align*}
\]
16-phenoxy-LXA₄: MS and MS/MS

16-phenoxy-LXA₄ free acid eluted isocratically with methanol/water/acetic acid (65:35:0.01, v/v/v) from a Prodigy 100 x 2 mm ODS-3 column (Phenomenex, Torrance, CA) at 0.2 ml/min.

MS: Base Peak

MS/MS

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16-phenoxy-LXA₄ MS/MS Fragmentation

\[ [\text{M-H}]^- = m/z \ 387.5 \]

\[
\begin{align*}
&\text{m/z 369: } [\text{M-H}]^- - \text{H}_2\text{O} \\
&\text{m/z 351: } [\text{M-H}]^- - 2\text{H}_2\text{O} \\
&\text{m/z 343: } [\text{M-H}]^- - \text{CO}_2 \\
&\text{m/z 325: } [\text{M-H}]^- - \text{H}_2\text{O}, - \text{CO}_2 \\
&\text{m/z 307: } [\text{M-H}]^- - 2\text{H}_2\text{O}, - \text{CO}_2 \\
&\text{m/z 293: } [\text{M-H}]^- - \text{HO} - \text{phenylethyl} \\
&\text{m/z 275: } m/z \ 293 - \text{H}_2\text{O} \\
&\text{m/z 271: } [\text{M-H}]^- - \text{CHOH(CH}_2)_3\text{CO}_2^- \\
&\text{m/z 251: } [\text{M-H}]^- - \text{phenylethyl} \\
&\text{m/z 233: } m/z \ 251 - \text{H}_2\text{O} \\
&\text{m/z 231: } m/z \ 293 - \text{H}_2\text{O}, - \text{CO}_2 \\
&\text{m/z 215: } m/z \ 251 - 2\text{H}_2\text{O} \\
&\text{m/z 213: } m/z \ 293 - 2\text{H}_2\text{O}, - \text{CO}_2 \\
&\text{m/z 207: } m/z \ 251 - \text{CO}_2 \\
&\text{m/z 189: } m/z \ 251 - \text{H}_2\text{O}, - \text{CO}_2 \\
&\text{m/z 177: } m/z \ 271 - \text{HO} - \text{phenylethyl} \\
&\text{m/z 135: } m/z \ 251 - \text{CHOH(CH}_2)_3\text{CO}_2^- \\
&\text{m/z 115: } \text{CHO(CH}_2)_3\text{CO}_2^- 
\end{align*}
\]
LXA₄: MS and MS/MS

LXA₄ free acid eluted isocratically with methanol/water/acetic acid (65:35:0.01, v/v/v) from a LUNA 150 x 2 mm C18-2 column (Phenomenex, Torrance, CA) at 0.2 ml/min.

MS: Base Peak

MS/MS

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Serhan Laboratory
7/2/99
LXA₄ MS/MS Fragmentation

$[\text{M-H}]^- = m/z\ 351.5$

$m/z\ 333: [\text{M-H}]^- - \text{H}_2\text{O}$
$m/z\ 315: [\text{M-H}]^- - 2\text{H}_2\text{O}$
$m/z\ 307: [\text{M-H}]^- - \text{CO}_2$
$m/z\ 289: [\text{M-H}]^- - \text{H}_2\text{O}, - \text{CO}_2$
$m/z\ 271: [\text{M-H}]^- - 2\text{H}_2\text{O}, - \text{CO}_2$
$m/z\ 251: [\text{M-H}]^- - \text{CHO (CH}_2)_4\text{CH}_3$
$m/z\ 235: [\text{M-H}]^- - \text{CHOH(CH}_2)_3\text{CO}_2^-$

$m/z\ 233: m/z\ 251 - \text{H}_2\text{O}$
$m/z\ 215: m/z\ 251 - 2\text{H}_2\text{O}$
$m/z\ 207: m/z\ 251 - \text{CO}_2$
$m/z\ 189: m/z\ 251 - \text{H}_2\text{O}, - \text{CO}_2$
$m/z\ 135: m/z\ 251 - \text{CHOH(CH}_2)_3\text{CO}_2^-$
$m/z\ 115: \text{CHO(CH}_2)_3\text{CO}_2^-$
LXB₄ free acid eluted isocratically with methanol/water/acetic acid (65:35:0.01, v/v/v) from a LUNA 150 x 2 mm C18-2 column (Phenomenex, Torrance, CA) at 0.2 ml/min.

