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Use of a Retroviral Vector for Expression of a Foreign Gene

Brent W. Miller and Aubrey R. Morrison

1. Introduction

Expression of foreign proteins in both cultured and intact mammalian cells has become essential to evaluating cellular biology and physiology including the proteins involved in the control of eicosanoid production. Many transient expression vectors are commercially available and are in widespread use. However, some cell types, such as the renal mesangial cell, have been difficult to transform with these vectors and require a different strategy to obtain stable expression of the exogenous gene. Viral vectors, including retroviruses, adenoviruses, and the Sinbis virus have been developed over the last decade for foreign gene expression in mammalian cells. This chapter will discuss the use of a replication-incompetent retroviral vector in cell culture to express an exogenous protein.

The molecular biology of retroviruses and retroviral vectors has been exhaustively reviewed elsewhere ***(1–3)***. The sophistication of these vectors and their use continue to evolve at a rapid pace including the recent description of retroviral vectors that contain a tetracycline-responsive promoter that allows investigation of proteins that may have a deleterious effect upon the cell ***(4,5)***.

The successful widespread use of retroviral vectors has relied on two advances over the last decade: the ability to determine and remove the *trans*-acting sequences from the genome (*gag, pol,* and *env* genes) while leaving the *cis*-acting sequences intact (viral long terminal repeat—the LTR—and the retroviral packaging signal). A typical wild-type virus and a replication-incompetent retroviral vector are diagrammed in **Fig. 1**; the development of "packaging cells" that provide the structural proteins deleted above (*gag, pol,* and *env*) and allow generation of a replication-incompetent viral particle with-

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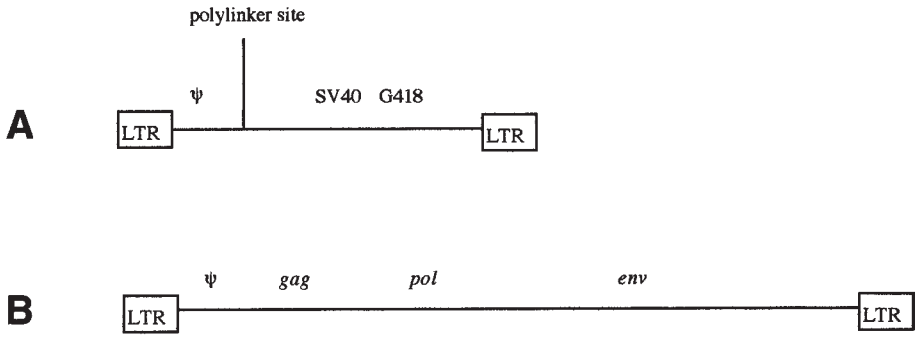

Fig. 1. Simplified schematic of **(A)** retroviral vector pLXSN and **(B)** a typical wild-type retrovirus. Note that the vector has all of the structural genes deleted and that the LTR are also mutated, thus making formation of a wild-type virus by recombination difficult, especially with the use of packaging cells rather than helper virus. The inserted gene in (A) is driven by the LTR promoter, whereas the G418 resistance is driven by the SV40 promoter y is the packaging signal necessary to package the RNA genome into a viral particle. The vector is contained within a bacterial plasmid conferring ampicillin resistance for easy manipulation.

out using a helper virus thus minimizing the risk of recombination creating a wild-type retrovirus ***(6).*** The use of packaging cells and an overview of the entire process are outlined in **Fig. 2**.

This chapter will provide a basic in vitro method for expressing a foreign protein in mammalian cell cultures ***(7)***. For whole tissue or animal expression, other sources are available ***(1,8–10)***.

2. Materials

2.1. Tissue Culture Supplies

1. Tissue Culture Hood with Biosafety Level II.
2. Six centimeter, 10 cm, 6-well, 12-well, and 24-well tissue culture dishes.
3. Fetal calf serum.
4. 0.45 µm syringe filters.
5. Sterile 10-cm syringes and 21-gage needles.
6. G418 or appropriate selection antibiotic.
7. Cell culture-grade thymidine, hypoxanthine, and methotrexate (for PA317 packaging cells) or other appropriate compounds for packaging cell selection determined by the specific packaging cell line chosen.
8. Sterile phosphate-buffered saline (PBS).
9. Sterile trypsin-EDTA (0.05%/0.02%).
10. Standard tissue culture media (Life Technologies' Dulbecco's modified eagle medium [DMEM] for fibroblasts, Life Technologies' RPMI-medium 1640 for mesangial cells).

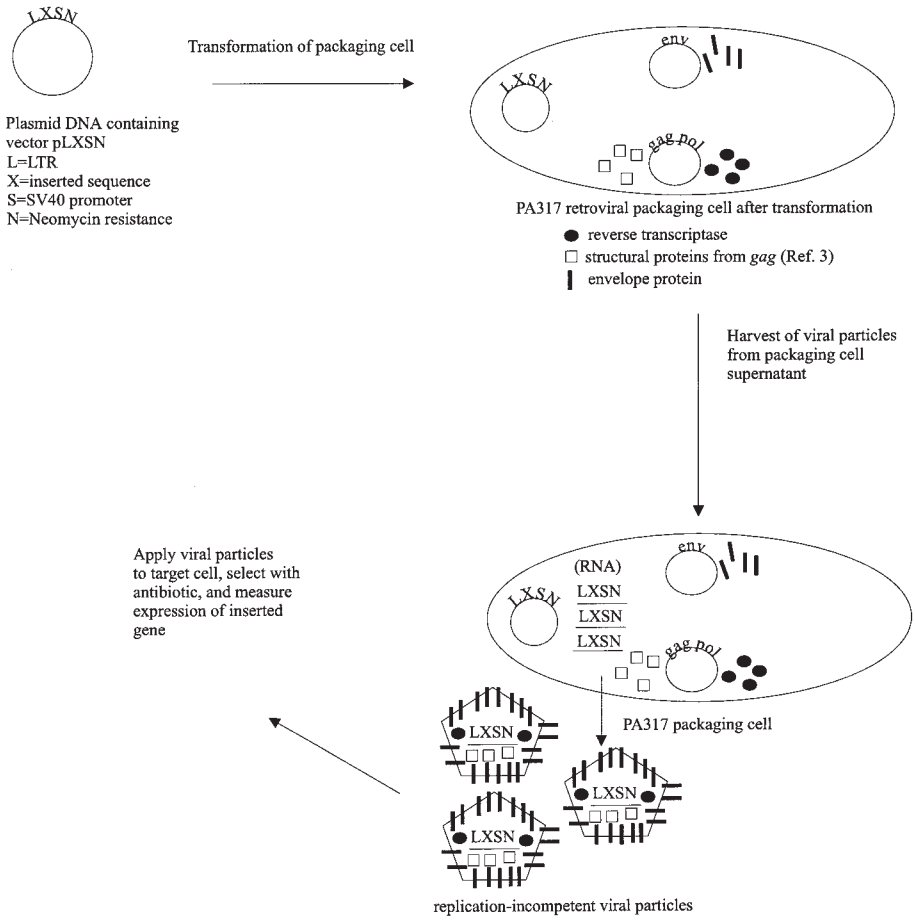

Fig. 2. Simplified diagram of use of a retroviral vector (pLXSN) to express an exogenous gene. Note that the viral RNA genome will be transcribed (by reverse transcriptase) and integrated into the host cell genome. All daughter cells will thus have the vector sequences.

2.2. Retroviral Vector

Many vectors are available with different promoters (LTR, CMV, SV40, and so on) and antibiotic resistance genes (G418, hygromycin, methotrexate, and so on). Most vectors allow expression of only one inserted gene because of size constraints of the genome and the difficulty with sustained reliable expression of the second gene, although some vectors now contain an internal ribosome entry site (IRES) sequence in an attempt to allow expression of two inserted genes in addition to the antibiotic resistance gene. Vector pLXSN developed

by A. D. Miller has been successfully used by many investigators including our laboratory *(7)* and is described in **Fig. 1**.

2.3. Retroviral Packaging Cell Line

In general, these are fibroblast cell lines containing episomal DNA encoding the structural proteins necessary to produce and package a replication-incompetent viral particle (**Note 1**). Many different cell lines are available (*see* http://www/atcc/org for details). The packaging cell line described in these experiments is PA317 (ATCC CRL-9078).

2.4. Materials Needed for Vector Construction and DNA Transformation (Note 2)

1. 2 *M* $CaCl_2$.
2. 2X HEPES-buffered saline, also called 2X HBS (280 m*M* NaCl, 10 m*M* KCl, 1.5 m*M* $Na_2HPO_4 \cdot 2H_2O$, 12 m*M* dextrose, 50 m*M* HEPES) stored in aliquots at –20°C.
3. Sterile glycerol.
4. Sterile vacuum grease.
5. Sterile glass cloning rings.
6. Appropriate restriction enzymes, modifying enzymes, and buffers.
7. Agarose, electrophoresis equipment, and photographic supplies.
8. Polybrene 0.8 mg/mL in PBS stored at –20°C.

2.5. Materials for Freezing Cells

1. Cell culture grade DMSO.
2. Cryogenic vials and markers.
3. Access to a –70°C freezer.
4. Liquid nitrogen storage facility.
5. Fetal calf serum.
6. Standard cell culture media determined by cell lines.

3. Methods

3.1. Construction of Retroviral Vector and Specific Packaging Cell Line

1. Clone gene of interest into polylinker site in vector and confirm orientation and sequence by restriction mapping or sequencing (*see* **Note 3**). Isolate and purify DNA (at least 10 µg will be needed for transformation).
2. Grow packaging cells to approx 50% confluence on 6-cm tissue culture dish. Each dish will require 10 µg of vector DNA.
3. Add 130 µL 2 *M* $CaCl_2$ to 20 µg of vector DNA, add sterile ddH_2O to a total volume of 1000 µL in a 1.5-mL microcentrifuge tube, and vortex.
4. Place 1 mL of 2X HBS in a 15-mL sterile conical tube. Add DNA-$CaCl_2$ mixture from **step 3** to 2X HBS dropwise at 1 drop/s while continuously gently vortexing. Incubate at room temperature for 30 min (**Note 4**).

5. Rinse cells twice with sterile PBS.
6. Add 1 mL DNA-HBS mixture to cells dropwise to each of two 6-cm plates containing the rinsed packaging cells. Incubate cells for 10 min at room temperature (**Note 5**). Add 3 mL media (DMEM with 10% fetal calf serum) and incubate at 37°C for 4 h (**Note 6**).
7. Glycerol shock of cells: Aspirate media from the dish and rinse twice with PBS. Add 0.9 mL 15% glycerol in 1X HBS and incubate for 2.5 min at 37°C. Aspirate glycerol-HBS and add 5 mL media and incubate overnight at 37°C.
8. Let the packaging cells grow to confluence (24–48 h). Change the media if necessary.
9. Split the cells with trypsin-EDTA and dilute 5:1 to 10:1 and replate in media containing G418 (500–1000 µg/mL) or the appropriate selection antibiotic (**Note 7**).
10. Let the cells grow for 1–2 wk at 37°C while changing the media approx every 72 h.
11. When colonies appear (**Note 8**), isolate individual clones with autoclaved glass cloning rings and sterile technique.
12. Mark the bottom of the tissue culture dish (on the outside) where the colonies are. Aspirate the media and rinse twice with PBS.
13. Place one end of the cloning rings in sterile vacuum grease and then place over the colonies. Add 100 µL trypsin-EDTA in cloning ring, incubate until cells lift off, and aspirate onto one well of 12- or 24-well plates containing the appropriate media.
14. Expand the individual clones and select a high-titer producing clone (**Note 9**).

3.2. Isolation of Replication-Incompetent Viral Particles and Expression in Target Cells (Note 10)

1. Grow the packaging cell line with the appropriate vector and control vectors from above to confluence on 6- or 10-cm tissue culture plates.
2. Remove media and rinse with PBS twice.
3. Add 1–5 mL of normal media without G418 (**Note 11**).
4. Incubate the cells for 12–24 h at 32 or 37°C (**Note 12**).
5. Remove supernatant with sterile needle and syringe and filter with 0.45 µ filter (**Note 13**). Dilute with normal media 1:3.
6. Add polybrene to supernatant at a final concentration of 8 µg/mL (**Note 14**).
7. Place 1–5 mL of supernatant on a 50% confluent dish of the target cell (**Note 15**). New supernatant containing fresh virus can be added at 12 h and may improve yields.
8. Grow overnight at 37°C.
9. Remove polybrene-containing media within 24 h and add media containing G418 (500–1000 µg/mL) or appropriate antibiotic at 24–72 h depending on cell type and doubling time.
10. Grow cells at 37°C until confluent and test for expression of inserted gene by immunoblotting (**Note 16**).

3.3. Storing Packaging Cells

1. Grow cells to approx 75% confluence on a 10-cm tissue culture dish.
2. Rinse twice with PBS.

3. Add trypsin-EDTA to remove cells from the plate. Add trypsin-EDTA-cell suspension to media containing serum to inactivate the trypsin.
4. Centrifuge cells in 50-mL tube for 5 min at 200*g*.
5. Remove supernatant, rinse with 10 mL media, and centrifuge again.
6. Remove supernatant and resuspend cells in 1 mL ($3–5 \times 10^6$ cells/mL) freezing media (20% fetal calf serum, and 8% DMSO in DMEM). Place in 2-mL cryogenic vials.
7. Place in –70°C freezer overnight in isopropanol-containing freezing device or alternatively in a styrofoam container wrapped in diapers (**Note 17**).
8. Place in liquid nitrogen storage the next day.

3.4. Thawing Packaging Cells

1. Remove from liquid nitrogen and quickly thaw in 37°C water bath.
2. Place in 10-cm tissue culture dish and add 9 mL regular media and incubate at 37°C.
3. Remove media 4–8 h later and replace with fresh media and incubate at 37°C (**Note 18**).

4. Notes

1. Two points need to be considered about the packaging cell line chosen. First, the ability of a viral particle to infect a cell is determined by its envelope protein, which must bind to a cell surface protein on the target cell. Thus, the host range for the vector is determined by the packaging cell and not the vector itself. Two types of packaging cells are described: ecotrophic and amphotropic. Ecotropic packaging cells will only produce viral particles capable of infecting the same cell type (typically murine with rat occasionally). Amphotrophic packaging cells will produce a viral particle capable of infecting a much broader host range including many mammalian cells. Second, the structural proteins are encoded on plasmids and thus are not completely stable. They should be selected in appropriate media on a monthly basis to keep the packaging cells functioning properly.
2. In order for the viral particle to be produced, the vector DNA with the desired foreign gene in place must be placed in the packaging cell line. Any mode of transformation can be used including calcium-phosphate, electroporation, of lipofectin. The calcium-phosphate protocol is included because it is inexpensive and generally successful.
3. The presence of viral LTRs in mammalian cells can cause abnormalities in gene expression that may not be related to the gene inserted in the vector; thus, several controls should be used ***(11)***. In addition to the normal cell without any vector, an "empty" vector without any insert and a "reverse-orientation" vector with the gene cloned in nonfunctionally should be constructed. This should provide adequate controls.
4. This solution should turn somewhat cloudy.
5. The success of this technique depends upon a fine DNA precipitate that can be visualized under a phase contrast microscope at this step and should not look like large clumps.

6. Remember that these cells are going to be grown for several weeks, so sterile technique is critical to success. Our incubators are dedicated to tissue culture (no bacteria, no yeast) and use 5% CO_2.
7. In our laboratory, G418 from multiple commercial sources has been extremely effective at lower concentrations (500 µg/mL for selection and 125–250 µg/mL for maintenance). This is important because G418 can be toxic to some cells (e.g., renal mesangial cells) and alter morphology even in the presence of the resistance gene. In nonbuffered media, G418 can also lower the pH by approx 0.10. Methotrexate can also cause alter characteristics of cells despite the presence of the resistance gene.
8. The colonies are clumps of cells derived from a single cell that has been transformed by the vector DNA (doubling time of fibroblasts is approx 18 h). The colonies are best visualized by the naked eye by temporarily aspirating the media and tilting the dish. Alternatively, they can be seen under a phase contrast microscope.
9. If the eventual target cells are hardy and easy to infect with the replication-incompetent particle (e.g., the typical fibroblast in prolonged cell culture) and the investigator will be satisfied with an efficiency of approx 10%, packaging cells do not necessarily need to be clonally selected and titered to obtain the highest titer-producing clone; however, one must realize this is a shortcut and may have consequences a later date. One reference ***(2)*** even suggests that virus can be harvested almost immediately after the vector transformation of the packaging cell though results with that method have been disappointing in our laboratory. The easiest way to titer the virus is to infect normal 3T3 fibroblasts as described in **Note 11** and express the titer as colony-forming units (cfu)/mL of supernatant. Other methods can be utilized ***(2)***. To obtain a high-titer clone, 10–60 colonies usually will need to be screened. Remember that the packaging cells now contain both the new vector and the previous plasmids containing genes encoding the viral structural proteins and other drug resistance genes (**Fig. 2**). Thus, low concentrations of the antibiotic that the vector encodes resistance to (in the example of pLXSN, it is G418) will need to be maintained and intermittent (approximately monthly) selection for 5 d of the antibiotic encoded by the plasmids containing the retroviral structural enzymes (in the example of PA317 cells this is methotrexate with hypoxanthine and thymidine—HAT media) will need to be performed. The precise concentrations of antibiotic and optimization of timing of administration will vary between different vectors and packaging cells. Retroviral vectors and packaging cells continue to evolve at a rapid pace and consultation with a person who has had success with the particular vector or packaging cell saves time and money.
10. Production of a wild-type virus is always a concern, but the advent of packaging cells that eliminate the need for helper-virus has markedly diminished the possibility of that event. Another possibility is the production a wild-type virus by recombination with an endogenous retrovirus. To minimize this risk, most current retroviral vectors have other mutations introduced in their genome and would require multiple recombinatorial events for this to happen.

11. Usually one-half (2.5 mL for a 6-cm plate, 5 mL for a 10-cm plate) of the normal amount of media is used to harvest the virus, but this can be experimented with to find the best volume. This supernatant can be further diluted 1:3 to place on the target cells. It is critical that the media added to the packaging cell line at this point is the same media used by the target cell. The host cell must be able to replicate in order for the viral genome (now containing the inserted gene and antibiotic resistance gene) to insert into the target cell's genome.
12. Some authors have found better viral particle production at 32°C.
13. Alternatively, the supernatant can be centrifuged to isolate viral particles ***(2)***. Viral particles have a short half-life (3–6 h); therefore, the supernatant must be used immediately. Some authors have had success at freezing or lyophilizing supernatants for later use but personal experience has shown at least a 10-fold diminution in effectiveness.
14. This step is controversial because polybrene is toxic to cells but allows for better virus–cell interaction. This step can be omitted if toxicity develops. It is key to not leave the polybrene on the cells for a long period of time (>24 h).
15. A fundamental property of the target cell must be that is actively replicating for a retroviral vector to work—this is how a retrovirus integrates into the host genome. Thus, any conditions that may enhance replication during the time the virus is added may improve results.
16. Because the viral genome is inserted into the host genome, polyclonal groups of cells are preferred by our laboratory to further minimize the possibility, although remote, of the process of insertion of the viral sequence into the host genome causing a biological effect rather than the gene of interest (this has not been a problem in our laboratory, but always seems to be raised when data is presented). The expression of the foreign protein is variable and if a distinct level is needed (say 40 times baseline instead of four times baseline) then isolating specific clones may be useful. To determine the presence of the control vectors is more problematic. Stable survival through antibiotic selection has been our method if the protein of interest can be demonstrated in the cells infected with the appropriate vector. Measuring production of a virus with helper virus is cumbersome. Isolating the RNA genome from the supernatant of packaging cell cultures and analyzing it by gel electrophoresis is now possible with rapid viral genome isolation kits that are in use clinically to isolate viral RNA from clinical samples (QIA-amp, Qiagen, Valencia, CA), but this only shows the appropriate viral genome and not the presence in the control cells.
17. The goal is to slowly lower the temperature of the cells by 0.5–1 degree/min.
18. The DMSO must be removed as quickly as possible and 4–8 h should allow the cells to attach to the dish. Many cells will not survive the thaw.

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2

Extraction of Eicosanoids from Biological Fluids, Cells, and Tissues

William S. Powell

1. Introduction

Many analytical methods for eicosanoids require extraction of these substances from biological fluids or media prior to analysis, and even when not essential, extraction can be used to increase both the specificity and sensitivity of assays. Extraction and chromatography of eicosanoids and other solutes depends on their partitioning between two phases that differ in polarity. Although all eicosanoids contain the negatively charged carboxylate anion, they are relatively hydrophobic molecules as a result of the presence of the 20 carbon chain. Exceptions to this are the cysteinyl-leukotrienes (cys-LTs), which have positively charged amino groups. Thus the partitioning of leukotrienes C_4, D_4, and E_4 and their metabolites between these two phases differs from that of other eicosanoids. This is reflected by different behavior of cys-LTs compared to other eicosanoids when they are subjected to extraction and chromatographic analysis ***(1)***. It should be noted, however, that *N*-acetyl-LTE_4, a major metabolite of cys-LTs in the rat, does not contain a free amino group, and thus behaves more like noncysteine-containing eicosanoids.

The traditional method for the extraction of eicosanoids involved extraction from acidified aqueous media into organic solvents such as diethyl ether or ethyl acetate. However, this method is time consuming and can be complicated by the formation of emulsions, making it difficult to separate the two phases. To circumvent these problems we developed methods for the solid-phase extraction of eicosanoids on columns containing the hydrophobic stationary phase octadecylsilyl-silica (ODS-silica). This approach can be used either to obtain extracts containing a mixture of various eicosanoids ***(2,3)*** or can be

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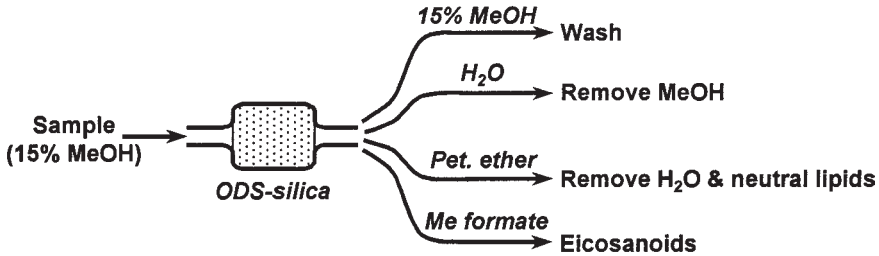

Fig. 1. Scheme illustrating the extraction of eicosanoids (not including cys-LTs) on an ODS-silica extraction cartridge, such as a C_{18} Sep-Pak. The sample is loaded onto the cartridge in 15% aqueous methanol, and the cartridge is washed with 15% methanol, water, and petroleum ether (pet. ether). Eicosanoids are then eluted with methyl formate. For extraction of samples containing cys-LTs, *see* **Subheading 3.1., step 5**.

coupled to reversed-phase high-pressure liquid chromatography (RP-HPLC) for the analysis of eicosanoids ***(4)***.

Eicosanoids can be extracted from biological fluids or media using cartridges containing ODS-silica (C_{18} Sep-Paks) ***(2,3)***. This method takes advantage of the fact that the manufacturing procedure employed to prepare certain types of ODS-silica does not result in the complete removal of all of the polar sites on the stationary phase, which thus retains some residual polar character. In this procedure the sample is loaded onto the stationary phase in medium containing a low percentage of methanol in water and eicosanoids are retained by a reversed-phase mechanism. Instead of eluting the eicosanoids with a higher concentration of methanol or acetonitrile, they are eluted by a water-immiscible organic solvent, methyl formate, after the cartridge has been washed with water and petroleum ether (**Fig. 1**). Unlike the adsorption process, elution of the sample appears to involve a normal-phase mechanism, since the eluting power of organic solvents is in the order of their polarity rather than their hydrophobicity (i.e., methyl formate > diethyl ether > chloroform > hexane) ***(3)***. It should be noted that this procedure should not be employed for the extraction of cys-LTs. Instead, it is possible to elute these substances by using pure reversed-phase elution (i.e., by increasing the concentration of the water-miscible component of the solvent [e.g., methanol]).

There are a number of advantages of solid-phase extraction over the traditional method of organic solvent extraction. This procedure is rapid, and the resulting extract is obtained in a small volume of a highly volatile solvent that can be readily evaporated under a stream of nitrogen. Furthermore, many contaminating materials are removed, thus reducing the possibility of interference in subsequent analytical procedures. The procedure is also economical, since the cartridges can be washed and reused several times. This technique is particularly useful for extraction of large amounts of media as in preparative

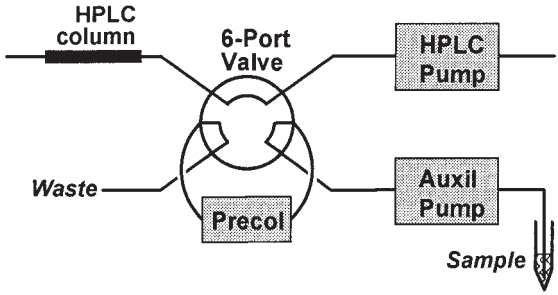

Fig. 2. Scheme illustrating the manual technique for the analysis of eicosanoids by precolumn extraction/RP-HPLC. The 6-port valve is shown in the "load" configuration in which the sample is being pumped onto the precolumn by the auxiliary pump. At the same time, the analytical column is being equilibrated by the HPLC pump.

experiments since the capacity of the ODS-silica is quite high and it is possible to concentrate the extract in a relatively small volume.

Another important advantage of solid-phase extraction is that it can be combined directly with RP-HPLC. Most of our current applications involve either direct analysis by HPLC, using UV/visible and/or radioactivity detectors, or combine HPLC with immunological techniques for the analysis of cys-LTs. Thus, we do not often use the solid-phase extraction techniques described in the previous paragraph, except in experiments designed to prepare relatively large amounts of eicosanoids for further study. Instead, biological samples are extracted on a precolumn cartridge (i.e., guard column) containing ODS-silica (**Fig. 2**). Substitution of such a precolumn for the loop of a conventional six-port injector permits the in-line extraction of a sample that is pumped onto the precolumn using an auxiliary pump with the injector in the "load" position. Once the sample has been loaded onto the precolumn and the latter is washed, the injector is switched into the "inject" position to initiate the chromatographic separation. This is an extremely useful procedure, since it can readily be automated (**Fig. 3**), reducing sample manipulation to a minimum and eliminating the requirement for manual extraction and evaporation of the extract. Because the procedure is very simple and requires few steps, the possibility for problems to arise owing to contamination or faulty technique is minimized. This method serves equally well for both cys-LTs and nonamino acid-containing eicosanoids.

2. Materials and Equipment

2.1. Solid-Phase Extraction of Eicosanoids

1. Stationary phase (ODS-silica): Waters C_{18} Sep-Pak cartridges (Sep-Pak Classic cartridges, part number 51910 or Sep-Pak Plus cartridges, part number 20515).
2. Glass syringes: 10 and 20 mL.

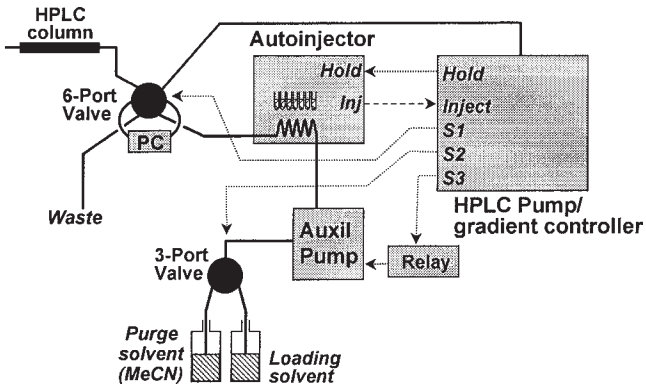

Fig. 3. Scheme illustrating the automated analysis of eicosanoids by precolumn extraction/RP-HPLC. The sample is pumped by the auxiliary pump from the sample loop in the autoinjector onto the precolumn connected to the 6-port valve while the analytical column is being equilibrated. The 3-port valve (or equivalent) controls the solvent being pumped by the auxiliary pump. S1, S2, and S3 are three timed-event switches on the gradient controller, which send on/off signals to the 6-port valve, the 3-port valve, and the relay, respectively. The autoinjector sends an "inject" signal to the gradient controller when its sample loop is placed in line with the auxiliary pump and the precolumn. The "hold" signal from the gradient controller to the autoinjector prevents the autoinjector from injecting further samples in the event of a power failure.

3. Solvents required for the extraction of eicosanoids:
   a. Methanol.
   b. Water (*see* **Note 1**).
   c. 15% Methanol in water.
   d. Petroleum ether (boiling range 38 to 52°C) (*see* **Note 2**).
   e. Methyl formate (Aldrich, 97% pure; redistilled) (*see* **Note 3**).
   f. 80% Ethanol in water.

2.2. Manual Technique for Precolumn Extraction of Eicosanoids Coupled to HPLC

1. Precolumn cartridge: Waters µBondapak C_{18} Guard-Pak inserts (Part number WAT088070) (*see* **Note 4**).
2. Precolumn cartridge holder: Waters Guard-Pak holder (part number WAT088141). For cartridges from other suppliers it will be necessary to use the appropriate cartridge holder.
3. 6-Port valve (e.g., Valco Model C6W) (*see* **Note 5**).
4. Auxiliary pump (*see* **Note 6**).
5. Solvents:
   a. Methanol;
   b. Loading solvent: 15% or 30% methanol containing 2.5 m*M* phosphoric acid;

   c. Purge solvent: acetonitrile;
   d. HPLC mobile phase (any appropriate mobile phase; *see* Chapter 5 and **refs. *4–6***).
6. HPLC system.
7. HPLC column (ODS-silica).

2.3. Automated Procedure for the Precolumn Extraction of Eicosanoids Coupled to HPLC

1. Precolumn cartridge: Waters µBondapak C_{18} Guard-Pak inserts (Part number WAT088070) or equivalent (*see* **Note 4**).
2. Precolumn cartridge holder: Waters Guard-Pak holder (part number WAT088141). For cartridges from other suppliers it will be necessary to use the appropriate cartridge holder.
3. Automated 6-port valve: Rheodyne 2-position 6-port fluid processor (*see* **Note 7**).
4. Automated 3-port valve or equivalent: Rheodyne Solvent Selector (*see* **Note 8**).
5. Autoinjector: Waters WISP or equivalent (*see* **Note 9**).
6. Auxiliary pump: (*see* **Note 6**).
7. Relay: This is required to control the auxiliary pump (*see* **Note 10)**.
8. Autoinjector vials and inserts: (*see* **Note 11**).
9. Solvents:
   a. Methanol;
   b. Loading solvent: 15% methanol containing 2.5 m*M* phosphoric acid;
   c. Purge solvent: acetonitrile;
   d. HPLC mobile phase: any appropriate mobile phase (*see* Chapter 5 and **refs. *4–6***).
10. HPLC system.

3. Methods

3.1. Solid-Phase Extraction of Eicosanoids on Sep-Pak Cartridges

1. Wet the ODS-silica: Prior to use, the ODS-silica in the cartridge should be wetted with an organic solvent, which must then be removed with water. Using a 20-mL glass syringe, apply methanol (10 mL) through the C_{18} Sep-Pak cartridge followed by water (10 mL). For the extraction of cys-LTs, recoveries can be improved by subsequently washing the Sep-Pak with 0.1% EDTA (5 mL) ***(7)***.
2. Preparation of sample: The concentration of methanol in the aqueous sample should be adjusted to 15% (*see* **Note 12**). This will require either the addition of methanol to aqueous samples, or the addition of water to samples from incubations that have been terminated by the addition of methanol. An internal standard can be added at this point if required (*see* **Note 13**).The samples should then be acidified to a pH of approx 3.0 (using pH paper) (*see* **Note 14**). Any particulate matter is then removed by centrifugation of the sample at 2000*g* for 10 min.
3. Apply the sample to the ODS-silica: The sample is applied to the C_{18} Sep-Pak using a 5 to 20-mL glass syringe. The capacity of the Sep-Pak is quite high and we apply up to 50 or 60 mL of sample to a single cartridge, depending on the nature of the experiment (*see* **Note 15**).

4. Wash the ODS-silica with the loading medium: To remove any unadsorbed material from the ODS-silica, the Sep-Pak is washed with 20 mL of water containing the same percentage of methanol as the loading medium (e.g., 15% methanol).
5. Wash the ODS-silica with water and petroleum ether: The Sep-Pak is washed with water (20 mL) followed by petroleum ether (10 mL) (*see* **Note 16**). This and the following step should not be performed for the elution of cys-LTs. These substances will be retained along with other eicosanoids by the ODS-silica, but, owing to their polar character, will not be eluted by methyl formate. After washing the Sep-Pak with 15% methanol, cys-LTs can be eluted with methanol ***(8)*** or 90% acetonitrile in 0.05% acetic acid, buffered to pH 6.4 by addition of ammonium hydroxide (*see* **Note 17**).
6. Elute eicosanoids with methyl formate: Eicosanoids are eluted by passing methyl formate (10 mL) through the Sep-Pak and collecting the eluate in a 15-mL glass tube. The methyl formate is then removed under a stream of nitrogen (*see* **Note 18**).
7. Regenerate the ODS-silica: The Sep-Pak can be reused several times, depending on the type of sample. For extraction of medium containing relatively small amounts of biological material each Sep-Pak can be used up to five times (*see* **Note 19**). However, for preparative experiments, they should be used only once. To regenerate the Sep-Pak, it should be washed with 80% ethanol in water (20 mL) followed by ethanol (20 mL), and water (20 mL) prior to reuse.

3.2. Precolumn Extraction/HPLC (Manual Method)

1. Setup of equipment: In addition to the equipment normally required for HPLC, this method requires only an auxiliary pump. The precolumn (i.e., guard column) normally used for HPLC can be substituted for the sample loop in the 6-port injector used with the HPLC (**Fig. 2**). An additional precolumn is not needed unless the sample injected contains a lot of extraneous material (e.g., urine) (*see* **Note 20**). The dead volume of the inlet and outlet tubing for the auxiliary pump should be not be too large to minimize the time required for the procedure. We normally use polypropylene tubing (id 0.04 in.) for the inlet and stainless steel tubing (id 0.02 in.) to connect the outlet of the pump to the six-port injector.
2. Preparation of sample: The sample is prepared in a manner similar to that for extraction using a Sep-Pak. The concentration of methanol in the sample should be adjusted to give the required amount (e.g., 15%) (*see* **Note 21**). It is not necessary to adjust the pH (we normally inject samples obtained from cell supernatants in physiological media containing added methanol at neutral pH). The medium should not contain an indicator that could interfere with the HPLC. Internal standards can be added at this time (*see* **Note 13**). The sample is then centrifuged. Slight modifications of this procedure can be used for analysis of body fluids such as urine (*see* **Note 22**) or bile (*see* **Note 23**).
3. Equilibration of precolumn: The auxiliary pump is used to pump the loading solvent (15% methanol containing 2.5 m*M* phosphoric acid) onto the precolumn (10 min at a flow rate of 3 mL/min) with the six-port injector in the "load" position. It is essential that the precolumn be properly equilibrated to prevent losses during the extraction procedure (*see* **Note 24**).

4. Loading the sample onto the precolumn: Once the precolumn has been equilibrated the inlet tube is placed in the tube containing the sample and all of the sample is pumped onto the precolumn.
5. Washing the precolumn: The inlet tube of the precolumn is quickly rinsed with a small amount of loading solvent and is then transferred to the loading solvent reservoir. The loading solvent is then pumped through the precolumn for 5 min at a flow rate of 3 mL/min. This is required both to pump the sample remaining in the solvent lines onto the precolumn as well as to wash the precolumn and remove undesirable polar materials.
6. Injecting the sample: The 6-port valve is switched from the "load" position to the "inject" position. This places the precolumn in line with the analytical column. The material adsorbed by the precolumn is eluted by the HPLC mobile phase onto the analytical column. The 6-port valve should be left in the "inject" position until the sample is removed by the mobile phase. This will take considerably less time than the chromatography on the HPLC column, but it will depend on the conditions used for the HPLC (e.g., how steep a gradient is used).
7. Purging the precolumn: Towards the end of the HPLC run or at the time that the HPLC column is being purged, the 6-port valve is turned back to the "load" position and the precolumn is purged with acetonitrile for 5 min at a flow rate of 3 mL/min.
8. Preparation for the next sample: Once the precolumn has been purged, it is equilibrated with the loading solvent (15% methanol) for 5 min as described in **Subheading 3.2.3.** in preparation for injection of the next sample (*see* **Note 24**).

3.3. Precolumn Extraction/HPLC (Automated Method)

1. Setup of equipment: fluid connections: The configuration of the equipment we use in our laboratory for the precolumn extraction/HPLC analysis of eicosanoids is illustrated in **Fig. 3**. With respect to the precolumn extraction step there are four major components: the autoinjector (Waters WISP), the automated 6-port and 3-port switching valves and the auxiliary pump. Solvent is supplied to the auxiliary pump via an automated 3-port valve, enabling the solvent to be switched between the loading solvent and the solvent used to purge the precolumn (acetonitrile). The auxiliary pump is used to pump the sample from the sample loop in the autoinjector onto the precolumn as well as to equilibrate and purge the precolumn. It may be necessary to increase the size of the sample loop of the autoinjector (*see* **Note 9**) The automated 6-port valve is configured exactly as shown in **Fig. 2** for the manual method described above.
2. Setup of equipment: electrical connections: The precolumn extraction/HPLC procedure is regulated by a series of electrical signals from the automatic injector and the gradient controller. In the system shown in **Fig. 3** these signals are as follows:
   a. Inject (autoinjector → gradient controller): initiates the "timed events" program on the gradient controller.
   b. Switch 1 (gradient controller → 6-port valve): positions the 6-port valve in either the "load" or the "inject" position.

c. Switch 2 (gradient controller → 3-port valve): positions the 3-port valve so that the intake for the auxiliary pump is either the loading solvent (e.g., 15% methanol) or the purge solvent (e.g., acetonitrile).
d. Switch 3 (gradient controller → relay): turns the auxiliary pump on or off via a relay switch.
e. Hold (gradient controller → autoinjector; optional): sends a signal to the automatic injector to ensure that it will not continue to inject samples in the event that other components of the system have been shut down by a power failure (*see* **Note 25**).

3. Preparation of samples: The samples are prepared as described for the manual method described above. However, the sample size will be limited by the capacity of the vials used in the autoinjector. With the Waters WISP we use a rack with a capacity for 48 vials of 4 mL each. The capacity of the vials will have to be taken into consideration if incubations are terminated by the addition of methanol, so that the amount does not exceed the concentration of methanol desired to load the sample (15–30%).
4. Procedure: Before starting, it is necessary to ensure that the precolumn has been purged with acetonitrile (5 min at 3 mL/min) and equilibrated with the loading solvent (e.g., 15% methanol; 10 min; 3 mL/min; *see* **Subheading 3.2.3.**). The lines between the solvents and the 3-port valve should be full of the appropriate solvents before starting this procedure (*see* **Note 24**). Once the samples have been loaded into the automatic injector the sequence is initiated by starting the automatic injector, which controls the total time allocated for each run. An example of the timing of events controlled by the automatic injector and the gradient controller for a 30 min chromatographic run is shown in **Table 1**.

4. Notes

1. It is important to purify the water to remove impurities that could accumulate on the Sep-Pak and then be eluted by methyl formate. We normally use water purified using the Milli-Q ultrapure water system (Millipore).
2. It is possible to substitute hexane or petroleum ether of a different boiling point range.
3. Other solvents of similar polarity, such as ethyl acetate can be substituted for methyl formate. However, ethyl acetate is considerably less volatile and will require more time for evaporation.
4. Cartridges containing ODS-silica from other suppliers can also be used. For extraction of eicosanoids from biological fluids, such as urine, it may be necessary to use a cartridge with a higher capacity. For example, for analysis of urinary cys-LTs by precolumn extraction/HPLC/radioimmunoassay we currently use an Adsorbosphere C_{18} precolumn (7.7 × 4.6 mm) (*see* **Note 22**). We have also used a Brownlee RP-18 ODS guard cartridge (15 × 3.2 mm) in a Brownlee NewGuard holder for similar purposes. It is also possible to purchase an unpacked precolumn and pack it manually with ODS-silica.
5. Any 6-port valve can be used. The injection port is simply replaced by the outlet from the auxiliary pump and the sample loop is replaced by the precolumn.

Table 1
Timed Events for Automated Precolumn Extraction/HPLC

Time (min)	Signal source	Action	Comments
Initial	Gr-cont	S1[a]: 6-port valve → "load" S2: 3-port valve → 15% MeOH S3: Auxiliary pump → off	System ready for loading sample onto precolumn
0.00	Autoinj	"Inject" signal received by gradient controller from autoinjector to start run	The autoinjector has finished loading the sample into its injection loop and has placed it in line with the auxiliary pump (*see* **Note 26**)
0.01	Gr-cont	S3: Auxiliary pump → on	The sample is pumped from the autoinjector onto the precolumn and the precolumn is washed with the loading solvent.
6	Gr-cont	S1: 6-port valve → "inject" S3: Auxiliary pump → off	The sample is injected from the precolumn onto the HPLC column (*see* **Note 27**)
33	Gr-cont	S2: 3-port valve → acetonitrile S3: Auxiliary pump → on	Pumps acetonitrile through the autoinjector loop
36	Gr-cont	S1: 6-port valve → "load"	The precolumn is purged (*see* **Note 28**)
41	Gr-cont	S2: 3-port valve → 15% MeOH	The precolumn is equilibrated
47	Gr-cont	S3: Auxiliary pump → off	
50	Autoinj	Inject next sample	The autoinjector starts to transfer the sample from the next vial to the sample loop. The sequence for analysis of the second sample is initiated (*see* **Note 29**)

[a]S1, S2, and S3, refer to three timed event switches on the gradient controller (*see* **Subheading 3.3.2.**). These switches can be programmed to be on, off, or to send a pulse. Note that the precolumn should initially be equilibrated with the loading solvent (15 or 30% methanol). The sequence is initiated by starting the autoinjector. Gr-cont, gradient controller; Autoinj, autoinjector.

6. We have used two types of HPLC pumps as auxiliary pumps. The first is a Milton Roy/LDC minipump which was purchased a number of years ago and appears to be no longer available. The second is a Waters model 501 pump. In principal any

HPLC pump can be used for this purpose. A simple, inexpensive pump is quite adequate, provided it is reliable. It does not have to pump against a very high back pressure and it does not matter if there is considerable pulsation in flow rate as is the case with the single-head Milton Roy minipump. Eldex Laboratories (Napa, CA) manufactures a series of inexpensive pumps that should be suitable for this application (e.g., Models A-60-S, AA-72-S, or CC-100-S).

7. We currently use the 6-port automated switching valve available on the Waters WAVS (Waters Automated Valve Station) for this purpose, but this instrument is no longer available. Instead, a Rheodyne 2-position 6-port fluid processor can be used. Part number PR700-100-01 permits control by contact closure, and can thus be controlled by the Waters gradient programmer. Other versions which can be controlled in different ways (e.g., by an RS232 connection) are also available from Rheodyne.
8. We currently use the 3-port automated switching valve available on the Waters WAVS for this purpose, but as this instrument is no longer available, an alternative will have to be used. The Rheodyne LabPRO Solvent Selector (PR100-105-01) should be suitable for this purpose. Alternatively, it may be possible to use the Rheodyne 2-position 6-port valve described in **Note 7**, using only three of the six available ports. In the latter case, the diameter of the flow passage through the valve is smaller than in the Solvent Selector, so there could potentially be a problem with solvent degassing at higher flow rates.
9. The WISP autoinjector has been modified by increasing the loop size from 2 mL to 4 mL by the addition of stainless steel tubing (1.23 m of 0.04 in. id tubing has a capacity of 1 mL). Although the autoinjector is equipped with a 2500 μL syringe, the syringe size is set to be 250 μL. This will result in delivery of 10 times the set volume into the sample loop. Thus, when the sample volume is set to 360 μL, the volume actually transferred from the sample vial to the injection loop will be 3.6 mL. Other autoinjectors may also be used, but they should be capable of injecting relatively large volumes (e.g., 3–4 mL). We also use a model 231 Gilson autoinjector (Gilson, Middleton, WI), which permits the injection of larger samples, depending on the rack employed. In the configuration we use, samples up to 9 mL can be injected automatically. The model we use requires a certain amount of programming, which can be somewhat time consuming but also provides a high degree of flexibility.
10. A relay is required to turn the auxiliary pump on and off. The auxiliary pump is plugged into an electrical socket controlled by the relay, which is in turn controlled by a switch on the gradient controller.
11. A variety of vials are available from different suppliers. The capacities of the vials vary depending on the design and it is important to use vials that have a sufficient capacity. For the Waters WISP we use shell vials without caps with a capacity of 4 mL. To maximize the proportion of the sample which is injected, a conical insert can be placed in bottom of the vial, or alternatively, a small quantity of glass beads, large enough so as not to block the syringe of the autoinjector, can be used. For the Gilson model 231 autoinjector we use 13 × 100 mm glass tubes with a capacity of 10 mL.

12. For the extraction of polar ω-oxidation products of prostaglandins, the concentration of methanol should be lowered to 5 or 10%. On the other hand, recovery of unmetabolized arachidonic acid is rather low (approx 50–60%) when the sample is loaded onto the Sep-Pak in 15% methanol. The recovery can be improved to over 80% by increasing the concentration of methanol to 30% ***(3)***. The recovery of arachidonic acid using solid-phase extraction is also enhanced by using neutral pH ***(4)***. For analysis of cys-LTs, it is also preferable to load the sample onto the cartridge in 30% methanol ***(6)***.
13. For HPLC analyses we normally use PGB_2 (25–200 ng) as an internal standard ***(9)***. In situations where PGB_2 interferes with some of the peaks of interest, as in the case of leukotrienes derived from 5,8,11,14,17-eicosapentaenoic acid, we add 19-hydroxy-PGB_1 ***(10)*** 19-hydroxy-PGB_2 ***(11,12)***, or 13-hydroxy-9,11-octadecadienoic acid (13-HODE) ***(13)***. For subsequent analysis of eicosanoids by mass spectrometry various deuterium-labeled standards can be added at this time.
14. Neutral pH can also be used, and is recommended for acid-sensitive samples or more hydrophobic compounds, such as arachidonate. If it is required to extract more polar eicosanoids, such as ω-hydroxy metabolites of prostaglandins, the concentration of methanol should be lowered to 10% if neutral pH is used.
15. For example, in preparative experiments designed to isolate relatively large amounts of lipoxygenase products after incubation of arachidonic acid with porcine leukocytes ***(14)***, we pass the sample in 60 mL of 15% methanol (corresponding to 4×10^9 cells) through a single Sep-Pak. In this case, unfractionated porcine leukocytes (75×10^6/mL) are incubated with arachidonic acid and A23187 and the incubations terminated by addition of methanol to give a final concentration of 15% methanol, followed immediately by centrifugation to remove the leukocytes. A 60-mL portion of the supernatant is then acidified and applied to a washed C_{18} Sep-Pak in three 20-mL aliquots using a 20-mL glass syringe. The Sep-Pak is then washed with 15% methanol, water, and petroleum ether, followed by elution of lipoxygenase products with methyl formate (10 mL).
16. The wash with water removes any methanol remaining on the ODS-silica. If this is not done, the subsequent wash with petroleum ether will remove any arachidonic acid and partially remove monohydroxyeicosanoids (HETEs) from the ODS-silica. The petroleum ether removes water from the Sep-Pak, thus reducing the time required to evaporate the solvent from the eicosanoid fraction subsequently obtained by eluting with methyl formate.
17. It should be noted that cys-LTs are rather unstable and readily decompose upon removal of solvents containing unbuffered acids such as acetic acid and especially trifluoroacetic acid. In this case, it is important to add a volatile base, such as ammonium hydroxide or trimethylamine, prior to evaporation of the solvent.
18. Methyl formate is a highly volatile (boiling point 34°C) organic solvent that is not miscible with water. However, it is sufficiently polar to dissolve any water remaining in the Sep-Pak after it has been washed with petroleum ether. This water will remain after most of the methyl formate has been removed. At this

point, the sides of the tube can be washed with ethanol and the remainder of the solvent removed under a stream of nitrogen.

19. For example, for extracts of media from up to 30×10^6 leukocytes in 1 mL we have reused a single Sep-Pak four times (i.e., five times in total).
20. To analyze urinary LTE_4 the sample is loaded onto an Adsorbosphere C18 guard cartridge (7.7 × 4.6 mm) in an All-Guard cartridge holder. In this case, the analytical column is further protected by an Aquapore RP-18 guard column placed between it and the 6-port valve.
21. For cys-LTs we have found that 30% methanol gives better recoveries than 15%. In fact 30% methanol can be used for most eicosanoids normally analyzed ***(6)***.
22. For the analysis of urinary LTE_4 the urine should be adjusted to alkaline pH (9.0), by the addition of sodium hydroxide prior to storage at –70°C. The free radical scavenger 4-hydroxy-TEMPO should also be added prior to storage ***(15)***. After thawing, the sample is centrifuged, filtered, and the pH adjusted to between 5.0 and 6.0. The sample is then loaded onto an Adsorbosphere C_{18} precolumn in an All-Guard cartridge holder (Alltech, Deerfield, IL) that has been equilibrated for 5 min at a flow rate of 3 mL/min with an aqueous solution of 0.1% acetic acid adjusted to a pH of 5.4 by the addition of ammonium hydroxide. In this case, the analytical column is further protected by a guard column placed between it and the 6-port valve. After the sample has been loaded onto the Adsorbosphere precolumn, it is washed with ammonium acetate, prepared as described above, for 2 min at a flow rate of 3 mL/min. The sample is then injected onto the analytical column. Between runs, the precolumn is purged with methanol for 5 min and equilibrated with ammonium acetate as described above.
23. For the analysis of cys-LTs in rat bile ***(16)***, methanol (0.8 mL) is added to 0.2 mL of bile to precipitate any protein. After 30 min the sample is centrifuged at 3500*g* for 15 min and the supernatant is filtered through a 0.22 µm filter. Distilled water is added to give a final concentration of 30% methanol and the pH is adjusted to 3.0 by addition of phosphoric acid. The sample is then loaded onto a precolumn that has been equilibrated with 30% methanol in 2.5 m*M* phosphoric acid as described for urine in **Note 22**. After the sample is loaded, the precolumn is washed for 2 min at a flow rate of 3 mL/min with 30% methanol in 2.5 m*M* phosphoric acid prior to injection of the sample onto the analytical column. Between runs the precolumn is purged with methanol and then equilibrated with 30% methanol as described above.
24. Since the solvent lines are kept in acetonitrile when the system is not in use, it is important to ensure that all of the acetonitrile is removed from the inlet tubing used for the loading solvent (15% methanol). The initial equilibration of the precolumn prior to the first chromatographic run should be for a longer time (10 min) to ensure that all of the acetonitrile is removed. For equilibration of the precolumn between individual runs in a series, it is possible to use a shorter time. If the dead volume of the tubing connected to the precolumn is too large, more time may be required for equilibration. It is advisable to test the conditions used to ensure that they are adequate. One way of doing this would be to load a radioactive sample

onto the precolumn using the appropriate conditions, followed by washing. The sample could then be eluted with a relatively small amount of acetonitrile and collected directly from the tube that normally connects the 6-port injector to the HPLC column. It should be noted that in calculating recoveries, quenching due to the solvent should be taken into account. Therefore an appropriate amount of the eluting solvent (i.e., acetonitrile) should be added to the counting vial used to count an aliquot of the sample applied to the precolumn.

25. In the event of a short power failure, it is possible that the automatic injector will continue to inject samples even though other components of the system have returned to initial conditions. Thus, the samples could be injected into the sample loop of the autoinjector, but would not be further processed and therefore would be lost. This has happened to us on several occasions in the past. To circumvent this problem, a "hold" signal can be sent from the gradient controller to the autoinjector which will prevent it from injecting additional samples.
26. The "inject" signal from the autoinjector signifies that the sample is starting to be transferred from the autoinjector to the precolumn, which can take several minutes. In addition, it is also necessary to equilibrate the precolumn with 15% methanol before actually injecting the sample from the precolumn onto the analytical column. The HPLC column can be equilibrated at the same time as the precolumn. The first 6 min of the gradient program for the HPLC column is therefore really an equilibration step, and if a gradient is to be used it should be started at 6 min, when the sample is injected onto the HPLC column.
27. A signal can be sent at this time from either the autoinjector or the gradient controller to the detector to commence the collection of data. The HPLC gradient should also be started at this time (*see* **Table 1**).
28. The HPLC column can be purged at this time. It is preferable to purge the precolumn independently of the analytical HPLC column to prevent any material eluted from the precolumn from contaminating the HPLC column.
29. If the run time on the autoinjector is set for 50 min it will start transferring the sample from the vial to its sample loop at this time. This will take approx 5 or 6 min to complete. The HPLC column can be equilibrated during this time. The "inject" signal will thus be sent to the gradient controller approx 55–56 min after the preceding "inject" signal. To purge the analytical column and stop the HPLC pump after all the samples have been analyzed the gradient programmer can switch the HPLC mobile phase to acetonitrile at 65 min, and then switch the flow rate to zero 10 min later.

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3

Assessment of Cyclooxygenase RNA Expression by Northern Hybridization

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1. Introduction

1.1. Preparation of Total RNA from Tissue Culture Cells or Tissue

The acid guanidinium-thiocyanate phenol chloroform (AGPC) procedure is the principal method used for one-step RNA extraction and is a predominant choice for isolation of total RNA from a wide variety of biological samples. (**Note 1**) ***(1,2)***. This procedure permits not only isolation of RNA from large number of samples, but also recovery of total RNA from small quantities of tissue or cells. Several widely used commercial RNA preparative kits are based on this AGPC procedure. Cultured cells and tissues are homogenized in a denaturing solution containing 4 *M* guanidine thiocyanate. The homogenate is mixed sequentially with 2 *M* sodium acetate (pH 4.0), phenol, and finally chloroform/isoamyl alcohol or bromochloropropane ***(3)***. The resulting mixture is centrifuged, yielding an upper aqueous phase containing RNA, while the lower organic phase and interphase contains protein and DNA. Following isopropanol precipitation, the RNA pellet is redissolved in a denaturing solution, reprecipitated with isopropanol, and washed with 75% ethanol.

RNA STAT-60 is a commercial kit for total RNA isolation utilizing the guanidine-based procedure ***(4)***. This RNA isolation reagent combines guanidinium thiocyanate, phenol, and buffer in a single monophase solution. A tissue or cell sample is homogenized in the RNA STAT-60 using a polytron or glass-Teflon homogenizer. Upon addition of choroform, the homogenate separates into two phases: the upper aqueous phase that contains the RNA and the lower organic phase and interphase that contain DNA and protein. The total RNA is precipi-

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tated from the aqueous phase by the addition of isopropanol, is washed with ethanol, and solubilized in water. This method offers an improved yield and shorter RNA isolation time *(5)*.

1.2. Analysis of Cyclooxygenase RNA Expression by Northern Hybridization by Agarose-Formaldehyde Gel Electrophoresis

mRNA preparations can be detected by northern blotting hybridization analysis (**Note 10**). Fractionated RNA is transferred from an agarose gel to a membrane support (such as nitrocellulose or nylon membrane) (**Note 11**). The resulting blots are studied by hybridization analysis with radio-labeled COX-1 or COX-2 DNA probes *(6)*. Because most RNAs are single-stranded, they form secondary structures very easily by intramolecular base-pairing and must therefore be electrophoresed under denaturing conditions. Denaturation is achieved by adding formaldehyde to the gel and loading buffers. We describe blotting (**Subheading 3.4.**) and hybridization (**Subheading 3.6.**) of RNA fractionated in an agarose-formaldehyde gel (**Subheading 3.3.**). Three sections are included in this protocol: electrophoresis of an RNA preparation under denaturing conditions in an agarose-formaldehyde gel, transfer of the RNA from the gel to a nylon or nitrocellulose membrane by upward capillary transfer, and hybridization analysis of the COX mRNA expression using a radiolabeled DNA probe. This procedure is arguably the quickest and most reliable method for northern analysis of specific sequences in RNA extracted from eukaryotic cells. Stripping hybridization probes from blots is also described.

2. Materials

2.1. Total RNA from Tissue Culture Cells

1. Denaturing solution: 4 *M* guanidinium thiocyanate, 25 n*M* sodium citrate, pH 7.0, 0.1 *M* 2-mercaptoethanol (2-ME) and 0.5% *N*-lauroylsarcosine (sarcosyl). Prepare a stock by dissolving 250 g guandinium thiocyanate in a solution of 293 mL H_2O, 17.6 mL of 0.75 *M* sodium citrate, pH 7.0, and 26.4 mL of 10% sarcosyl at 65°C with stirring. This stock solution can be stored at least 3 mo at room temperature. A working solution is prepared by adding 0.36 mL 2-mercaptoethanol (2-ME)/50 mL of stock solution. The working solution can be stored for one month at room temperature.
2. 2 *M* Sodium acetate, pH 4.0: Add 16.42 g of sodium acetate to 40 mL H_2O and 35 mL of glacial acetic acid. Adjust solution to pH 4.0 with glacial acetic acid and the final volume to 100 mL with H_2O. The solution is 2 *M* with respect to the sodium ions.
3. Water-saturated phenol: Dissolve 100 g of phenol crystals in H_2O at 60–65°C. Aspirate the upper water phase and store up to 1 mo at 4°C.
4. RNA STAT-60, supplied by TEL-TEST "B", Friendswood, TX.
5. 49:1 (v/v) Chloroform/isoamyl alcohol or bromochloropropane.
6. 100% Isopropanol.
7. 75% Ethanol: prepared with diethyl pyrocarbonate (DEPC)-treated water.

8. 0.5% SDS in DEPC-treated water.
9. DEPC-treated water: add 0.2 mL of DEPC into 100 mL of water. Shake vigorously to get DEPC into water. Autoclave the water to inactive the remaining DEPC.

2.2. Cyclooxygenese RNA Expression

1. 10X MOPS running buffer: 0.4 *M* MOPS [3–(*N*-morpholino)-propanesulfonic acid], pH 7.0, 0.1 *M* sodium acetate and 0.01 *M* EDTA.
2. 12.3 *M* (37%) formaldehyde, pH >4.0.
3. Deionized formamide.
4. RNA sample buffer: 10 mL formamide, 3.5 mL 37% formaldehyde, and 2 mL 10X MOPS running buffer.
5. RNA loading buffer: 50% glycerol, 1 m*M* EDTA and 0.4% bromophenol blue.
6. 20X SSC: 3 *M* NaCl (175 g/L), 0.3 *M* Na_3citrate·$2H_2O$ (88 g/L). Adjust pH to 7.0 with 1 *M* HCl.
7. 10% sodium dodecyl sulfate (SDS).
8. 10 mg/mL ethidium bromide.
9. 50X Denhardt's solution: 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g BSA (Calbiochem Fraction V), H_2O to 1000 mL. Filter and store at –20°C.
10. Salmon sperm DNA.
11. 2X SSC/0.1% (w/v) SDS.
12. Prehybridization solution: 0.4 mL 50X Denhardt's solution, 1 mL 10% SDS, 2.5 mL 20X SSC, 5 mL formamide, 1 mL H_2O, and 0.1 mL salmon sperm DNA.
13. Hybridization solution: 0.2 mL 50X Denhardt's solution, 1 mL 10% SDS, 2.5 mL 20X SSC, 5 mL formamide, 1.2 mL H_2O, and 0.1 mL salmon sperm DNA.
14. COX-1 and/or COX-2 cDNA: The full-length COX-1 and COX-2 cDNA are excised from the plasmid pBluescript-COX-1 and pBluescript-COX-2. Excised cDNAs are puried from 1% agarose gel by GeneClean kit and stored at –20°C.
15. Random hexanucleotides.
16. Klenow fragment.
17. 0.5 m*M* dNTP mix (minus dATP): 0.5 m*M* dGTP, dCTP, and dTTP.
18. 10X Klenow fragment buffer; 0.5 *M* Tris-HCl, pH 7.7, 0.1 *M* $MgCl_2$, 10 m*M* DTT, and 0.5 mg/mL BSA.
19. [α-^{32}P]dATP.
20. DNA probe labeling reaction mix: 2.5 µL 0.5 m*M* dNTP mix (minus dATP), 2.5 µL 10X Klenow fragment buffer, 5 µL 3000 Ci/mmol [α-^{32}P]dATP and 1 µL Klenow fragment (4–8 U).
21. 0.5 *M* EDTA.
22. Stripping solution (1% SDS, 0.1X SSC, 40 m*M* Tris-HCl, pH 7.5).
23. Gene Clean kit supplied by BIO 101 (Vista, CA).
24. Quick Spin™ Columns (G-25 Sephadex Columns for RadioLabeled DNA purification) supplied by Boehringer-Mannheim (Indianapolis, IN).
25. Horizantal gel electrophoresis apparatus (International Biotechnologies, New Haven, CT). Wash with DEPC water before use.
26. GeneScreen hybridization transfer membrane (Du Pont, Boston, MA).

27. Sealable bags.
28. UV transilluminator (Stratagene, La Jolla, CA).
29. Vacuum oven.
30. UV crosslinker, such as UV-Stratalinker-1800 (Stratagene).
31. Hybridization oven and tubes.
32. DEPC-treated water: Add 0.2 mL of DEPC into 100 mL of water. Shake vigorously to get DEPC into water. Autoclave the water to inactive the remaining DEPC (**Note 12**).

3. Methods

3.1. Single-Step Total RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform (AGPC) Protocol

1. For cultured cells: After removal of the culture media, add 1 mL of denaturing solution/5–10 × 10^6 cells directly to the culture dish or flask. Swirl several times and let drain on an incline until liquid is no longer viscous. Scrape the viscous homogenate to the edge of the flask with a rubber policeman and transfer it to a nap top tube (**Notes 1–4**).
2. For tissue: Immediately after removal from the animal, mince the tissue on ice and homogenize with 1 mL of denaturing solution per 50–100 mg tissue with a few strokes in a glass-Teflon or Polytron homogenizer (**Note 5**).
3. After transfer of the homogenate into a polypropylene tube, add 0.1 mL of 2 *M* sodium acetate, pH 4.0, 1 mL of phenol (water saturated), and 0.2 mL of chloroform-isoamyl alcohol mix (49:1), and mix by inversion after the addition of each reagent (**Note 6**). Shake the final suspension vigorously for 10 s and cooled on ice fro 15 min (The volumes used here are per 1 mL denaturing solution) ***(2)***.
4. Centrifuge at 10,000*g* for 20 min at 4°C. After centrifugation, RNA is present in the upper aqueous phase whereas DNA and protein are present in the interphase and phenol phase. Transfer the aqueous phase to a new tube, mix with 1 mL of isopropanol, and incubate the sample at –20°C for at least 1 h to precipitate RNA. Centrifuge at 10,000*g* for 30 min at 4°C. Discard supernatant and dry inside of tubes.
5. Redissolve the RNA pellet by addition of 0.3 mL denaturing solution, vortex briskly, incubate sample tube at 65°C for 5 min, vortex again, and cool the sample. Transfer sample into a Eppendorf tube. Precipitate the RNA with 0.3 mL of isopropanol at –20°C for 1 h. Centrifuge for 30 min at 4°C at 10,000*g*. Discard supernatant and dry inside of tubes.
6. Resuspend the RNA with 0.3 mL of cold 75% ethanol, vortex, incubate 15 min. Centrifuge 10 min at 4°C at 10,000*g* and discard supernatant and dry the inside tube. Dry the RNA pellet in air for 15 min (**Note 7**).
7. Resuspend the dried RNA pellet in DEPC-treated H_2O or 0.5% SDS solution. Quantitate RNA by reading A_{260} and A_{280}. Store sample frozen at –80°C.

3.2. Total RNA Isolation by RNA STAT-60™ Method

1. For cultured cells (**Notes 2** and **3**), add 1 mL of RNA STAT-60 per 5–10 × 10^6 cells directly in the culture dish or flask (**Note 8**). Pass the cell lysate several

times through a pipet. Scrape the viscous lysate to the edge of the flask with a rubber policeman and transfer it to a tube for homogenization (**Note 4**).

2. For the tissue, add 1 mL of RNA STAT-60 per 50–100 mg tissue and homogenized with a few strokes in a glass-Teflon or Polytron homogenizer (**Note 5**) *(5)*.
3. Following homogenization (**Note 9**), keep the homogenate for at least 5 min at room temperature to completely dissociate the nucleoprotein complex. Then, add 0.2 mL of chloroform/1 mL of the RNA STAT-60. Cover the tube tightly, shake vigorously for at least 15 s. Let the samples sit at room temperature for 3 min. Centrifuge the homogenate at 12,000*g* for 15 min at 4°C. After centrifugation, the homogenate separates into two phase: a lower red phenol chloroform phase and colorless upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and protein are in the inter-phase and organic phase. The volume of the aqueous phase is about 60% of the volume of RNA STAT-60 used for homogenization.
4. Transfer the aqueous phase to a fresh tube and mix with isopropanol (0.5 mL of isopropanol per 1 mL RNA STAT-60 used for homogenization) (**Note 9**). Store samples for 30 min at –20°C (**Note 8**). Centrifuge at 12,000*g* for 30 min at 4°C. A visible RNA precipitate forms as a white pellet at the bottom of the tube.
5. Carefully remove the supernatant and wash the RNA pellet once with cold 75% ethanol (DEPC-treated 75% ethanol is stored at –20°C before use). Add at least 1 mL of 75% ethanol per 1 mL of the RNA STAT-60 used for the initial homogenization (**Note 9**). Vortex and centrifuge the samples for 15 min at 12,000*g* at 4°C.
6. Discard the ethanol and dry the RNA pellet for 15 min at room temperature (**Note 7**). Dissolve the RNA pellet in DEPC-treated water or in 1 m*M* EDTA, pH 7.0, or 0.5% SDS solution by passing the solution a few times through a pipet tip. An incubation for 10–15 min at 55–60°C may be helpful to dissolve RNA pellet. Quantitate total RNA by reading A_{260} and A_{280}. Store isolated RNA at –80°C.

3.3. Agarose/Formaldehyde Gel Electrophoresis

1. Dissolve 1.0 g agarose in 73 mL boiling water and cool to 60°C in a water bath.
2. When the flask has cooled to 60°C, place in a fume hood and add 10 mL of 10X MOPS running buffer and 16.2 mL of 37% formaldehyde.
3. Pour the gel and allow it to set. Remove the comb, place the gel in the gel tank, and add sufficient 1X MOPS running buffer to cover to a depth of ~1 mm.
4. Adjust the volume of each RNA sample (20–30 µg) to 5 µL with water, then add 20 µL of RNA sample buffer and 5 µL of RNA loading buffer.
5. Mix by vortexing, microcentrifuge briefly (5–10 s) to collect the liquid, and incubate 10 min at 65–70°C. Cool the sample on ice. Microcentrifuge and load the samples onto the gel.
6. Run the gel at ~100 V for several hours (usually 4 h) or at ~20 V overnight until the bromphenol blue dye has migrated one-half to two-thirds the length of the gel.
7. Remove the gel and cut off the lanes that are to be stained. Place this portion of the gel in an RNase-free glass dish, add 20 µL of 0.5 µg/mL ethidium bromide in 10X SSC, and allow to stain for overnight.

8. Examine gel on a UV transilluminator to visualize the RNA and photograph with a ruler laid alongside the gel so that band positions can later be identified on the membrane.

3.4. Transfer of RNA from Gel to Membrane

1. Place unstained portion of gel in an RNase-free glass dish and soak with several changes of sufficient DEPC-H_2O to cover the gel for 20 min to remove formaldehyde. (The portion of the gel that will be blotted is not stained with ethidium bromide as this can also reduce transfer efficiency). Cut off the bottom left-hand corner of the gel. This serves to orient the gel during the succeeding operations.
2. Cut a piece of nylon membrane just large enough to cover the exposed surface of the gel. Pour 10X SSC in an RNase-free glass dish and wet the membrane completely from beneath. Soak the nylon membrane for 20 min in 10X SSC (**Note 13**).
3. Place a piece of RNase-free glass or plastic plate on a large baking dish to form a transfer support for the gel. Fill the dish with 10X SSC (~500–1000 mL) until the level of the liquid closes almost to the bottom of the support.
4. Cut one piece of Whatman 3MM paper that is the width of the gel and is long enough for the ends to hang over the support plate and into the dish of transfer buffer.
5. Place the gel on the filter paper and squeeze out air bubbles by rolling a glass pipet over the surface.
6. Cut four strips of plastic wrap and place over the edges of the gel to prevent buffer from "short-circuiting" around the gel rather than passing through it.
7. Place the wetted membrane on the surface of the gel. Try to avoid getting air bubbles under the membrane; remove any that appear by carefully rolling a glass pipet over the surface.
8. Flood the surface of the membrane with 10X SSC. Cut five sheets of Whatman 3MM paper to the same size as the membrane and place on top of the membrane.
9. Cut stack of paper towels to the same size as the membrane and place on top of the Whatman 3MM paper to a height of ~10 cm.
10. Lay a glass plate on top of the structure and add a weight (e.g., 2 kg) to hold everything in place. Leave overnight. As the paper towels become wet, they should be replaced.
11. Remove paper towels and filter papers and recover the membrane and flattened gel. Mark in pencil the position of the wells on the membrane and ensure that the up-down and back-front orientations are recognizable.
12. Rinse the membrane in 2X SSC, then place it face up on a sheet of Whatman 3MM paper and allow to dry.
13. Bake the membrane in a vacuum oven for 30 min at 80°C, then fix RNA by crosslinking in a UV-Stratalinker-1800 (Stratagene) with 1200 kJ (**Note 14**).
14. Check transfer efficiency by staining the gel in ethidium bromide as described above (**Note 15**).

3.5. Radiolabeling COX-1 and COX-2 Probe by Random Oligonucleotide-Primer Synthesis

1. The double-stranded full-length COX-1 and COX-2 cDNA are excised and purified as described in **Subheading 2.2.**
2. Mix 25 ng of COX-1 or COX-2 cDNA with 1–5 µg of random hexanucleotides and DEPC-H_2O in a final volume of 14 µL. Denature DNA by heating for 5 min at 95°C and briefly chill on ice.
3. Add 11 µL of the DNA probe labeling reaction mix to the denatured DNA and incubate at 37°C for 2–4 h.
4. Stop the reaction by adding 1 µL of 0.5 *M* EDTA.
5. Purify ^{32}P-labeled COX-1 or COX-2 DNA by Quick Spin™ Columns.
6. Remove 1 µL of purified radiolabeled DNA and measure radioactivity. Normally, the radioactivity should be ~1–10 × 10^6 cpm/µL.

3.6. Hybridization Analysis

1. Place the membrane RNA-side-up in a heat-sealable polyethylene bag. Pipet prehybridization solution into the bag and seal the edges of the bag. Place the bag into hybridization tube and incubate for at least 6 h at 42°C in the hybridization oven with rotation (**Note 16**).
2. Remove prehybridization solution and pipet the desired volume of hybridization solution into the bag.
3. Denature ^{32}P-labeled COX-1 or COX-2 DNA by heating for 10 min at 100°C. Transfer to ice and add to hybridization bag as soon after denaturation as possible. Add 5 × 10^8 cpm ^{32}P-labeled COX-1 or COX-2 DNA into the bag that contains hybridization solution. Seal the bag. Place the bag into hybridization tube and incubate for 16–48 h at 42°C in the hybridization oven with rotation (**Note 17**).
4. Pour off hybridization solution. Place the membrane into a baking dish and wash with 200 mL of 2X SSC with rotation 5 min at room temperature twice.
5. Place the membrane in the hybridization tube, add 200 mL of 2X SSC/0.1% SDS and incubate 3 min at 60°C with rotation twice in hybridization oven.
6. Remove final wash solution and rinse membrane in 2X SSC at room temperature. Blot excess liquid and cover in UV-transparent plastic wrap.
7. Perform autoradiography by exposure overnight to Kodak XAR film at –70°C with intensifying screens.

3.7. Removal of Probes from Northern

1. To strip the membrane, boil the membrane for 2–5 min in stripping buffer or sterile H_2O. Pour out solution, then rinse the membrane with DEPC-H_2O at room temperature.
2. Place membrane on filter paper to remove excess solution. Wrap membrane in plastic wrap and perform autoradiography to verify probe removal.

4. Notes

1. Hands and dust are the major source of the RNase contamination. Use gloves and keep tubes closed. Use sterile, disposable polypropylene tubes.

2. Washing cells before addition of denatured solution should be avoided because this procedure may result the increase of possibility of mRNA degradation.
3. Tissue sample volume should not exceed 10% of the volume of denaturing solution that is used for homogenization.
4. The homogenization procedure can be carried out in sterile, disposable, round-bottom polypropylene tubes with caps. Before using, test if the tubes can withstand centrifugation at 10,000*g* with the mixture of denaturing solution and phenol/chloroform.
5. Fresh tissue is preferable for RNA isolation. Alternatively, tissue should be frozen immediately in liquid nitrogen and stored at –70°C. In the latter case, tissue should be pulverized in liquid nitrogen and homogenized, using a Polytron or Waring blender, in denaturing solution without thawing.
6. Chloroform can be substituted for with bromochloropropane in this procedure, since Bromochloropropane is less toxic than chloroform and its use for phase separation decreases the possibility of contaminating RNA with DNA.
7. Do not dry the RNA pellet completely since it significantly decreases its solubility.
8. For isolation of RNA from small amount of cells or tissue, homogenize samples in 0.8 mL of RNA STAT-60, transfer the homogenate to an Eppendorf tube and follow the isolation protocol with the exception of RNA precipitation, which should be carried out for 60 min at –20°C.
9. Procedure can be interrupted at one of the isopropanol precipitations or at the ethanol wash step. Following homogenization by RNA STAT-60 (before addition of chloroform), samples can be stored at –70°C for at least 2 wk. However, unlike RNA STAT-60 method, samples should not be kept in denaturing solution for more than 30 min.
10. The contaminating RNases in solutions and glassware and the concomitant difficulties in ensuring that an RNA preparation remains reasonably undegraded throughout the electrophoresis, blotting, and hybridization manipulations can make it difficult to obtain good hybridization signals with RNA. To inhibit RNase activity, all solutions for northern blotting should be prepared using sterile deionized water that has been treated with DEPC. In addition, RNA should be electrophoresed in gel tanks not previously used for DNA separations. The tank plus accessories should be washed by DEPC-treated water thoroughly before use.
11. DEPC is a suspected carcinogen and should be handled carefully. Investigators should wear gloves for all procedures involving radioactivity and should be careful not to contaminate themselves or their clothing. When working with ^{32}P, investigators should frequently check themselves and the working area for radioactivity using a hand-held radiation monitor.
12. Nitrocellulose and nylon membranes are equally effective for northern hybridization analysis, although high backgrounds are likely with nylon membranes if the protocol is not followed carefully.
13. Use clean blunt-ended forceps instead of gloved hands to handle nitrocellulose and nylon membranes.
14. UV crosslinking is recommended for nylon membrane to allow for covalent attachment and enables the membrane to be re-probed several times. The membrane must

be completely dry before UV crosslinking; check the manufacturer's recommendations, which may suggest baking for 30 min at 80°C prior to irradiation.

15. After transfer, membranes can be stored dry between sheets of Whatman 3MM filter paper for several months at room temperature. For long-term storage they should be placed in a desiccator at room temperature or 4°C.
16. For pre-hybridization and hybridization, ~1 mL prehybridization/hybridization solution/5 cm^2 of membrane should be added into the plastic bag.
17. To control for variability in the loading quantity of RNA, all membranes are probed with GAPDH cDNA to determine the steady state levels of GAPDH gene-related sequences and used to normalize mRNA for COX-1 and COX-2.

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4

Immunohistochemical Analysis of Cyclooxygenase Expression in Human Skin

ShaAvhree Y. Buckman, Alane T. Koki, Dorothy A. Edwards, and Alice P. Pentland

1. Introduction

Immunocytochemical analysis is an effective method for providing a differential assessment of protein expression and localization in tissue samples. A variety of methods exist for immunocytochemical analysis including: immunofluorescence, immunoperoxidase, and streptavidin–biotin linked methods. Furthermore, both paraffin embedded and frozen tissue samples can be utilized for immunohistologic analysis.

The focus of this chapter is to describe the streptavidin–biotin method routinely utilized in our laboratory for immunohistochemical analysis. This technique has the advantages of increased sensitivity, visualization of staining by simple light microscopy, and the ability to counterstain the tissue to enable clear assessment of positive staining. Unlike fluorescence mediated immunohistological methods, this procedure produces slides which are insensitive to light and are relatively stable during storage at room temperature.

The main principle underlying this technique is the ability of streptavidin, a 60 kDa protein isolated from *Streptomyces avidinii*, to bind biotin, a water soluble vitamin (244 D). The strong binding affinity ($K_d = 10^{-15}$) between streptavidin and biotin allows for reduced nonspecific background staining.

The procedures described in this chapter provide detailed methods for embedding frozen tissue, preparing frozen sections, and staining, using the streptavidin–biotin mediated stain. Briefly, frozen sections are first treated to inhibit endogenous biotin and avidin, then sections are incubated sequentially with primary and biotinylated secondary antibodies. The biotinylated second-

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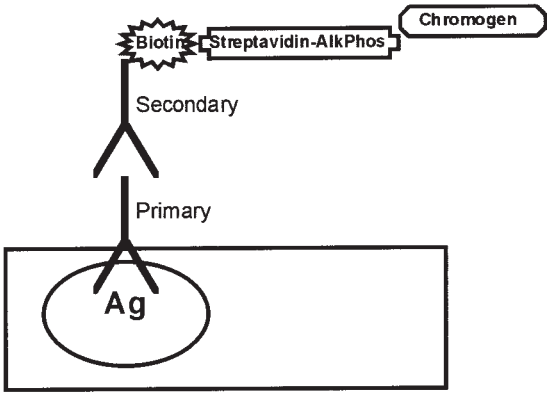

Fig. 1. Diagram of the chromogen development reaction.

ary antibody is then incubated with a complex consisting of streptavidin conjugated to alkaline phosphatase. The alkaline phosphatase is the enzyme necessary for the chromogen development reaction, which deposits positive staining where the primary antibody is bound to tissue (**Fig. 1**) ***(1,2)***.

A critical aspect of this multistep technique is maintaining the uniformity and integrity of the frozen sections. Generally, morphologic features are better preserved in paraffin rather than frozen sections. However, frozen sections have enhanced preservation of antigenicity and provide greater reproducibility of results. Whenever immunohistological techniques are employed, it is imperative to include necessary negative and positive control slides to ensure staining reliability. Furthermore, adjustments to primary and secondary antibody concentrations must be made to optimize staining and reduce background interference in each type of tissue and species studied.

Epidermal keratinocytes generate a large quantity of prostaglandin products and provide a viable histologic candidate for investigation of cyclooxygenase expression during inflammatory response and neoplastic invasion ***(3)***. Unfortunately, human skin remains a particular challenge for histologists who require uniform frozen sections. The goal of this chapter is to help define a clear, reproducible method for generation of frozen sections and analysis by a streptavidin–biotin method for the immunolocalization of cyclooxygenase-1 and cyclooxygenase-2 in the epidermis (**Fig. 2**).

2. Materials

1. Tissue Tek II Optimal cutting tissue compound (OCT) (Fisher Scientific, Pittsburgh, PA): OCT is a water soluble embedding media that freezes quickly and provides an excellent sample matrix for cryostat sectioning at temperatures

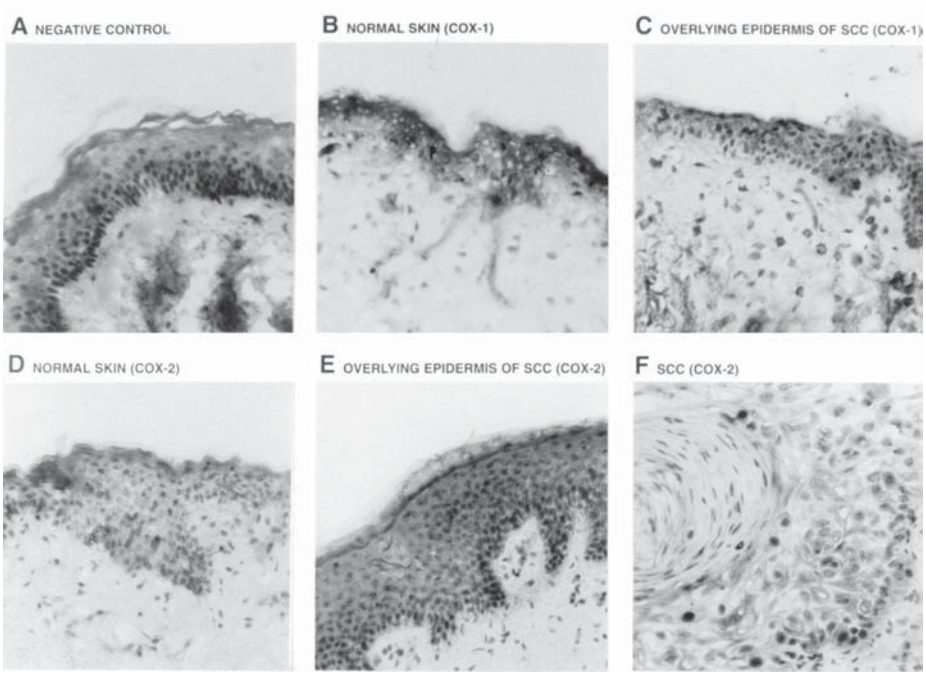

Fig. 2. Immunohistochemical localization of COX-1 and COX-2 in human skin explants from normal tissue and squamous cell carcinomas. **(A)** Negative control. **(B)** Normal skin (COX-1). **(C)** Overlying epidermis of squamous cell carcinoma (COX-1). **(D)** Normal skin (COX-2). **(E)** Overlying epidermis of squamous cell carcinoma (COX-2). **(F)** Squamous cell carcinoma (COX-2).

<–10°C. OCT will appear opaque and white when it is sufficiently frozen to begin sectioning.