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Serhan Laboratory
7/2/99
LXB₄ MS/MS Fragmentation

\[
[M-H]^- = m/z 351.5
\]

\[
\begin{align*}
 m/z 333: & \quad [M-H]^- - H_2O \\
 m/z 315: & \quad [M-H]^- - 2H_2O \\
 m/z 289: & \quad [M-H]^- - H_2O, - CO_2 \\
 m/z 271: & \quad [M-H]^- - 2H_2O, - CO_2 \\
 m/z 251: & \quad [M-H]^- - CHO(CH_2)_4CH_3 - CH_2COHCHOH(CH_2)_4CH_3 \\
 m/z 233: & \quad m/z 251 - H_2O \\
 m/z 215: & \quad m/z 251 - 2H_2O \\
 m/z 221: & \quad [M-H]^- - CHOCHOH(CH_2)_4CH_3 \\
 m/z 207: & \quad m/z 251 - CO_2 \\
 m/z 189: & \quad m/z 251 - H_2O, - CO_2 \\
 m/z 163: & \quad [M-H]^- - CO_2, - CH_2COHCHOH(CH_2)_4CH_3 \\
 m/z 129: & \quad CH_3CO(CH_2)_3CO_2^- \\
 m/z 115: & \quad CHO(CH_2)_3CO_2^-
\end{align*}
\]
5(S)-methyl-LXB₄: MS and MS/MS

5(S)-methyl-LXB₄ free acid eluted isocratically with methanol/water/acetic acid (65:35:0.01, v/v/v) from a Prodigy 100 x 2 mm ODS-3 column (Phenomenex, Torrance, CA) at 0.2 ml/min.

MS: Base Peak

\[ [M-H]^+ = a = m/z \ 365 \]

MS/MS

Clary B. Clish, Ph.D.
Serhan Laboratory
7/2/99
5(S)-methyl-LXB₄ MS/MS Fragmentation

\[ [\text{M-H}^-] = m/z ~ 365.5 \]

- \( m/z ~ 347 \): [M-H]⁻ - H₂O
- \( m/z ~ 329 \): [M-H]⁻ - 2H₂O
- \( m/z ~ 303 \): [M-H]⁻ - H₂O, - CO₂
- \( m/z ~ 285 \): [M-H]⁻ - 2H₂O, - CO₂
- \( m/z ~ 265 \): [M-H]⁻ - CHO (CH₂)₄CH₃
- \( m/z ~ 247 \): \( m/z ~ 265 \) - H₂O
- \( m/z ~ 235 \): [M-H]⁻ - CHOCHOH(CH₂)₄CH₃
- \( m/z ~ 229 \): \( m/z ~ 265 \) - 2H₂O
- \( m/z ~ 217 \): \( m/z ~ 235 \) - H₂O
- \( m/z ~ 203 \): \( m/z ~ 265 \) - H₂O, - CO₂
- \( m/z ~ 129 \): CH₃CO(CH₂)₃CO₂⁻
HPLC Methods:

For RP-HPLC (DAD) analysis and fractionation 10-350 µl of the \(^3\)H-LXA\(_4\)-methyl esters were taken to dryness under a stream nitrogen and immediately resuspended in HPLC mobile phase (15 µl). Compounds were analyzed with a Hewlett Packard 1100 Series Diode Array Detector (DAD) equipped with a binary pump and eluted on a Beckman C18 column (250 x 4.5 mm, 5 µm) using a mobile phase composed of methanol/water/acetate (60/39.99/0.01, v/v/v; flow rate 1.0 ml/min) as phase 1 (0-15 min), a linear gradient (0-30%) with methanol/acetate (99.99/0.01, v/v) as phase 2 (15-55 min) and a linear gradient (30-100%) of methanol/acetate (99.99/0.01, v/v) as phase 3 (55-60 min). Indicated fractions (0.3-1.0 ml) were directly hand collected from the DAD flow cell and immediately diluted in 3 volumes of methanol and 0.1 ml methyl format. Fractions were taken to dryness under a stream nitrogen and resuspended immediately in 200 µl of EtOH. Collected fractions were immediately used in binding assays. For analysis in an isocratic HPLC system compounds were eluted on a Phenomenex LUNA C18-2 microbore column (150 x 1 mm, 5µm) using a mobile phase composed of methanol/water/acetate (60/39.99/0.01, v/v/v; flow rate 0.1 ml/min).