2. HistoPrep Plastic Base Molds (Fisher, Scientific): Ideally, the dimensions of the mold should be approx 0.5–1.0 cm larger and deeper than the borders of the tissue to allow sufficient room for the embedding media to fill the mold and allow adequate matrix support. Frozen sectioning can also be difficult if the molds are excessively large for the specimen. Base molds should be kept at room temperature prior to tissue embedding.
3. Leica Cryostat (Leica, Deerfield, IL): The cryostat is a refrigerated rotary microtome. Although there are many good cryostats sold by various companies, our laboratory prefers the use of the Leica Jung CM 3000. Using this cryostat, all controls are accessible from outside the cabinet and it can be either mechanically or hand operated.
4. Accu-Edge Low Profile Disposable Blades: These blades are particularly useful for sectioning skin. Blade sharpness is usually well maintained for up to 100 sections (i.e., 10 sections each from 10 blocks).

5. Superfrost Plus Slides (Fisher Scientific): These pre-cleaned, commercially available slides have a charged surface that facilitates strong binding of the tissue section to the glass surface.
6. Five-Slide Transport Boxes (Fisher Scientific).
7. Several Wheaton glass staining dishes (20 slide capacity) with removable horizontal slide racks (Fisher Scientific).
8. Plastic Micro Slide Folder (VWR, Pittsburgh, PA) or Sequenza Slide Rack and Disposable Cover Plates (Shandon-Lipshaw, W. Chester, PA).
9. Paraffin Pen or similar water repellent marker (PAP Pen, Research Products, Mt. Prospect, IL).
10. Shur/Mark Histology Marking Pens (for writing on slides without loss of ink during solvent incubation) (Fisher Scientific).
11. Primary Antibody (e.g., COX-1 and COX-2 antibodies suitable for immunohistochemistry are available through Oxford Biochemical [Oxford, MI], Santa Cruz Biotechnology [Santa Cruz, CA], and Cayman Chemical [Ann Arbor, MI]).
12. Biotinylated goat antirabbit or goat antimouse secondary antibody (purchase from Zymed Labs, San Francisco, CA).
13. Washing solutions: Phosphate-buffered saline (PBS) 10 m*M* PBS, pH 7.4: For 1 L combine 0.26 g KH_2PO_4, 2.17 g Na_2HPO_4-$7H_2O$, 8.71 g NaCl, and 800 mL dH_2O. Adjust to pH 7.4 and bring to 1 L vol with dH_2O.
14. Blocking solutions: PBS-Blocking Buffer (200 mL): Add 1% BSA, 0.3% Triton X-100, 0.2% saponin to 200 mL of PBS. Filter-sterilize this solution prior to use 10% normal goat serum (10 mL): Add 1 mL of normal goat serum (Sigma, St. Louis, MO) to 9 mL of PBS. Sterile filter prior to use. Can be stored at 4°C for 2–3 wk. This solution should smell fresh when ready for use.
15. Avidin/Biotin blocking solution: (purchase from Vector Labs, Burlingame, CA).
16. Streptavidin–alkaline phosphatase (purchase from Lipshaw—Immunon, Pittsburgh, PA).
17. Vector red (Vector Laboratories): This substrate should be prepared according to kit instructions.
18. Acetate buffer, 0.1 *M*:
    a. 0.1 *N* acetic acid (pH 5.2): Prepare by mixing 3.0 mL of glacial acetic acid with distilled water and adjust volume to 500 mL total water (0.1 *M*).
    b. 0.1 *M* sodium acetate, Trihydrate: Dissolve 13.6 g of sodium acetate trihydrate in 1000 mL of distilled water.

    Prepare acetate buffer (0.1 *M*, pH 5.2) by mixing 105 mL of 0.1 *N* acetic acid with 395 mL of 0.1 *M* sodium acetate. All of these solutions can be stored at room temperature.
19. Buffered methyl green solution: Dissolve 2 g of methyl green (Sigma) in 100 mL of 0.1 *N* acetate buffer, pH 5.2. Adjust pH to 4.2–4.5. Place solution in a glass-separating funnel and extract solution with chloroform until the chloroform layer is without violet color. The supernatant should be pure methyl green solution. Expires 2 yr after preparation.

20. 1% Alcian blue solution: Dissolve 1 g of alcian blue in 100 mL of acetate buffer. Add a thymol crystal, filter, and refrigerate. Should expire 2 yr after preparation.
21. Methyl green/alcian blue counterstain: Add 100 mL of 1% alcian blue solution to 100 mL of methyl green solution, add 200 mL of acetate buffer, and mix thoroughly. Solution may require filtering prior to use. Store at room temperature.
22. Mounting media: GVA Mount can be obtained from Zymed Immunochemicals or Permount (Fisher Scientific).

3. Methods

3.1. Preparation of Frozen Sections

For performing special immunohistological techniques, superior results can often be obtained by using fresh frozen tissues in lieu of routinely processed and paraffin-embedded specimens. The use of frozen tissues offers two clear advantages: results can often be obtained from fresh tissues in a short time period and antigenic perservation is often superior to that obtained in tissues that are formalin-fixed and parrafin-embedded. Furthermore, cryosections are often the method of choice for the study of highly labile or soluble proteins.

Preparing skin specimens for immunohistological analysis consists of several steps: collecting and freezing the tissues, embedding and orienting the specimen, and sectioning and fixation prior to analysis. In **Subheading 3.1.1.** we will describe how frozen tissues can be stored for extended periods without antigenic or morphological loss.

3.1.1. Tissue Preparation and Embedding

1. Fresh tissues should be immediately cut into pieces that are no larger or thicker than 4–5 mm. If one is not interested in subcutaneous fat, the skin is more easily handled if trimmed to the level of the deep dermis. Specimens should then be frozen in OCT immediately following biopsy.
2. Once the sample is cut into the desired dimension, a small amount of Tissue-Tek OCT is placed in the appropriate size plastic base mold (room temperature).
3. Orient the specimen with forceps in the mold so the specimen is placed on its side with the epidermis and dermis touching the bottom of the plastic mold. The mold is then placed on dry ice, and the specimen surrounded with more OCT to completely fill the mold. The specimen is sufficiently frozen when the surrounding OCT appears opaque. Tissues should not be left on dry ice more than 5 min once frozen.

3.1.2. Frozen Sectioning

1. Check the cryostat to make sure that it is clean and free of dust (*see* **Note 1**).
2. Transfer the embedded frozen block to the microtome and allow it to equilibrate to the microtome temperature (*see* **Note 2**).
3. Put a small amount of OCT sufficient to cover the grooves of the chuck (the stand that holds the frozen tissue block for sectioning). Make sure that the chuck has been

prechilled to the microtome temperature. To remove the frozen block from the mold, carefully rub the back of the plastic mold with a finger to slightly melt the embedding media, and carefully pop the frozen block from the plastic mold. Place the frozen block on to the chuck and allow it to harden in the microtome for 10–15 min.

4. Place the chuck onto the cryostat, carefully aligning the chuck and tightening screws to hold the specimen in place.
5. Adjust the knife and holder angle and begin cutting the block to remove excess OCT overlying the frozen section (termed "facing the block") until the specimen is clearly visible and a nice cross-section of both epidermis and dermis is obtained for each section (*see* **Note 3**).

3.1.3. Mounting Frozen Sections

1. Take a clean superfrost plus slide (kept at room temperature) and hold it a fraction of an inch over the frozen section on the knife. The section will adhere directly to the warm slide and transfer itself from the cold knife to the slide (*see* **Note 4**).
2. Dry cryosections thoroughly for a minimum of 10 min at room temperature in a dessicated chamber or in a freeze-dryer for 4–18 h.
3. Sections can either be fixed and used immediately following drying or stored at –80°C.
4. Prepared sections can be stored in air-tight plastic slide holders at –80°C for up to 1 wk. Antigenic and morphologic preservation is sometimes lost if cut sections are stored for longer periods of time.

3.1.4. Tissue Storage

1. Unused frozen blocks can be safely stored by covering the cut face of the block with OCT.
2. The unused sample can either be removed from or stored on the chuck. The specimen must be wrapped tightly in Saran Wrap and then in aluminum foil to avoid dehydration of the tissue. Using this method, the blocks can be stored at –80°C for up to 1 yr.

3.2. Immunostaining of Frozen Sections Using Streptavidin–Alkaline Phosphatase

1. Allow frozen sections stored at –80°C to warm completely to room temperature in plastic chambers (Usually 1–2 h) (*see* **Notes 5** and **8**).
2. Transfer slides to horizontal glass slide racks and immerse in a glass dish containing chilled acetone (100%). Fix slides in acetone for 10–15 min at –20°C. Remove slides from the bath and allow to air dry (*see* **Notes 6** and **7**).
3. Transfer slides to PBS and wash two times 5 min each wash with gentle agitation (a rocker can be used for this step). Slides for washes should be immersed in fresh buffer for each wash. Do not reuse the buffer. After this point, slides can be set up in the Sequenza slide rack apparatus or the sections can be circled with a PAP pen to localize the solutions for incubation (*see* **Note 9**).
4. Block endogenous biotin and avidin (Vector Laboratories blocking kit). Incubate 15 min with avidin D followed by a 5 min wash in 1X PBS. Incubate 15 min in the biotin blocking solution. Follow with a 1X 5 min wash in PBS.

5. Incubate sections in PBS-blocking buffer for 30 min at room temperature. This buffer should be made fresh and filtered through a 0.45 µm filter prior to use (*see* **Note 10**).
6. Tap PBS-blocking buffer off the slides and incubate in the 10% normal goat serum blocking solution (*see* **Note 11**).
7. Apply primary antibody and incubate overnight at 4°C (e.g., anti-COX-2 rabbit polyclonal). The primary antibody can be diluted in PBS-blocking buffer. The correct concentration of the primary antibody must be empirically determined, however a 1:1000 dilution of the antibody is sometimes a good reference point (*see* **Note 12**).
8. Tap primary antibody off the slides and wash them three times for 10 min in fresh PBS.
9. Incubate slides in the appropriate biotinylated goat antiserum secondary for 45 min (e.g., use biotinylated goat antirabbit antisera for a polyclonal rabbit primary antibody) (*see* **Note 13**).
10. Wash slides three times for 10 min in PBS.
11. Incubate in streptavidin–alkaline phosphatase for 45 min at room temperature.
12. Wash slides three times for 10 min in PBS.
13. Add vector-red (enough to cover each section completely, approx 4–5 drops per slide) and incubate for approx 30–45 min at room temperature. Development progress can be noted by the generation of a red deposit on the slides that can be visualized by eye or light microscopy. (Development time should be monitored by visualization but generally can be estimated to occur within this time period. Improved staining intensity can occur by incubating slides with the substrate in the dark). (*See* **Note 14**.)
14. Wash slides three times for 10 min in PBS.
15. Counterstain slides by immersing them in the methyl green/alcian blue mixture for approx 10 min.
16. Wash in distilled water until counterstain is the intensity desired.
17. Mount slides in GVA mount (or a similar aqueous mounting media) by applying a drop of mounting media to the area overlying the tissue section and gently applying a coverslip. Allow slides to dry undisturbed for approx 3 h and then visualize staining by light microscopy (**Fig. 2**).

4. Notes

1. Routine maintenance and cleaning of the cryostat is essential for obtaining consistently high quality sections. Dust and debris are common culprits of knife artifacts and poor morphological preservation of the tissue.
2. In contrast to other tissues that are typically cut at –20°C, skin without fat is ideally cut at cryostat temperatures between 13 and 17°C. Tissues that contain significant amounts of subcutaneous fat cuts best between –20 and –25°C (ideal temperatures usually vary inversely with increasing fat content). Equivalent temperatures of the microtome and cabinet, knives, and other tools (i.e., forceps, brushes, and so on) are absolutely essential for ease of sectioning and acquiring quality sections.

3. Good sections depend on correct tissue and microtome temperature, adequately prepared tissues, sharp knives, proper orientation of the embedded specimen, and most importantly, appropriate knife angle. Listed below are some troubleshooting tips for sectioning problems:

Common problems	Causes
Frozen section does not form	Knife too warm
Compressed frozen sections	Cryostat and knife too warm
Wrinkled sections	Dull knife or knife angle
Sections adhere to knife	Knife edge dirty (clean with 70% ethanol)

4. If there is a problem adhering frozen sectioned tissue to glass, homemade subbed slides can be utilized. These slides can be made by preparing a chrome alum gelatin solution by heating 1 L of distilled water to 60°C and completely dissolving 3.0 g of gelatin, type A, 275 bloom on a magnetic stirrer. Stir in 0.5 g of chromium potassium sulfate (the solution should turn pale blue). Add a few crystals of thymol as a preservative. Dip precleaned slides in the warm gelatin solution and let the slides stand and air dry at room temperature for several hours. Once the coated slides have dried completely, they can be stored in a dust-free container at room temperature for extended periods.
5. To avoid repeated freeze/thaw cycles for prepared sections, the airtight plastic slide holders are ideal for storing limited numbers of replicate slides. When ready to use, the plastic holders should be removed from the freezer and remain sealed until completely thawed to room temperature. Opening the holders prior to complete thawing may cause ice crystal formation and result in poor sample morphology.
6. Although frozen sections can be fixed in room temperature acetone, cold methanol, cold ethanol, or cold 0.5–1% formalin, we have obtained the best antigenic and morphological preservation with cold acetone in skin specimens from a variety of different species.
7. If not used immediately, fixed sections (which have been adequately dried) can be stored in airtight plastic holders at –80°C for up to 1 wk.
8. Slides for immunohistochemical analysis should always be prepared at least in duplicate to guard against accidental specimen loss. Always prepare extra positive and negative control slides when initiating immunohistochemical analysis. Positive control tissue should be a sample that is known to contain the antigen to be stained. In order to assess the nonspecific binding caused by the other reagents utilized, prepare a negative control slide that is treated with either nonimmune serum diluted to the same concentration as the primary antibody or the diluent for the antibody alone.
9. Please note that these incubations can be performed by a variety of methods. Some investigators use paraffin pens (PAP pens) to circle around the sections and thus keep the various incubation solutions localized on the tissue. We have found it most useful to use slide incubation chambers that maintain a thin layer of solution covering each section. All washes can also be performed in these appa-

ratuses. Shandon Lipshaw sells a Sequenza slide incubation rack that is excellent for these applications when used in conjunction with their disposable slide coverplates.

10. This step is critical for cell permeabilization and if necessary, 0.2% saponin can be added to this mixture.
11. This step is utilized to reduce nonspecific background on the slides. If you use a secondary antibody raised in another species other than goat, adjustments to this incubation buffer should be made accordingly.
12. These incubations should be performed in a humidity chamber. If using the Sequenza apparatus, these incubations can be performed directly in the slide holders. However, this can also be created by taking a plastic enclosed flat slide holder (VWR Scientific) and lining the bottom with dampened paper towels. The slides are then placed in the holders overlying the paper towels and incubated for the appropriate time period. (Alternatively, slides can be incubated in large Petri dishes with dampened paper towel placed on the bottom of the dish). This procedure helps to eliminate the probability of slides drying during long incubation periods.
13. The absolute titer of the antibody will have to be determined by experimentation. However, Zymed Laboratories sells biotinylated secondary antibodies at a standard dilution that can be used initially. If necessary it can be further diluted to reduce nonspecific background binding.
14. The advantages of this method are that the Vector Red chromagen is highly fluorescent and therefore can be visualized with rhodamine, fluorescein, or AMCA excitation filters. This method may allow for visualization of a more amplified signal than observed by light microscopic techniques.

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Separation of Hydroxyeicosatetraenoic Acids and Leukotrienes Originating from Kidney Tissue by C-18 Reverse-Phase High-Pressure Liquid Chromatography

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1. Introduction

Metabolism of arachidonic acid results in formation of eicosanoids including prostaglandins (PGs), thromboxanes (Txs), monohydroxyeicosatetraenocinoids (HETEs), leukotrienes (LTs), lipoxins (Lxs), epoxy metabolites, and hepoxilins *(1)*. Most of these compounds are formed in minute quantities in response to specific stimuli and participate in modulation of the vascular tone or of the inflammatory process. We have been studying the formation of arachidonic acid metabolites in kidney tissue (mainly glomeruli) following immune injury. Normal glomeruli synthesize very small quantities of HETEs and LTs. Following immune injury, glomerular synthesis and levels of these eicosanoids increase *(2)*. Leukotrienes (B_4, C_4, and D_4), 5-HETE, and 12-HETE can be separated, detected, and quantitated by several analytical techniques, including immunoassay, bioassay, gas chromatography-mass spectrometry (GC-MS), thin-layer chromatography (TLC), ultraviolet spectroscopy, and high pressure liquid chromatography (HPLC).

HPLC has been used to separate eicosanoids with great success. It provides an economical platform for a variety of chromatography supports (stationary phase) and detection systems with a great flexibility. Typical duration of a chromatographic cycle using HPLC is relatively short with high reproducibility and sensitivity. Separation of the components in a mixture is achieved by partitioning them between a stationary phase and a mobile liquid phase. The small particle size of the stationary phase provides a large surface area for

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separation of closely related molecules. Particles of the stationary phase can be modified to serve as the basis of separation with one specific characteristic, such as affinity for a ligand, ion exchange, size exclusion, hydrophobic interaction, and adsorption on a normal or reverse phase. The composition, ionic strength, pH, and polarity of the mobile phase can also be designed for specific chromatographic separation. The biological sample containing the eicosanoids to be separated is dissolved in a defined mixture of an organic solvent with water (i.e., methanol:water) solvent and loaded on a column containing the chromatographic support pre-equilibrated with the same solvent:water mixture. Retained compounds are eluted by a mobile phase that consists of the same solvent:water mixture (isocratic elution) or by a mobile phase that consists of the same solvent:water solution that becomes progressively enriched with one or more organic solvents of differents polarity (gradient elution). Mixing of solvents takes place in a pre-column chamber. Structural properties of eicosanoids (i.e., presence of conjugated double bonds) render them characteristic light absorption properties in the ultraviolet/visible/infrared range. These features are used in their detection and quantitation. In order to detect the eluted compounds by the light absorption method, the eluate is passed through a flow cell positioned between a monochromator and a photodetector. The signal generated is recorded using a data integration system.

Reverse Phase HPLC (RP-HPLC) is the most widely used chromatographic technique. The term reverse phase is derived from the fact that in RP-HPLC the mobile phase is more polar than the stationary phase, which is an inert hydrocarbon. Only hydrophobic interactions between the solute and the stationary phase are allowed in RP-HPLC by including acid modifiers (acetic acid, trifluroacetic acid, or phosphoric acid) in the mobile phase. In contrast to RP-HPLC, conventional chromatography is based on the use of a polar stationary phase and a relatively less polar mobile phase.

Retention characteristics of most eicosanoids on a reverse phase column are determined by the numbers of hydroxyl groups, oxo groups, and double bonds they possess. The position of the hydroxyl radical in HETEs determines the polarity of the molecules and their chromatographic separation. Leukotrienes also separate on the basis of polarity. Thus LTB_4 is separated owing to two hydroxyl groups. Cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) behave quite differently than other eicosanoids because of a positively charged amino group in addition to the negatively charged carboxyl group, that is common to all eicosanoids. The presence of the cysteinyl group introduces another variable that is used for separation of LTC_4, LTD_4, and LTE_4 by selecting different acidic modifiers of the mobile phase as well as different stationary phases. Relative amounts of solvents (e.g., methanol and acetonitrile) in the mobile phase also introduce selectivity differences.

Products formed by the arachidonic acid 5-lipoxygenation pathway possess at least two conjugated double bonds and have characteristic ultraviolet (UV) absorption that makes these compounds suitable for HPLC separation and detection with a UV detector. Thus the presence of a cis-trans conjugated diene in HETEs enables this group of compounds to absorb light near 232 nm. Similarly, LTs gain their characteristic absorption of light near 270 nm owing to the presence of a conjugated triene. In contrast, with the exception of 12-hydroxy-5,8,10-heptadecatrienoic acid, none of the major products of the cyclooxygenase pathway possess conjugated double bonds and, therefore, can not be detected at low levels on the basis of absorbance in the UV range.

Most of the available HPLC methods use radioligands or employ multiple chromatography cycles to separate eicosanoid. This makes methodology both uneconomical and time consuming. Here we describe separation of leukotrienes (LTB_4, LTC_4, and LTD_4) and HETEs (5, 12, or 15-HETE) in one chromatographic cycle by making use of differences in their polarity and UV light absorption characteristics. A C_{18} reverse phase column is used for separation. First, an isocratic solvent flow of solvent is employed to elute leukotrienes which are detected at 280 nm. In the latter part of the chromatography cycle a gradient of two solvents is used to elute HETEs that are detected at 232 nm.

2. Materials

The laboratory should be equipped with all basic instruments and facilities including pH meter, waterbath, microbalance, magnetic stirrer, liquid scintillation counter, vacuum line, and nitrogen tank (*see* **Notes 1–3**).

1. High-pressure liquid chromatography system: The HPLC system in our laboratory is from Shimadzu Scientific Instruments (Columbia, MD). It consists of a system controller (SCL-10A), two pumps (LC-10AS), and a UV-VIS detector (SPD-10AV) with programmable wavelength setting options and a computerized data handling system (CR-501 Chromatopac) with recording and programming capabilities. The system has a manual injection port and can be connected to a fraction collector (Cygnet, ISCO, Lincoln, NE). (*see* **Notes 4** and **5**).
2. Reverse phase column: We use a C-18 reverse phase column Prodigy ***(3)*** ODS (Phenomenex, Torrence, CA). The column is 250 mm × 4.6 mm (id) and is packed with 5 µm 99.9% pure silica particles having 100°A pores. According to the manufacturer, this column had not been previously tested for its capability to separate eicosanoids (*see* **Notes 6–8**).
3. Solvents: We use HPLC grade methyl alcohol and tetrahydrofuran, and analytical grade acetic acid. Ultrapure water is used to prepare a 0.1% solution of EDTA that is filtered through a 0.5 µm filter and degassed. Methanol and tetrahydrofuran are filtered and degassed separately. Filtration and degassing of each solvent

separately prior to mixing minimize losses owing to evaporation and the potential change in composition of the mixture (*see* **Notes 9–14**).

4. Preparation of solvents A and B: Tetrahydrofuran is used as solvent A. Solvent B is prepared by mixing 25 parts of tetrahydrofuran, 30 parts of methanol, 45 parts of 0.1% EDTA in water, and 0.1 parts of acetic acid (v/v). The pH of this mixture is adjusted to 5.5 with 20% ammonium hydroxide solution. The final mixture is degassed and filtered through a 0.5 µm filter. A similar solvent system has been used by other investigators ***(3)*** (*see* **Notes 9–14**).
5. Degassing: All solvents are degassed by stirring the solution on a magnetic stirrer under vacuum created by connecting the flask to a laboratory vacuum line.
6. Connecting pumps to solvent reservoirs and pre-equilibration of the column: A binary gradient system is used in this separation method. Pump A is connected to a reservoir of filtered and degassed HPLC grade tetrahydrofuran. Pump B is connected to a buffered mixture of tetrahydrofuran/ methanol/ water containing 0.1% EDTA/acetic acid (25:30:45:0.1 v/v) (*see* **Note 15**).
7. Sample loading: A 1-mL capacity injection loop is connected in the injection port. Samples are prepared from tissue or cell preparations and dissolved in solvent B. The column is pre-equilibrated in the same buffer. One-milliliter aliquots of the sample dissolved in solvent B is loaded using a syringe filter after removal of all bubbles (*see* **Note 16**).
8. Standards: LTC_4, LTB_4, LTD_4, 5-HETE, 12-HETE, and 15-HETE standards can be obtained from Cayman Chemical Company (Ann Arbor, MI) or other manufactures and used at 250 ng/mL concentration in solvent B (*see* **Note 17**).
9. Checking recovery: Recoveries are estimated by "spiking" tissue or cell homogenates with [^{3}H]-LT or [^{3}H]-HETE (New England Nuclear, Boston, MA) before the eicosanoid extraction step. Prepare a "spike" solution of a tritiated eicosanoid from the stock solution. 100 µL of "spike" solution should contain at least 2000 cpm. Recoveries should be greater than 65% (*see* **Note 18**).

3. Method

The following is a method established in our laboratory for separation of HETEs and LTs after their extraction from glomeruli of rat kidneys.

3.1. Isolation of Glomeruli and Extraction of Eicosanoids

1. Anesthetize a male Sprague-Dawley rat (180–200 g) using metofane (methoxyfurane) vapor. Remove both kidneys through an abdominal incision and place them in serum free RPMI-1640 medium in a plastic Petri dish on crushed ice.
2. Cut out the renal cortex from the medulla and mince the cortex into small pieces with a blade. Spread the minced cortex on a stainless steel sieve (150 mesh/ 106 µm) using a spatula (just like spreading butter over a slice of bread). This movement should exert a mild and even pressure and mechanically separates glomeruli from tubules. Collect the material that has appeared on the flipside of this sieve and transfer it to fresh RPMI-1640. Stir up this suspension to homogeneity using a long tipped Pasteur pipet.

3. Pour this suspension of glomeruli on to a 200 mesh (75 μm) sieve. This sieve allows tubules to pass through the pores of the sieve while it retains glomeruli. Passage of tubules is facilitated by squirting RPMI-1640 directly on to the sieve using a long tipped Pasteur pipet.
4. Recover retained glomeruli by aspirating them using a long tipped Pasteur pipet and transfer them to a cone-tipped polypropylene tube. Let glomeruli stand at 4°C (crushed ice) for approx 15 min. This step further separates suspended glomeruli from tubules (glomeruli will pellet quickly like sand particles, but tubules will lag behind because of oscillating movements). Remove supernate (mostly tubules) and use pellet (glomeruli) for the subsequent steps described below. The pellet should now consist of a preparation that is ~95% glomeruli. This can be assessed by microscopy of a 5-mL aliquot spread over a glass cover slide (*see* **Notes 19** and **20**).
5. Incubate the isolated glomeruli in serum free RPMI-1640 with 1 μ*M* of the calcium ionophore A12387 for 45 min. Arachidonic acid is inefficiently metabolized via the lipoxygenase pathway in glomeruli and the presence of the ionophore optimizes production and detection of the HETEs and LTs ***(4)***.
6. At the end of the ionophore incubation, add 3 vol of cold absolute ethyl alcohol and mix the contents of the tube vigorously overnight at 4°C. Centrifuge the ethanolic mixture at 3000*g* for 15 min at 4°C to precipitate the denatured cells and cell debris and evaporate the supernate under vacuum using a Speed-Vac or similar equipment. Flush each dried tube with a stream of nitrogen and keep it closed at –70°C. Reconstitute the dried ethanolic extract in 1 mL of the buffered HPLC solvent mixture B and inject into the injection port of the HPLC system.

3.2. HPLC

1. Equilibrate the column with at least three column volumes of solvent B. Program the HPLC equipment for elution of eicosanoids using a gradient mode in the following manner that describes the typical conditions we use. It is possible different equipment require slightly different settings.
2. First select gradient mode of elution and set the flow rate at 1.0 mL/min in pump B. This will pump 100% of solvent B through the column. Set the deuterium lamp at 280 nm as the default wavelength with 0.0001 absorbance for full scale range settings for the detector. Some systems may require manual change of wavelengths. Set the solvent gradient and wavelength parameters as follows:

Time (min)	% B	Wavelength (nm)
25.00	100	232
45.00	80	232
50.00	80	232
55.00	100	232
55.10	100	280
55.20	stop	

In order to avoid giving two commands at the same time we have used a short interval between change of wavelength and stop command.

3. Run the equipment at 100% B (1 mL/min) with detector at 280 nm and obtain a smooth baseline. If the baseline shows a drift, check for a cause. It is necessary to have a noise free baseline before loading a sample. It is essential to check the baseline with every new batch of solvents and buffers. In the elution program described here the baseline shifts with the change of wavelength from 280 to 232 nm and we have to manually suppress the baseline fluctuations by repeatedly pressing the baseline-zero switch. Some HPLC systems are equipped with a programmable baseline zeroing capability.
4. Run a chromatogram of standards every time you switch to a new batch of solvents. It is our practice to also run a blank chromatogram by injecting solvent B only. It is also advisable to wash the column with solvent B between samples. We generally wash the column with at least one column volume of solvent B before loading the next sample.
5. Dissolve the sample containing the eicosanoids to be separated in 1.0 mL of solvent B and load into a 1-mL capacity injection port loop keeping the injection port in the "load" position (or equivalent sign on your machine), to allow the sample to stay in the injection port. The solvent present in the port is forced out through the outlet and the sample replaces the solvent. Then start the cycle by turning the injection port knob to the "run" position (or equivalent sign on your machine) which allows the sample to flow to the column. This will trigger the programmed cycle of solvent flow and wavelength changes.
6. Leukotrienes, LTC_4, LTD_4, and LTB_4 are separated during the isocratic phase (0–25 min) and detected at 280 nm. We found retention times (r_T) for these eicosanoids to be 10.48 ± 0.1 min, 14.97 ± 0.28 min, and 21.44 ± 0.16 min, respectively ($n = 4$ runs).
7. Hydroxyeicosatetraenoic acids, 15-, 12- and 5-HETE, elute during the gradient phase at 41.43 ± 0.20 min, 44.22 ± 0.51, and 47 ± 0.46 min, respectively, and are detected at 232 nm. A typical chromatogram is shown in **Fig. 1**.
8. Collect eluates as 1-mL fractions. If an online fraction collector is not available, these fractions can be collected manually. Collected samples should be dried under vacuum and either stored at –20°C or processed for quantitation by radioimmunoassay or other methods.
9. We use an online plotter/integrator (Chromatopac, Shimadzu Scientific) to obtain a hardcopy of the chromatogram. Parameters like peak area detection sensitivity, attenuation, and chart-speed need to be programmed. These could vary for each recording system and need to be verified. We used the following settings: chart-speed 2.5 mm/min; attenuation 5; and minimum peak area of 10,000.
10. At the completion of the chromatographic cycle, re-equilibrate the column with three column volumes of solvent B. Wash the column with solvent A at the end of several runs. Store the column in methanol (*see* **Notes 21–27**).

4. Notes

1. The cleanness of your glassware/plasticware will determine the quality of your results. All containers that come in contact with your sample or solvents should be cleaned to obtain maximum degree of residue free surfaces and they should be stored covered in dust free area.

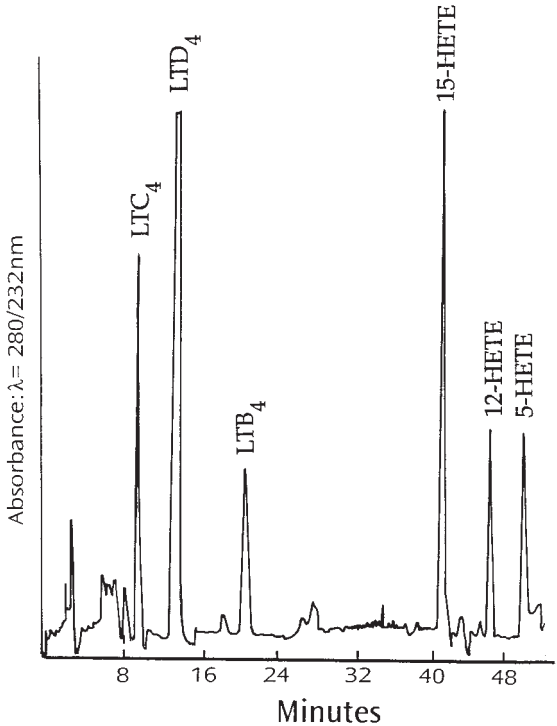

Fig. 1. Chromatogram of hydroxylicos atetraenoic acids, and 15-, 12-, and 5-HETE.

2. Glass containers must be of the highest quality glass. Glass syringes used for injection of samples should be cleaned thoroughly between samples with methanol followed by solvent B.
3. All plastic containers and tubing should be resistant to solvents used and should not leach out. Remember that every contaminant gets concentrated during sample processing and the error gets multiplied several folds during data analysis.
4. The HPLC system available to you may be from a different manufacturer and have differences in details of the machine. However, they should all be able to get similar results. If your laboratory has had an HPLC system for some time, there should be a locally available expert who is very familiar with the system. Please seek this person's advice for questions related to your specific equipment.
5. Leaks are a common occurrence in HPLC based techniques. Keep standard fittings and unions available. Finger tight plastic fittings have become available, they are resistant to common solvents and high pressure, and are easy to use.
6. The column you select should be free of uncoated silica and the size of particles should be small. At the present time, analytical columns with 5–10 μm particle size from various manufacturers can be obtained at about $300–500.

7. The stationary phase of reverse phase columns are prepared by binding nonpolar hydrocarbon chains with silanol groups of silica particles. However, parts of silica particles remain unreacted and can act as binding sites with relatively higher polarity. In order to minimize the effect of the unreacted binding sites, RP-HPLC is generally carried out at low solvent pH. Low pH suppresses ionization of solutes and prevents interaction with unreacted regions of silica particles.
8. It is a common practice to use a guard-column between the injection port and the analytical column. The guard column (precolumn) is generally 5–10 cm long and it should be filled with particles containing the same hydrophobic chain as the analytical column. The guard column protects the analytical column and it generally does not affect the separation characteristics. When washing the guard column, make sure it is disconnected from the analytical column.
9. Polarity of the solvent is a very important factor in obtaining a good separation by RP-HPLC. Solvent characteristics can be changed by adjusting the pH and/or adding salts and chelators.
10. UV-cutoff of a solvent is the wavelength below which the solvent absorbs the incoming light. It is an important factor in deciding the applicability of a given buffer system with a UV-VIS detector. Sometimes UV-absorbance is caused by impurities and additives which should be minimized by distilling the solvent or by passing it through an ion-exchanger, silica, or C-18 column. Water as an impurity can be removed by passing the solvent through a molecular sieve or anhydrous sodium sulfate. Alcohol contamination can be removed by distillation or by extraction with water followed by drying over anhydrous sodium sulfate or a molecular sieve.
11. Impure water is a common source of particulate contamination. Impurities in water accumulate at the head of the column and result into "ghost-peaks." Ultrapure water should be filtered using 0.5 μm filters.
12. Each solvent can be degassed separately before mixing. The solvent can be poured through a 0.5 μm filter into a flask placed on a magnetic stirrer and connected to a vacuum line. A Teflon coated bar should keep the solvent stirred. At first you will see a large number of bubbles which will decrease gradually. Stop the stirrer, disconnect the vacuum line when bubbles are no longer visible, and use the solvents A and B.
13. Filtration and degassing of each solvent, separately and prior to mixing, helps in minimizing evaporation losses of solvents that have low boiling points. This prevents changes in final buffer composition.
14. Impurities and preservatives, like antioxidants in the solvent, can alter chromatographic characteristics. Make sure your solvents have been treated/distilled to remove light absorbing contamination besides the particulate material. This is easily accomplished by passing the solvent through a C-18 cartridge. Change in temperature may also cause the baseline to drift.
15. Always prime your pumps to remove the air in the line. It is imperative to make sure that you do not run your pumps dry.

16. The syringe used for injecting sample aliquots need to be cleaned thoroughly between samples. Wash the syringe three times by aspirating solvent B, then methanol twice, and finally solvent B again. Store the syringe after washing with methanol.
17. Standards should be freshly made. If there are old unused bottles of standards in your freezer, ascertain their purity before using. Standard solutions should be stored under nitrogen.
18. Take all precautions to prevent radioactivity spills. The "spike" solution should be loaded on the column and eluted using identical conditions. Eluates should be dried under nitrogen. Add a liquid scintillation counting fluid and record the radioactivity counts on an available beta counter. Always use a control to measure the background noise which should be deducted from "spike" counts before calculating recoveries.
19. Sieves used should be previously cleaned and dried. If you have just rinsed your sieves, wash with RPMI-1640.
20. Repeated washing with RPMI will help in removing the debris and tubules from the glomerular preparation.
21. Separation of two solutes depends on the resolving power of the chromatographic column. This is determined by the size of the particles of the stationary phase, length of the column, solvent flow rate, composition of the mobile phase, pH of the mobile phase, presence of salts and temperature. Each of these parameters can be varied to obtain the optimum resolution. If you get variations in retention time of solutes, or if two well separated peaks start to appear incompletely separated, check the mobile phase composition and look for leaks in the system.
22. Flow rates of solvents determine the total duration of the chromatographic cycle, clarity of the separation and width of peaks. Minor adjustments in flow rates can improve the sharpness of peaks in a moderately well separated chromatogram.
23. Owing to low boiling points of solvents most RP-HPLC separations are performed without including temperature as a variable. Thus most chromatographic separations are carried out at room temperature. Where applicable, increased temperature can improve column capacity and efficiency of the chromatographic separation. However, this requires use of expensive "column ovens" or other regulators of column temperature.
24. A decrease in pressure across the column causes a drop in the solubility of gases dissolved in the solvent resulting in formation of bubbles. High inlet pressure can further aggravate this problem. Bubbles passing through the detector cell appear as "spikes" on the chromatogram. Fluctuations in the detector response, poor resolution and changes in the flow rates are associated with bubbles. These can be avoided by thorough degassing of all solvents.
25. If you notice a baseline with noise, check the ground connections of the recorder and detector. This will correct high frequency short term noise ("grass"). If the noise persists for long time (long-term noise), most likely your solvents are not free of contaminants.
26. An upward or downward drift in the baseline is generally a result of changing composition of the mobile phase. Gradient elution often shows a drift with increasing

proportion of organic component. It is a common observation that the baseline drifts as the proportion of an organic solvent increases.

27. The final chromatogram obtained presents the result of fine tuning between the nature of the sample, amount of the sample loaded, and the chromatographic conditions designed by you.

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6

Localization and Distribution of Cyclooxygenase-2 in Brain Tissue by Immunohistochemistry

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1. Introduction

Prostaglandin production is dependent on the presence of an enzyme called prostaglandin G/H synthase, better known as cyclooxygenase, that catalyzes the first steps in the conversion from arachidonic acid. A cDNA for cyclooxygenase was first cloned from sheep seminal vesicles in 1988 ***(1,2)***. In 1991, we and others reported the cloning of a second isoform of cyclooxygenase, now known as COX-2, that is rapidly induced in response to growth factors and proinflammatory cytokines and downregulated by glucocorticoid hormones ***(3–6)***.

With the discovery that two isoforms of cyclooxygenase exist, a new appreciation of the diversity of prostanoid mediated processes has emerged ***(7,8)***. Molecular and immunological reagents provide tools for examination of cyclooxygenase expression and regulation, and when applied to histological preparations either as *in situ* hybridization or immunohistochemistry, provide details of tissue and cell-specific expression. Such studies reveal remarkable specialization of COX-1 and COX-2. For example, in the rat kidney COX-1 expression is limited to medullary collecting ducts and interstitial cells, whereas COX-2 is expressed in the macula densa and its levels are regulated by sodium intake ***(9)***.

The brain is a complex organ with many unique cellular constituents including neurons, glia, ependymal cells, and the vascular smooth muscle cells and endothelium of blood vessels and capillaries. Neurons comprise a diverse array of structural and transmitter phenotypes. The more numerous glia include astrocytes, the supporting cells of the central nervous system, resident macrophage-like cells known as microglia, and oligodendrocytes, which are responsible for myelination of neuronal axons.

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Both COX-1 and -2 are expressed in brain tissue and various studies have demonstrated regulated COX-2 expression in specific neuronal populations ***(10–12)***, astrocytes ***(13,14)***, microglia ***(15,16)***, and perivascular cells ***(17)***. Interest in central nervous system expression of cyclooxygenase has been heightened by epidemiological studies suggesting potential benefits of nonsteroidal anti-inflammatory drugs in the treatment and prevention of Alzheimer's disease ***(18–20)***. In addition, COX-2 selective inhibitors have been found to be neuroprotective following brain ischemia ***(21)***.

Immunohistochemistry essentially consists of incubating an antibody with tissue sections under conditions that allow specific interactions with antigen to occur while blocking nonspecific tissue binding. This is followed by incubation with a second antibody reactive with the first. The second antibody is generally modified such that its presence can be detected by means of an enzymatic reaction, fluorescent dye, or a third "linking" reagent that itself carries an enzyme or fluorescent dye. In the present case we use a biotinylated secondary antibody so that a streptavidin (which binds biotin with very high affinity) conjugated enzyme (horseradish peroxidase) or fluorescent marker (Texas Red) could be used. This provides greater amplification of signal than a two-component system consisting only of primary and secondary antibody. Although simple in principle, immunohistochemical methods must be empirically determined for each antigen:antibody interaction and will also vary with the tissue utilized.

In this chapter we present immunohistochemical methods currently employed in our laboratories to examine cyclooxygenase-2 expression in the rat brain. We have organized **Subheading 3.** into the following subsections: tissue fixation and sectioning (**Subheading 3.1.**); pretreatment and antibody incubation (**Subheading 3.2.**); signal detection (**Subheading 3.3.**); and controls and data collection (**Subheading 3.4.**). In addition, we provide an alternate method for detection using immunofluorescence. This approach has proven particularly useful for double-label immunohistochemistry.

2. Materials

1. Fixation: Rinsing solution: 0.5% (w/v) sodium nitrite and heparin (2 U/mL) in 0.1 *M* Na phosphate buffer, pH 7.2, prepared immediately before use. Paraformaldehyde fixative: 4% paraformaldehyde in 0.1 *M* Na phosphate buffer, pH 7.2. This fix should be prepared on the day of use and cooled to 4°C (*see* **Note 4**). Alternate acidic-glutaraldehyde fixative: 2.5% glutaraldehyde (v/v), 1% acetic acid (v/v), 10 m*M* Na-m-periodate, and 40 m*M* Na phosphate, pH 4.0, prepared the day of use ***(22)***. Surgical instruments for perfusion. Anesthetics appropriate for animal species being used.
2. Materials/supplies for tissue sectioning and staining: microtome with freezing stage or cryostat; microtome knife; isopentane (2-methyl butane) or 100%

ethanol; Cryoprotectant solution: 30% sucrose (w/v), 30% (v/v) ethylene glycol, 1% (w/v) polyvinyl pyrolidone-40 and 50 m*M* Na phosphate buffer, pH 7.2 ***(23)*** (*see* **Note 6**); netwells with 74 µm mesh and 12-well tissue culture plates (Corning, Elmira, NY) (**see Notes 7** and **9**); shaking platform.