TLC Methods:

Compounds (1-50 µl) were loaded on a TLC plate (Whatman LK6D Silica Gel 60Å, 20x20 cm, 250 µm) under nitrogen and eluted (50 min, 4 °C) in a mobile phase composed of the ethyl acetate/2,2,4 Trimethyl Pentane/acetate/water (110:50:20:100, v/v/v/v, organic phase).
Ligand Binding Assay

- **Binding buffer:** DPBS ++ (with Ca²⁺ and Mg²⁺)

- **Washing buffer:** 10 mM Tris/HCl (pH 7.5)

- **Cold ligand:** Prepare serial dilution (1 nM to 1 mM) in ethanol
  
  Use 1 µl / incubation (1 ml)

- **Hot ligand:** Take out aliquot of HPLC fraction #2
  
  Evaporate ethanol under nitrogen gas and resuspend in binding buffer
  
  (100 µl per incubation)

  **HPLC fraction #2 (~ 1 µM, specific activity ~ 9.2 Ci / mmol)

- **Cells:** detach HEK293 cells with DPBS − (w/o Ca²⁺ and Mg²⁺), centrifuge at 1,000 rpm
  
  for 10 min and resuspend cells at 1x 10⁶ / 100 µl binding buffer

- **Reaction:**

  1. To each Eppendorf tube, add the followings:
     
     800 µl binding buffer
     
     1 µl cold ligand
     
     100 µl hot ligand
     
     100 µl HEK293 cells
     
     ( **Vortex cold and hot ligands in binding buffer for 10 sec before adding cells)

  2. Incubate on ice for 40 min

  3. Separate the bound and unbound ligand by filtration through Whatman GF/C glass microfiber filter (Whatman Cat. No. 1822025, Fisher Cat. No. 0987432A)

  4. Wash the filters with 5 ml ice-cold washing buffer for three times

  5. Determine the radioactivity on filters by β-counter (Beckman)

7-20-99

Nan Chiang, Ph.D.

Serhan Lab.
Analysis of Commercially Prepared Lipoxin Compounds

We received commercial preparations I and II on 4/29/99. The UV spectra of the solutions were measured and revealed wavelengths of maximal absorbance in the range of 302-303 nm for both preparations. In contrast, the characteristic wavelength of maximal absorbance is 300-301 nm for authentic LXA_4. Next the samples were taken for LC/MS/MS analyses. The commercial preparation I had a major peak at RT 10.2 min (Fig. 1) with a molecular anionic mass of 351.5, which is the same mass as the LXA_4 molecular anion (see Fig. 3). Commercial preparation II showed one major peak at RT 12.2 min (Fig. 2), of mass 351.5. A co-injection of authentic LXA_4 with commercial preparation II revealed a greater than one minute difference in retention time between the two compounds, as LXA_4 eluted at 13.3 minutes in this system (Fig. 4A). Commercial preparation II showed a difference in the relative intensities of its MS/MS product ions (Fig. 4B) relative to authentic LXA_4 (Fig. 4C). In particular, the m/z 251 fragment which arises from a cleavage of the C14-C15 bond is approximately 75% less intense for commercial preparation II. The samples were then analyzed on a second, distinct HPLC system that was equipped with a diode array UV/VIS detector (Fig. 5). Again, commercial preparation II eluted at a shorter retention time than authentic LXA_4 and the compound’s λ_max occurred at a longer wavelength. Taken together, these data indicate that these commercially prepared lipoxins, are isomers of authentic LXA_4.
Commercial Preparation I

(A) SIM chromatogram of m/z 351.5, [M-H]- of LXA₄ isomer. (B) UV A₃00nm chromatogram. (C) UV A₂70nm chromatogram.

Commercial Preparation II

(A) SIM chromatogram of m/z 351.5, [M-H]- of LXA₄ isomer. (B) UV A₃00nm chromatogram. (C) UV A₂70nm chromatogram.

LXA₄

(A) SIM chromatogram of authentic LXA₄. (B) UV A₃00nm chromatogram. (C) UV A₂70nm chromatogram.

Figure 1. LC/MS/MS chromatogram of commercial preparation I. (A) SIM chromatogram of m/z 351.5, [M-H]- of LXA₄ isomer. (B) UV A₃00nm chromatogram. (C) UV A₂70nm chromatogram.