3. Primary antibody: mouse antirat cyclooxygenase-2 monoclonal antibody from Transduction Laboratories (Lexington, KY). Several other suppliers also sell anti-COX-2 antibodies. The specificity of each for COX-2 should be determined (*see* **Table 1**). Store aliquots at –80°C or diluted 1:1 with glycerol at –20°C. Avoid freeze-thawing the antibody more than two or three times.
4. Secondary antibodies: biotin-labeled goat antimouse IgG antibody from Jackson ImmunoResearch Laboratory (West Grove, PA), multiple-label quality. Similar biotin-labeled antimouse antibodies are available from numerous suppliers. Store aliquots at –80°C or diluted 1:1 with glycerol at –20°C. Avoid freeze-thawing the antibody more than two or three times.
5. Horseradish-peroxidase (HRP)-labeled avidin: The Elite-ABC kit from Vector labs (Burlingame, CA) was used in this study. HRP-labeled avidin or -streptavidin conjugates are available from many vendors. From the Elite-ABC kit, dilute 2 µL solution A and 2 µL solution B per mL of 0.15 *M* Na phosphate buffer, pH 7.2. Allow 20–30 min for the ABC complex to form before placing the solution over tissue sections.
6. Streptavidin-Texas Red: For immunofluorescence detection of COX-2, use streptavidin-labeled with Texas Red (Jackson ImmunoResearch Laboratory) Store diluted 1:1 with glycerol at –20°C in the dark. Similar fluorescent-labeled reagents are widely available.
7. Washing and incubation solutions
   a. 0.15 *M* Na phosphate buffer, pH 7.2. Dissolve 20.16 g NaH_2PO_4 anhydrous and 61.36 g Na_2HPO_4 anhydrous in 4 L of distilled water. Adjust to pH 7.2 if necessary.
   b. 0.15 *M* Na phosphate buffer, pH 7.2, with 0.4% Triton X-100 (v/v). This later buffer is used for the majority of incubation steps.
   c. 175 m*M* Na acetate and 10 m*M* imidazole, pH 7.0: Dissolve 23.82 g Na acetate and 0.68 g imidazole in 1 L of distilled water. Adjust pH to 7.0.
8. HRP substrate mixture: 0.03% DAB (w/v), 100 m*M* Ni(II) sulfate-$7H_2O$, 125 m*M* Na acetate, 10 m*M* imidazole, and 0.01% hydrogen peroxide (v/v). Prepare immediately before use.
   a. Dissolve 8.506 g Na acetate and 0.34 g imidazole in 500 mL of distilled water. **Do not** adjust pH. This solution can be made earlier and stored at 4°C until ready for use.
   b. Dissolve 3 mg diaminobenzidine (DAB) in 10 mL of solution (a) with gentle stirring. **Note: DAB is potentially carcinogenic: handle with care! DAB tablets may be used to minimize handling.**
   c. Add 281 mg nickel (II) sulfate-heptahydrate (FLUKA, Ronkonkoma, NY) to solution (b) and dissolve completely with gentle stirring.

d. Immediately before use, add 3.3 µL of 30% hydrogen peroxide to solution (c).

Table 1
Specificity of Various Commercially Available Antibodies for Cyclooxygenases[a,b]

Ab Source and catalog no.	Antigen	Type	Rat COX-1	Rat COX-2	Human COX-1	Human COX-2	Mouse COX-2	IHC
Cayman (160116)	Murine COX-2 peptide	Rabbit polyclonal	–	+++	–	++	+	+/– (rat)
Cayman (160112)	Human COX-2 peptide	Mouse monoclonal	–	–	–	+++	–	NT
Oxford (PG 16)	COX-1 peptide	Rabbit polyclonal	–	–	++	–	NT	+ human)
Oxford (PG 27)	Human COX-2 peptide	Rabbit polyclonal	–	++	–	+++	NT	+/++ (rat)
Santa Cruz (N-20; sc-1746)	Rat COX-2 peptide	Goat polyclonal	–	+++	–	++	+	NT
Transduction laboratories (C22420)	Carboxyl half of rat COX-2	Mouse monoclonal	++	++	++	++	+	+++ (human and rat)

[a]Immunoreactivity for specific COX antigens was determined by standard western blot procedures ***(24)*** using a 1:1000 dilution of each antibody and horseradish-peroxidase conjugated secondary reactive with the appropriate species at 1:5000. Visualization was accomplished by Enhanced Chemiluminescence (ECL; Amersham) detection. Purified recombinant rat COX-1 and COX-2 and human COX-2 antigens were obtained from Phyllis Whiteley, Roche Bioscience (Palo Alto, CA). Human COX-1 immunoreactivity was detected in a protein lysate of purified human platelets. For these samples "++" indicates a detection limit of 10 ng recombinant protein or 10 μg of lysate, and "+++" indicates that 2 ng recombinant protein was detected. Murine COX-2 was detected in 5 μg protein lysate of LPS and γ interferon stimulated RAW 264.7 cells; no additional quantification was performed. NT, not tested.

[b]Immunohistochemistry (IHC) was carried out essentially as described in the text, using 4% paraformaldehyde fixed brain tissue. Despite its lack of specificity, the Transduction Laboratories antibody consistently performed better in immunohistochemical analyses; hence the "+++".

9. Slides and coverslipping: Glass slides treated to allow adherence (e.g., VectaBond, Superfrost; *see* **Note 10**); glass coverslips.
   Mounting medium:
   a. For sections reacted with DAB, coverslip with DPX (FLUKA) or an equivalent.
   b. For sections prepared for immunofluorescence, coverslip with ProLong (Molecular Probes, Eugene, OR) or equivalent in order to inhibit fading of the fluorescent marker.

3. Methods

3.1. Tissue Fixation and Sectioning

1. Following deep anesthesia with 90 mg/kg ketamine plus 6 mg/kg xylazine, rats are perfused with two solutions. First, perfuse animals via the ascending aorta with ≈ 50 mL of a rinsing solution containing sodium nitrite and heparin to remove the blood. Next, perfuse animals with 200 mL of either of two fixatives: (a) 4% paraformaldehyde, or (b) acidic-glutaraldehyde (*see* **Note 5** about choice of fixative). Remove brains and postfix for 2 h at 4°C in either fixative (a) or (b). The surgical procedures and amounts of solutions for perfusion will differ between species (*see* **Note 3**).
2. Following fixation, rinse the tissue briefly in 0.15 *M* Na phosphate buffer to remove any residual fixative. Then incubate the tissue for 24–48 h in 30% sucrose (w/v) in 0.15 *M* Na phosphate buffer, pH 7.2. After the tissue block has sunk to the bottom, blot the tissue dry of sucrose solution and rapidly freeze it by immersion in isopentane (2-methyl butane) chilled on dry ice. **Caution: isopentane is highly flammable. Ethanol chilled on dry ice may be used in place of isopentane for freezing.** Tissues can be sectioned immediately or stored at –80°C. For sectioning, cut 25–30 µm sections using a sliding microtome equipped with a freezing stage. Remove sections from the knife with a red sable brush and place them in 0.15 *M* Na phosphate buffer prior to immunohistochemical processing. For long term storage, place sections in Cryoprotectant solution (*see* **Note 6**). After equilibration in this solution, sections may be stored at –20°C for months to years with little loss of staining.

3.2. Tissue Pretreatment and Antibody Incubations

1. Place tissue sections in netwells in clean 12-well plates containing 2.5–3 mL of 0.15 *M* Na phosphate buffer and rinse four times for 5 min each. In this and all subsequent steps, gently agitate the sections on a shaking platform (≈ 60 rpm). Gently move sections between solutions by moving the netwell. Following the rinsing steps, dehydrate and rehydrate the sections by incubating for 5 min each through an ethanol series: 50%, 70%, 95%, 100%, 95%, 70%, and 50% (v/v in distilled water). Rinse sections three times for 5 min each in 0.15 *M* Na phosphate buffer with 0.4% Triton X-100 (v/v). Triton X-100 is a detergent that enhances penetration of the antibody solutions and reduces non-specific binding.
2. To block nonspecific binding of the antibody reagents, preabsorb sections for 30 min at room temperature in 10% normal goat serum (v/v) in 0.15 *M* Na phosphate

buffer with 0.4% Triton X-100 (v/v). Note that the normal serum used is the same species as the secondary antibody to be used later (*see* **Subheading 3.2.4.**).

3. Incubate sections with gentle agitation for 24–48 h at 4°C in the primary antibody solution (*see* **Note 9**). The primary antibody is a mouse antirat COX-2 IgG1 monoclonal diluted 1:1000 in 0.15 *M* Na phosphate buffer with 0.4% Triton X-100 (v/v) and 1% normal goat serum (v/v). Following incubation, rinse the sections eight times for 3 min each in 0.15 *M* Na phosphate buffer with 0.4% Triton X-100 (v/v).
4. Gently agitate sections with biotin-labeled secondary antibody for 2–3 h at room temperature. The secondary antibody is a goat anti-mouse IgG diluted 1:3000 in 0.15 *M* Na phosphate buffer with 0.4% Triton X-100 (v/v) and 1% normal goat serum (v/v). Following incubation, rinse sections eight times for 3 min each in 0.15 *M* Na phosphate buffer with 0.4% Triton X-100 (v/v).

3.3. Signal Detection

3.3.1. Diaminobenzidine

1. For a permanent record of the COX-2 staining, the Elite-ABC method with a nickel-enhanced diaminobenzidine reaction is used. Following rinsing of the sections in **Subheading 3.2.4.**, transfer the netwell containing the tissue sections into the prepared Elite-ABC complex and agitate gently for 2 h at room temperature. Remove and save the Elite-ABC solution for use below in **step 2**. Rinse the sections eight times for 3 min each in 175 m*M* Na acetate + 10 m*M* imidazole buffer, pH 7.0. This buffer is used because it does not precipitate the nickel substrate in **step 2**.
2. Prepare the HRP substrate solution (*see* **Subheading 2., step 8**) and immediately place on the sections. Incubate 2–8 min at room temperature with agitation. If necessary, examine the sections under a dissecting microscope to determine the appearance of specific staining. To stop the reaction once the specific COX-2 staining is visible, but before the background staining becomes severe, transfer netwells to 0.15 *M* phosphate buffer, pH 7.2. This nickel-enhanced DAB reaction is very sensitive, however, the reaction proceeds rapidly and must be monitored carefully. To the HRP substrate solution, add the Elite-ABC solution saved from **Subheading 3.3.1.1.** above. Set aside and allow the Elite-ABC-HRP to completely degrade the remaining DAB. This method has been demonstrated to be better than Clorox treatment in destroying the potentially carcinogenic DAB.
3. Following the DAB reaction, rinse the sections briefly in 0.15 *M* Na phosphate buffer. Mount the sections on glass slides treated to allow adherence (*see* **Note 10**), air dry, dehydrate through alcohols and xylene, and coverslip with DPX or equivalent.

3.3.2. Texas Red

1. In some instances (e.g., double-labeling immunohistochemistry), it may be preferable to use a fluorescent marker to localize COX-2 (*see* **Note 11**). Following rinsing of the sections in **Subheading 3.2., step 4**, transfer the netwell containing the tissue sections into Streptavidin-Texas Red diluted 1:5000 in 0.15 *M* Na phos-

phate buffer with 0.4% Triton X-100 (v/v). Incubate the sections in the dark with gentle agitation for 2–3 h at room temperature.

2. Rinse the sections six times for 5 min each in 0.15 *M* Na phosphate buffer. Avoid exposure to light. Mount on prepared glass slides and coverslip with ProLong (prepared according to the manufacturer's protocol) before the sections have completely dried. Set the slides aside in the dark and allow the ProLong to dry. The dried ProLong dramatically inhibits the fading of the fluorophore when viewed in the fluorescent microscope. When dry, store the slides at 4°C in the dark.

3.4. Controls and Data Collection

1. A number of controls regarding the specificity of the antibodies should be included. Most important are controls for the primary antibody. First, the staining by the primary antibody may be established by omitting the antibody. This does not establish its specificity, however. To determine specificity, the primary antibody may be pre-incubated with the antigen (final concentration of 10^{-5} *M*) for 1–4 h prior to incubation with tissue sections. This preabsorption step should eliminate specific staining for COX-2. Because of the similarity of COX-1 and COX-2, it may be necessary to perform a Western blot to determine specificity of the primary antiserum (*see* table in **Notes**).
2. An additional important control is the use of a positive control tissue, one in which you know there will be staining. In the present case, we induced the expression of COX-2 by injecting young adult rats with kainic acid (18 mg/kg, IP) to induce seizures. Rats were perfused 24 h later (*see* **Fig. 1**).
3. To accurately determine changes in expression of COX-2, all tissues to be compared should be processed identically. Prepare master mixes of all antibody solutions, then divide among the tissue samples. Similarly, all washes should be identical in length and temperature. The use of netwells simplifies this procedure. Finally, the diaminobenzidine reaction should be identical for all samples. A master mix should be prepared and the length of the reaction should be the same for all samples. The reaction should be stopped before the staining intensity begins to plateau. The described DAB reaction is linear over at least 10 min in our hands. Lastly, the photography of the tissue sections and printing of the photomicrographs should be identical. The use of automatic exposure meters on the microscope camera should be avoided as this will compensate for differences in staining intensity. Similarly, final printing of the photomicrographs in the darkroom should be completed with identical exposures and contrast.

4. Notes

1. See **Table 1** for information on the specificity and utility in IHC for some commercially available cyclooxygenase antibodies.
2. The methods described here should be applicable to most other tissues and species. We have successfully used these methods on both mouse and human brain sections with similar results. The major care in transferring the method to other species will be in determining the specificity of the primary antiserum.

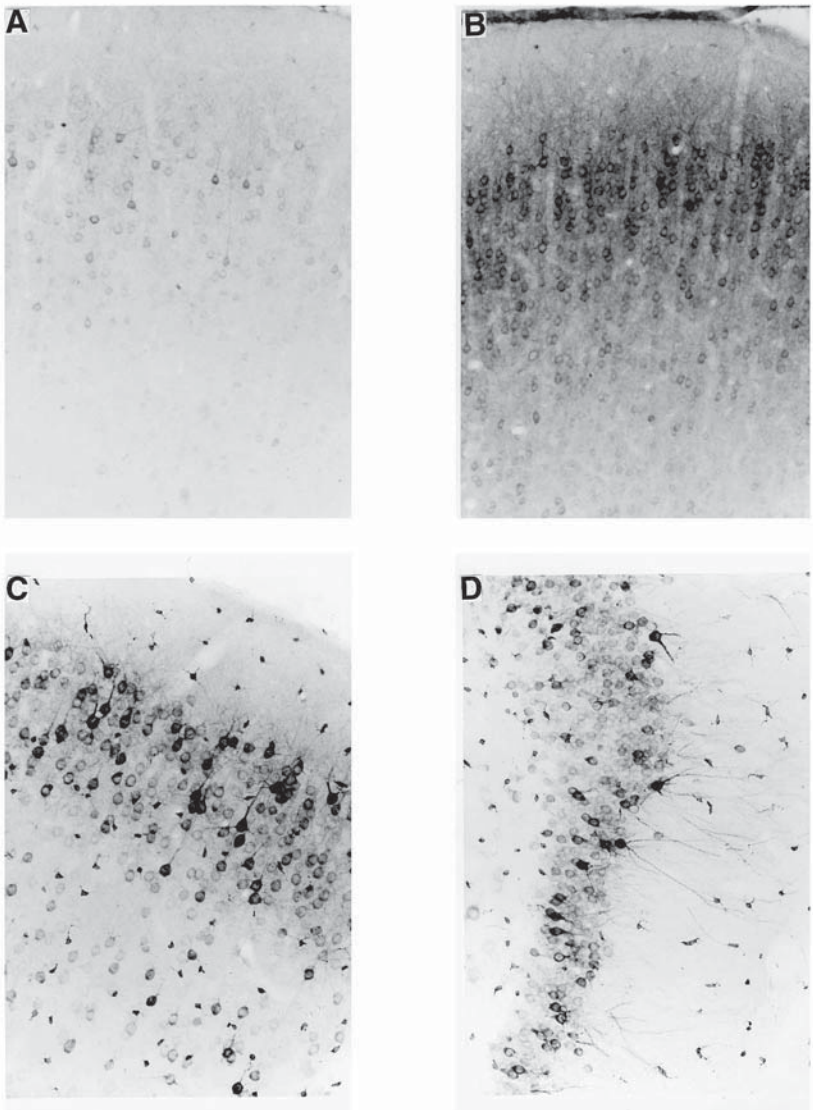

Fig. 1. Induction of COX-2 and comparison of fixatives. **(A)** and **(B)** show immunostaining of rat neocortex perfused with 4% paraformaldehyde using the Transduction Laboratories antibody at a 1:1000 dilution. Control rat neocortex (A) shows modest cyclooxygenase immunoreactivity in some neurons, but 24 h following seizure induction with kainate, robust COX-2 staining is observed in many neurons (B). **(C)** and **(D)** represent sections from control rat tissue perfusion-fixed with acidic glutaraldehyde and subjected to the protocol presented in this chapter. Note the strong staining of neurons and their processes (C is from neocortex, D from dentate gyrus) relative to 4% paraformaldehyde-fixed control tissue (compare to A). Original magnification was ×20.

3. Although techniques for animal perfusion are relatively simple, we feel that they should be performed and demonstrated by someone with previous experience rather than described. It is particularly important to adhere to local guidelines of appropriate animal care. Furthermore, the technique differs depending on the species used and tissues collected. For example, in our studies of rat brain we limit fixation to the head and upper extremities by clamping the descending aorta.
4. To prepare 4% paraformaldehye fixative, dissolve 40 g of paraformaldehyde and 0.6 g NaOH with rapid stirring in 500 mL of distilled water by heating to no more than 60°C. Remove from heat and add 500 mL of 0.2 *M* Na phosphate, pH 7.2. Cool to room temperature and if necessary adjust the pH to 7.2 with glacial acetic acid. Filter the solution through Whatmann No. 1 paper to remove particulates and cool to 4°C prior to use in perfusion. To avoid exposure to aldehydes, perform all operations, including animal perfusion, in a fume hood. This recipe can be scaled to prepare larger or smaller amounts of fixative as required.
5. There is a significant difference in the staining intensity for COX-2 depending on the method of fixation. Although there is moderate staining retained with standard 4% paraformaldehyde fixation, staining is markedly increased with the use of the acidic-glutaraldehyde fixative (*see* **Fig. 1**). It is not clear why the acidic fixative is superior, however, McKanna and Zhang have demonstrated the usefulness of this fixative for staining lipocortin 1 ***(22)***. We have not tested this fixative with other antigens, but there is a concern that the high glutaraldehyde concentration may denature other antigens. An alternative method is the perfusion of the animal with 4% paraformaldehyde and then postfixing only those tissues to be stained for COX-2 in the acidic-glutaraldehyde fix. Simple postfixation in this fix enhances COX-2 staining, though it does not reach the quality of tissue perfused with the same fixative.
6. The use of a cryoprotectant to store sections has proven very convenient. Normally we fill the wells of a multiwell culture plate (e.g., 24-well plate) with cryoprotectant solution, and then place sections in the wells as they are cut. Sections are allowed to equilibrate for 24 h at 4°C prior to storage at –20°C.
7. Although with modifications this method can be applied to sections already mounted on glass slides, we strongly recommend the use of free-floating section immunohistochemistry whenever possible. The use of net-wells greatly simplifies this process. Free-floating immunohistochemistry provides more uniform tissue penetration and results in stronger signals. It also allows one to successfully stain thicker sections (up to 40 µm in our hands).
8. A dehydration step prior to COX-2 immunohistochemistry appears to be important for antigen detection. The current method employs a gradual dehydration and rehydration series in ethanol. Others have reported success using ice-cold 100% methanol for 20 min ***(14)***.
9. The incubation times given are highly variable. With the antibody dilutions used here, longer incubation times are more appropriate. Many laboratories use much higher antibody concentrations, but incubate in the primary antibody for only 1–2 h. Additionally, the choice of buffers may also be varied. The sodium phosphate buffer

listed here may be replaced with PBS or Tris buffers with similar success. The sodium acetate-imidazole buffer used for the DAB reaction is necessary since the nickel substrate precipitates in Tris or phosphate buffers. Finally, the molarity of each buffer has been adjusted to near 300 mOsm in order to be suitable for electron microscopy.

10. Glass slides must be treated to allow for proper tissue adherence during the mounting process. We typically use pretreated slides, such as Superfrost brand, available from Fisher Scientific (Pittsburgh, PA), or coat standard glass slides with VectaBond (Jackson ImmunoResearch Laboratory) using instructions that come with the product. Other treatments such as coating with poly-D-lysine or gelatin-Chrom-alum are also suitable.
11. The current immunofluorescent method involves the use of a biotin-labeled secondary antibody followed by streptavidin-Texas Red. This method allows for the use of a dilute secondary antibody, thus reducing background, and a fluorophore-labeled streptavidin which amplifies the signal. Other fluorophores linked to streptavidin may be used in place of Texas Red; however, the sensitivity of Texas Red offers an advantage. For double-labeling immunofluorescence, the second primary antibody can be detected using the current method or the secondary antibody may be directly labeled with the fluorophore. The second antigen to be observed is best visualized with a fluorophore such as FITC that is distinct in its excitation/emission wavelengths from the first antigen's fluorescent marker. This minimizes problems of interpretation in potential double-labeling of cells. Finally, the use of a mounting medium that inhibits fading of the fluorophores enhances visualization and photography. A number of companies sell antifading mounting media.

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7

Assessment of Cyclooxygenase Protein Expression by Western Blotting

Zhonghong Guan and Aubrey R. Morrison

1. Introduction

Western blotting provides an effective, sensitive, and specific method for the identification and quantitation of cyclooxygenase protein expression in an unknown complex protein sample. Electrophoresis is used to separate complex mixtures of proteins. Denaturing discontinuous one-dimensional gel electrophoresis separates proteins only based on molecular size as they move through a SDS-polyacrylamide gel (SDS-PAGE) toward the anode with the smaller proteins migrating faster and bigger proteins running slower. The SDS-PAGE is a separating gel topped by a stacking gel and secured in an electrophoresis apparatus. Sample proteins are solubilized by boiling in the presence of SDS and equal amounts of the protein solution are loaded into a gel lane, and the individual proteins separated electrophoretically. 2-Mercaptoethanol (2-ME) and dithiothreitol (DTT) are added to reduce disulfide bonds.

After electrophoresis, proteins are electro-transfered from polyacrylamide gels to blot transfer membranes. Protein on the transfer membranes are stained to estimate the transfering efficiency. Even and efficient transfer of most proteins is possible with semidry blotting that is a convenient alternative to tank transfer systems. Instead of being placed vertically into a tank filled with a large amount of transfer buffer, the gel is held horizontally between transfer buffer-saturated filter paper that is in contact with the electrodes. The electrodes are close together, giving high field strengths and rapid transfer. After transfer, the membrane can be stained to verify the transfer efficiency.

Immobilized proteins are probed with cyclooxygenase-specific antibodies to identify and quantitate any COX protein expression. The membrane is immersed in blocking buffer to fill all protein-binding sites with nonreactive

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protein or detergent. Then, it is incubated in a primary antibody solution containing the COX antibody directed against the COX protein. The blot is washed and exposed to an enzyme-antibody conjugate directed against the primary antibody. COX protein expression is then identified by chromogenic or luminescent visualization of the COX/anti-COX antibody/ anti-anti-COX antibody/ enzyme complex bound to the membrane. Amersham enhanced chemiluminescence (ECL) western blotting detection kit is a sensitive and convenient system for the immunodetection.

The primary and secondary antibodies can be almost completely removed from membrane by stripping of bound antibodies and reprobed several times if necessary.

2. Materials

1. 10X Phosphate buffered saline (PBS): 160 g NaCl, 4 g KCl, 28.8 g Na_2HPO_4, 4.8 g KH_2PO_4. Add H_2O to 2 L. Adjust pH to 7.6 and autoclave.
2. Whole cell extract (WCE) buffer: HEPES-NaOH, pH 7.7), 0.3 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* ethylenediaminetetra-acetic acid (EDTA), 1% Triton X-100, 0.5 m*M* dithiothreitol (DTT), 20 m*M* β-glycerophosphate, 100 μ*M* $NaVO_4$, 2 μg/mL leupeptin and 100 μg/mL phenylmethylsulfonyl fluoride (PMSF).
3. Copper (II) sulfate pentahydrate 4% solution (Sigma, St. Louis, MO).
4. Bicinchoninic acid solution (Sigma).
5. Micoplate reader (Model 450, Bio-Rad, Hercules, CA)
6. 10% separating gel solution (10 mL):

40% Acrylamide stock (acrylamide:bis-acrylamide, 29:1)	2.5 mL
1.5 Tris-HCl, pH 8.8	2.5 mL
10% SDS	0.1 mL
10% Ammonium persulfate	0.1 mL
H_2O	4.8 mL
TEMED	10 μL

7. 5% stacking gel solutions (10 mL):

40% Acrylamide stock (acrylamide:bis-acrylamide, 29:1)	1.25 mL
0.5 Tris-HCl, pH 6.8	2.5 mL
10% SDS	0.1 mL
10% Ammonium persulfate	0.1 mL
H_2O	6.05 mL
TEMED	10 μL

8. H_2O-saturated isobutyl alcohol: The H_2O-saturated isobutyl alcohol is prepared by shaking isobutyl alcohol and H_2O in a separatory funnel. The aqueous (lower) phase is removed. This procedure is repeated several times. The final upper phase is H_2O-saturated isobutyl alcohol.
9. Protein molecular-weight-standard mixture (Bio-Rad).
10. 6X SDS sample buffer:

0.5 Tris-HCl, pH 6.8	7 mL
Glycerol	3.6 mL

SDS	1 g
DTT	0.93 g
Bromphenol blue	1.2 mg

Add H_2O to 10 mL. Store in 0.5-mL aliquots at –70°C.

11. 1X SDS electrophoresis buffer:

Tris base	3.02 g
Glycine	14.4 g
SDS	1 g

Add H_2O to 1000 mL.

12. Electrophoresis apparatus set: Mini-PROTEIN II cell (Bio-Rad) or Mighty Small II unit (Hoefer Pharmacia Biotech) with clamps, glass plates, casting stand, buffer chambers, 0.75-mm spacers and 0.75-mm comb with 10 teeth (**Notes 1–4**).
13. Constant-current or constant-voltage power supply (Bio-Rad).
14. Six sheets of Whatman 3MM filter paper cut to size of gel and saturated with transfer buffer.
15. Semi-dry transfer unit (Bio-Rad).
16. Transfer buffer:

Tris base	18.2 g
Glycine	86.5 g

Add H_2O to 4 L. Then add 1.2 L methanol and bring to 6 L with H_2O (200 mL MeOH/L of buffer).

17. Polyvinylidene difluoride (PVDF) transfer membrane (Immobilon-PTM; Millipore).
18. Ponceau S solution: Dissolve 0.5 g Ponceau S in 1 mL glacial acid. Adjust to 100 mL with H_2O.
19. Tris buffered saline (TBS), pH 7.6

Tris base	2.42 g
NaCl	8 g
1 *M* HCl	3.8 mL

Add H_2O to 1000 mL and adjust pH to 7.6.

20. TBS-T: 0.05% Tween-20 in TBS.
21. Blocking buffer: 10% nonfat dry milk in TBS-T.
22. Anti-COX-1 or COX-2 antibody (Cayman Chemical, Ann Arbor, MI).
23. Goat antirabbit IgG antibody coupled to horseradish peroxidase (Amersham, Arlington Heights, IL).
24. Plastic container for blotting membranes.
25. ECL Western blotting detection reagent (Amersham).
26. Stripping buffer: 100 m*M* 2-mercaptoethanol, 2% SDS and 62.5 m*M* Tris-HCl, pH 6.7.

3. Method

3.1. Preparation of Samples

3.1.1. Lysis of Mammalian Cells

1. Confluent cells grown in monolayer are treated with required pharmacological compounds.

2. Cells are then washed twice with ice-cold phosphate-buffered saline (PBS) and drain thoroughly by aspiration.
3. Add whole cell extract buffer (WCE) and rock the cells for 30 min in the cold room to lyse the cells. Scrape the viscous lysate into a microfuge tube with a policeman.
4. Centrifuge the samples for 20 min at maximal speed (Eppendorf microfuge) in the cold room.
5. Collect the supernatant and store in –80°C. Discard the pellet.

3.1.2. Measurement of Protein by Sigma Bicinchoninic Acid Protein Assay Kit

1. Prepare a protein standard curve described below in order to determine the protein concentration of unknown samples.

g BSA/10 µL	µL of 10 mg/mL BSA stock	µL of H_2O
0	0	40
5	2	38
10	4	36
20	8	32
40	16	24
80	32	8

2. Prepare the required amount of protein determination reagent by adding one part of copper (II) sulfate pentahydrate 4% solution to 50 parts of bicinchoninic acid solution.
3. Mix 190 µL of protein determination reagent with 10 µL of protein standard or unknown protein sample and load the mixture on 96-well assay plate.
4. Incubate plate at 37°C for 30 min.
5. Read the plate and determine the absorbance at 562 nm in a Micoplate reader.
6. Calculate the protein concentration (mg/mL) of unknown samples according to the protein standard curve.

3.2. Electrophoretic Separation of Protein by SDS-PAGE

3.2.1. Pour the Separating Gel

1. Assemble the glass-plate sandwich of the electrophoresis apparatus according to manufacturer's instructions using two clean glass plates (washed completely with H_2O and ethanol) and two 0.75-mm spacers.
2. Lock the sandwich to the casting stand.
3. Prepare the 10% separating gel solution. After adding the specified amount of 10% ammonium persulfate (APS) and TEMED to the degassed solution, stir gently to mix. Use the solution immediately.
4. Using a Pasteur pipet, apply the separating gel solution to the sandwich along an edge of one of the spacers.
5. Using another Pasteur pipet, slowly cover the top of the gel with a layer (~1 cm thick) of H_2O-saturated isobutyl alcohol, by gently layering the isobutyl alcohol against the edge of one of the spacers.
6. Allow the gel to polymerize 30–60 min at room temperature.

3.2.2. Pour the Stacking Gel

1. Pour off the layer of H_2O-saturated isobutyl alcohol and rinse with H_2O three times to completely remove isobutyl alcohol. (**Note 5**).
2. Prepare the stacking gel solution. Use the solution immediately after adding the APS and TEMED to keep it from polymerizing in the flask.
3. Using a Pasteur pipet, slowly allow the stacking gel solution to trickle into the center of the sandwich along an edge of one of the spacers until the solution in the sandwich arrives at the top of the plates.
4. Insert a 0.75-mm Teflon comb into the layer of stacking gel solution (be careful not to trap air bubbles in the tooth edges of the comb). If necessary, add additional stacking gel to fill the spaces in the comb completely.
5. Allow the stacking gel solution to polymerize 30–60 min at room temperature. (**Notes 6** and **7**).

3.2.3. Prepare the Sample and Load the Gel

1. Dilute a portion of the protein sample to be analyzed 5:1 (v/v) with 6X SDS sample buffer and boil 5 min in a sealed screw-cap microcentrifuge tube. (**Notes 8** and **9**).
2. Carefully remove the Teflon comb without tearing the edges of the polyacrylamide wells. After the comb is removed, wash the wells with 1X SDS electrophoresis buffer.
3. Using a Pasteur pipet, wash and fill the wells with 1X SDS electrophoresis buffer.
4. Attach gel sandwich to upper buffer chamber according to the manufacturer's instructions.
5. Fill lower buffer chamber with the recommended amount of 1X SDS electrophoresis buffer.
6. Place sandwich attached to upper buffer chamber into lower buffer chamber.
7. Partially fill the upper buffer chamber with 1X SDS electrophoresis buffer so that the sample wells of the stacking gel are filled with buffer.
8. Load the protein samples (using the loading tips) into sample wells by carefully applying the sample as a thin layer at the bottom of the wells. Load control wells with molecular weight standards. Add an equal volume of 1X SDS sample buffer to any empty wells to prevent spreading of adjoining lanes.
9. Fill the remainder of the upper buffer chamber with 1X SDS running buffer so that the upper platinum electrode is completely covered. Do this slowly so that samples are not swept into adjacent wells.

3.2.4. Running the Gel

1. Connect the power supply to the cell and run at 15 mA of constant current for each 0.75 mm thick gel, until the bromphenol blue tracking dye enters the separating gel. Then increase the current to 20 mA for each gel (**Notes 10–13**).
2. When the bromphenol blue tracking dye reach the bottom of the separating gel, disconnect the power supply.

3.2.5. Disassemble the Gel

1. Discard electrode buffer and remove the upper buffer chamber with the attached gel sandwich.
2. Orient the gel so that the order of the sample wells is known, remove the sandwich from the upper buffer chamber.
3. Use the exposed spacer as a lever to pry open the glass plate carefully, exposing the gel.
4. Carefully remove the gel from the lower plate. Cut a small triangle off one corner of the gel so the lane orientation is not lost.

3.3. Immunodetection of Cyclooxygenase Protein Expression

3.3.1. Transfer of Proteins from SDS-PAGE Gel to Transfer Membrane

1. Soak and equilibrate the gel into transfer buffer for 30 min.
2. Prepare transfer membrane: Cut PVDF membrane to same size as gel plus ~2 mm on each edge. Sock the membrane in methanol for 20 s, then place it into transfer buffer and equilibrate for 15 min. (Do not let membrane dry at any time. If this occurs, wet once again with methanol and transfer buffer as described above.)
3. Cut two sheets of the extra thick filter paper (Bio-Rad) to the same size of the gel. Equilibrate the filter paper with transfer buffer for 15 min.
4. Place one sheet of filter paper saturated with transfer buffer on the anode.
5. Place equilibrated transfer membrane on top of filter paper. Remove all bubbles between membrane and filter paper by rolling a test tube over surface of membrane.
6. Place gel on top of membrane. Carefully roll a test tube over surface of gel to insure intimate contact between gel and membrane and to remove any interfering bubbles.
7. Complete the transfer stack by putting the remaining sheet of filter paper on top of gel. Roll out bubbles as described above.
8. Place top electrode onto transfer stack. Connect high-voltage leads to the power supply. Apply 15–18 V constant voltage to initiate protein transfer. Transfers of 1 h are generally sufficient. (Once assembled, do not move the top electrode. This can shift the transfer stack and move the gel relative to the membrane. Some transfer will occur as soon as the gel contacts the membrane, and any shifting of the transfer stack after assembly will distort the transfer pattern.) (See **Notes 14** and **15**.)
9. After transfer, turn off power supply and disassemble unit. Remove membrane from transfer stack.

3.3.2. Reversible Staining of Transferred Protein

1. Place protein transferred PVDF membrane in Ponceau S solution to stain for 5 min at room temperature.
2. Destain 2 min in water and observe the protein bands on the membrane to verify the transfer efficiency.
3. Completely destain membrane by soaking in an additional 10 min in several changes of water.

3.3.3. Western Immunodetection

1. Place membrane in plastic container with 10 mL blocking buffer (enough to completely cover membrane) (**Notes 16** and **17**). Incubate 1 h at room temperature or 4°C overnight with agitation on an rocking platform.
2. 1:2000 dilution of COX primary antibody in 10 mL of blocking buffer (dilution may vary with specific antibody) (**Note 18**).
3. Pour off blocking buffer. Replace with diluted primary antibody and incubate 4 h at room temperature or 4°C overnight with constant agitation.
4. Pour off primary antibody solution and wash five times by agitating with 20 mL TBS-T, 10–15 min each time.
5. 1:4000 dilute goat antirabbit (or antispecies specific) IgG antibody coupled to horseradish peroxidase in blocking buffer.
6. After washing membrane, add diluted goat antirabbit (or species specific) IgG antibody coupled to horseradish peroxidase, and incubate 1–2 h at room temperature with constant agitation.
7. Pour out secondary antibody solution and wash 5 times by agitating with 20 mL TBS-T, 10–15 min each time.
8. Detection by using enhanced chemiluminescence (ECL) kit:
   a. Mix 10 mL of ECL detection solution 1 and 2.
   b. Drain the excess TBS-T buffer from the washed membrane and place it on the piece of saran wrap, protein side up.
   c. Add the mixed detection reagent to the protein side of the membrane. Incubate for 1 min at room temperature without agitation.
   d. Drain off excess detection reagent and wrap membrane in saran wrap.
   e. Place the membrane, protein side up, in the film cassette. Place a sheet of film on top of the membrane, close the cassette and expose for one min or time required for optimal detection.

3.4. Stripping of Western Blotting

1. Submerge the membrane in stripping buffer, Incubate at 50°C for 30 min.
2. Wash the membrane twice for 10 min in TBS-T at room temperature using large volumes of wash buffer.
3. Reprobe the membrane as described above (**Subheading 3.3.**).

4. Notes

1. Never remove or insert high-voltage leads unless the power supply is turned off. Always grasp high-voltage leads one at a time with one hand only. Never insert or remove high-voltage leads with both hands.
2. Always start with the power supply turned off. Make sure that the power supply is off and the voltage control is zero first. Then hook up the gel apparatus: normally, connect the red high-voltage lead to the red outlet and the black high-voltage lead to the black outlet. Turn the power supply on with the controls set at zero and the high-voltage leads connected, then turn up the voltage, current, or

power to the desired level. Reverse the process when the power supply is to be turned off.

3. Power supplies usually have more than one pair of outlets. If more than one gel is connected directly to the outlets of a power supply, then these gels are connected in parallel. In a parallel circuit, the voltage is the same across each gel.
4. Thicker gels require more current which creates more heat when it is running. A 1.5-mm gel can be thought of as consisting of two 0.75-mm-thick gels run in parallel. Unless temperature control is available in the gel unit, a thick gel should be run more slowly than a thin gel.
5. Residual isobutyl alcohol can reduce resolution of the protein bands; therefore, it must be completely removed. The isobutyl alcohol overlay should not be left on the gel longer than 2 h.
6. If the gels polymerize too fast, the amount of ammonium persulfate should be reduced by one-third to one-half. If the gels polymerize too slowly or fail to polymerize all the way to the top, use fresh ammonium persulfate or increase the amount of ammonium persulfate by one-third to one-half.
7. After a separating gel is poured, it may be stored with an overlay of the same buffer used in the gel. Immediately prior to use, the stacking gel should be poured; otherwise, there will be a gradual diffusion-driven mixing of buffers between the two gels, which will cause a loss of resolution.
8. Prior to adding the sample buffer, keep samples at 0°C. Add the SDS sample buffer directly to the 0°C sample (still on ice), vortex and transfer directly to a 100°C water bath for 5 min. Do not leave the sample in SDS sample buffer at room temperature without first heating to a 100°C to inactivate proteases.
9. Before loading the samples, preparing the samples at the equal amount of protein, approximately the same concentration and loading volume to each well will ensure that all lanes are the same width and that the proteins run evenly. The samples will layer on the bottom of the wells because the glycerol added to the sample buffer gives the solution a greater density than the electrophoresis buffer. The bromphenol blue in the sample buffer makes sample application easy to follow visually.
10. Uneven heating of the gel causes differential migration of proteins, with the outer lanes moving more slowly than the center lanes (so-called smiling). Increased heat transfer eliminates smiling and can be achieved by stirring the lower buffer with a magnetic stirrer and by running the gel in the cold room or on the ice. Alternatively, decrease the heat load by running at a lower current.
11. If the tracking dye band is diffuse, prepare fresh buffer and acrylamide monomer stocks. If the protein bands are diffuse, increase the current by 25–50% to complete the run more quickly and minimize band diffusion. If there is vertical streaking of protein bands, decrease the amount of sample loaded on the gel, further purify the protein of interest to reduce the amount of contaminating protein applied to the gel, or reduce the current by 25–50%.
12. If the protein bands spread laterally from gel lanes, the time between applying the sample and running the gel should be reduced in order to decrease the diffu-

sion of sample out of the wells. Alternatively, the acrylamide percentage can be increased in the stacking gels from 4% to 4.5% or 5% acrylamide, or the operating current should be increased by 25% to decrease diffusion in the stacking gel.

13. If the protein bands are uneven, the stacking gel may not have been adequately polymerized. This can be corrected by deaerating the stacking gel solution thoroughly or by increasing the amount of ammonium persulfate and TEMED. Another cause of distorted bands is high salt concentration in the protein sample, which can be removed by dialysis, gel filtration, or precipitation. Skewed protein bands can be caused by an uneven interface between the stacking and separating gels, which can be corrected by starting over and being careful not to disturb the separating gel while overlaying with isobutyl alcohol.
14. Transfer efficiency depends on many factors, such as gel concentration and thickness, protein size, shape, and net charge. Below is a guideline for 0.75-mm-thick SDS-PAGE gels transferred by semi-dry blotting.

Percent acrylamide (separating gel)	Size range transferred (~100% efficiency)
5–7	29–150 kD_a
8–10	14–66 kD_a
13–15	<36 kD_a
18–20	<20 kD_a

The transfer buffer can be modified to increase efficiency. Lowering the concentration of methanol will also improve the recovery of proteins from the gel. Adding SDS to a concentration of 0.1% in the transfer buffer improves the transfer of all proteins out of the gel, particularly those above 60–90 kD_a in size. Gel cross-linking and thickness also have a profound effect on the transfer efficiency. In general, 0.5–0.75-mm-thick gels will transfer much more efficiently than thicker gels. Gels with a higher acrylamide percentage will also transfer less efficiently.

15. Multiple gels can be transferred using semidry blotting. Simply put a sheet dialysis membrane (Bio-Rad) equilibrated with transfer buffer between each transfer stack. Transfer efficiency is dependent on the position of the transfer stack in the blotting unit and for critical applications transferring one gel at one time is recommended. The gel next to the anode tends to be more efficiently transferred when blotting more than one gel at a time.
16. A variety of agents are currently used to block binding sites on the membrane after blotting These include Tween-20, nonfat dry milk, BSA, and serum. A 0.05% (v/v) solution of Tween-20 in TBS (TBS-T), a convenient alternative to protein-based blocking agents, is recommended for chromogenic development of PVDF membranes. In contrast to dry milk/TBS blocking solution, TBS-T is stable and has a long shelf life at 4°C. Furthermore, TBS-T generally produces a clean background.
17. Insufficient blocking or nonspecific binding of the primary or secondary antibody will cause a high background stain. Try switching to another blocking agent; protein blocking agents may weakly cross-react. Lowering the concen-

tration of primary or secondary antibody should decrease background and improve specificity.

18. The specificity of COX-1 and COX-2 antibodies is critical for the interpretation of the results. Some commercial COX-1 antibodies are found to cross-react with COX-2 protein. Therefore, positive controls using purified COX-1 and COX-2 protein are recommended in this protocol.

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8

Assessment of Arachidonic Acid Distribution into Phospholipids of Inflammatory Cells

Alfred N. Fonteh

1. Introduction

Arachidonic acid located at the *sn-2* position of glycerophospholipids is a precursor of an important class of lipid mediators termed eicosanoids ***(1,2)***. The major eicosanoids include leukotrienes, prostaglandins, and thromboxanes. In inflammatory cells, the bulk of AA is esterified into phospholipids (PE, PC, and PI/PS). Arachidonate is further localized in specific subclasses based on different *sn-1* substituents of the glycerol backbone of PC and PE. In mast cells and neutrophils, the major subclasses of PC are 1-acyl- and 1-alkyl-linked, whereas 1-acyl- and 1-alk-1-enyl-linked subclasses constitute the bulk of arachidonate in PE ***(3–5)***. PI is composed mainly of 1-acyl-linked species in these cells.

Inflammatory cells under resting conditions, maintain very low levels of free AA. The levels of AA are maintained by rapid acylation and remodeling activities between phospholipid subclasses. Initially, free AA is converted to arachidonoyl CoA by arachidonyl CoA synthetase at the expense of ATP. Arachidonoyl CoA is then rapidly incorporated into 1-acyl-2-lyso phospholipids by CoA dependent acylation reactions. Once in the 1-acyl-subclass, arachidonate is remodeled into 1-ether phospholipid subclasses by Co-A independent transacylase (CoA-IT) activity. This rapid incorporation into 1-ester-linked subclasses followed by the CoA-IT orchestrated remodeling into 1-ether-linked subclasses, accounts for the uneven distribution of arachidonate in phospholipid subclasses ***(6)***.

Recent studies suggest that the cellular content and the amount of AA in lipid bodies is increased many fold when inflammatory cells are exposed to very high AA concentrations and upon migration of these cells into sites of

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inflammation. These lipid bodies are composed mainly of triglycerides, suggesting that TGs may represent a novel AA storage pool. For example, inflammatory cells in circulation contain >90% of their arachidonate in phospholipids. Upon migration of these cells into the lung, there is a fourfold increase in AA content of the cells concomitant with a significant increase of AA in TGs *(7)*. The proposed pathway for the incorporation of AA into triglycerides involves the formation of a various intermediates *(6)*.

The aforementioned studies suggest that AA is not evenly distributed among glycerolipid classes. Moreover, AA is being constantly remodeled between various glycerolipid pools. Furthermore, the cellular location of AA is greatly influenced by disease conditions. In addition to the phospholipid subclasses described above, at least 20 arachidonate containing molecular species exist in phospholipids (PC, PE, and PI) of human neutrophils and mast cells. Therefore, to accurately determine the distribution of AA within glycerolipids and to understand the role of AA metabolism in diseases, it is essential that the classes, subclasses, and molecular species are isolated and identified. An effective means of separating phospholipids utilizes high performance liquid chromatography employing columns with different silica bonded phases. For cells in culture, radiolabeling with tritiated AA followed by multiple steps of HPLC offers a way of identifying arachidonate distribution within phospholipids. For cells that can not be radiolabeled to isotopic equilibrium, the distribution of AA into glycerolipids is best determined by gas chromatography in combination with mass spectroscopy. This chapter reviews the HPLC methods that we use in our laboratory to determine AA distribution into phospholipids of inflammatory cells. First, cells are labeled with [^{3}H]-AA followed by organic extraction of all glycerolipids. Subsequently, glycerophospholipids are separated into individual classes using normal phase HPLC (**Fig. 1**). Molecular species of individual glycerophospholipid classes can be further isolated by reverse phase HPLC. Alternatively, individual classes of glycerophospholipids (PC and PE) are subjected to phospholipase C digestion followed by derivatization of the resulting diglycerides. Derivatized diglycerides can then be separated into their respective subclasses (1-acyl-, 1-alkyl-, and 1-alk-1-enyl) by normal phase HPLC. Once isolated, the subclasses can be further separated into several molecular species by reverse phase HPLC.

2. Materials

1. Radiolabels: [5,6,8,9,11,12,14,15–^{3}H]-Arachidonic acid (76 Ci/mmol) obtained from New England Nuclear Products (Boston, MA). [1-^{14}C]-Arachidonic acid obtained from American Radiolabeled Chemicals (St. Louis, MO).
2. Phospholipid standards: PC, PE, PI, PS, and phosphatidic acid purchased from Avanti Polar Lipid (Birmingham, AL). Molecular species standards (1,2-

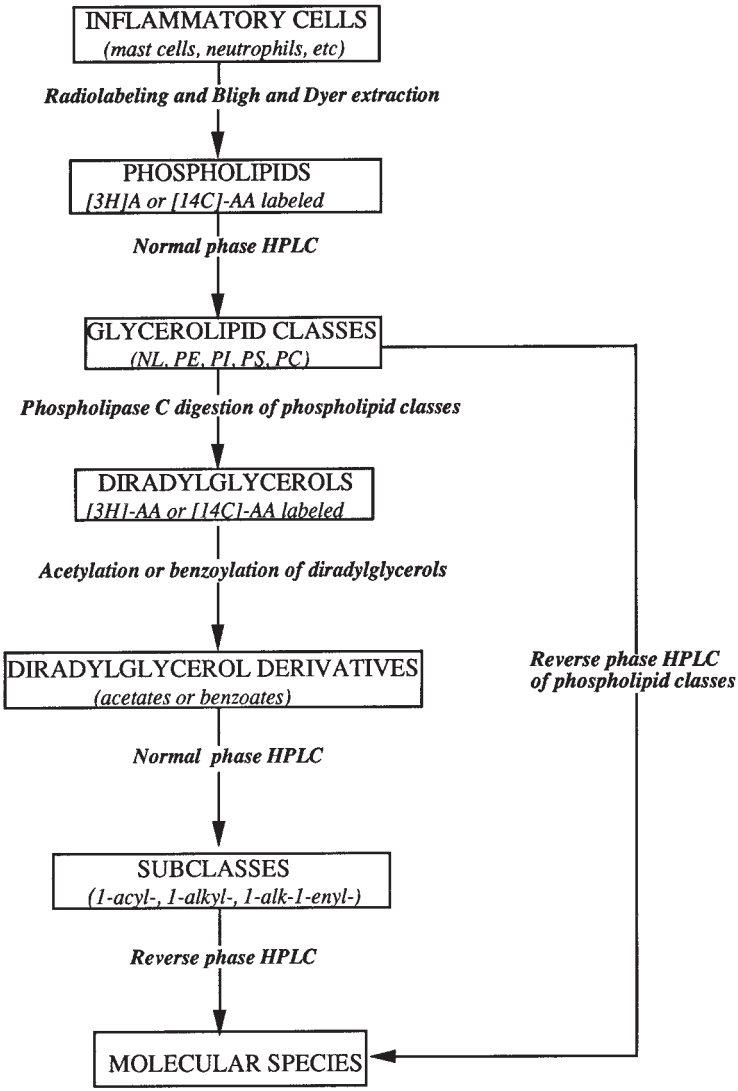

Fig. 1. Determination of the distribution of arachidonate into phospholipid classes (PE, PI, PS, PC), subclasses (1-acetyl, 1-alkyl, 1-alk-l-enyl-linked), and molecular species.

dirachidonoyl-*sn*-GPC,1-palmitoyl-2-arachidonoyl-*sn*-GPC, 1-stearoyl-2-arachidonoyl-*sn*-GPC and 1-alkyl-2-arachidonoyl-*sn*-GPC) purchased from Avanti.

3. Equipment: Dual pump HPLC systems with gradient capability and UV detectors (Diode Array and Multiwavelength UV) purchased from Hewlett Packard or Waters Chromatography, respectively.

4. Enzymes and cell culture reagents: Phospholipase C *(Bacillus cereus,* Type XI), fetal bovine serum (FBS), nonessential amino acids and essential amino acids purchased from Sigma (St. Louis, MO). Hanks Balanced Salt Solution (HBSS) obtained from Mediatech. RPMI 1640 culture medium obtained from Gibco-BRL (Grand Island, NY). Six percent Dextran 70 in 0.9% sodium chloride for neutrophil sedimentation purchased from Abbott Laboratories (North Chicago, IL). Ficoll-Paque for gradient centrifugation purchased from Pharmacia Biotech (Piscataway, NJ).
5. Derivatizing agents:
   a. Acetylation grade acetic anhydride and pyridine obtained from Alltech/Applied Science Associate (Deerfield, IL). Acetic anhydride and pyridine are irritants that should be handled with gloves under a ventilated fumehood.
   b. Benzoyl chloride utilized for the synthesis of diglyceride benzoates purchased from Sigma (St. Louis, MO). Toxic benzoyl chloride should be handled under a fumehood with cloves.
6. HPLC solvents: Analytical grade or HPLC grade solvents (hexane, cyclohexane, cyclopentane, methyl-*t*-butyl ether, 2-propanol, acetonitrile, ethanol, water and benzene) purchased from Fisher Scientific (Norcross, GA). Benzene is a known carcinogen and should be used under the hood under well-ventilated conditions.
7. HPLC columns:
   a. Normal phase HPLC column for classes. Supelcosil LC-Si, 5 µm, 25 cm × 5.0 mm ID (Supleco Inc., Bellafonte, PA). Store column in hexane and avoid exposure to aqueous solvents.
   b. Reverse phase HPLC column for subclasses. Ultrasphere ODS, 5 µm, 25 cm × 4.6 mm ID from Rainin Instrument Co. (Worburn, MA). Store column in methanol/water (70:30 [v/v]) or in acetonitrile.
   c. Reverse phase HPLC column for molecular species. Supelcosil LC 18, 25 cm × 2.1 mm from Supleco. Store column in methanol/water (80:20 [v/v]) or acetonitrile.
8. HPLC solvents:
   a. Normal phase HPLC solvent A: Make 25 m*M* phosphate buffer by dissolving 1.7 g gold labeled monobasic potassium phosphate in 500 mL HPLC water and adjust the pH to 7.4 using 1 *M* sodium hydroxide. Composition of solvents A is hexane/2-propanol/ethanol/25 m*M* phosphate buffer (490:367:100:30 [v/v/v/v]). Prepare this solvent by first adding 2-propanol to 25 mm potassium phosphate and then add hexane and ethanol. Use a magnetic stirrer to mix solvents for 4–6 h and then filter solvents through 0.2 µm FH Millipore filter (Millipore, Bedford, MA). Add 200 µL glacial acetic acid to the filtered solvent mixture. For normal phase solvent B, make the composition of all the other components except increase the amount of phosphate buffer from 30 mL (3%) to 50 mL (5%) and add 600 µL glacial acetic acid after filtration.
   b. Solvent for separating subclasses:

      Acetate derivatives of subclasses are resolved into subclasses on normal phase silica column using a mobile phase mixture of cyclopentane/hexane/methyl-*t*-butyl ether/acetic acid at 73:34:3:0.8, (v/v/v/v), whereas diradylglycero-

benzoates are separated into subclasses using cyclohexane/2-propanol, 99.85:0.15, (v/v).

c. Solvent for separating molecular species:

1-Alkyl-2-acyl- and 1-alk-1-enyl-2-acyl-*sn*-glyceroacetates are separated into molecular species using a solvent mixture of acetonitrile/2-propanol/methyl-*t*-butyl ether/water at ratios of 63:28:7:2 (v/v/v/v) while diacyl glyceroacetates molecular species are resolved using the same solvents at ratios of 73:18:8:2 (v/v/v/v). Diacyl-, 1-alkyl-2-acyl- and 1-alk-1-enyl-2-acyl-*sn*-glycerobenzoates are separated into their respective molecular species by isocratic elution with acetonitrile/2-propanol mixed in ratios 70:30, 63:37, and 65:35, respectively.

For separating phospholipid classes (PC, PE, PI, PS) into molecular species, make 20 m*M* choline chloride in methanol/water/acetonitrile, 90.5:7:2.5 (v/v/v). The solvent is prepared by dissolving choline chloride in water followed by filtration using a 0.45 mm filter (Millipore) before adding acetonitrile and methanol. For PS, elute molecular species using 30 m*M* choline chloride in methanol/25 m*M* monobasic potassium phosphate/acetonitrile/acetic acid, 90.5:7:2.5:0.8 (v/v/v/v). Apply PC and PE onto columns using 500 µL elution buffer and PI and PS using 25–50 µL chloroform.

9. Solid phase extraction column and solvents. Bond Elute silica solid phase extraction columns supplied by J. T. Baker (Phillipsburg, NJ). Solvents of various compositions (hexane/diethyl ether) are made immediately before use utilizing analytical grade or HPLC grade solvents purchased from Fisher.

3. Methods

*3.1. Isolation and Radiolabeling of Inflammatory Cells with [^{3}H]-AA and/or [^{14}C]-AA- (*see *Notes 1–3)*

1. Human neutrophils are isolated from venous blood from healthy donors by dextran sedimentation followed by Ficoll-Paque density gradient centrifugation *(7)*. Platelets, monocytes, and alveolar macrophages are obtained as previously described *(7)*. Bone marrow mast cells are obtained from cell culture of bone marrow from CB/J mice cultured in RPMI 1640 (Gibco-BRL, Grand Island, NY) medium supplemented with 10% FBS, 50 m*M* 2-mercaptoethanol, 2 m*M* glutamine, 0.1% (v/v) penicillin/streptomycin, and 50% WEHI supernatant fluid as a source of IL-3.
2. For pulse labeling, maintain cells in HBSS at 10 million/mL. Label cells by adding [^{3}H]-AA complexed to HSA (0.25 mg/mL) in 200 µL HBSS for 30 min at 37°C. Remove cells from the supernatant fluid by centrifugation. Remove unincorporated label by washes (2×) of cells with HBSS containing 0.25 mg/mL HSA.
3. For equilibrium labeling experiments, label cells under sterile conditions as described in **step 2**. Subsequently, place cells in RPMI culture medium for 24–48 h to ensure equilibrium distribution of [^{3}H]-AA into phospholipid species.
4. Double label studies. For double labeling, first pulse label cells with [^{14}C]-AA (1 µCi/40 × 10^6) complexed to 200 µL HSA (0.25 mg/mL) in HBSS for 0.5 h.

Remove cells from supernatant fluids by centrifugation and then placed cells in culture medium for 24 h. During this chase period, [^{14}C]-AA is remodeled into 1-ether-linked phospholipids such that the distribution of the radiolabel reflects the mass distribution of arachidonate in the same phospholipids (isotopic equilibrium). Subsequently, remove cells from growth medium and label with [^{3}H]-AA (1 μCi/ 20 × 10^6 cells) complexed to HSA (0.25 mg/mL) for 0.5 h at 37°C. Remove unincorporated label by washing the cells twice with HBSS containing 0.25 mg/mL.

3.2. Extraction of Glycerolipids

The most widely lipid extraction methods use chloroform and methanol ***(8,9)***. Addition of organic acid (three drops of formic acid) during this extraction condition is critical in protecting arachidonate containing phospholipids and in ensuring reproducible, high recovery of glycerolipids. In our laboratory, glycerolipids are extracted from cells by the method of Bligh and Dyer ***(9)*** (*see* **Note 4**).

1. Maintain cells in HBSS at 5 million/mL. Acidify the cell suspension by adding three drops of 9% formic acid and then add 2 vol of methanol and 1 vol of chloroform to the cells making sure that the mixture forms a monophase. If a monophase is not formed, add 1–3 drops of methanol until a clear monophase solution accompanied by bubbling is obtained.
2. Add two more volumes of chloroform and then break the monophase by adding 1 mL of water. Vortex mixture briskly and centrifuge at room temperature to separate the lower glycerolipid rich chloroform phase from the aqueous upper phase.
3. Using a Pasteur pipet, carefully transfer the lower phase into clean borosilicate glass culture tubes. Re-extract the aqueous phase using 2 vol of chloroform. Chloroform can be removed from the extract using a stream of nitrogen.

3.3. Chromatographic Analysis of Glycerolipids

3.3.1. Isolation of Glycerolipid Classes by HPLC

HPLC and TLC are the most used methods in isolating glycerophospholipid classes ***(10,11)***. When high recoveries are required for further analysis, HPLC is the method of choice since recoveries >85% are easily obtained. In our laboratory, glycerolipid classes are isolated by normal phase HPLC using a modification of the method of Patton et al. ***(12)***. This method differs from the original method in that we are using a phosphate gradient to completely and rapidly resolve the major glycerophospholipids extracted from cells. Although our standard method employs a 5 min phosphate gradient, a better resolution of PI and PS can be achieved by extending this gradient to 25 min. In addition to running a phosphate buffer gradient, we have also adjusted the amount of acid that is utilized in the starting buffer. We have noticed that the 1-alk-1-enyl-2-

acyl-*sn*-GPE subclass is susceptible to break down under acid conditions employed by the original method. We have reduced the acid in solvent A from 0.06% (v/v) to 0.02% (v/v) in order to avoid the breakdown of this subclass (*see* **Notes 5** and **6**).

1. Remove solvents of the Bligh and Dyer extract using a stream of nitrogen and suspend glycerolipid extract in 500 µL injection solvent (hexane/2-propanol/water, 4:5.4:0.3 [v/v/v]).
2. Load extract suspension onto an Ultrasphere Silica column and elute with solvent A (hexane/2-propanol/ethanol/phosphate buffer, pH 7.4, acetic acid [490:367:100:30:0.2 v/v]) for 5 min at a flow rate of 1 mL/min.
3. Increase the phosphate content of the eluting solvent from 3 to 5% over 5 min and maintain this solvent composition until all the major phospholipid classes are eluted from the column (30–40 min). The 5 min gradient time can be increased to 25 min to obtain a better resolution between PI and PS.
4. Monitor the elution of lipids by UV spectroscopy at 205 nm. Collect fractions (1 mL) for liquid scintillation counting (*see* **Note 7**).

3.4. Phospholipase C Hydrolysis and Derivatization

In order to completely resolve phospholipid molecular species, it is desirable to first isolate the individual subclasses. To achieve this goal, the polar head groups of the phospholipids have to be removed. Once the polar head groups have been removed, chemical modifications that further reduce the polarity of the DGs are performed. Examples of chemical modifications that are performed include acetylation, benzoylation, dinitrobenzoylation, or anthroylation ***(13–16)***. For cells that have been radiolabeled, separation can be rapidly accomplished by acetylation and quantitation by liquid scintillation counting. Otherwise the UV absorption of the DGs is increased by attaching a chromophore such as a benzoate (UV 230), dinitrobenzoate or anthroyl ethers (UV 254), and the enhanced absorbance utilized for quantitation.

1. Remove solvent from PC and PE fractions obtained from normal phase HPLC (**Subheading 3.3.**) using a stream of nitrogen.
2. Dissolve lipids in 1 mL anhydrous ethyl ether. Add 1 mL 0.1 *M* Tris-HCl, pH 7.4, containing 5–10 U or 25–30 U of *Bacillus cereus* phospholipase C for PC and PE, respectively. Mix samples at room temperature for 2.5 or 6 h for PC and PE, respectively (*see* **Note 8**).
3. Two methods can be used to extract the diglyceride products. In the first method, we utilize a hexane/ether (50:50 [v/v]) mixture to extract DGs. Extract diglycerides by adding 2 mL hexane. Mix thoroughly and transfer the DG-rich top hexane/ether layer into clean borosilicate glass tubes. Re-extract using 4 mL hexane/ether (1:1 [v/v]) and combine the extracts. The second method of extraction utilizes the Bligh and Dyer extraction procedure (**Subheading 3.2.**). Dry off the

diethyl ether layer using a stream of nitrogen after the phospholipase C activity and then extract lipids as described above.

4. Synthesis of Diradylglycerides acetates ***(13)***: Remove organic solvents from diglycerides using a stream of nitrogen. Convert diradylglycerides to diradylglyceroacetates by adding acetic anhydride/pyridine, 9:1 (v/v) (*see* **Note 9**). Maintain reaction at 37°C for 12 h. Remove the derivatizing agent using a stream of nitrogen.
5. Synthesis of diradylglyceride benzoate ***(15)***: Remove organic solvents from diglycerides using a stream of nitrogen. Convert diradylglycerides to diradlyiglycerobenzoates in 1 mL benzene/benzoyl chloride (9:1 [v/v]). Add 100 µL pyridine and incubate overnight in airtight borosilicate glass tubes. Benzoylation can also be performed rapidly using 1 mL redistilled pyridine/benzoyl chloride (20:1 [v/v]) for 15 min at room temperature. Ensure that air is removed from the reaction tubes by capping under nitrogen (*see* **Note 9**). To reduce background absorbance and to ensure that subclasses and molecular species are well resolved, all unreacted reagent (benzoyl chlorides) has to be removed from the samples (*see* **Note 10**). After derivatizing, add 100 µL H_2O to samples and dry vigorously under a stream of nitrogen. Add 1 mL ammonium hydroxide and 2 mL hexane and vortex (~20 s). Remove the upper DG-rich hexane layer and re-extract (2X) using 2 mL of hexane. Add 5 mL 2% acetic acid to the hexane extract and vortex vigorously. Centrifuge (220*g*, 10 min at room temperature) and remove upper hexane layer. Remove hexane under a stream of nitrogen and resuspend sample in 1 mL of hexane. Filter sample using 0.2 µ syringe filter (Nalgene) and then wash the filter with 1 mL hexane.
6. Removal of derivatizing agents (*see* **Note 10**): To perform solid phase extraction of derivatized diglycerides, extract derivatized diglycerides using hexane/ether (1:1 [v/v]) and then remove the solvents using a stream of nitrogen. To obtain a diglyceride rich fraction, suspend lipids in 4 mL of hexane before application onto a Bakerbond silica gel extraction column (J. T. Baker, Phillipsburg, NJ) that has been conditioned with 4 mL of hexane. Wash the column with hexane and then elute the derivatized diglyceride rich fraction using 10% diethyl ether in hexane.

3.5. Determination of Arachidonate Distribution into PC and PE Subclasses

1. HPLC of DG acetates ***(13)***: Dissolve diradyl-*sn*-glyceroacetates prepared as described above in 50–100 µL hexane. Load the samples onto a normal phase column that had been previously equilibrated with elution solvent. Isolate subclasses using a flow rate of 2 mL/min at 36°C. Collect 1-min fractions and determine the amount of radioactivity in subclasses by liquid scintillation counting after removal of organic solvents (*see* **Note 7**). For samples that are not radiolabeled, detect the elution of subclasses by monitoring the UV absorption at 205 nm.
2. HPLC of DG benzoates ***(15)***: For samples that can not be radiolabeled to isotopic equilibrium, it may be necessary to increase the UV detection limit of the DGs by attaching a chromophore such as benzoate (UV, 230 nm). Although benzoylation

is as rapid as acetylation of DGs, it is more difficult to extract the derivatized products from the reaction mixture. However, solid phase extraction or a combination of base/acid treatment has been found to reduce the background and enhance resolution of DG benzoates (*see* **Subheading 3.4., step 6**). Once derivatizing agents have been removed, dry sample under a stream of nitrogen and resuspend in 0.2 mL cyclohexane. Load samples onto a normal phase column previously equilibrated with cyclohexane/2-propanol, (99.85:0.15, [v/v]) and elute subclasses at 1 mL/min at room temperature. Monitor elution of subclasses by UV spectroscopy (230 n*M*) and also collect 1-mL fractions. Determine the distribution of radioactivity into subclasses after organic solvent removal by liquid scintillation counting.

3.6. Molecular Species Distribution

1. Isolation of DG acetate molecular species. After removal of solvents from subclasses (**Subheading 3.6.2.**), separate into molecular species as described by Nakagawa and Horrocks ***(13)***. Collect 1 mL fractions and determine radioactivity by liquid scintillation counting (*see* **Note 7**).
2. Isolation of DG benzoate molecular species. After removal of solvents from subclasses (**Subheading 3.6.**), separate into molecular species as described by Blank et al. ***(15)***. Collect 1 mL fractions and determine radioactivity by liquid scintillation counting (*see* **Note 7**).
3. Separation of molecular species of glycerophospholipids ***(12)***: This procedure is less labor intensive than the DG methods since phospholipase C digestion and derivatization are not needed. A potential drawback is that baseline resolution is not attainable. However, all the major AA-containing molecular species of PE, PC, and PI can be identified. We have attempted to improve the resolution of molecular species by using a microbore column. Before biologic samples are injected onto the column, the elution times for authentic standards of PC or PE molecular species are obtained. Further confirmation of peak composition requires fatty acid analysis. Fatty acid analysis in our laboratory is done by GC/MS after alkaline hydrolysis and conversion of fatty acids to their pentafluorobenzyl derivatives ***(17)*** (*see* **Note 11**). Using a C18 microbore column, our studies suggest that mast cells and human neutrophils have at least 20 different arachidonate-containing molecular species.
   a. After normal phase HPLC, remove solvents from combined PE or PC or PI/PS fractions using a stream of nitrogen.
   b. Dissolve PE or PC fractions in absolute ethanol (100–200 µL) and the PI/PS fraction in chloroform. PE and PC fractions can also be suspended in elution solvent (250–500 µL) without compromising resolution of molecular species.
   c. Load samples onto reverse phase column that had been previously conditioned with eluting solvent (**Subheading 2.**) at 40°C (*see* **Note 12**).
   d. Elute molecular species at 0.42 mL/min using an isocratic gradient of methanol/acetonitrile/water containing 30 m*M* choline chloride and collect 1-min fractions. Allow solvents to evaporate in a ventilated fumehood and deter-

mine the distribution of radioactivity in each molecular species by liquid scintillation counting.

4. Notes

1. For cells that have been pulse labeled with [^{3}H]-AA, the amount of radioactivity in phospholipid classes, subclasses, and molecular species does not reflect the abundance or mole quantities of arachidonate in these species. For cells that have been labeled with [^{3}H]-AA to isotopic equilibrium, the radioactivity closely mimics the mole quantity distribution of AA amongst glycerolipid classes. When labeling to isotopic equilibrium, a pulse-chase procedure is recommended. First, the cells are incubated with radiolabeled AA for ~30 min and then they are placed in culture media that contains high levels of cold AA for as long as is required for the radiolabel to attain isotopic equilibrium. This period varies for different cell types and depend upon their capacity to proliferate. Fast growing cells will invariably remodel the labeled AA faster and attain isotopic equilibrium quicker than cells that are growing slowly. Typically, 24–48 h are required for mast cells to attain isotopic equilibrium ***(4)***.
2. During radiolabeling of inflammatory cells with [^{3}H]-AA, we use very high specific activity label (76 mCi/mmol). Under these conditions, the bulk of the label is incorporated into phospholipids. When low specific activity labels are used such that cells are exposed to high amounts of AA (>10 μ*M*), the incorporation of AA into glycerolipids is altered such that triglycerides become the major arachidonate-containing pool ***(6)***. It is necessary to maintain low amounts of [^{3}H]-AA when one in interested in determining the distribution of AA under resting conditions.
3. Double labeled experiments ***(5)***. As stated in the introduction, AA is not evenly distributed into phospholipid classes and subclasses. Free AA is initially incorporated into 1-ester-linked subclasses before it can be shifted into 1-ether-linked subclasses. During cell activation, there is an increase in this remodeling process concomitant with the release of AA. These processes catalyzed by CoA-IT and PLA_2, respectively, makes it difficult to accurately determine changes in AA distribution during cell activation. Using a double-labeled approach, one can better understand the changes that occur in AA distribution during cell activation. After double labeling, one can determine the distribution and movement of AA during cell activation by measuring the quantities and calculating the ratios of [^{14}C] to [^{3}H] in each subclass. In addition to determining the movement and distribution of arachidonate, this double labeling strategy can be utilized to determine the phospholipid pools that are mobilized for the biosynthesis of eicosanoids ***(5)***.
4. The Bligh and Dyer procedure extracts almost all glycerolipids from cells and tissues ***(9)***. The acidification of the cell suspension solution using 9% formic acid protects unsaturated fatty acids such as AA and ensures high recovery and reproducible extraction of glycerolipids. The reaction should be carried out at room temperature and all centrifugation steps should be carried out in a warm centrifuge in order to increase the efficiency and enhance the recovery of glycerolipids.

5. Addition of phosphate buffer to organic solvents required for phospholipid class isolation by normal phase HPLC results in salt precipitation. After addition of phosphate buffer, allow dissolution of the precipitate by mixing the solvents using a magnetic stirbar. Filter off any precipitate using a Millipore filter before pumping the solvents through the HPLC. Filtration prevents blockage of the HLPC tubings and high pressure problems. If the HPLC system is to be stored for longer periods of time, store the normal phase column in hexane, unhook the column from the HPLC pumps and flush the system with HPLC grade water to avoid the precipitation of phosphate buffers in the tubings and flow cell of the detector. Before hooking up the column, flush out the water with hexane to avoid exposing the normal phase column to water. This assures good resolution of phospholipids classes and long column life.
6. 1-Alk-1-enyl-2-acyl-*sn*-GPE also known as plasmalogen PE is sensitive to acid conditions. Avoid the break down of this subclass by decreasing the amount of acid added to normal phase solvent A. Addition of 200 μL acetic acid/L solvent instead of 600 μL/L is usually enough to protect the plasmalogen subclass without adversely affecting the resolution of glycerophospholipids.
7. To obtain accurate radioactivity counts, it is essential that all organic solvents are removed before scintillation counting to avoid quenching. Solvents are readily removed using a stream of nitrogen before the addition of scintillation fluid.
8. PLC hydrolysis of PC is more effective than that of PE. This problem is solved by adding 3–4 times more units of phospholipase C and hydrolyzing PE for a longer period of time than is required for PC. For PC isolated from 5 million cells, 10 U of PLC for 2.5 h at room temperature are the conditions for hydrolysis.
9. Derivatizing agents (pyridine, acetic anhydride, benzoyl chloride) are sensitive to moisture. Store agents in a desiccator at all times. After usage, flush out residual moist air with nitrogen. In our laboratory, these reagents are normally bought in ampoules in sizes (5–10 mL) that are suitable for derivatizing samples from one experiment. Usage of a newly opened ampoule ensures that very anhydrous reagents are utilized in derivatizing our diglycerides.
10. For best resolution of PE and PC subclasses, make sure that all pyridine and acetic anhydride are removed from derivatized diglycerides before application onto an HPLC column. After derivatization, add 1 mL of water and 1 mL of methanol followed by 4 mL hexane. Vortex samples very well and extract top hexane layer. Back extract the hexane layer by adding 1 mL of methanol followed by 1 mL of water. Repeat the back extraction (3X) to ensure that most of the pyridine and acetic anhydride are extracted from the hexane layer. Solid phase extraction described in **Subheading 3.** and other extraction procedures that use base/acid (ammonium hydroxide/2% acetic acid) combinations to destroy the benzoylcholride will assure that a clean sample is obtained for further analysis.
11. For inflammatory cells that can not be radiolabeled to isotopic equilibrium with [^{3}H]-AA, mole quantities of AA are best determined by gas chromatography/mass spectroscopy. Our laboratory utilizes negative ion chemical ionization GC/MS (Hewlett Packard mass spectrometer [HP 5989 A] interfaced to an Hewlett Packard series II gas chromatograph [HP 5890]) and deuterated internal

standards to determine the mole quantities of arachidonate in phospholipid classes, subclasses, and molecular species ***(17)***. Other mass spectroscopy methods are described elsewhere ***(18–21)***.

12. Phospholipid molecular species resolve well at 40°C. It is essential that the column be maintained at this temperature using a temperature jacket or a water bath. In preparing the solvents for molecular species of phospholipid classes, make sure that the choline chloride is totally dissolved in water. Filter this solution before adding the methanol/acetonitrile component.

Acknowledgments

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9

Determination of Phospholipase A_2s Expression in Mast Cells by Reverse-Transcriptase Polymerase Chain Reaction

Alfred N. Fonteh, William Reed, and James M. Samet

1. Introduction

The release of arachidonic acid (AA) from membrane phospholipids by phospholipase A_2 (PLA_2) is a critical step in the generation of lipid mediators by mast cells. AA released by PLA_2 activity is metabolized to bioactive compounds, such as prostaglandins, thromboxane, and leukotrienes. During the last decade, several mammalian PLA_2s have been shown to play a role in the mobilization of AA from phospholipids. At least ten different PLA_2 types have been described ***(1,2)***. In general, these consist of secretory, cytosolic, or membrane bound activities.

A secretory low molecular weight PLA_2s ($sPLA_2$, ~14 kDa) and a cytosolic high-mol-wt ($cPLA_2$, ~100 kDa) have received the most attention regarding the mobilization of AA from mast cells. $sPLA_2$s display a very rigid three-dimensional structure owing to the presence of multiple disulfide bridges. These enzymes have an absolute requirement for Ca^{2+} for their catalytic activity. At least five different groups of $sPLA_2$s have been cloned and sequenced ***(1–5)***. When provided with phospholipids substrates (vesicles or as *Escherichia coli* membranes) containing several fatty acids at the *sn-2* position, $sPLA_2$s hydrolyze a variety of unsaturated fatty acids, including linoleic acid, oleic acid, and arachidonic acid. However in whole cells, including mast cells, there is evidence that $sPLA_2$ can selectively mobilize AA during cell activation ***(6,7)***. During antigen activation of mast cells, there is a rapid increase in $sPLA_2$ activity concomitant with the appearance of free AA in the supernatant fluid. Specific activity measurements suggest that AA that is released into the supernatant fluid is different from AA that remains cell associated. Further evidence

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for a role of $sPLA_2$ in extracellular AA mobilization is provided by studies that show that the incubation of mast cells with extracellular PLA_2 results in the selective release of AA into the supernatant fluid ***(6)***. This selective release of AA by $sPLA_2$ is proposed to be mediated through specific receptors on the surface of mast cells. Another $sPLA_2$ termed PS-specific has been described in mast cells ***(8)***. The PS-specific PLA_2 has not been cloned and thus it is not known whether this represents a novel class of PLA_2 enzymes.

In addition to $sPLA_2$, considerable research effort has been devoted to intracellular AA-metabolizing enzymes ($cPLA_2$) in mast cells. $cPLA_2$ has been purified, cloned and sequenced from different cell lines ***(9–11)***. Its structure is shown to be completely unrelated to $sPLA_2$s. Likewise, its substrate specificity and requirement for calcium ions are different from those of $sPLA_2$. When supplied with vesicles containing different phospholipids with a variety of fatty acids, $cPLA_2$ selectively hydrolyzes AA-containing phospholipids. In the presence of low concentrations of calcium ions (0.1–1 μm) and during cell activation, $cPLA_2$ appears to translocate from the cytosol to membrane fractions of mast cells ***(12)***. The translocation to a membrane domain and the activation of $cPLA_2$ is accompanied by phosphorylation ***(12,13)***. Our studies suggest that $cPLA_2$ may be responsible for generating AA that is cell associated ***(14)***. In addition, levels of $cPLA_2$ are closely linked to cytokine treatment of cells. For example, treatment of mast cells with stem cell factor will result in an increase in the expression of $cPLA_2$ ***(14,15)***.

Recently, a calcium-independent PLA_2 ($iPLA_2$) activity has been described in a macrophage-like cell line ***(16)***. This enzyme has been sequenced and cloned and shown to be a protein of ~80 kDa. $iPLA_2$ is proposed to be involved in controlling AA incorporation and remodeling in inflammatory cells ***(17,18)***. Future studies are required to determine whether this enzyme is present in mast cells and if so, whether it plays in role in AA metabolism.

From the above studies, it is clear that there are at least three different types of PLA_2s in mast cells. In this chapter, we present methods by which the major mast cell PLA_2 ($sPLA_2$ and $cPLA_2$) activities are determined in our laboratories. In addition, we provide detailed instructions on how the expression of mRNA of these enzymes can be monitored by RT-PCR.

2. Materials

1. General reagents:
   a. Absolute ethanol, sterile water, 5 *M* NaCl, are supplied by Sigma (St. Louis, MO).
   b. Diethyl pyrocarbonate (DEPC) purchased from Sigma. Treat distilled water with DEPC (0.1%) and leave overnight at room temperature. Autoclave for 20 min. Use this DEPC-treated water to make up solutions required for RNA extraction and for cleaning all glassware and plastic ware that will be utilized

for RNA isolation. Note that DEPC is a suspected carcinogen. Wear protective clothing and gloves, work in a fumehood and exercise standard laboratory safety procedures.

c. Make 5X Tris-borate-EDTA (TBE) by adding 54 g Tris base, 27.5 g boric acid and 20 mL of 0.5 *M* EDTA (pH 8.0) in one liter of sterile water.
d. High strength molecular biology grade agarose is obtained from Sigma.
e. PLA_2 substrates: [^{3}H]-AA labeled *E. coli* membranes are purchased from New England Nuclear while 1-palmitoyl-2-[1-^{14}C]-arachidonoyl-*sn*-GPC is purchased from Amersham (Arlington Heights, IL).
f. PLA_2 activity buffers: $sPLA_2$ activity is determined in 50 m*M* Tris-HCl buffer, pH 8.3, containing 5 m*M* $CaCl_2$ and 0.1 *M* NaCl. $cPLA_2$ activity is determined in 10 m*M* HEPES buffer, pH 7.4, containing 80 m*M* KCl, 1 m*M* EDTA, 4.7 m*M* $CaCl_2$, 5 m*M* DTT and 0.5 mg/mL fatty acid free human serum albumin (HSA).

2. Reagents for RNA isolation:
   a. Cell lysis buffer: 4 *M* Guanidine thiocyanate (Boehringer Mannheim, Indianapolis, IN) in 50 m*M* sodium citrate, pH 7.1 (Sigma) containing 0.5% Sarkosyl (Sigma). One m*M* dithiothreitol (DTT) (Sigma) is added to the lysis buffer just prior to use. Do not deviate from the specified pH because guanidine thiocyanate may release a toxic gas (hydrogen cyanide) when acidified.
   b. 5.7 *M* CsCl/0.1 *M* EDTA, pH 8.0 is purchased from Sigma. The CsCl solution should have a density of 1.8 g/mL and should be filtered through a 0.2 µm filter after preparation.
   c. Polycarbonate centrifuge tubes (7 × 20 mm) and TL-100 ultracentrifuge are supplied by Beckman (Palo Alto, CA). Sorvall TH641 swinging bucket rotor and Sorvall OTD55B ultracentrifuge are purchased from DuPont (Wilmington, DE).
3. Reverse transcriptase (RT) reagents:
   a. 10X RT-Buffer supplied with RT enzyme or made up of 100 m*M* Tris, pH 9.3, containing 500 m*M* KCl, 30 m*M* $MgCl_2$, and 1 mg/mL BSA from Sigma.
   b. dNTP mix (5 m*M* each of dATP, dCTP, dGTP, and dTTP) prepared from individual nucleotide triphosphate stocks from Pharmacia (Piscataway, NJ).
   c. RNasin supplied by Promega (Madison, WI).
   d. Random hexamers obtained from Pharmacia.
   e. Moloney murine leukemia virus reverse transcriptase supplied by Gibco-BRL (Grand Island, NY).
4. Polymerase chain reaction (PCR) reagents:
   a. 10X PCR buffer supplied with Taq polymerase or made up of 100 m*M* Tris, pH 9.3, containing 500 m*M* KCl, 30 m*M* $MgCl_2$, and 1 mg/mL BSA all purchased from Sigma.
   b. dNTP mix (same as that used for reverse transcription).
   c. Taq polymerase purchased from Boehringer-Mannheim (Indianapolis, IN).
   d. Sense and antisense primer (*see* **Table 1**) mix (5 µ*M* each primer) designed as described below are synthesized by the UNC Chapel Hill DNA synthesizing facility or the Wake Forest University School of Medicine DNA Core Laboratory.