Figure 2. LC/MS/MS chromatogram of commercial preparation II. (A) SIM chromatogram of m/z 351.5, [M-H]- of LXA₄ isomer. (B) UV A₃00nm chromatogram. (C) UV A₂70nm chromatogram.

Figure 3. LC/MS/MS chromatogram of authentic LXA₄. (A) SIM chromatogram of m/z 351.5, [M-H]- of LXA₄. (B) UV A₃00nm chromatogram. (C) UV A₂70nm chromatogram.

C.B. Clish, Ph.D. Serhan Lab 5/6/99

HPLC conditions:
Mobile phase: 65/35/0.01 MeOH/H₂O/HOAc (v/v/v), Flow rate = 200 µl/min
Column: LUNA C18-2 (150 x 2 mm, 5µ), temperature = 30 °C
Figure 4. Co-injection of commercial preparation II with authentic LXA₄. (A) SIM chromatogram of m/z 351.5, [M-H]⁻ of LXA₄ and its isomers, and UV A₃₅₀ nm and A₃₇₀ nm chromatograms. (B) MS/MS product ion spectrum of commercial product II at retention time 12.2 min. (C) MS/MS product ion spectrum of authentic LXA₄ at retention time 13.3 min.
Figure 5. HPLC analysis utilizing an HP1100 diode array detector. (A) Co-injection of commercial preparation II with authentic LXA₄. (B) Overlay of UV spectra for commercial preparation II at retention time 12.8 min and LXA₄ at retention time 14.1 min. (C) Peak purity analysis of commercial preparation II peak. (D) Peak purity analysis of authentic LXA₄.
Table 1. Crossreactivity of 15-epi-LXA₄ antiserum to lipoxins and related eicosanoids.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ value</th>
<th>Crossreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-epi-LXA₄</td>
<td>50 pg</td>
<td>100%</td>
</tr>
<tr>
<td>15-epi-LXA₄ methyl ester</td>
<td>60 pg</td>
<td>83.0</td>
</tr>
<tr>
<td>LXA₄</td>
<td>8 ng</td>
<td>0.63</td>
</tr>
<tr>
<td>LXB₄</td>
<td>40 ng</td>
<td>0.13</td>
</tr>
<tr>
<td>15-βنشر-methyl LXA₄</td>
<td>20 ng</td>
<td>0.25</td>
</tr>
<tr>
<td>15-epi-LXB₄ methyl ester</td>
<td>30 ng</td>
<td>0.17</td>
</tr>
<tr>
<td>5S-HETE</td>
<td>20 ng</td>
<td>0.25</td>
</tr>
<tr>
<td>12S-HETE</td>
<td>&gt;100 ng</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>15S-HETE</td>
<td>40 ng</td>
<td>0.13</td>
</tr>
<tr>
<td>15R-HETE</td>
<td>4 ng</td>
<td>1.25</td>
</tr>
</tbody>
</table>

IC₅₀ values represent the amount of the compound required to displace 50% of the maximum binding with the HRP-linked 15-epi-LXA₄. The crossreactivity of the antiserum to an individual compound was expressed as (IC₅₀ value of 15-epi-LXA₄ / IC₅₀ value of the compound) × 100%.
Table 2. LXA₄ and 15-epi-LXA₄ MS/MS daughter ion relative intensities

<table>
<thead>
<tr>
<th>MS/MS daughter ion (m/z)</th>
<th>LXA₄ intensity</th>
<th>15-epi-LXA₄ intensity</th>
<th>LXA₄/15-epi-LXA₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>333</td>
<td>80.8 ± 4.3</td>
<td>96.4 ± 2.8*</td>
<td>0.8</td>
</tr>
<tr>
<td>315</td>
<td>44.9 ± 4.8</td>
<td>51.1 ± 3.7</td>
<td>0.9</td>
</tr>
<tr>
<td>307</td>
<td>100.0 ± 0.0</td>
<td>97.7 ± 2.8</td>
<td>1.0</td>
</tr>
<tr>
<td>289</td>
<td>73.7 ± 2.6</td>
<td>79.3 ± 6.3</td>
<td>0.9</td>
</tr>
<tr>
<td>271</td>
<td>19.2 ± 3.2</td>
<td>25.2 ± 4.9</td>
<td>0.8</td>
</tr>
<tr>
<td>251</td>
<td>69.2 ± 2.0</td>
<td>80.2 ± 2.4*</td>
<td>0.9</td>
</tr>
<tr>
<td>235</td>
<td>74.2 ± 9.1</td>
<td>92.5 ± 3.9</td>
<td>0.8</td>
</tr>
<tr>
<td>233</td>
<td>38.7 ± 6.5</td>
<td>44.6 ± 1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>215</td>
<td>4.4 ± 1.0</td>
<td>9.9 ± 0.3*</td>
<td>0.4</td>
</tr>
<tr>
<td>207</td>
<td>13.3 ± 2.0</td>
<td>13.6 ± 3.3</td>
<td>1.0</td>
</tr>
<tr>
<td>189</td>
<td>27.3 ± 1.8</td>
<td>36.8 ± 4.1</td>
<td>0.7</td>
</tr>
<tr>
<td>135</td>
<td>4.9 ± 1.4</td>
<td>7.0 ± 2.8</td>
<td>0.7</td>
</tr>
<tr>
<td>115</td>
<td>65.0 ± 2.4</td>
<td>76.5 ± 4.4*</td>
<td>0.9</td>
</tr>
</tbody>
</table>