Table 1
Oligonucleotide Primers forHuman and Rodent PLA_2s

	GenBank accession	Product size (bp)	Sense primer	Antisense primer
Cytosolic PLA_2				
cPLA2 (human)	M68874 M72393	338	GCCTTGGTGAGTGATTCAGC	AGAGTCACTTGGCCTTGCAG
Secretory PLA_2				
Group I (human)	M21054 E02268	205	GACCCCTTCTTGGAATAC	CTACAGGTGATTGCCGAG
Group II (human)	M22430	241	AAGGAAGCCGCACTCAGTTA	GCAGCAGCCTTATCACACTC
Group I (rat)	D00036	341	AACTCCTTCTGCTGGCTGCT	CGGTCACAGTTGCAGATGAA
Group II (rat/mouse)	D00523 M25148	178	GGTGGCAGAGGATCCCCC	CGGCAGCTTTATCGCACT
Group V (rat/mouse)	MMU18119 RRUO7798	282	CGCAGCGCCTTCTTCTCCTA	GGTGGCCAGGTTCTCCTTGA

Primers were designed as described in methods using published sequences for human and rodent PLA_2s as templates.

5. Gel running, staining reagents, and equipment:
   a. 2 µg/mL ethidium bromide solution for staining gels is supplied by Sigma. Ethidium bromide breaks down in solution over time and should be replaced weekly. Ethidium bromide is a mutagen and suspected carcinogen, gloves must be worn when handling solutions and stained gels. Contaminated light boxes and staining trays should be cleaned regularly to minimize exposure to ethidium bromide.
   b. Power source and gel electrophoresis equipment are purchased from Bio-Rad (Hercules, CA).
   c. UV transilluminator box is supplied by Fisher Biotech (Pittsburgh, PA) and a digital camera purchased from Kodak (Rochester, NY). Type 55 P/N films are obtained from Polaroid (Cambridge, MA). Radiation from a UV transilluminator can cause serious damage to unprotected eyes and skin. Wear protective gear, such as a facemask and safety glasses.
   d. A set of 1-D gel analysis macros is available from NIH Image, a public domain image analysis software program. Both NIH Image and the macros can be downloaded from the NIH Image home page (http://rsb.info.nih.gov/nih-image/). There are several commercially available 1-D gel image analysis software packages that are crossreferenced as related web sites on the NIH Image home page.
   e. Glass fiber columns for PCR product purification (QIAquick PCR purification kit) are purchased from Qiagen Inc. (Chatsworth, CA).
6. PLA_2 primers: Sequences for primers for human and rodent PLA_2s are shown in **Table 1** (*see* **Note 1**). These primers were designed using cDNA sequences obtained by searching the GenBank data base using the ENTREZ browser service of the National Center for Biotechnology Information (NCBI), which is available via the World Wide Web (www.ncbi.nih.nlm.gov/entrez/). Oligonucleotide primer design is performed most reliably using a computer program that identifies suitable primer sequences based on the sequence of the target cDNA. There are a number of commercially available programs that will analyze sequence files downloaded from NCBI, and at least two primer selection World Wide Web sites (http://www.genome.wi.mit.edu and http://alces.med.umn.edu/vgc.htm). All of these programs select primers with a number of physical characteristics that are within specified limits. These characteristics include the estimated temperature at which the primer melts off the template (T_m), the primer's % GC content, the absence of secondary structure (hairpin formation), the absence of significant interprimer dimerization and the estimated specificity of the primer for the target cDNA. Default settings for acceptable limits of these characteristics are specified. They are relatively stringent and can be relaxed if necessary.

3. Methods

3.1. Mast Cell Culture

1. Culture mast cells in RPMI 1640 culture medium (Gibco-BRL) supplemented with 10% (v/v) fetal calf serum, 50 µ*M* 2-mercaptoethanol, 1% essential amino

acids, 1% nonessential amino acids, 2 m*M* L-glutamine, 5 μg/mL gentamycin, and 1% (v/v) penicillin/streptomycin.

2. Periodically (2 times/wk) enrich the culture medium with a 50% WEHI supernatant fluid as a source of cytokines.
3. After 3 wk in culture, count cells and determine viability by Trypan blue exclusion.

3.2. Demonstration of Multiple PLA_2 Activities in Mast Cells

1. Secretory PLA_2: Treat mast cells (~10–20 million) with 0.18 *M* sulfuric acid (0.5 mL) overnight at 4°C ***(4)***. Neutralize acid extract and determine PLA_2 activity with or without 5 m*M* dithiothreitol (DTT) treatment using [^{3}H]-AA labeled *E. coli* membranes or microsomes ***(19,20)***.
2. Cytosolic PLA_2: Prepare cytosol from 10–20 million mast cells. Determine PLA_2 activity using 50 μg cytosolic protein and 400 pmol sonicated vesicles of 1-palmitoyl-2-[1-^{14}C-] arachidonoyl -*sn*-GPC as substrate. Preincubate the cytosol with 5 m*M* DTT in order to eliminate residual $sPLA_2$ activity and then initiate $cPLA_2$ activity by adding the substrate. Determine $cPLA_2$ activity as previously described ***(15)***.

3.3. RNA Isolation (see Notes 2 and 3)

The most widely used methods of total RNA isolation are the guanidinium thiocyanate/acid phenol/chloroform method and methods that employ the binding of RNA to glass fibers in the presence of chaotropic salts ***(21,22)***. Although they are faster and more convenient than the method described here, they are more likely to yield total RNA that is contaminated with chromosomal DNA. Since mRNAs for $sPLA_2$s are relatively rare, their presence and abundance in cells is most effectively studied by semiquantitative RT-PCR, which is confounded by chromosomal DNA contamination of the total RNA under investigation. Consequently we use a miniaturization of the CsCl sedimentation procedure of Chirgwin et al., which starting with 5×10^5 mast cells or more yields total RNA that are sufficiently free of chromosomal DNA ***(23)***.

1. Remove culture media from mast cells after pelleting by centrifugation (250*g*, 10 min, 4°C). Rinse cells twice at room temperature using phosphate buffered saline (PBS). It is important to remove as much fluid from the cells as possible prior to lysing the cells to avoid diluting the lysis solution.
2. Lyse the cells in a minimal volume (100 μL) lysis buffer (*see* **Note 4**). Shear cells by passing through (4X) a sterile disposable 22-gage syringe that reduces the viscosity of the lysate by shearing chromosomal DNA.
3. Gently load the lysate on a 100-μL cushion of CsCl in a Beckman (Palo Alto, CA) 7 × 20-mm polycarbonate tube. Pellet RNA at 80,000 rpm at 15°C for 2 h in a TLA-100 fixed-angle rotor in a Beckman TL-100 ultracentrifuge (250,000*g*). For larger samples, the TLS55 swinging bucket rotor can be used in the Beckman

TL-100 instrument by centrifuging at 55,000 rpm for 4 h. For even larger samples, the DuPont (Wilmington, DE) Sorvall TH641 swinging bucket rotor can be used in the Sorvall OTD55B ultracentrifuge by centrifuging at 35,000 rpm at 15°C overnight (100,000*g*). During centrifugation, proteins float on whereas DNA is focused into a band in the center of the CsCl cushion.

4. After centrifugation, gently remove the supernatant containing protein and DNA by aspiration. Ensure that the DNA does not contaminate the RNA pellet which is tightly affixed to the wall of the centrifuge tube. After DNA is removed, drain excess supernatant fluid by inverting the centrifuge tube over clean tissue paper and then carefully blot the sides of the tube with a sterile cotton swab (Q-tip) to remove any DNA-contaminated residual fluid on the upper surface of the tube wall. The RNA pellet is clear so it is difficult to locate visually. Since it has a different refractive index than the polycarbonate tube, the RNA appears as a refractive spot on the tube wall. Tubes containing RNA should be kept on ice at all times from this point on. In order to facilitate solubilization of the RNA pellet, it is advisable to briefly leech the CsCl by gently washing the pellet without disturbing it with 100 µL of 80% ethanol kept at –20°C. This step may be omitted when using the Beckman 7 × 20 mm tubes or when the samples are very small. Frequently, the RNA pellet becomes visible only after it is rinsed in cold 70% ethanol. Extra care should be taken to avoid dislodging the RNA pellet when removing the ethanol with a pipet tip.
5. Solubilize RNA in 100 µL room temperature TE (10 m*M* Tris, pH 8.1, containing 1 m*M* EDTA) by gently working the pellet with a sterile pipet. RNase-free TE as a 100X stock is obtained from Sigma (*see* **Notes 2** and **3**).
6. The RNA is then transferred to a clean microfuge tube, 3 vol of 100% ethanol (Sigma) and a final concentration of 80 m*M* NaCl (from a 5 *M* stock) are added and the RNA is precipitated at –20°C overnight. RNA precipitation can be expedited by placing the tube at –80°C for 1 h.
7. Recover RNA by centrifuging in a microfuge at >12,000*g* at 4°C for 30 min. The microfuge tubes should be placed with the hinged edge of the cap facing out to aid in locating the pellet. Discard the supernatant and wash the pellet with ice-cold 80% ethanol before sedimentation at >12,000*g* at 4°C for 10 min. Completely remove supernatant fluid by pipetting and briefly dry the tube by incubation at 37°C with the tube cap open. Dissolve RNA in a small volume of room temperature TE (20–50 µL), depending on the amount of cells extracted. Drying the pellet too long will greatly reduce the solubility of the RNA.
8. Determine optical density of the sample at 260 nm using the formula 1.0 OD = 40 µg/mL in a quartz cuvet. Micro cuvets capable of giving accurate readings of 35 µL or smaller volumes are now available from a number of suppliers. A fraction of the sample is usually sacrificed for reading the OD. However, it is possible to recover the sample for use if it was read in a DEPC-treated cuvet (*see* **Notes 2** and **3**). RNA may be stored frozen in TE at –80°C for a few weeks or precipitated in ethanol and stored indefinitely.

3.4. Reverse Transcription

This procedure for the estimation of mRNA abundance by sequential reverse transcription and DNA amplification (RT-PCR) is based upon the method of Kawasaki et al. ***(24)***. It is assumed that mRNA sequences are proportionally represented in the first strand cDNAs synthesized by reverse transcription, so that the relative amount of a cDNA sequence is proportional to the relative amount of the corresponding mRNA in the cell. The reverse transcription (RT) reaction synthesizes cDNA transcripts from every transcribable RNA in the sample (*see* **Note 5**).

1. Typically we use 100 ng of total RNA in 10 µL TE as the template in a reaction volume of 50 µL that contains 5 µL RT-buffer, 10 µL dNTP mix, 1.25 µL RNasin, 0.2 A_{260} U random hexamer and 500 U Moloney murine leukemia virus reverse transcriptase.
2. Initiate the reaction by mixing and placing the sample tube at 39°C for 1 h. Terminate the reaction by heating the tube to 94°C for 4 min to denature the reverse transcriptase. Chill and then freeze samples at –20°C or use for amplification.

3.5. cDNA Amplification

The procedure for PCR amplification of cDNA is based on the method of Saiki et al. ***(25)***.

1. Perform the PCR in a total volume of 50 µL consisting of the following components: 5 µL of 10X PCR buffer, 0.5 µL of 5 m*M* dNTP mix, 1.25 U Taq polymerase, 2:l of primer mix (5 µ*M* each), and 2 µL of the RT reaction containing the first strand cDNAs (*see* **Note 6**).
2. As verification that the amplification products are derived from RNA rather than chromosomal DNA contaminating the RNA, use an aliquot of the total RNA equivalent to the amount present in the cDNA template as a template in a parallel amplification. For example, use 4 ng of total RNA as template in the no RT control amplification when 100 ng of total RNA is input to the RT reaction.

3.6. Quantitative PCR

1. Make a dilution series of a standard DNA that is amplified in parallel with the unknowns. The standard can be a plasmid containing the cDNA of interest or amplified cDNA of interest which has been isolated from primers and deoxynucleoside triphosphates. Since the concentration of the DNA can be determined by UV spectrophotometry, the standards can be used to relate the amount of product DNA to the amount of target cDNA input to the amplification.
2. Without an absolute standard, generate a relative standard using a dilution series of first-strand cDNAs synthesized from an abundant source of the mast cell ($1–2 \times 10^7$ cell) RNA, of interest.

3.7. cDNA Amplification and Product Isolation

1. Perform amplification using the following "touchdown" protocol: maintain PCR mix at 94°C for 2 min (1X), then 10 cycles of denaturing for 15 s at 92°C, annealing for 30 s at 61°C less 0.5°C per cycle, and polymerization for 1 min at 72°C. After the first ten cycles, the annealing temperature is held constant at 56°C for the remaining cycles (*see* **Notes 7** and **8**).
2. For semiquantitative estimates of cDNA abundance, take 10–15 µL samples of the each reaction (standards and unknowns) at 2–3 stages of the amplification (e.g., after 27, 30, and 33 cycles) and analyze by electrophoresis through 2% alkaline agarose gels (*see* **Notes 9** and **10**).
3. Load a DNA ladder of double-stranded (ds) DNAs of known size in one lane of the gel to check that each product is of the expected size. A ladder with about 100 bp increments in the sizes of the dsDNAs is suitable for 2% gels.
4. Run the agarose gel at constant voltage (100–150 V) while monitoring the migration of the bromophenol blue dye, which comigrates with dsDNAs of ~50 bp (*see* **Note 11**).

3.8. Product Quantitation (see Notes 9 and 10)

1. Stain agarose gels in 2 µg/mL ethidium bromide (Sigma) for 3 min with gentle agitation. Destain the gel in distilled water for 15–20 min.
2. Photograph the gel under UV illumination with a conventional digital camera or Polaroid Type 55 P/N film.
3. Scan Polaroid negatives using a densitometer. Convert the digitized Polaroid images or images taken with a digital camera to a format compatible with a 1-D gel image analysis software package. Determine the integrated intensities (volume integration) of the amplification products resolved as single bands of the predicted PCR product size.
4. Use the integrated intensities of products resulting from the amplification of the standard cDNAs to construct a standard curve relating the relative amount of DNA input to the amplification to the integrated intensity of the resulting products for each amplification stage. Obtain an estimate of the relative amount of PLA_2 cDNA in each unknown sample by interpolation using the standard curve (*see* **Note 12**).
5. Also quantify cDNA derived from a housekeeping gene whose expression is not expected to vary in response to cellular stimulation (e.g., actin or glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) in parallel to the PLA2 cDNA. Use the level of a housekeeping gene to normalize for sample to sample variation in the total amount of amplifiable cDNA.

3.9. Identification of Products

1. Confirm the identity of amplification products by sequencing one strand of the product DNA. Prior to sequencing, separate products from amplification primers and nucleoside triphosphates using a glass fiber filter column.

2. Determine the concentration of the products by UV spectrophotometry.
3. Use 10 ng/100 bp length in a standard dideoxy chain termination sequencing reaction. Also use one of the amplification primers to prime the sequencing polymerization. Products with sequences that have 95% or greater sequence identity to the target cDNA are considered confirmed as arising from the target cDNA (*see* **Note 13**).

4. Notes

1. Whenever possible it is advisable to take the organization of the gene of interest into consideration when designing primers, since this enhances their selectivity and usefulness. Many mammalian genes are interrupted by intervening sequences (introns). The presence of introns can be used to design primers that have a reduced likelihood of yielding spurious products derived from chromosomal DNA contaminating the total RNA. In the case of the human group I and II $sPLA_2$s it was possible to select primers that span one or more introns, since the sequence of large regions of these two genes have been published. The sense primer for group I $sPLA_2$ hybridizes to exon 2 and the antisense primer hybridizes to exon 3. Instead of the 206 bp product that results from amplification of the group I $sPLA_2$ cDNA, amplification of the gene with these primers yields a 1006 bp product which includes the 800 bp intron B. Similarly, the group II primers hybridize to exons 3 and 5, yielding a 2704 bp DNA from the gene instead of the 242 bp product derived from the cDNA. Short polymerization incubations (1 min) in the amplification protocol disfavor exponential amplification of the larger products derived from the gene, thus making the primers somewhat more selective for the cDNA of interest. This strategy can be confounded by the presence of pseudogenes, spliced variants of a gene that arise by reverse transcription of mRNA and integration into the chromosome. The presence of pseudogenes contaminating total RNA can be checked by amplification of RNA that has not been reverse transcribed.
2. All materials should be procured from reliable chemical or biotechnology supply houses. Purchase only the highest grade available, usually referred to as Ultrapure or Molecular Biology Grade. The manufacturer must test for contaminating RNase activity. Most suppliers provide documentation of enzyme lot activity that includes specifications for contaminating RNase and other activities. All reaction stock components are made up in advance, aliquoted and stored frozen until used. Freeze-thaw cycles are avoided when dealing with most solutions of nucleotides, proteins and buffers.
3. A major concern in the preparation and handling of RNA is degradation by RNases. RNases are extremely resilient enzymes that are ubiquitous in the environment. RNases should be assumed to be present on human skin, in every solution, all utensils and on every surface in the laboratory. While baking in an oven at 300°C for 60 min does destroy RNases autoclave, filtration, and sterilization procedures are ineffective. Remember that sterile does not mean RNase-free. Thus, it is vital that procedures to avoid the introduction of RNases be rigorously

followed at every step during the extraction, purification and handling of RNA. Clean disposable gloves should be worn at all times and changed frequently to avoid transferring RNases into samples. Plastic ware such as pipets tips and centrifuge tubes is generally assumed to be RNase-free as long as it was machine-molded and machine-packaged, and never touched by human hands, gloved or otherwise. A safe way to prepare microfuge tubes is to pour them from a bulk-packed bag into a baked glass container and then autoclave them.

Similarly, the quality of the water used to dissolve reagents and dilute samples is critical to the success of RNA analyses. It bears repeating that it is not sufficient to use autoclaved double deionized water as RNases are notoriously resistant to heat inactivation. An effective way to inactivate RNase is to treat water with 0.1% DEPC for overnight. One potential problem with this procedure is that DEPC can form adducts with RNA; therefore, it is important to completely hydrolyze unreacted DEPC by autoclaving before using DEPC-treated water. It is possible to treat inorganic solutions with DEPC (e.g., NaCl) but it should not be used to decontaminate solutions of organic compounds (e.g., Tris, and so on). A freshly prepared 0.1% DEPC solution can also be used to decontaminate plastic or glass (e.g., a cuvet) provided the treatment is followed by extensive rinsing with decontaminated water. RNase-free water can now be purchased from several suppliers and the savings in time and worry may well be worth the cost.

4. The number of cells required for RNA extraction will depend on the cell type and on the technique to be utilized for analysis. RT-PCR can be performed reliably starting with as little as 50 ng of total RNA. We typically use 5 million mast cells per condition, which usually yields sufficient total RNA for a several RT reactions. Since each standard RT reaction produces enough cDNA for 25 PCR reactions, this is more than adequate for most purposes.
5. Note that although cDNA is much more stable than RNA, many of the precautions taken to avoid introducing RNases are also effective in avoiding crosscontamination of samples, a major concern in PCR amplification reactions.
6. To avoid multiple pipetting of very small volumes and to avoid the risk of contamination, it is desirable to make a "master mix" that contains all components for the RT and the PCR reactions. After aliquoting the master mix into thin wall tubes or thin wall microtiter plates designed for use in a thermal cycler, RT or PCR is initiated by adding the RNA extract or the cDNA, respectively.
7. A "touchdown" amplification protocol increases the stringency of the template selection in the early phase of the amplification when the competition with the target cDNA for primers is greatest ***(26)***. In later cycles, stringency is not as important, since the vast majority of template is amplification products. Under these conditions primer annealing to first strand cDNAs is heavily disfavored and annealing to and amplification of the products produced in the earlier cycles are heavily favored. Reducing the annealing temperature during the latter part of the amplification increases the amplification efficiency.
8. Primer pairs may exhibit sensitivity to the Mg^{2+} concentration of the amplification reaction, although we have only rarely observed this. We always use 3 m*M*

MgCl in our amplification buffer. If a primer pair fails to yield a product or yields multiple products, it should be tested using a range of Mg^{2+} concentrations between 1 and 4 m*M*.

9. Perform quantitation as close as possible to the amplification stage at which the product from an unknown sample is first detected. Differences in cDNA abundance between samples requires quantitation of the unknowns at different amplification stages, since templates with a higher abundance of the target cDNA will yield detectable products at an earlier stage of the amplification.
10. For estimation of mRNA abundance, it is convenient to select primers that produce amplification products of between 100 and 400 base pairs. Products smaller than 100 base pairs are difficult to resolve by typical alkaline agarose gel electrophoresis and their identity is difficult to confirm by reamplification using nested primers. Larger products amplify less efficiently and take longer to polymerize which unnecessarily increases the time it takes to perform amplification
11. The designation of 0.5X for the working strength of TBE is a historical artifact. Originally 1X was used, and later 0.5X was found to provide adequate buffering for most routine uses. In cases where large currents are needed, 1X TBE should be used. Sufficient agarose for a gel (usually 100 mL, depending on the size of the apparatus) is melted in a glass container in a microwave for 2 min. Care must be used, as the hot melted agarose can cause severe burns. The gel is then cast, combs are put in place and the gel is allowed to set until it reaches room temperature. Gels can also be made ahead of time and stored wrapped in plastic in the refrigerator for a week. The running TBE buffer should be of equal strength to that used in the gel and enough should be poured to just cover the gel slab.
12. The levels of PLA_2 mRNA and PLA_2 isotypes in mast cells varies depending on the amount of cytokines that the cells are exposed to. When mast cells are maintained in culture media that is supplemented with IL-3 or with stem cell factor (SCF), $cPLA_2$ expression is increased many fold. By contrast, SCF treatment results in less $sPLA_2$ expression ***(14,27)***.
13. During sequencing to confirm the presence of the expected products, some sequence variation is expected resulting from errors introduced by Taq DNA polymerase during the amplification and misreads of the sequence. Sequence within 20 base pairs of the sequencing primer is excluded, since this region is unusually noisy. Product identity can also be confirmed by reamplification of the products using one or two additional oligonucleotide primers that prime the product DNA (nested primers). This approach is much less informative and can be more expensive than direct sequencing of the product DNA.

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10

Enzyme immunoassays (EIA) of Eicosanoids

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1. Introduction

Radioimmunoassay (RIA) and enzyme immunoassay (EIA) are used for detecting very small amounts of a hormone, drug, or other such material in blood (serum), tissue, urine, or culture media. Both radioimmunoassay and enzyme immunoassay techniques for the measurement of eicosanoids are based on the principles of the competitive substrate binding assay. More specifically, these assays involve the competition (for binding sites on an antibody) of a known concentration of a labeled eicosanoid with an unknown quantity of the same unlabeled eicosanoid. The antibody used has been generated against the specific eicosanoid to be measured. EIA is similar to RIA as an assay except that instead of using a radiolabeled compound, the competing antigen is labeled with a specific enzyme.

The advantage of the EIA over the RIA is that the EIA provides a better method of measuring eicosanoids without losing specificity or sensitivity. Furthermore, enzyme labeled compounds can be stored for longer periods than radiolabeled compounds, without a significant loss of activity. The volumes of sample needed to perform EIA are much smaller than those needed for RIA, leading to a more economical and possibly efficient assay. This is especially the case if multiple determinations from the same sample are required.

At present, commercially available EIA kits provide all the necessary reagents to perform 100 determinations (approx 30 samples in duplicate and standards). If one needs to assay the sample a second time owing to unacceptable variation between duplicates or insufficient dilution of sample, another kit must be purchased. There are kits for 500 determinations but once the tracer is reconstituted in EIA buffer, it is only stable for 1 wk. Thus, if EIA for a particular eicosanoid is going to be performed on a routine basis, it is more economical to

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develop the assay in the laboratory rather than repeatedly purchasing commercial kits. This chapter focuses on the development of EIA in the laboratory using commercially available antibodies and enzyme-tracers. This is not an extensive review of the literature of EIA, but is intended as a guide to setting up and validating an EIA in the laboratory.

2. How Does EIA Work?

EIA involves an antigen–antibody reaction as written below.

$$\text{Ag} + \text{Ag}^* + \text{Ab} \rightarrow \text{Ag/Ag}^*\text{Ab complex} + \text{free Ag} + \text{free Ag}^* \quad (1)$$

Ag = standard eicosanoid of known concentration or unknown level of eicosanoid in the sample
Ag* = fixed amount of eicosanoid conjugated to the tracer enzyme
Ab = antibody produced against the eicosanoid

The measurement of the eicosanoid depends on the competition between the tracer and the eicosanoid in the sample, for a fixed number of specific binding sites on the antibody. The smaller the amount of eicosanoid in the sample, the greater the amount the tracer available to compete for and bind to the binding sites on the specific antibody. The success of this immunobased assay is predicated on two assumptions:

i. There is a fixed concentration of Ab that offers a relatively fixed number of Ab receptor sites for the eicosanoid Ag.
ii. The antibody is highly specific for the eicosanoid being measured.

After incubation of the sample and the labeled eicosanoid tracer for a set amount of time, it is necessary to separate bound tracer from the free tracer. In most commercial EIA this is done by simply emptying the 96-well plate. This is possible because the plates are precoated with antirabbit gamma G immunoglobulin (IgG). Thus, if the eicosanoid antibody is a polyclonal rabbit antibody, the specific polyclonal antibody will bind to the antirabbit IgG coating the wells. The free tracer can then be dumped off and the enzyme binding coloring reagent is added. The color developing reagent specifically binds to the eicosanoid–enzyme tracer. Depending on the color development reagent used, absorbance at a specific wavelength can be measured using a commercial platereader. To quantitate the amounts of eicosanoids, absorbance of samples are compared with the values for absorbance obtained with a series of standards of known concentration.

A flow chart of the steps involved in the assay is provided below:

1. Precoat plates with antirabbit IgG overnight.
2. Wash plates with wash buffer.
3. Saturate plates by filling with EIA buffer.

4. Cover plates with plastic film.
5. Store coated plates at 4°C.
6. Sequentially add specific antibody, tracer, sample (or standards).
7. Incubate the mixture for a fixed amount of time depending on tracer used.
8. Wash plate to separate the bound from free eicosanoid or antigen.
9. Add the color development reagent and allow to incubate.
10. Read at the specified absorbance.

2. Materials

1. Assay plates: Ninety-six well NUNC (Maxisorb) plates are the standard plates used in enzyme immunoassay. However, other high binding plates are available. It is advisable to use flat-bottomed plates as they allow for more reproducible results. The use of round bottom wells tends to introduce variability in the measurement of absorbance.
2. Pipets: For greater efficiency, a multichannel (8 or 12) pipetter should be used to dispense the antibody, the tracer and the color development reagent. Alternatively, a repeating pipettor can be used if a multichannel unit is not available. For washing the plates, a fully automated or semiautomated 96-well dispenser is highly recommended. Plastic troughs suitable for pipetting with a multichannel pipetter are commercially available.
3. Plate reader: A 96-well spectrophotometric plate reader is essential for reading of absorbances. The Biotek or Softmax plate readers with filters that enable absorbance measurements of 405, 450, and 630 nm are the most commonly used plate readers.

3. Methods

3.1. Plate Coating

Although precoated plates are commercially available, they are expensive. As mentioned earlier, if it is intended to perform assays on a routine basis or in large quantities, it is more cost effective to purchase the component parts of the assay.

Polyclonal antirabbit IgG is the most common antibody coating for the EIA plates. At present, mouse monoclonal antirabbit IgG is also available for coating plates. The latter may afford greater reproducibility as various lots of polyclonal antirabbit IgG may differ in their binding affinity. The protocol for coating the plates is as follows:

1. The antibody is diluted in potassium phosphate buffer (50 m*M*) to give a concentration of 10 µg/mL per well. Each well should be coated with no more than 200 µL of antirabbit IgG. Polyclonal goat antirabbit IgG is most frequently used for this purpose. An alternative buffer that can be used is 100 m*M* sodium bicarbonate buffer (pH 8.2).
2. The plates are left overnight at 4°C. At the end of this incubation period, the plates are then inverted onto paper towels to remove unbound antirabbit IgG.

3. Plates are then washed three times with wash buffer (300 µL/well; *see* **Subheading 3.1.2.** for composition of wash buffer). Wells can then be filled with 300 µL EIA buffer (*see* **Subheading 3.1.1.**) to completely saturate them. This latter step is vital because the EIA buffer contains albumin, which blocks any nonspecific binding to the antirabbit IgG. Plates can then be sealed with plastic cover slips and stored at 4°C for approx 4 wk.

3.1.1. Composition of EIA Buffer

Use only ultrapure, deionized water that is free of organic contaminants, when making up buffers. In potassium phosphate buffer (100 m*M*), add NaCl (0.4 m*M*), tetrasodium EDTA (85 m*M*), sodium azide (0.01%) and bovine serum albumin (BSA) (0.1%). All concentrations in parenthesis indicate final concentrations. The sodium azide is an antioxidant to preserve the buffer in storage. It also prevents growth of bacteria in the presence of albumin.

3.1.2. Composition of Wash Buffer

1. 0.05% Tween-20 in 100 µ*M* potassium phosphate buffer.

3.2. Standards, Samples, and Blanks

The standard curve consists of values for:

1. Maximum binding (B_0; tracer [50 µL], antibody [50 µL], and EIA buffer [50 µL]).
2. Nonspecific binding: tracer (50 µL) and buffer (100 µL).
3. Standards of known concentrations of eicosanoids: The standards are prepared by serial dilution of a stock solution with EIA buffer. The standards should range in concentration from 2 ng/mL to approx 5 pg/mL (1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9). All components of the standards should be performed in duplicate. The total volume in all wells mentioned above should be 150 µL. A standard curve should be run on every plate.

It is strongly recommended that at least two dilutions of each sample be run concurrently. This is because samples at one dilution may not fall in the linear portion of the standard curve. All samples should have the same volume of 50 µL for reproducibility. A 50% dilution of a sample may be achieved by adding one-half the original volume of the sample and adding the appropriate amount of buffer to achieve a final volume of 50 µL.

When pipetting reagents or samples into your plate, the following steps in sequence are recommended.

1. Addition of standards and samples;
2. Addition of specific eicosanoid antibody;
3. Addition of tracer (50 µL, except for NSB wells). This regimen should be adhered to closely to increase reproducibility.

In addition to the above components of the standard curve and samples, at least four wells should be left unfilled. Two wells will be used to measure total

enzyme activity (TA) and two wells will serve as blanks. Addition of tracer (and color development reagent) to the TA wells and color development reagent to the blanks is performed AFTER incubation of the standards and samples.

After addition of the reagents, the plates are covered with a plastic film and left to incubate at 4°C for a fixed period of time depending on the enzyme tracer that is used. For acetylcholine esterase, the incubation time is approx 18 h, however if peroxidase is used as the enzyme tracer, incubation time is not longer than 2 h. At the end of the incubation, plates are washed three times with wash buffer. This is an important step to separate and remove free molecules leaving behind the bound complexes. It is imperative to wash the plate thoroughly.

After the plates have been washed, the tracer (5 µL each well) is added to TA wells. The blank wells will not have any reagent in them. Blanks are an indication of the absorbance of the color development reagent in the absence of tracer. This should normally be negligible and is automatically subtracted from all other absorbance values by the plate reader. The type of color development reagent used depends on the type of enzyme tracer conjugated to the eicosanoid. The color development reagent used to visualize binding of the acetylcholine esterase tracer to the antibody is "Ellman's reagent" (0.54 m*M* 5,5'-dithiobis(2-nitrobenzoic acid)). This is added to 0.69 m*M* acetylcholine iodide in phosphate buffer (50 m*M*). The Ellman's reagent can readily be purchased but if one wishes to make their own reagent, it is advisable to make it fresh and wrap it in aluminum foil as the solution is light sensitive. If the eicosanoid is conjugated to horseradish peroxidase, it is common to use various commercially available peroxidase substrates, such as K-Blue® substrate (stabilized 3,3',5,5' tetramethylbenzidine plus hydrogen peroxide), to develop the color. The plates are read at a specified wavelength (405 nm for acetylcholine esterase; 650 nm for peroxidase substrate). If possible, it is recommended that the plate be read at two wavelengths, the optimum wavelength for acetylcholine esterase enzyme tracer and 630 nm. Absorbances measured at 630 nm will automatically be subtracted to correct for nonspecific absorbances. The latter can occur if the plate is scratched. Reading the plate at two wavelengths is not normally performed when using horseradish peroxidase as the enzyme tracer because the absorbance is read at such long wavelengths. A frequent question concerning the performance of the EIA is how long one allows for color development. It is our experience that the best standard curves are generated when the development of color is allowed to proceed until an absorbance for B_0 is obtained between 0.5 and 0.8.

It is important to note that the acetylcholine esterase enzyme is not a suicide enzyme, i.e., it does not auto-inactivate when turning over. If a mistake is made in the process of color development, e.g., absorbance is allowed to go above 1.0,

unequal amounts of Ellman's reagent were added to each well or if the plate was tipped over after addition of the Ellman's reagent, it is possible to wash the plate (with wash buffer) add the Ellman's again and read the plate after color development. On the other hand using the horse radish peroxidase enzyme, incubation times are much shorter and the assay is less time consuming.

3.3. Preparation of Antibody and Tracer

The proper dilution of antibody or tracer is critical to the success of the assay. It is imperative to follow the manufacturer's recommendation for the optimal dilution factor of the antibody and tracer. A wide range of specific antibodies and tracers can be purchased. If a noncommercial antibody is being used, it should be diluted to a concentration at which the sensitivity is greatest without being too dilute. If the concentration of the antibody is too high, this will increase the cost of the assay, needlessly waste the reagent and produce an assay with diminished sensitivity. Poor sensitivity results because all the binding sites have to be saturated before displacement of the tracer by the unknown, occurs. Commonly the antibody is titrated to a concentration that binds 40–60% of the enzyme tracer. This concentration of antibody can easily be established by incubating 50 µL of different concentrations of antibody, 50 µL of tracer (at a constant concentration) and 50 µL of EIA buffer. The antibody concentrations can be diluted starting with a 1:100 dilution to 1:51,200 by serial doubling dilutions. It is advisable to wait for the well(s) with the greatest color development (should be the wells with the most dilute antibody) to reach an optical density of 1.0 before taking the final reading. The dilution that produces approximately 50% binding of tracer to antibody is estimated, by plotting bound/maximum binding – NSB × 100 (where maximum binding is the absorbance of wells with the most dilute antibody). Once the dilution that produces 50% binding of tracer is determined, additional dilutions around the 50% binding concentration can be tested to achieve maximal sensitivity of the assay.

4. Quantitation of Results

The data from the assayed plates should be expressed as $(B - NSB/B_0 - NSB) \times 100\%$. A sample standard curve is given below where NSB is 0.005.

Concentration (pg/mL)	Absorbance	$\%B/B_0$
0	0.556	100
3.9	0.504	90.6
7.8	0.483	86.7
15.6	0.455	81.6
31.2	0.403	72.2
62.5	0.319	57.0
125	0.231	41.1

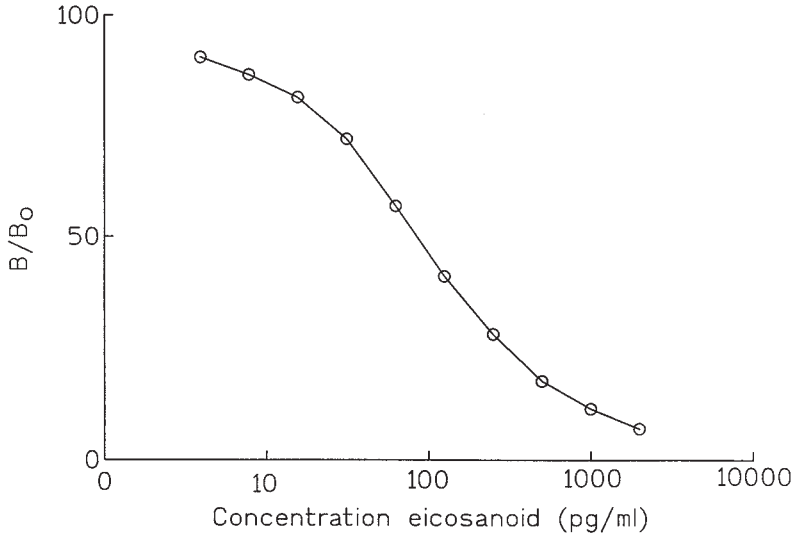

Fig. 1. Depiction of an example of a typical standard curve for an eicosanoid.

250	0.160	28.1
500	0.102	17.6
1000	0.068	11.4
2000	0.043	7.0

The values obtained are graphed to produce a standard curve. Levels of eicosanoids in samples can be read directly from the graph of the standard curve (**Fig. 1**). Several programs, such as Graphpad Prism or Inplot4 (DOS program) and the IBM-based EIA data analysis, can perform the required function and are easy to use. It is imperative that all samples fall within the linear portion of the standard curve. Generally, this is usually between 15 and 80% of maximum binding (B_0). If the sample(s) did not fall within this range, then it should be diluted appropriately and reassayed. If the level of eicosanoid in the undiluted sample (50 µL of neat sample) falls between 80 and 100% of maximum binding, it is considered as below the limits of detection. For the purpose of statistics, the concentration that corresponds to 80% binding is assigned to the sample. If a numerical value is imperative, the sample needs to be extracted and concentrated (*see below*). It is also important to note the value obtained for the nonspecific binding. If the nonspecific binding is relatively high, there are at least four reasons for this:

1. The water used to make buffer is impure;
2. The tracer is too old;
3. The plate was not coated correctly;

4. Plate was not washed thoroughly after coating or after incubation of samples. It is advisable to repeat the samples with new buffer and tracer as well as a different plate, if nonspecific binding is greater than 10% of B_0.

Total activity of the tracer is an important diagnostic tool. If only the tracer has any absorbance then antibody probably was not added.

5. Interference

Any substance that blocks the binding of antigen (either tracer or eicosanoid) with antibody is considered to interfere with the assay. An easy way to ascertain if there is interference in your assay is to measure absorbances of samples spiked with known quantities of the eicosanoid, e.g., 200, 100, and 50 pg/mL. If the absorbances do not increase linearly, interference is most likely present in the assay. Estimation of linearity can be made through linear regression. At the very least, a correlation coefficient (r) of 0.98 should be observed. If interference is suspected, the most common method of eliminating interfering substances from samples is through the use of an extraction procedure. It can also be used to concentrate samples. Extraction of eicosanoids from plasma is always necessary. Eicosanoid extraction is not always necessary for urine samples and is never needed for culture media supernatant. A detailed description of various extraction procedures is presented in Chapter 4. A summary of the steps used in our laboratory, are presented below.

1. 500-µL aliquot of sample is removed and 2 mL of ethanol added to the mixture. Mixture is vortexed and allowed to stand at 4°C for 10 min. It must be noted here that the volume of plasma suggested here is simply one we have found to be useful and used routinely. Other volumes of plasma may be used depending on the expected concentration of eicosanoid in the sample and a simple correction of the solvent volumes to match the ratios suggested here can be applied.
2. Samples are centrifuged at 1500*g* for 10 min to remove proteins.
3. Supernatant is poured into clean scintillation vials.
4. 8 mL of 0.1 *M* phosphate buffer, pH 4.0, is added and the mixture vortexed.
5. Using a 10-mL syringe, the mixture is passed through a Sep-Pak column (C18) preconditioned with ethanol (5 mL) and water (5 mL).
6. Column is washed with water (5 mL) and hexane (5 mL). The hexane may be difficult to push through the column. A smaller volume of hexane will probably be adequate, but this needs to be checked against the percentage recovery (*see below*).
7. Sample is eluted with methanol/water (1:1 or 70:30%), or ethyl acetate (1% methanol). The choice of elution solvent is both a matter of preference as well as providing conditions that have yielded good recovery of the compound in your laboratory or in the literature. Through experience, it has been found that eluting the column twice with 15 min between elutions is a most effective way to maximize recovery.
8. The samples are dried down under nitrogen gas or centrifuged under vacuum. After samples are evaporated to dryness, 500 µL EIA buffer is added to reconsti-

tute the sample(s). If the sample needs to be concentrated, then a smaller volume is used to reconstitute the sample.

The major disadvantage of any extraction procedure is the loss of compound through the process. To determine the recovery of the compound, a known amount of radiolabeled eicosanoid is added to the plasma aliquot before extraction. After the extraction process, half the original aliquot (in this case 250 μL) is taken for counting. To obtain percentage recovery the following simple formula is used:

$$\text{Counts after extraction} \times 2/\text{Total counts added} \times 100 = \%\ \text{Recovery} \quad (2)$$

This procedure should be repeated four to five times and an average % recovery obtained. This percentage can then be used to correct for all the samples extracted by the procedure, taking into account any concentration increases or dilution factors that were used during the extraction procedure. Alternatively, a more accurate method to determine % recovery would be to spike all the samples with a known quantity of radiolabeled eicosanoid. However, this would negate one of the advantages of EIA which is that, it is a nonradioactive assay.

If a radioactive isotope of the eicosanoid is not available, a cold spike method can be used. In this procedure, two identical aliquaots are taken. To one aliquot add a known amount of the eicosanoid. Then extract both samples as described above. The percentage recovery is calculated from:

$$\frac{\text{concentration of spiked sample} - \text{concentration of unspiked sample}}{\text{concentration of eicosanoid spike}} \times 100 \quad (3)$$

This method assumes that the two samples were handled identically and there was no intrinsic variability between them. Thus, there is an inherent disadvantge to this particular method of estimating the recovery.

6. Evaluation of Assays

Extensive validation of enzyme-immunoassays and radioimmunoassays have been performed and reviewed ***(1–4)***. A brief summary of important issues to be considered when establishing an assay in the laboratory are presented below.

6.1. Sensitivity of the Assay

This is defined as the least amount of compound which can confidently be reproducibly detected by the assay system. This is often described as the amount of eicosanoid which will displace 15% of the eicosanoid tracer binding to the antibody, (i.e., $B/B_0 = 85\%$) and usually corresponds to the highest point of the linear portion of the standard curve. If assay conditions are optimal, (e.g., ultrapure water is used, plates are coated correctly, and so forth) then sensitivity is dependent on the quality of the manufacturer's antibody.

6.2. Precision of the Assay

The precision of the assay is defined as the extent to which a measurement of replicates of a sample of equal volume, agree with the mean of the replicates of the sample. Mathematically, this is conveniently expressed by the coefficient of variation (CV) for the sample results. The % CV is equal to SD/Mean × 100. The CV can be calculated for intraassay variability and for interassay variability. The most convenient method to evaluate intra-assay variability would be to measure a sample and/or one standard (e.g., a standard that falls in the linear portion of the standard curve) in five different wells. A CV value >10% is considered poor. There are several ways to measure interassay variability. An easy way is to measure the eicosanoid level (in duplicate) of one particular sample in other assays for the same eicosanoid. Another easy method to measure interassay variability would be to calculate the EC50 for all the standard curves ($B/B_0 = 50\%$) performed on each plate.

Generally, precision is dependent on the quality of the technical performance of the assay, i.e., the pipetting technique and washing of the plates. Thus, it is important to pay attention to technique and to keep all conditions constant for all assays.

6.3. Accuracy

This measure is probably the single most difficult parameter to evaluate when validating your EIA. Accuracy is defined as the extent to which the mean of an infinite number of measurements of a substance is similar to the exact amount of the substance present. Under ideal conditions one can assess the accuracy of the assay by measuring the amount of eicosanoid in samples and comparing that with exactly known amounts of the eicosanoid. The best way of determining the accuracy would be to weigh accurate amounts of the eicosanoid in the best analytical balance available and dissolve it in ethanol. The eicosanoid can then be blown dry under nitrogen and reconstituted in EIA buffer to be assayed. The difference between the measured concentration through EIA, and the actual amount of eicosanoid can be expressed as percentage error (measured amount – actual amount/actual amount × 100). Another way to measure accuracy which might be convenient is to compare the concentrations measured by EIA with those measured by mass spectrometry. Differences between the two measurements will invariably be a result of inaccuracies in the measurement by EIA.

6.4. Known Changes in Eicosanoids

As a last check on the validity of your assay, it is important to see if there is an increase in eicosanoid concentration in biological sample(s) that have been exposed to conditions known to increase eicosanoid production as compared to nonstimulated controls or if use of an inhibitor of arachidonic acid metabolism

will decrease the production of the eicosanoid being measured. For example, if measuring the production of PGE2 from cultured endothelial cells, the cells can be stimulated with lipopolysaccharide (LPS), an agent that is well known to increase eicosanoid production in endothelial cells. It is also highly recommended that indomethacin (10 μ*M*) be added to some of the wells with the LPS and to some of the controls to see if both stimulated and/or basal PG levels are decreased. Please note that this is only an example of possible validation procedures. There are many other models where eicosanoids are known to increase, such as in experimentals models of hypertension or models of acute inflammation. The choice of which biological sample to use for validation of the assay essentially depends on availability and convenience.

7. Summary

Enzyme immunoassay of eicosanoids is a nonradioactive, highly sensitive method of determining the concentration of eicosanoids in biological samples. Although relatively easy to use, the assays require a high level of precise pipetting technique and familiarity with critical points in the assay procedure. Although assay kits complete with plates, buffers, antibodies, tracers, and color development reagents are available, it is more economical to develop the assay within the laboratory if the assay is to be performed routinely.

The only major disadvantage with EIA is that the investigator is limited to measuring eicosanoids with commercially available enzyme-tracers and antibodies. The labeling of particular eicosanoids by enzyme tracers is rarely, if ever, performed outside of industry. Growing of antibodies is conducted in many laboratories but is beyond the scope of this chapter. It requires a significant level of commitment of time and resources to establish the specificity of the antibody (i.e., does the antibody cross-react with eicosanoids of similar structure). Furthermore, this will not solve the problem of availability of eicosanoid-tracer. On the other hand, it must be noted that with the exception of the cytochrome P-450 metabolites of arachidonic acid, most of the major eicosanoids that are biologically active and are known to play regulatory roles in physiology and/or pathology, have commercially available antibodies and enzyme-tracers.

Acknowledgments

We wish to acknowledge the helpful discussions with Frank F. Sun.

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11

Transcellular Regulation of Eicosanoid Biosynthesis

Karsten Gronert, Clary B. Clish, Mario Romano, and Charles N. Serhan

1. Introduction

The development of new and improved analytical methods for the study of eicosanoid biosynthesis has enabled researchers 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, and chemoattractants. It thus has 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 lipoxygenase (LO) activity observed in platelets is that of 12-LO, although 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 A_4 (LTA_4), LTB_4, and 5S,12S-diHETE ***(4)***. On the other hand, neutrophils upon activation release >45% of their LTA_4 to neighboring cells ***(5)***, which is rapidly converted by platelets to both LTC_4 and lipoxins (LXA_4 and LXB_4) ***(6,7)***. The release of

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LTA_4 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, 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 chapter will outline and review the salient features in these in vitro models and highlight crucial cellular and analytical components in the experimental design, which should allow the reader to expand this approach to other cell–cell interactions in specific scenarios of interest.

2. Materials

1. Most of the major eicosanoids are commercially available from a variety of vendors such as Cayman Chemical Co. (Ann Arbor, MI), Biomol (Plymouth Meeting, PA), and Oxford Biomedical Research (Oxford, MI), and [^{3}H]- or [^{14}C]-labeled eicosanoids are available from DuPont NEN (Boston, MA). 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 of all stocks initially upon receipt and—as a standard laboratory protocol—throughout the use of the compound (*see* **Note 1**).
2. Buffers and Reagents: Dulbecco's phosphate-buffered saline (DPBS) plus Ca^{2+} and Mg^{2+} ($DPBS^+$) or minus Ca^{2+} and Mg^{2+} ($DPBS^-$), RPMI 1640, Hanks' balanced salt solution (1 or 10X) 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 m*M* of citric acid, 75 m*M* of trisodium citrate, 136 m*M* of dextrose. This solution is prepared in water and sterilized by filtration. Prepare a concentrated EDTA (500 m*M* 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_2PO_4 (0.055 g), dextrose (0.5 g), and HEPES

Table 1
Cytokines or Agonists That Induce Key Enzymes or Proteins in the Eicosanoid Biosynthetic Pathway

Cytokine/agonist	Enzyme/protein	Cell type
IL-1 TGF-β TNF-α	Cytosolic phospholipase A_2	Neutrophil Monocyte Mast cell
GM-CSF TGF-β IL-3	5-lipoxygenase	Monocytes Neutrophil
IL-4 IL-13	15-lipoxygenase	Tracheal epithelia Monocytes
Growth factors	12-lipoxygenase	Platelet
IL-1β TNF Endothelial growth factors Tumor promoters LPS	Cyclooxygenase II	Tracheal epithelia GI epithelia Neutrophil Monocyte Vascular endothelia
Growth factors c-kit	LTC_4 synthase	Platelet Endothelia
GM-CSF c-kit	Five lipoxygenase activating protein (FLAP)	Neutrophil Monocyte

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

Table 2
Agonists that Stimulate Formation of Novel Lipoxygenase Derived Products During Cell–Cell Interactions

Cell–cell interactions	Receptor bypass	Product	Reference
From healthy donors			
Neutrophil-platelet	Ca^{2+} Ionophore	LTC_4	***4***
Neutrophil-platelet	Urate crystals	LTC_4	***34***
Granulocyte-platelet	Ca^{2+} Ionophore	LTC_4, LX	***35, 36***
Neutrophil-endothelial cell	Ca^{2+} Ionophore	LTB_4, LTC_4	***37***
Monocyte-platelet	Ca^{2+} Ionophore	LTC_4	***38***
Leukocyte-endothelial cell	Ca^{2+} Ionophore	LTC_4	***40***
Granulocyte-lung tissue	Ca^{2+} Ionophore	LX	***39***
Alveolar macrophage-epithelia	Ca^{2+} Ionophore	LTB_4, LX	***41***
Neutrophil-epithelial cell	IL-1 (+ASA), Ca^{2+} Ionophore	15-epi-LX	***10***
Cell–cell interactions	**Receptor mediated**	**Products**	**Reference**
From healthy donors			
Neutrophil-platelet	fMLP, Thrombin	LTC_4, LX	***23***
Neutrophil-platelet	GM-CSF and fMLP, Thrombin	LTC_4, LX	***6***
Neutrophil-platelet	GM-CSF and fMLP, Thrombin or PDGF	LX	***25***
Neutrophil-endothelial cells	GM-CSF and fMLP, TNF	LTC_4	***30***
Neutrophil-endothelial cells	IL-1,TNF or LPS and fMLP or PMA,Thombin (+ASA)	15-epi-LX	***9***
Cell type or cell–cell interactions	**Disease**	**Products**	**Reference**
Diseased donors or disease model			
Neutrophils	Asthma	LX, LTB_4	***41***
Neutrophils	Rheumatoid arthritis	LX, LTB_4	***42***
Macrophage-astrocytes	HIV	LX, LTB_4, LTD_4	***43***

5. Cell culture for in vitro incubations:
   a. Endothelial cells: Human umbilical vein endothelial cells (HUVEC) are available from American Type Culture Collection (ATCC; 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, Lexington, MA), 50 µg/mL endothelial mitogen (Biomedical Technologies, 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 nontransformed 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 ATCC. 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 cm^2 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* **refs.** ***17,18***); therefore, the human acute monocytic leukemia cell line THP-1 (ATCC) 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 (FBS) and 2-mercapthoethanol (50 m*M*). Note that these monocytes are nonadherent 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 variety of vendors (*see* **Subheading 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 (*see* Chapter 14). However, appropriate controls have to be designed to ensure that observed quantities of immunoreactive 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* Chapter 4 and **Note 5**). In addition, analysis is limited to simple quantitation of a single eicosanoid per assay without offering any structural detail on the compound.

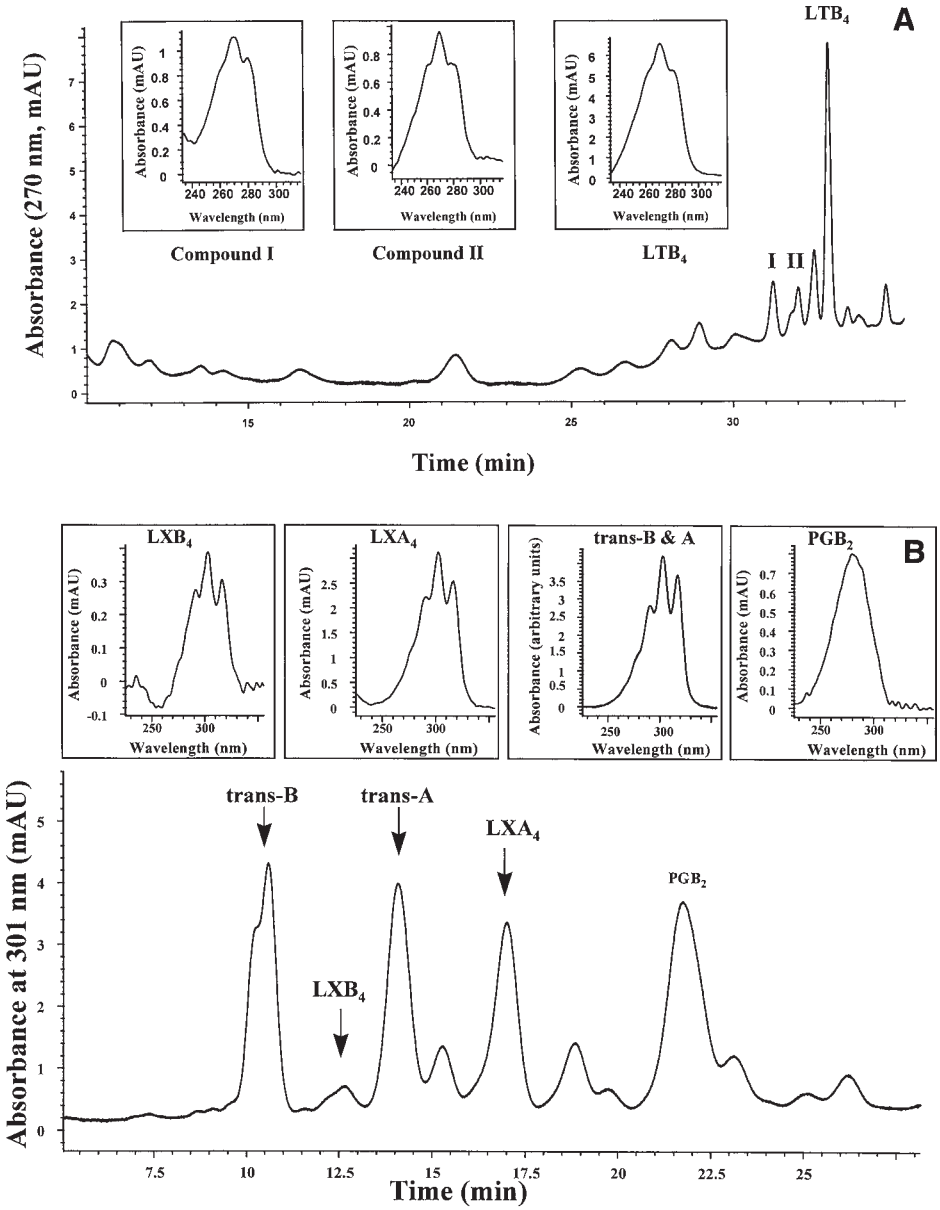

Fig. 1. Transcellular biosynthesis of lipoxins during platelet-PMN interactions. **(A)** Representative RP-HPLC chromatogram of endogenous products obtained from human PMN exposed to LPS (1 µg/mL) and stimulated with platelet-activating factor (PAF, 300 n*M*) and fMLP (100 nM). Products were analyzed 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 × 1 mm, 5 µm) using

b. Reversed phase high pressure liquid chromatography (RP-HPLC) coupled with either ultraviolet, refractive index, electrochemical, or mass spectrometric detection (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 λ_{max} of ~270 nm [diHETEs] or λ_{max} of ~280 nm [peptide-containing leukotrienes] and lipoxins carry a conjugated tetraene chromophore with a λ_{max} of ~300 nm; *see* **Fig. 1**) that permits their identification and quantitation by spectrophotometric analysis (*see* Chapter 5 and **ref. 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 prostanoid specific immunoassay, electrochemical detection, or electrospray tandem mass spectrometry.
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 workup.
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. 2**). This method represents the most power-

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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 LTB_4 and the non-enzymatic hydrolysis products of the epoxide LTA_4, denoted as compound I (6-trans-LTB_4) and compound II (6-trans-12-epi-LTB_4), are shown in the insets. **(B)** RP-HPLC chromatogram of products obtained from human platelets incubated with LTA_4. Platelets (1.6×10^8 cells) suspended in PBS containing 0.1% BSA were incubated with 20 μ*M* LTA_4 and 5 μ*M* A23187 for 20 min at 37°C. Trans-A denotes retention times of 11-trans-LXA_4 and 6S-11-trans-LXA_4, and trans-B denotes 8-trans-LXB_4 and 14S-8-trans-LXB_4. The specific UV chromophores of LTA_4-derived lipoxins and the internal PGB_2 standard are shown in the insets.

ful 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 (*see* Chapter 6). Furthermore, this method has the distinct advantage that eicosanoids do not have to be volatilized as in GC/MS, so derivatization is not required.

3. Methods

3.1. Transcellular Biosynthesis of Lipoxins and Leukotrienes During Platelet and PMN Interactions

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 chapter outlines specific examples of incubation scenarios, providing a framework (*see* **Fig. 3**) for the evaluation of 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. 4**). This model of transcellular

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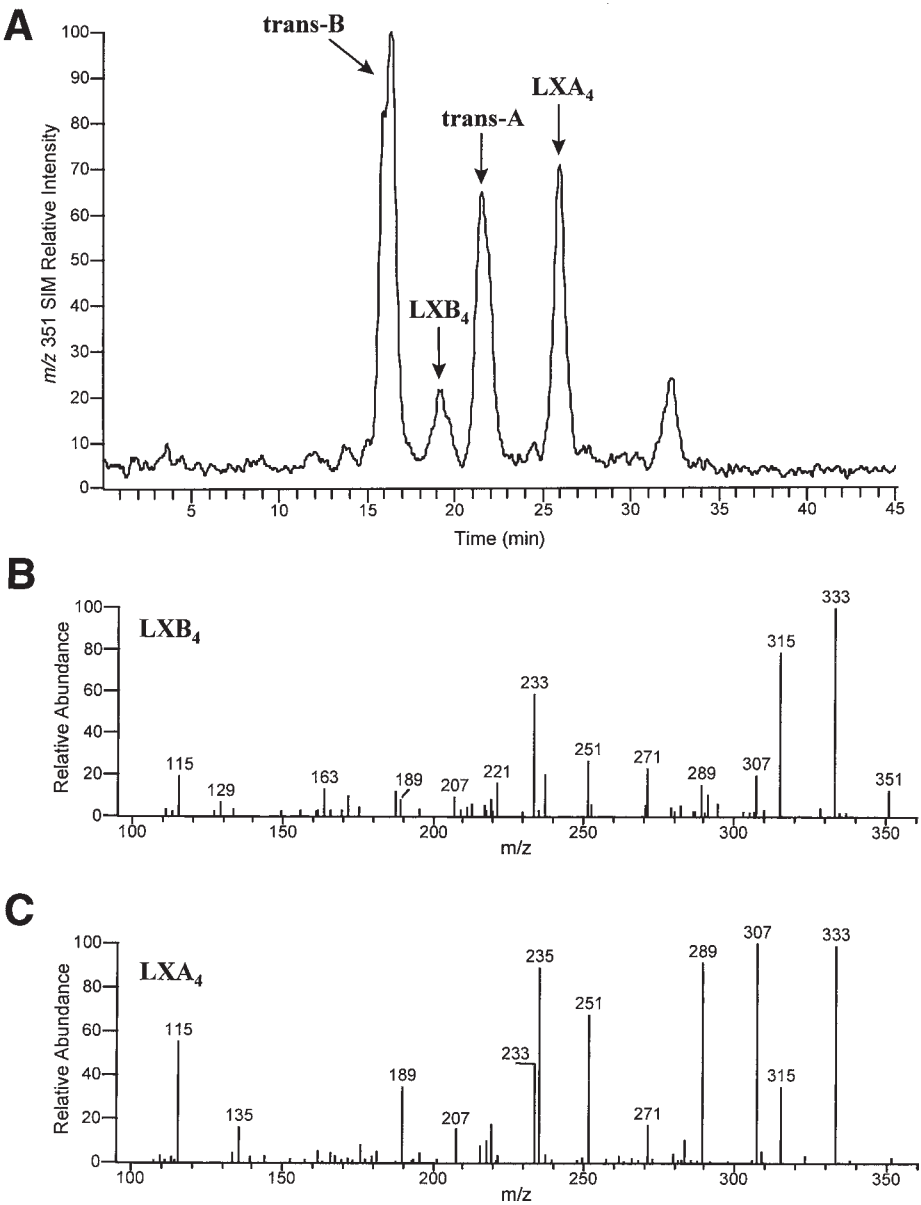
Fig. 2. LC/MS/MS analysis of platelet and LTA_4 derived lipoxins. LC/MS/MS (liquid chromatography-tandem mass spectrometry) was performed on a Finnigan LCQ quadrupole ion trap mass spectrometer system equipped with an electrospray atmospheric pressure ionization probe. A SpectraSYSTEM AS3000 autosampler 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 × 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 A_4 and B_4). **(A)** Representative LC/MS/MS *m/z* 351 SIM (selected ion monitoring) profile of products obtained from human platelets incubated with LTA_4. Products were obtained and isolated as in **Fig. 1B**. **(B)** Diagnostic MS/MS spectrum of LXB_4 peak from profile shown in A. MS/MS spectrum of LXA_4 peak from profile shown in (A).

LXB_4 Fragments: The MS/MS spectrum of LXB_4 shows prominent ions of diagnostic value at *m/z* 333 [351 - H_2O], 315 [351 - $2H_2O$], 307 [351 - CO_2], 289 [351 - H_2O, - CO_2], 271 [351 - $2H_2O$, - CO_2], 251 [351 - $CHO(CH_2)_4CH_3$], 233 [351 - H_2O, - $CHO(CH_2)_4CH_3$], 221 [351 - $CHOCHOH(CH_2)_4CH_3$], 207 [351 - CO_2, - $CHO(CH_2)_4CH_3$], 189 [351 - H_2O, - CO_2, - $CHO(CH_2)_4CH_3$], 163 [351 - CO_2, - $CH_2COHCHOH(CH_2)_4CH_3$], 129 [$CH_3CO(CH_2)_3COO^-$], and 115 [$CHO(CH_2)_3COO^-$] ***(32,33)***.



LXA_4 Fragments: The MS/MS spectrum of LXA_4 shows prominent ions of diagnostic value at *m/z* 333 [351 - H_2O], 315 [351 - $2H_2O$], 307 [351 - CO_2], 289 [351 - H_2O, - CO_2], 271 [351 - $2H_2O$, - CO_2], 251 [351 - $CHO(CH_2)_4CH_3$], 235 [351 - $CHO(CH_2)_3COOH$], 233 [351 - H_2O, - $CHO(CH_2)_4CH_3$], 207 [351 - CO_2, - $CHO(CH_2)_4CH_3$], 189 [351 - H_2O, - CO_2, - $CHO(CH_2)_4CH_3$], 135 [351 - $CHO(CH_2)_3COOH$, - $CHO(CH_2)_4CH_3$], and 115 [$CHO(CH_2)_3COO^-$] (cf. ***[28,33]***).

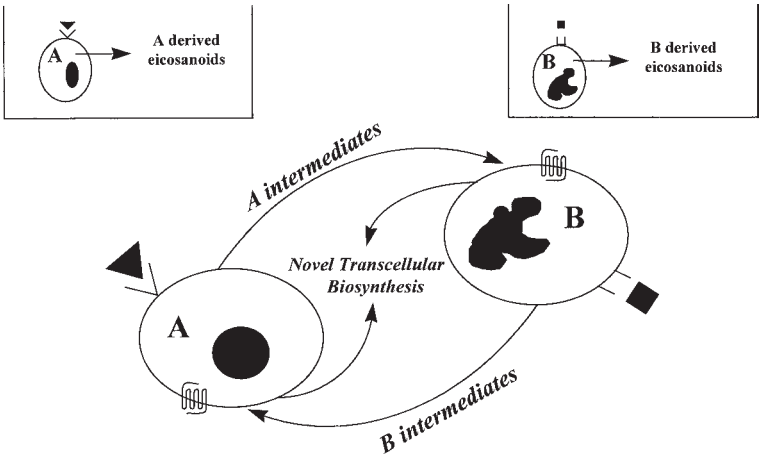


Fig. 3. General outline for investigating transcellular biosynthesis. General scheme illustrating the experimental approach to investigate transcellular biosynthesis. Pivotal experimental components are identified and include the initial characterization of cell type specific product profiles with receptor-triggered activation. In coincubation scenarios, the bidirectional transfer of intermediates from both cell types A and B must 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.

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. 1** and **2**).

3.1.1. Neutrophil Isolation (Modified from ***ref.*** **20***)*

To ensure clear evaluation of eicosanoid profiles by criteria outlined in **Fig. 3**, 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.

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 wk. Collect blood in 60-mL syringes containing acid citrate dextrose (7.0 mL) as an anticoagulant.

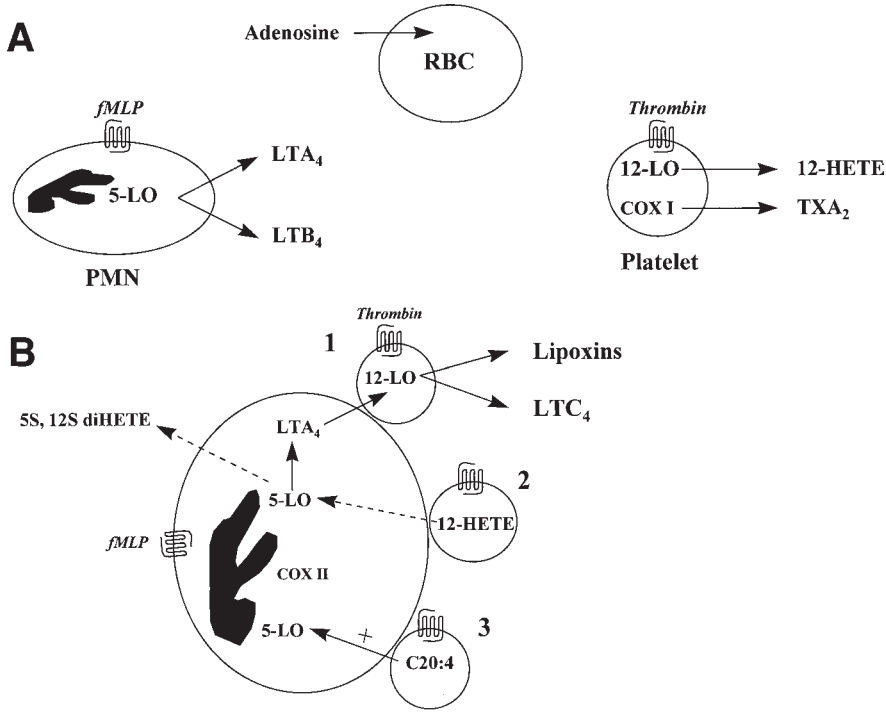


Fig. 4. Transcellular biosynthesis with peripheral blood cells. **(A)** Predominant products generated by isolated neutrophils (PMN) and platelets that are activated via specific serpentine receptors (i.e., thrombin and fMLP) are indicated. Isolated erythrocytes (RBC) do not generate significant amounts 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 LTA_4 that is transformed by adhering platelets to the novel products lipoxins and LTC_4 by 12-lipoxygenase and LTC_4 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 LTB_4 and LTA_4 generation.

2. Transfer 30–40 mL of blood to 50-mL Falcon tubes and spin at 800 rpm (134*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 **Subheading 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 (**Caution: do not vigorously shake or mix**). Centrifuge for 30 min at 1500 rpm (514*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×) and immediately mix suspension by inverting the tube (mix well). Spin the tubes at 1200 rpm (329*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 ~70×10^6 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 approx 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 m*M*; this will chelate calcium to prevent platelet activation.
2. Centrifuge at 2000 rpm (914*g*) for 10 min and carefully remove the supernatant using a transfer pipet.
3. Wash the pellet with approx 2.0 mL HEPES-Tyrode buffer using a transfer pipet (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*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 $CaCl_2$ (1 m*M*).
7. Enumerate platelets, preferably with an automated counter such as a Coulter counter (Coulter Electronics, Hialeah, FL). Adjust the cell number to ~700×10^6 platelets/mL (or desired cell number).

3.1.3. Incubation Conditions

3.1.3.1. Treatment of Cells Prior to Coincubation *(Optional)*

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 protocols for each particular cell to be investigated. As a specific example, the protocol for radiolabeling platelet C20:4 pools is given below (*see* **refs. *4,22***).

1. Preparation of [^{3}H] sodium arachidonate (*see* **Note 7**).
   a. Convert [^{3}H]-C20:4 (≈50 µCi, specific activity 60–100Ci/mmol) to the sodium salt by taking the suspension to dryness with a stream of nitrogen, makingsure 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* Na_2CO_3 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 m*M* of Tris, 134 m*M* of glucose, and 0.01% of delipidated bovine serum albumin, pH 7.4. Determine the total activity of the [^{3}H] 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 × 10^9 platelets for the labeling protocol and dilute the platelet suspension to a final concentration of 0.2 × 10^9 cells/mL. Transfer the platelet suspension to a disposable screw-capped plastic container and add the 3 mL [^{3}H]-sodium C20:4.
   b. Incubate the platelets in a covered water 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*g* for 15 min at 4°C.
   d. After centrifugation, collect the supernatant that 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 [^{3}H]-C20:4 (**item 1, step c**) by scintillation counting and calculate the percent of incorporated C20:4.
   e. Resuspend the platelets in HEPES-Tyrode buffer (minus calcium) at the desired number and keep the cells at 4°C until use. The percent of incorporated ^{3}H-C20:4 should be approx 50–80% (a value that is both donor-dependent and varies with the state of activation of the cell suspension).

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. 4**), then expose platelets to aspirin (100–500 µ*M*) or indomethacin (100 µ*M*) for 20 min before the incubations ***(23)***.

If the objective is to eliminate or evaluate the impact of platelet-derived 12-LO products in the coincubation product profiles, then expose platelets to 12-LO inhibitors, such as esculetin (100 µ*M*), for 10–20 min before coincubations ***(23)***.

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 p*M*, 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.**).

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 erythrocytes (*see* **Fig. 4A**). 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).

3.1.3.2. Platelet/Neutrophil Coincubations

1. Combine aliquots of platelets (0.5 mL, 35×10^7 cells) and PMN (0.5 mL, 35×10^6 cells) into 12-mL Falcon tubes (*see* **Note 8**). To compare the coincubation 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 (100 n*M*), a potent PMN agonist, and thrombin (1 U/mL), a potent platelet agonist (*see* **Notes 3** and **4**; **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 vol 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**). Samples in general should be extracted within 24–48 h, even though eicosanoids 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* **Figs. 5** and **6**). 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 gener-

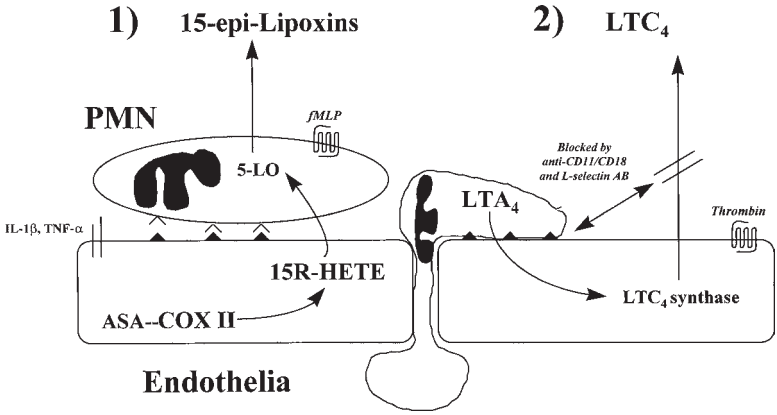


Fig. 5. Vascular endothelia and PMN interactions in the presence of aspirin. 1) Cytokine exposure induces COX II expression in vascular endothelial cells. This isozyme is acetylated by aspirin, and cell activation via serpentine receptors (i.e., thrombin) generates 15R-HETE that is transformed by the 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 LTA_4 that is transformed by endothelial LTC_4 synthase to the novel product LTC_4. This transcellular biosynthesis is diminished by antibodies specific for the adhesion molecules L-selectin and beta-2 integrins (CD 11/CD18).

ated during these cell–cell interactions include both lipoxins and aspirin-triggered lipoxins by PMN and LTC_4 by endothelial cells (*see* **Figs.** 5 and 6).

3.2.1. Endothelia/PMN Interactions ***(9)***

This section provides an evaluation of vascular transcellular biosynthesis during endothelia and leukocyte interactions with aspirin-treated vascular endothelial cells.

1. Day 1: Confluent monolayers of HUVEC (*see* **Subheading 2., step 5, item a**) are exposed to either IL-1β (1 ng/mL) to induce cyclooxygenase II (*see* **Table 2**, **Note 11**) or, for nonaspirin scenarios, to media alone for 24 h.
2. Day 2:
   a. Isolate PMN (*see* **Subheading 3.1.1.**). Adjust the cell suspension to 100×10^6 PMN/mL (or desired cell number). The desired ratio of PMN to endothelial cells is 8:1 for biosynthesis of aspirin-triggered lipoxins (*see* **Subheading 3.1.3.1., Note 12**, and **Table 1** for optional treatment, i.e., cytokines or inhibitors, of cells).
   b. After 24 h, 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 μ*M*; *see* **Note 13**) or vehicle

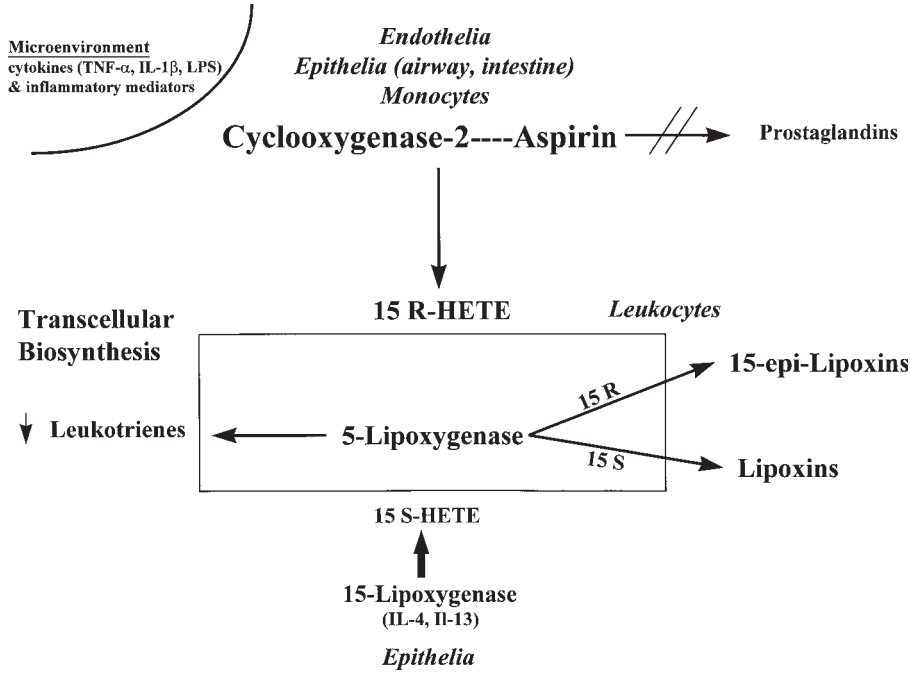


Fig. 6. Novel aspirin-triggered pathway in PMN interactions with epithelia and endothelia. Endothelia, airway epithelia, or enterocyte exposure to cytokines in an inflammatory milieu induces the expression of cyclooxygenase II. In the presence of aspirin, this isozyme is acetylated. This covalent modification 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-LXA_4. 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.

(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 (50×10^6 cells, 0.5 mL) to each monolayer of endothelial cells (approx 6×10^6 HUVEC/75 cm^2 flask). After adding cells for coincubation, add agonists (e.g., 100 n*M* of fMLP) and coincubate the cells without mixing for 30 min (*see* **Note 9**).

f. Terminate coincubation with the addition of 2 vol of cold methanol (–20°C) containing appropriate standards such as PGB_2 (100 ng) (*see* **Note 10**). To optimize 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 24–48 h, even though eicosanoids can be stored for a longer period of time in the methanol stop solution.

3.2.2. Epithelium/PMN Interactions ***(10)***

This section provides an 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. 5**). 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. 6** and **7**). 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, LTA_4 hydrolase, COX II, and PMN 5-LO (*see* schematic outline in **Fig. 6**). Transcellular products generated during these cell–cell interactions are aspirin-triggered lipoxins by PMN (**Fig. 6**), and LXA_4 as well as LTB_4 by epithelia (**Fig. 6**).

1. Day 1: To induce COX II, expose A549 cells to IL-1β (1 ng/mL) or, for a nonaspirin 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. 7**) 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)***.
2. Day 2:
   a. Isolate PMN (*see* **Subheading 3.1.1.**). Adjust the cell numbers in suspension to 100×10^6 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* **Subheading 3.1.3.1.**, **Table 1**).
   b. After 24 h, 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 μ*M*) (*see* **Note 13**) or vehicle (ethanol) alone for 20 min prior to coincubation with PMN.

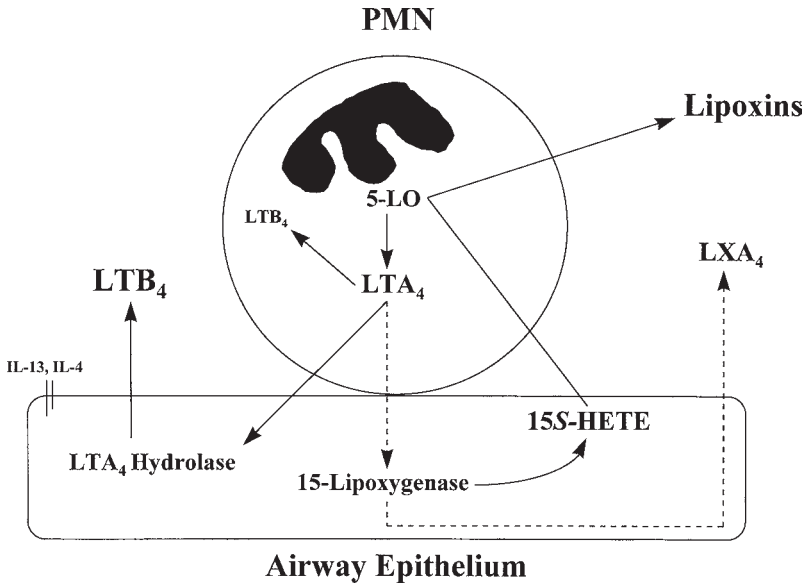


Fig. 7. Bidirectional transfer of eicosanoid intermediates during PMN and epithelia interactions. Exposure epithelia to cytokines (i.e., IL-13, IL-4) induces the expression of 15-LO. In subsequent interactions with adjacent leukocytes, the 5-LO-derived intermediate LTA_4 is transformed by LTA_4 hydrolase and 15-LO of epithelial cells to LTB_4 and the novel product LXA_4, 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 LXA_4 and LXB_4.

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 μ*M*), a nonspecific inhibitor of COX (*see* **Note 13**); and/or 17-octadecynoic acid (500 μ*M*), 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 5 min at 37°C.
d. Expose epithelial cells to C20:4 (20 μ*M*, 1 min) (*see* **Note 14**).
e. Add PMN (~80×10^6 cells, 0.8 mL) to monolayers of epithelial cells (approx 10×10^6 cells/75 cm^2 flask). After addition, stimulate cells with A23187 (5 μ*M*; activates both cell types) and coincubate the cells without mixing for 30 min (see **Note 9**).

f. Terminate coincubations by the addition of 2 vol 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 24–48 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)***

This section describes transcellular biosynthesis during leukocyte-leukocyte interactions and provides an 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 (*see* **Fig. 6**).

1. Day 1: Expose monocytes to LPS (1 μg/mL) to induce COX II or, for nonaspirin scenarios, expose cells to media alone (*see* **Note 11**) for 16 h 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 ***(25,27)***.
2. Day 2:
   a. Isolate fresh PMN (typically 3 h post venipuncture taking cell isolation into consideration; *see* **Subheading 3.1.1.**). Adjust the cell number to 120×10^6 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 ***(28)*** (*see* **Subheading 3.1.3.1.** and **Table 1** for optional treatment of PMN, cytokines and inhibitors).
   b. After 16 h, pellet the nonadherent monocytes. Remove the media and resuspend the monocytes at 20×10^6 cells/mL in $DPBS^+$.
   c. Expose monocytes to either aspirin (300 μ*M*) (*see* **Note 13**) or vehicle (ethanol) alone for 20 min.
   d. Pellet monocytes at 1000 rpm (228*g*, 10 min), remove buffer, and resuspend cells at 20×10^6 monocytes/mL.
   e. Coincubate the cells with freshly isolated PMN by combining 1 mL of the PMN (120×10^6 cells) and 1.0 mL of the monocyte (20×10^6 cells) suspension.
   f. Equilibrate the cells for 5 min at 37°C.
   g. Addition of agonist: Coactivate the cell suspension by adding 20 μ*M* of C20:4 and 5 μ*M* of calcium ionophore (activates both cell types) (*see* **Notes 4** and **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 vol of cold MeOH (–20°C) containing appropriate standards (*see* **Note 10**).
i. Store samples (–20 to –80°C) for extraction and workup (*see* **Note 5** for extraction). Samples in general should be extracted within 24–48 h, even though eicosanoids can be stored for a longer period of time in the methanol stop solution.