MS/MS spectra of LXA₄ and 15-epi-LXA₄ standards were measured with a Finnigan LCQ (see "Methods"). Intensity values represent the mean MS/MS daughter ion relative intensities ± SEM from n = 3 (* p < 0.05, two-tailed Student’s t-test).
Figure Legends

Fig. 1 Specificity of 15-epi-LXA₄ antiserum and direct comparison with LXA₄ antiserum.

15-epi-LXA₄ (1-1,000 pg), several lipoxin analogs (A) or individually related HETEs (B) were incubated with standard amounts of HRP-labeled 15-epi-LXA₄ and 15-epi-LXA₄ antiserum in 96-well plates precoated with goat anti-rabbit IgG as described in “Methods”. On the vertical axis, % B/B₀ represents the bound enzyme activity in the presence (B) of unlabeled compound relative to its absence (B₀). All data are expressed as the mean ± SE of the mean (SEM) from n=3.

Fig. 2 LC-MS/MS of synthetic LXA₄ and 15-epi-LXA₄.

Mass spectra of synthetic LXA₄ (A) and 15-epi-LXA₄ (C), and MS/MS analysis of LXA₄ (B) and 15-epi-LXA₄ (D) was performed with a Finnigan LCQ LC/MS/MS as described in “Methods”.

Fig. 3 Generation of 15-epi-LXA₄ by activated PMN.

Human PMN (10 x 10⁶) were incubated with 5 μM of A23187 at 37°C for 30 min in the presence of 15R-HETE (10 μM) or vehicle alone. For the kinetics of 15-epi-LXA₄ production (inset), incubations were collected at indicated time points and cells were separated from supernatant by rapid microcentrifugation. Incubations were terminated by adding two volumes of ice-cold methanol and the samples were purified by extraction with solid phase C18 cartridges. Serial dilutions of the samples were incubated with standard amounts of HRP-labeled 15-epi-LXA₄ and 15-epi-LXA₄ antiserum in 96-well plates for ELISA analysis as described in "Methods". The values are expressed as the mean ± SEM from n=3. Statistical difference was obtained (* p<0.01) by comparing values from incubations with stimulated PMN alone and with stimulated PMN plus 15R-HETE.

Fig. 4 15-epi-LXA₄ generation by costimulation of human PMN and THP-1 cells.

THP-1 cells (10 x 10⁶) were incubated with LPS (1 μg/ml) for 16 h and treated with ASA (300 μM) for 20 min prior to coincubation with or without PMN at a cell ratio of 1:6 (THP-1:PMN) for 30 min at 37°C in the presence of A23187 (5 μM) and AA (20 μM). The coincubations were performed at different cell ratios in the presence or absence of AA (20 μM) or A23187 (5 μM)
The incubations were terminated and the samples were extracted for ELISA analysis as in Fig. 1. The values are expressed as the mean ± SEM from n=3. Statistical differences were obtained when compared to values from incubations at a cell ratio of 1:0 (*p<0.01, **p=0.01) and compared to values from incubations at a cell ratio of 1:6 with vehicle alone (+p < 0.01, ++p=0.04).

**Fig. 5 PGHS-2 is expressed by THP-1 cells and mouse peritoneal lavage cells:**
Western blot analysis.

Mice were injected with LPS (1.25 mg/kg body weight). THP-1 cells were treated with LPS (1 μg/ml) at 37°C. Sixteen hours later, the THP-1 cells and cells from peritoneal lavage were collected and suspended in lysis buffer. The whole cell lysates (20 μg/lane) were subjected to SDS-PAGE and transferred to PVDF membrane by electro blotting. The blot was probed using an anti-human PGHS-2 polyclonal antibody (1,100 dilution) and developed as described in "Methods". **Lane 1** contains cell lysates from untreated peritoneal lavage and **Lane 2** the cell lysates from LPS-treated peritoneal lavage. **Lane 3** contains the untreated THP-1 cells and **Lane 4** the LPS-treated THP-1 cells. Molecular weight markers are indicated by arrows.

**Fig. 6 Peritoneal inflammatory exudates from ASA-treated mice generate 15-epi-LXA₄.**

Mice were injected with LPS (1.25 mg/kg body weight) to induce PGHS-2 and 16 h later they were treated with vehicle or (a) one bolus dose of ASA (0.125 g/kg body weight) by intraperitoneal injection for 30 min prior to casein-initiated induction of neutrophil infiltration for 4 h. (b) Consecutive doses of ASA were given in a separate group of animals that received ASA (0.125 g/kg body weight) by intraperitoneal injection 30 min prior to casein injection and before sacrificing the mice. The lavage exudates were collected and stimulated with 5 μM of A23187 (30 min, 37°C). The values are expressed as the mean ± SEM from three independent experiments. The values from both treatment groups were statistically different (*p=0.05 for group a, **p=0.03 for group b) from those obtained with animals that did not receive ASA (vehicle). The samples were extracted as described in "Methods". BW, body weight; IP, intraperitoneal injection.
Fig. 7 Indomethacin but not 17-ODYA inhibits 15-epi-LXA₄ generation by mouse peritoneal exudates.

Mice were injected with LPS (1.25 mg/kg body weight). Sixteen hours later, they were treated with ASA (0.125 g/kg body weight) and 5 mg indomethacin, ASA (0.125 g/kg body weight) and 0.7 mg 17-ODYA or ASA alone prior to induction of neutrophil infiltration by intraperitoneal injection of casein. The peritoneal lavage samples (18 x 10⁶ PMN/mouse) were incubated further with 300 μM of ASA and indomethacin, 300 μM ASA and 5 μM 17-ODYA or ASA alone and stimulated with A23187 (5 μM) as described in "Methods". The reactions were stopped and the samples were extracted. The values are expressed as the mean ± SEM from three independent experiments. Values for indomethacin plus ASA treatment were statistically different from ASA treatment alone (*p < 0.01) and ASA plus ODYA (+p=0.02). BW, body weight; IP, intraperitoneal injection

Fig. 8 Proposed scheme for generating 15-epi-LXA₄ by murine inflammatory exudates.

Arachidonic acid is converted to 15R-HETE predominantly by ASA-acetylated PGHS-2 in macrophage and epithelial cells. Cytochrome P450 might have only a minor contribution to 15R-HETE generation in the mouse. 15R-HETE then undergoes transcellular conversion by 5-LO in PMN to 15-epi-LXA₄ via a 15-epi-5(6)-epoxytetraene intermediate.
Index Terms:

aspirin

inflammation

lipid mediators
(A) MS of LXA₄ at retention time: 14.7 min

(B) MS/MS of LXA₄ \([\text{M-H}]^- (m/z \ 351)\)
Experimental Timelines:

(a) one bolus dose of ASA

(b) consecutive doses of ASA

ASA administration
Experimental Timelines:

(1.25 mg/kg BW)
LPS

+/- Indo  2% Casein (IP)  Harvest peritoneal lavage:
+/- Indo (5 min)
+/- 17-ODYA, +ASA (5 min)

+ ASA (0.125 g/kg BW)
+/- 17-ODYA

---

15-epi-LXA₄ (ng/5 ml lavage of each mouse)

<table>
<thead>
<tr>
<th></th>
<th>ASA</th>
<th>Indomethacin</th>
<th>17-ODYA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>9</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>
Inflammatory Exudate

Monocytes / Macrophages  ASA  PGHS-2  Epithelial cells  Cytochrome P450

Vascular endothelial cells  inducible  Major  Minor

15R-HETE  Transcellular Biosynthesis

5-LO

Leukocytes  (PMN)

15-epi-LXA₄