4. Notes

1. Eicosanoid stock should be stored considering manufacturer's instructions (usually –20 to –80°C). Most bioactive eicosanoids 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. This is especially relevant when evaluating isoprostanes (*see* Chapter 1). 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 eicosanoid stock is used, pass a gentle stream of nitrogen into the vial before closing. This will slow the oxidative degradation of eicosanoids. Determine the concentration of lipoxin, leukotriene, and some 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 LTA_4 (*see* **Fig. 4B**) 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 purity and, for example, percent isomerization of an eicosanoid (*see* Chapter 5).
2. 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.
3. The total amount of vehicle (ethanol or dimethyl sulfoxide) added to your cell incubation or coincubations should not exceed 0.1%. Higher concentrations of alcohol or dimethyl sulfoxide can affect cell integrity (i.e., enzyme activity and membrane integrity). It is therefore desirable to prepare stocks of stimuli or inhibitors at 1000–5000× the final concentration so that only small aliquots are added directly to cell suspensions.
4. 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 stimulate individual cells and cell coincubations with specific ligands that bear functional surface membrane receptors in these cells (*see* **Table 2**).
5. Extraction protocol optimized for lipoxins and aspirin-triggered lipoxins ***(23,29)***: Place the incubation suspension at –20°C for at least 20 min (protein precipita-

tion). Centrifuge the incubation suspension at 2000 rpm (800*g*) for 20 min at 4°C. Collect the supernatant in a 50-mL tube and dilute the sample with at least 5 vol of pure water. Rapidly adjust the pH of this aqueous suspension to pH 3.5 with HCl (1 *N*). 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 (e.g., LTC_4, LTD_4, LTE_4) 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 (i.e., 100 μL). For further consideration of eicosanoid extraction, *see* Chapter 4.

6. The pivotal leukotriene and lipoxin intermediate in platelets (i.e., 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. 1A**). Stability of LTA_4, and therefore transcellular biosynthesis, is greatly enhanced by albumin or other stabilizing agents, such as liposomes, which serve as a carrier for this lipid and other eicosanoids.
7. 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, whereas platelet pools would be labeled with [^{3}H]-C20:4. This dual labeling permits the identification of the cellular source of eicosanoids in a cell–cell interaction profile.
8. The ratio and amounts of platelet-derived LTC_4 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 LTA_4-derived transcellular product, and at a ratio of 1:100 approach PMN-generated LTB_4 levels. To obtain significant amounts of LTC_4, ratios of 1:100 (PMN:platelets) are indicated.
9. 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 ***(30)*** have demonstrated that inhibition of P-selectin and integrin (CD11/CD18)-mediated adhesion during PMN and endothelia interactions greatly inhibits transcellular eicosanoid biosynthesis.
10. 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. PGB_2 usually meets those criteria for analysis of lipoxins and leukotrienes, whereas 13-HODE is used when analyzing mono-HETEs, and 19-OH-PGB_2 is suitable for analysis of omega or beta oxidation products. Depending on the sensitivity of your analytical assay, 50–200 ng (*see* **Fig. 1B**) are added to the incubations.
11. 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 ***(10,16)***.

12. PMN should be used immediately after isolation (i.e., ~3 h 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 ***(31)***, 5S,15S-diHETE and lipoxins, which are normally associated with transcellular biosynthesis, are generated endogenously from a single cell type (PMN).
13. Aspirin hydrolyses rapidly in an aqueous environment. Therefore, prepare aspirin stocks in ethanol 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.
14. Cyclooxygenase and lipoxygenase 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 eicosanoid 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.

5. Summary

Models for in vivo scenarios of transcellular biosynthesis provide invaluable information about the regulation of eicosanoid biosynthesis that is likely to occur during multicellular events 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. 3**) and advance the emerging area of transcellular biosynthesis of bioactive lipid mediators.

Acknowledgments

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12

Assessment of Cellular Localization of the Thromboxane A_2 Receptor by Immunocytochemistry

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1. Introduction

1.1. Overview of Immunocytochemistry

Transmembrane receptors play a crucial role in communication between cells. By coupling to intracellular proteins and second-messenger systems, these molecular entities allow for the transduction of a biochemical message across the cell membrane. Early investigations of receptor-mediated signaling pathways in nervous system tissue were originally limited to staining techniques for neurotransmitters, retro- and orthograde transport of labeled substances or enzymes, and identification of cells by specific stains (e.g., silver impregnation, Nissl stain, and so on). The introduction of immmunocytochemistry in brain research led to the discovery of numerous neurochemical pathways ***(1)***. In this regard, immunocytochemistry offers laboratories the ability to visualize receptors at a tissue, cellular, and subcellular resolution, thereby providing a powerful tool for understanding the nature and organization of receptor-based signaling mechanisms. Readily available immunological and biochemical probes for markers of cellular differentiation, cytoskeletal proteins, and signal transduction cascade components extend these techniques, allowing for an assessment of the dynamics of receptor function. Furthermore, improvements in commercial monoclonal (MAb) and polyclonal (PAb) antibodies, and the widespread use of extremely sensitive multistep developing kits have considerably simplified immunocytochemical staining procedures. Finally, advances in tissue culture technology have allowed for the isolation

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and manipulation of homogenous populations of nervous system cells as well as immortalized cell lines. Taken together, these important technical advances have led to a dramatic refinement in the procedures used for receptor localization and characterization. When preparing to undertake an immunocytochemical approach to receptor localization, it is important to consider the following four general principles and their attendant variables.

1.2. General Principles of Immunocytochemistry

1.2.1. Type of Antibody

Choice of either monoclonal or polyclonal primary antibody is an extremely important aspect of this experimental approach. Whereas monoclonal antibodies, because of their high specificity and low background staining, are often preferred in immunocytochemical studies, polyclonal antibodies generally give high signal strength. This characteristic derives from the fact that PAbs contain multiple antibodies directed against different epitopes of the target protein. On the other hand, these antibodies frequently produce higher background staining. Normally, this nonspecific immunoreactivity can be reduced by preparation of the IgG fraction and subsequent immunoaffinity purification of the target antibodies. A separate consideration is that although different antibody purification procedures can result in a higher specific activity, they can also lead to a substantial loss of total antibody protein. Consequently, there are no fixed rules regarding the degree of specific antibody activity which should be achieved. In general, if a laboratory is beginning immunocytochemical experiments with a newly developed MAb or PAb, it is advisable to experiment with crude antibody fractions (e.g., serum, ascites, or supernatant) before embarking on a purification scheme, in order to observe changes in the labeling pattern as the antibody preparation is refined.

1.2.2. Direct vs Indirect Labeling

It is possible to perform immunocytochemical experiments using a single antibody step in which the primary antibody itself is labeled with a detectable marker. Direct labeling has become more accessible to investigators with the widespread use of MAb technology. Given a potentially unlimited supply of high-specificity antibody, it is possible for a laboratory to prepare large quantities of immunoglobulin probes coupled to enzymes, fluorescent markers (e.g., fluoroscein-5-isothiocyanate [FITC]) or to biotin. The ability to biotinylate MAbs further expands the range of possible markers, given the wide availability of avidin-enzyme (e.g., horseradish peroxidase [HRP], alkaline phosphatase [AP]) or avidin-fluorochrome (e.g., Cy3, FITC, TRITC, R-Phycoerythrin) conjugates. Furthermore, direct labeling of primary antibodies allows for double- and triple-stainings, enabling investigators to colocalize different receptors, or

identify a specific receptor-bearing cell type in a heterogeneous cell culture or tissue slice. Direct labeling using MAbs offers a high ratio of specific to nonspecific signal, and requires only a single antibody incubation step. On the other hand, direct labeling sacrifices the amplification that is possible with indirect techniques, so the overall signal may be lower, especially in specimens with a relatively scarce target antigen. In addition, covalent modification of the antibody during the labeling procedure may affect the ability of the antibody to recognize its target epitope.

Indirect labeling relies on unmodified primary antibodies that are bound, in subsequent steps, to labeled secondary antibodies. The labeled secondary antibodies provide for both amplification, due to their ability to bind multiple epitopes on the primary antibody, and detection, via their label (i.e., fluorochrome, enzyme conjugate, biotin, or gold). Indirect detection systems commonly rely on antibodies prepared against the IgG from the animal species used to produce the primary antibody. In the case of the thromboxane A_2 (TXA_2) receptor antibodies (*see* **Subheading 1.3.**), all were produced in rabbit, and therefore rely on goat-antirabbit (GAR) IgG antibodies for facilitating visualization. The use of indirect labeling allows for a wide range of possible detection methods, as well as additional amplification of the immunoreactivity when employing a biotinylated secondary antibody.

The main advantage of indirect labeling lies in the ability to detect very low concentrations of cellular or tissue antigens because of the multiple secondary antibodies that bind to each bound primary antibody molecule. In addition, covalent modification of the primary antibody is not necessary, thus preserving activity. A wide range of secondary antibodies, produced against numerous animal species and coupled to a variety of markers, are commercially available and relatively inexpensive. Also, several avidin–enzyme or avidin–fluorochrome conjugates are available for enhanced labeling. It should be noted, however, that the use of a secondary antibody has the potential for increasing nonspecific labeling compared to direct labeling techniques.

1.2.3. Fluorescence vs Chromogenic Labeling

Immunofluorescence is a widely utilized technique because of its enormous flexibility and for its ability to label both living and fixed cells. The relatively recent explosion of new fluorescent probes with distinct emission spectra offers investigators tools for labeling of single or multiple targets with a high degree of sensitivity and spatial resolution. In addition, fluorescence labeling allows the use of laser confocal microscopy technology for enhanced visualization at the subcellular level. It should be noted that fluorescence microscopy requires specialized equipment, and specimens are vulnerable to photobleaching and degradation of signal during prolonged storage.

In contrast, the deposition of a colored substrate following labeling with an antibody–enzyme conjugate permits high sensitivity staining that is stable for long periods of time. In addition, enzyme labeling techniques have been well defined and are widely applicable to both cell culture specimens and intact tissues. Finally, enzymatic detection of immunoreactivity only requires access to a light microscope, making it accessible to broader range of laboratories. The examples provided in this review utilize both types of labeling techniques, allowing the reader to choose the system most appropriate for his or her needs.

1.2.4. Cultured Cells vs Intact Tissue

The decision to use an intact tissue or a cell culture preparation depends on the specific goal of the experiment and the degree of resolution that the investigator requires. Immunocytochemical analysis of intact tissue provides data on the macro- and microscopic localization of a particular antigen, allowing for a more global appreciation the distribution and potential function of the target. The use of cultured cells allows for labeling to be correlated with specific aspects of cellular morphology, as as well allowing for subcellular localization, especially when employing laser confocal microsopy. In this respect, our investigation of thromboxane A_2 receptors in the CNS began with immunolabeling of intact rat brain and spinal cord preparations and progressed to labeling of cultured oligodendrocytes and human oligodendroglioma cells in an attempt to identify the specific cellular component of white matter that expressed the TXA_2 receptor (*see* **Subheading 4.**).

Immunolabeling of intact tissues is a widely used procedure that has been well documented in a variety of publications. Two basic approaches to tissue preparation are commonly employed: The whole animal or an intact organ is perfused with a fixative, and the desired tissues are cryoprotected, frozen, and sectioned, or the tissue is surgically removed, frozen, and sectioned, but remains unfixed. The former method preserves tissue morphology and allows for long-term storage of specimens, whereas the latter is superior for the preservation of antigenic epitopes. In either case, tissue sections (5–20 µm in thickness) may be stained as "free-floating sections" in solution or attached to microscope slides prior to immunostaining. The protocols outlined below are easily adapted for use with tissue sections.

1.3. Immunocytochemical Localization of the Thromboxane A_2 Receptor in the CNS

Numerous eicosanoid receptors have been localized in central nervous system (CNS) tissues and cell types, including members of the EP, FP, and TP families *(2)*. Evidence pointing to the existence of thromboxane A_2 receptors

in CNS components was first provided by radioligand binding experiments using cultured rabbit astrocytes and human astrocytoma cells ***(3)***. However, the use of radioligands for specific localization of receptor sites within the CNS has historically been hindered by a high degree of nonspecific labeling. This technical limitation stems from the difficulty in using highly lipophilic thromboxane ligands in a lipid rich tissue, such as the CNS. Based on these considerations, separate approaches have been taken for the localization and study of TXA_2 in the CNS. Thus, the use of affinity chromatography procedures ***(4)*** and the development of anti receptor antibodies ***(5)*** have allowed for the localization of thromboxane receptors in intact adult rat brain and spinal cord tissue (**Fig. 1**). These antireceptor antibodies have also recently been used to identify TXA_2 receptors in glomeruli and renal tubules ***(6)***, as well as in oligodendrocytes, the myelinating cell of the CNS ***(7)***.

Currently, only polyclonal antibodies are available for immunodetection of the TXA_2 receptor. These have been produced by injecting rabbits with either purified human platelet TXA_2 receptor protein (for the production of TxAb) ***(5)*** or with KLH-conjugated decapeptides derived from the predicted sequence of the cloned thromboxane A_2 receptor ***(8)***. Two such peptide sequences are [C]-AVLRRLQPRL (residues 314–323) and [C]-HAALFEWHAV (residues 89–98) ***(9)***. These specific peptides were chosen based on their degree of antigenicity and hydrophobicity as determined by a computer-assisted sequence analysis program (Sequaid, Kansas State University) and were used to generate two antipeptide antibodies, i.e., P_1Ab and P_2Ab, respectively. Regarding the purification and characterization of these TXA_2 receptor antibodies, the IgG fraction of each antibody was first purified using DEAE Affi-Gel Blue chromatography (Bio-Rad Laboratories, Hercules, CA). In the case of the antipeptide antibodies (P_1Ab and P_2Ab), the IgG was then subjected to further purification using immobilized cognate peptide coupled to Affi-Gel 501 organomercurial agarose (Bio-Rad Laboratories) ***(9)***. Finally, all three antibodies were tested for specific immunoreactivity by immunoblot and immunocytochemical analysis.

Numerous protocols for receptor immunolocalization have evolved since each researcher modifies methodologies for their specific use at the light, laser confocal, and electron microscopic level. In addition, high-quality reviews and book chapters detailing immunocytochemical procedures are widely available ***(10–13)***. The present chapter compiles the protocols that are currently used in our laboratory and that have consistently yielded positive results. This discussion will therefore be limited to receptor localization using light and fluorescence microscopy, monolayer cell cultures and antithromboxane A_2 receptor antibodies. Nevertheless, the following methods should suit most applications and be compatible with the majority of primary antibodies.

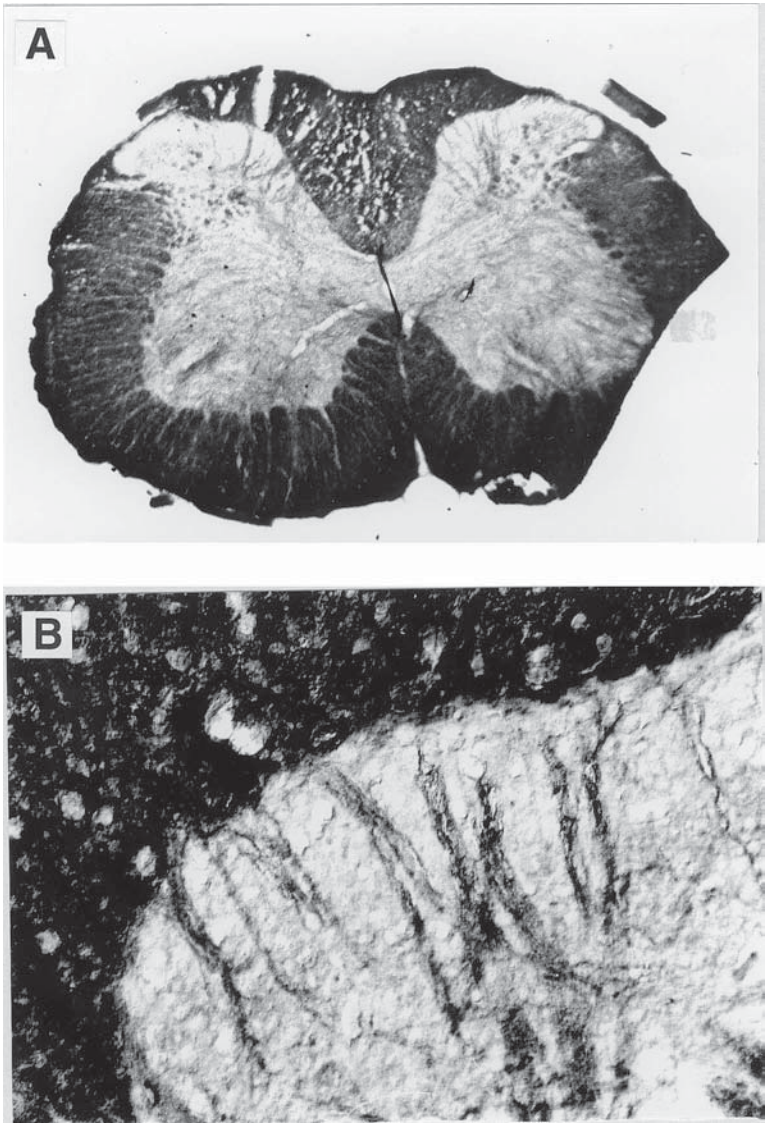

Fig. 1. Immunohistochemistry of adult rat brain spinal cord. **(A,B)** Labeling pattern of TxAb (1:100) in an intact spinal section from an adult Sprague-Dawley rat. Cryostat sections (40 μm) were incubated with primary antibody overnight at 4°C and developed using an HRP-substrate (B) Higher magnification view of the white/gray matter border, demonstrating positive immunostaining of distinct white matter tracts entering the gray matter.

2. Materials

Please note that where possible, Sigma (St. Louis, MO) catalog numbers are given, however other suppliers are available for many of these reagents.

1. Human oligodendroglioma cells (HOG) ***(14)*** and primary culture neonatal rat brain oligodendrocytes ***(15)***, cultured under standard adherent conditions on glass cover slips (No. 1 thickness—0.13–0.16 mm, Fisher Scientific, Cat. #12–545–80, 12 mm in diameter) coated with poly-L-lysine (Sigma, P-9155) in 24-well tissue culture plates (*see* **Note 1**).
2. Phosphate-buffered saline (PBS): 140 m*M* NaCl, 3 m*M* KCl, 8 m*M* Na_2HPO_4, 2 m*M* NaH_2PO_4, pH 7.4. Tris-buffered saline (TBS) may also be used: 25 m*M* Tris-HCl, 140 m*M* NaCl, 3 m*M* KCl, pH 7.4 (*see* **Note 2**).
3. Permeabilization solution: 0.2% Triton X-100 (Sigma, T-9284) in PBS.
4. Fixative solution: 4% paraformaldehyde (Sigma, P-6148) in PBS (*see* **Note 3**).
5. Blocking solution: 10% normal goat serum (Sigma, G-9023), 0.1% Tween-20 (Sigma, P-7949) in PBS.
6. PBS + T: PBS + 0.1% Tween-20.
7. PBS + T/NS: PBS + 0.1% Tween-20 + 1% normal goat serum.
8. Primary antibody for thromboxane A_2 receptor (TxAb or P_2Ab) diluted in PBS+T/NS.
9. Primary antibodies for oligodendrocyte markers: antimyelin basic protein (anti-MBP, Cat. #AB-908, Chemicon, Temecula, CA) or antigalactocerebroside (anti-GalCer, Sigma, G-9152) diluted in PBS + T/NS.
10. Secondary antibody: goat-antirabbit IgG (H + L) conjugated to either FITC (Sigma, F-9887) or biotin (Sigma, B-8895) diluted in PBS + T, preabsorbed to rat brain acetone powder (Sigma, B-4257) (*see* **Note 4**).
11. Chromogenic substrate: diaminobenzidine tetrahydrochloride (Sigma, D-5637), hydrogen peroxide, and nickel chloride (Sigma, N-5786) or cobalt chloride (Sigma, C-2644).
12. Mounting medium for either fluorescence (e.g., FluoroGuard [Cat. #170–3140 Bio-Rad Laboratories, Hercules, CA]; Mowiol 4–88 [Cat. #475904, Calbiochem, San Diego, CA]) or chromogenic labeling (e.g., Permount® Cat. #SP15–500, Fisher Scientific, Fair Lawn, NJ) (*see* **Note 6**).

3. Methods

3.1. Preparation of Cells and Coverslips (see Note 1)

1. 12-mm coverslips are sterilized by autoclaving and placed in a sterile 24-well culture plate for pretreatment and seeding with cells.
2. Coverslips are pretreated with 0.01% poly-L-lysine for 2–24 h and then washed twice with sterile PBS and allowed to air dry.
3. Cells are seeded onto coverslips and grown in the appropriate medium to subconfluent density.
4. Growth medium is removed by aspiration and the cells are washed twice for 5 min each with PBS (room temperature).

3.2. Fixation, Permeabilization, and Blocking (see Notes 2 and 3)

1. Fixative solution (500 µL of 4% PFA or 1% GA) is added to the cells and incubated at room temperature for 10–15 min. Glutaraldehyde fixation should be performed in a well-ventilated fume hood.
2. Cells are then washed twice for 5 min each with PBS.
3. Permeabilization, if desired, is performed by incubating fixed cells with 0.2% Triton X-100 in PBS for 2 min at room temperature. Some antigens will not be exposed without longer (up to 15 min) exposures to Triton.
4. Cells are washed four times over 5 min with PBS + T.
5. If endogenous peroxidase activity is to be blocked, cells are incubated with 0.3% H_2O_2 for 10–20 min at room temperature. Following this incubation, it is critical that the coverslips are thoroughly washed with PBS + T because failure to do so may completely abolish the immunostaining. Note that this step is not necessary if immunofluorescence is to be utilized.
6. Cells are incubated with blocking solution for 30–60 min at room temperature.

3.3. Primary Antibody Incubation

Blocking solution is removed by aspiration and primary antireceptor antibody solution (300–400 µL for 24-well plates) is added without rinsing the coverslips. Incubation can be accomplished in two hours at room temperature, or can proceed overnight at 4°C. Coverslips should be left in the tissue culture plate for this step, and the edges of the plate wrapped in Parafilm if incubation is to proceed for more than 4 h. If the volume of antibody solution is limited, coverslips can be incubated in volumes as low as 50–100 µL (*see* **Note 4**). Depending on whether receptor is to be visualized by fluorescence or by the deposition of a chromogenic substrate, refer to **Subheading 3.4.1.** or **3.4.2.**

3.4. Secondary Antibody Incubation (see Note 4)

3.4.1. Immunofluorescence

1. Incubation with the primary antibody is terminated by removing the antibody solution (*see* **Note 8**) and washing the specimen three times with PBS + T for 5 min each at room temperature.
2. Cells are incubated with the appropriate, species-specific, secondary antibody, diluted in PBS + T to a concentration of 1:50–1:150. Incubation is allowed to proceed for 1 h at room temperature. The secondary antibody should be conjugated to FITC (or another fluorescent marker) it is strongly recommended that it be preabsorbed with rat brain acetone powder (*see* **Note 4**).
3. Unbound secondary antibody is removed, followed by three washes of 5 min each with PBS + T and one wash with PBS.
4. Following the final wash, coverslips are mounted (*see* **Subheading 3.5.** and **Note 6**). It is important that the cover slip not be allowed to dry during this process.
5. Cells are observed with a conventional epifluorescence microscope. Photomicrographs are taken using Kodak Tri-X PAN 400 ASA film (*see* **Note 7**). Phase-

contrast photomicrographs of immunofluorescently labeled cells are helpful in establishing localization of fluorescent signal within the cellular architecture. These specimens are suitable for analysis using a laser confocal microscope, which allows for a higher spatial resolution of receptor localization.

3.4.2. Chromogenic Detection

1. Incubation with the primary antibody is terminated by removing the antibody solution (*see* **Note 8**) and washing the specimen three times with PBS+T for 5 min each at room temperature.
2. Cells are incubated with the appropriate, species-specific, secondary antibody, diluted in PBS + T buffer to a concentration of 1:100–1:400. Incubation is allowed to proceed for 1 h at room temperature. The secondary antibody may be preconjugated to biotin, which allows for an additional amplification step involving avidin- or streptavidin-conjugated HRP (*see* **step 3**). Nonspecific background labeling is eliminated by preabsorbing the secondary antibody with rat brain acetone powder (*see* **Note 4**). Unbound secondary antibody is removed by washing two times for 5 min each with PBS + T, followed by one wash with PBS, at room temperature.
3. If a biotinylated secondary antibody has been employed, an avidin or streptavidin conjugate (e.g., Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) is used to couple the detection reagent to the bound secondary antibody. The reagent should be prepared 30 min in advance according to the manufacturer's instructions. In this example, the ABC Kit is prepared in PBS + T and applied to the specimen for 30 min at room temperature. Following this incubation, specimens are washed two times for 5 min each with PBS + T, followed by one wash with PBS.
4. Enzymatic activity is developed using 0.5 mg/mL diaminobenzidine tetrahydrochloride (DAB) in 0.03% H_2O_2 (final concentration) in PBS and $NiCl_2$ or $CoCl_2$ (0.04% final concentration) is added as an enhancing agent. Immunoreactivity is allowed to develop until a brown/black color appears in the specimen (2–20 min). The reaction is terminated by rinsing the specimen in PBS followed by distilled water.

3.5. Mounting

3.5.1. Immunofluorescence

Following the final rinse, cells labeled by immunofluoresence should be mounted in a medium (*see* **Note 6**) that will protect the specimen against photobleaching during the prolonged exposures required for visualization and photography ***(16)***. Place a small drop of mounting medium on a clean optical-quality glass microscope slide and, using a fine forceps, gently mount the coverslip on the slide, avoiding the presence of any trapped air bubbles. As this is not a permanent mount, coverslips can be secured to the slide with small drops of rubber cement or nail polish. Slides should be stored in the dark at 4°C. The slide can be observed with a nonimmersion lens within an hour or so, but should be left to "set" overnight before use with oil immersion lenses.

3.5.2. Chromogenic

Labeling visualized by the deposition of a chromogenic substrate should be dehydrated through ethanol and xylene following the final distilled water rinse. Soak the coverslips twice (for 10 s each) in the following: 70, 95, and 100% ethanol, followed by xylene. Allow the coverslips to dry and mount using a permanent mounting medium (e.g., Permount). Cells can be visualized by light microscopy within 1 h, but should be allowed to dry overnight for best results. Slides should be stored in the dark, preferably at –20°C.

3.6. Results and Interpretation

3.6.1. Results of Ommunocytochemistry Using Cultured Oligodendrocytes and Human Oligodendroglioma Cells

Our initial experiments involved immunostaining of intact brain and spinal cord using the P_2Ab, which is directed against the first extracellular loop of the TXA_2 receptor. As can be seen in **Fig. 1**, the white matter of the spinal cord is predominantly labeled following immunostaining. At a higher magnification, it is possible to see labeled tracts penetrating the gray matter of the spinal cord. Similar patterns of staining limited to white matter tracts were also observed in the striatum, internal capsule, and optic chiasm ***(9)***.

Based on these observations, we hypothesized that the positive immunoreactivity was caused by the presence of TXA_2 receptors in some aspect of the myelin sheath. This notion was validated, to a limited extent, by the presence of a 55 kDa immunoreactive protein in a purified myelin preparation subjected to immunoblot analysis with TxAb ***(9)***. In order to further investigate these findings, neonatal rat brain oligodendrocytes were cultured and immunocytochemical analysis was performed. Prior to immunostaining with an antithromboxane receptor antibody, cell lines were characterized by labeling them using an antibody directed against a myelin protein (e.g., anti-MBP) or oligodendrocyte-specific marker (e.g., antigalactocerebroside) (**Fig. 2A,B**).

As can be seen in **Fig. 3A**, staining of permeabilized oligodendrocytes with TxAb results in labeling of both the cell bodies and the numerous cytoplasmic processes. Of particular interest, there appears to be a continuous ring of positive immunoreactivity surrounding the cell body as opposed to the interrupted, or puntate labeling pattern seen on the processes. The HOG cell line, which was derived from a surgically removed human oligodendroglioma, has been shown to express at least two oligodendrocyte-specific proteins (i.e., a 15 kDa form of myelin basic protein and 2'3'-cyclic nucleotide phosphodiesterase) ***(14)***. As demonstrated in **Fig. 3B**, these cells also express the TXA_2 receptor. The labeling pattern, however, is dominated by punctate staining, with an increased density of immunoreactivity localized in the nonnuclear regions of the cells.

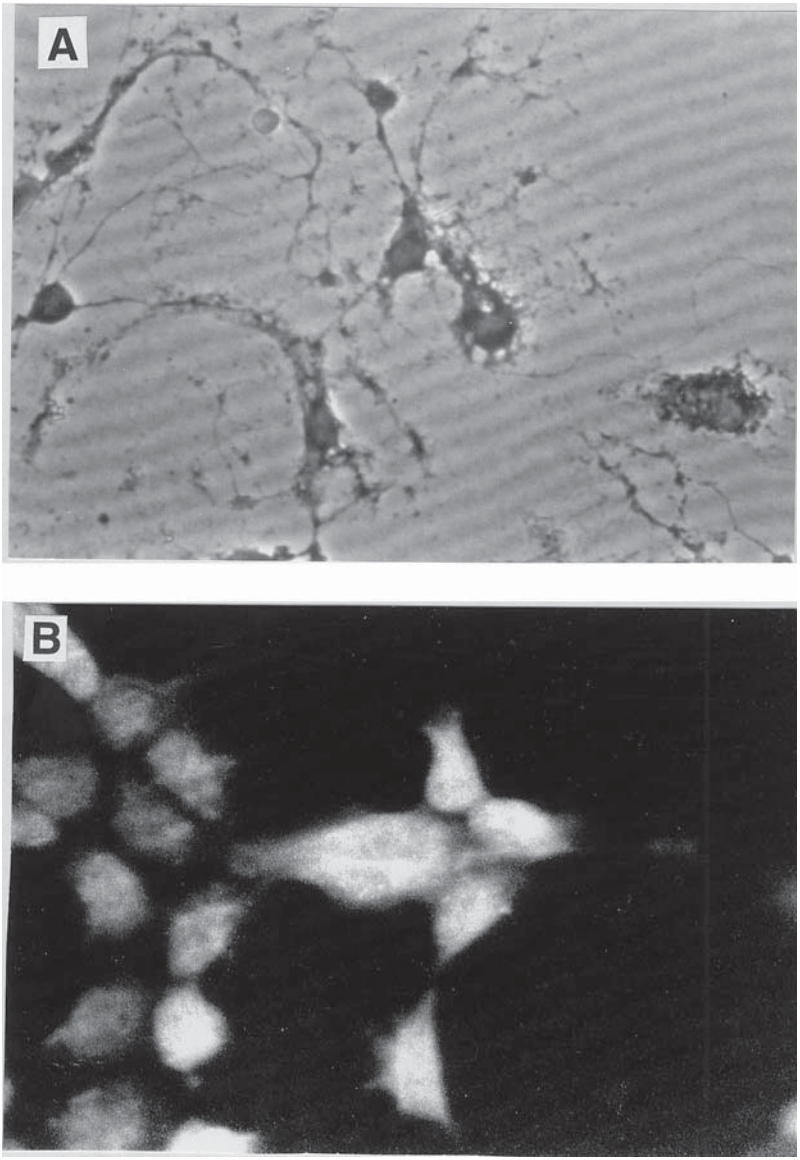

Fig. 2. Immunocytochemical characterization of oligodendrocytes and HOG cells. **(A)** Neonatal rat brain oligodendrocytes were isolated and labeled with an antimyelin basic protein IgG (anti-MBP, 1:50). Immunoreactivity was visualized using an HRP-conjugated goat-antirabbit IgG and metal-enhanced DAB as the enzyme substrate (×400 magnification). **(B)** Human oligodendroglioma (HOG) cells were immunofluorescently labeled with an anti-galcer IgG (1:100). Labeling was visualized using a FITC-conjugated goat-antirabbit IgG (×400 magnification). Reprinted with permission from **ref.** *7*.

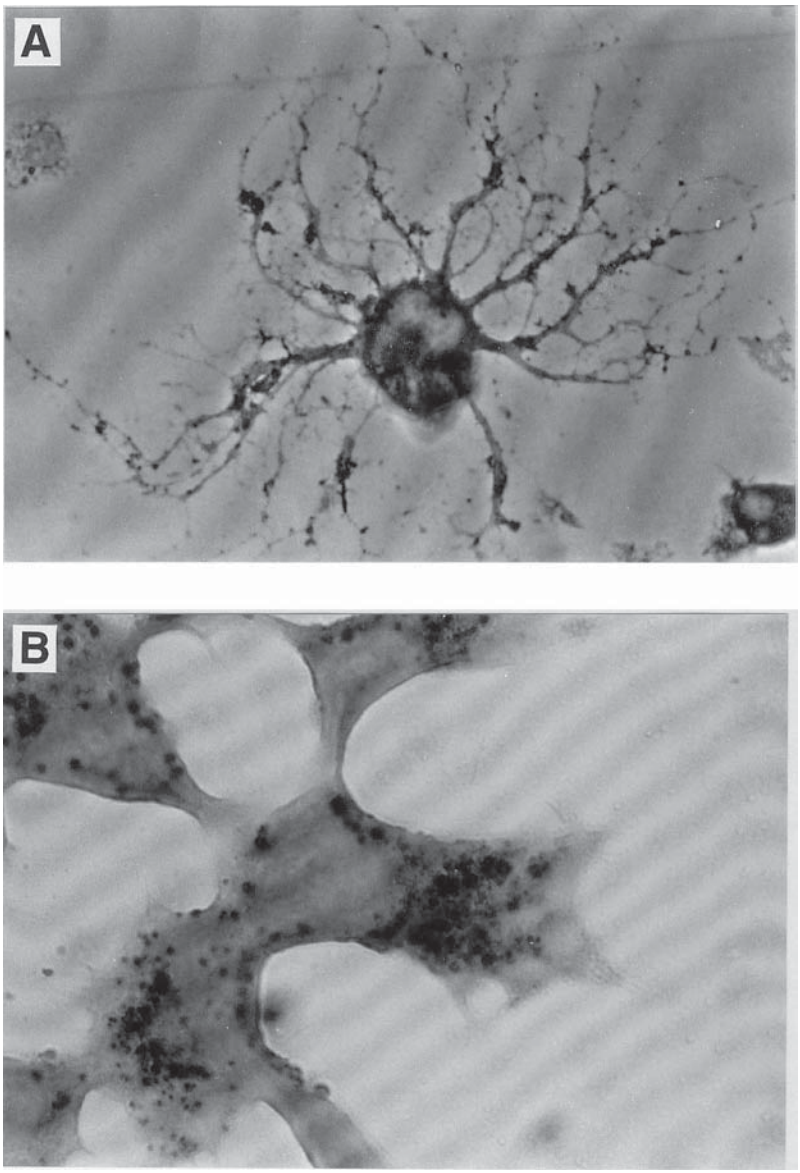

Fig. 3. Immunocytochemical demonstration of TXA_2 receptors on oligodendrocytes and HOG cells. **(A)** Neonatal rat brain oligodendrocytes were fixed, permeabilized, and immunocytochemically labeled with TxAb (1:100). Metal-enhanced DAB was used as the chromogen. Distinct staining can be seen on the cell body as well as in the cytoplasmic processes (×1000 magnification). **(B)** Human oligodendroglioma (HOG) cells were similarly stained with TxAb (1:100) and developed with metal-enhanced DAB (×1000) magnification).

These findings, therefore, strongly supported our hypothesis that TXA_2 receptors are present in the myelin sheath, in that oligodendrocytes are the sole myelinating cells of the CNS *(7)*.

3.6.2. Interpretation of Results

Interpreting the results of immunocytochemical experiments requires a thorough knowledge of both the procedures used, and the inherent limitations of this technique. The ability to state, with a high degree of certainty, that a receptor or other biochemical entity is localized to a particular cell or tissue depends on several factors: utilization of a rational and thorough series of control experiments, characterization of the cell or tissue system using unique antigens or markers of cellular differentiation, and a demonstration of antibody specificity. In addition, it is possible to gain addition insight into the nature of the antigen in question by observing the immunostaining pattern in permeabilized and nonpermeabilized systems. Finally, validation of the results by biochemical techniques (e.g., immunoblotting) is also recommended.

3.6.2.1. Controls

It is important to perform the appropriate controls with each immunocytochemical experiment, especially when attempting to characterize a novel antigen, or when working with a new antibody preparation. An important control when using a polyclonal serum is the preimmune serum from the same animal used for antibody production. If this control is not available, pooled normal serum from the same species may be used, provided that it is matched to the same concentration and dilution as the primary antibody being utilized. Similiarly, if a purified IgG is being employed, a preimmune IgG should be used as the control. When using an MAb, it is possible to use hybridoma supernatants from control cell lines, or ascites fluid from animals immunized with control or unrelated hybridomas. It is also possible to use commercially available preimmune purified antibody fractions, matched to the correct Ig subtype.

In order to determine the extent of non-specific staining by the secondary antibody, when performing indirect labeling studies, one should substitute an appropriately diluted protein control (e.g., BSA or nonfat dry milk at the same protein concentration as the diluted antibody) in place of the primary antibody. In addition, it is also important to determine the extent to which endogenous enzyme activity increases background staining when using a peroxidase- or alkaline phosphatase-based system. This is accomplished by substituting a protein control for both the primary and secondary antibody. Tissues rich in biotin may show increased nonspecific labeling when using a biotinylated secondary antibody with an avidin-label conjugate for signal amplification. In this case, one should perform a control experiment in the absence of the avidin conjugate.

Finally, when examining the expression of an antigen in a novel location, it is often useful to perform duplicate experiments on cells or tissue systems known to both express (positive control) and not express (negative control) the antigen of interest. With regard to the thromboxane A_2 receptor, all of the antibodies employed were previously tested against human blood platelets as a positive control, and against Swiss 3T3 fibroblasts as a negative control (data not shown).

3.6.2.2. Determination of Cell Surface Localization vs Intracellular Localization

Permeabilization of the cell with an agent, such as Triton X-100, saponin, or lysophosphatidylcholine (LPC), can aid investigators in determining the degree to which the antigen of interest is localized to the plasma membrane. As can be seen in **Fig. 4C,D**, treatment of HOG cells with 0.2% Triton X-100 prior to incubation with the primary antibody leads to a diffuse labeling pattern across the plasma membrane, versus nonpermeabilized cells. Because TxAb is a polyclonal antibody raised against the entire native receptor protein, this increased labeling is most likely caused by the availability of intracellular epitopes. Highly specific MAbs raised against intracellular epitopes should require membrane permeabilization in order to demonstrate immunoreactivity.

3.6.2.3. Determination of Antibody

Another valuable control experiment is available when utilizing antibodies raised against synthetic peptide antigens. Preabsorbing the antibody with the cognate peptide should abrogate specific immunoreactivity. This experiment can be performed using crude serum, an IgG fraction, or an affinity-purified preparation. Briefly, a concentrated stock solution of the synthetic peptide should be prepared in a biologically compatible solvent. Diluted antibody preparations are then incubated with the peptide at a final concentration of 0.1–1.0 m*M*, overnight at 4°C with gentle mixing. It is important to prepare a parallel antibody solution in the same manner, without the addition of peptide, as a control. As can be seen in **Fig. 4**, P_2Ab labeling of HOG cells (**Fig. 4A**) is almost completely abolished when the antibody is preabsorbed with the synthetic peptide (**Fig. 4B**). It should be noted that this particular control may not work in all cases, owing to differences between the native antigen and the conformation of the synthetic peptide antigen in solution.

3.6.3. Limitations of Immunocytochemistry and Validation by Protein Biochemistry Techniques

Although immunocytochemistry is an extremely valuable technique for localizing cellular and tissue antigens, it is limited by the number of technical

considerations, the ability of the antigen to withstand fixation and sample preparation, and possible differences in immunoreactivity between native antigen *in situ* and the denatured antigen or synthetic peptide used for antibody preparation. Furthermore, immunocytochemical techniques cannot provide accurate quantitative data, or information as to the degree of activity of the immunological target. For these reasons, it is important to validate experimental results using protein biochemistry techniques, such as SDS-PAGE and immunoblotting of proteins from the cell or tissue sample. This allows the investigator to determine the molecular mass of the labeled antigen, as well as providing an additional mechanism for verifying antibody activity. When examining cell surface receptors, radioligand binding allows for quantitation of expressed active receptor, and determination of binding kinetics.

4. Notes

1. Culture conditions and coverslips:
   a. Culture conditions: We find that plating cells on 12-mm round glass coverslips held in a 24-well plate allows for a large number of samples to be prepared simultaneously. In addition, incubation volumes are relatively small, providing for the conservation of primary and secondary antibody solutions. Larger coverslips (15–20 mm) can be easily accommodated by 6- or 12-well plates. This technique, in addition to being convenient, allows investigators to easily prepare simultaneous controls using cells of the same passage, plated at the same density. In addition, using this system it is possible to experimentally manipulate cellular function within a homogenous culture of cells subjected to the same environmental and growth conditions, thus extending the power of immunocytochemical investigations.
   b. Coverslips: Glass coverslips are resistant to all organic solvents and exhibit low autofluorescence, making them ideal for both immunofluorescence and chromogenic detection. Autoclaving of coverslips in a small glass beaker is convenient. Because of the delicate nature of glass coverslips, they should be handled with a sterile fine-tipped forceps, taking care to apply minimal pressure only to the edge of the coverslip. Pretreatment of glass surfaces with attachment factors, such as poly-L-lysine, facilitates cell adhesion and enhances plating efficiency, especially in low serum concentrations. Poly-L-lysine should be prepared at a concentration of 100 mg/L (0.01%) in distilled water and sterilized by filtration. Add 0.5 mL of poly-L-lysine to each of the wells with coverslips. Remove the poly-L-lysine solution after 2–24 h and wash twice with sterile PBS. Allow the plates and coverslips to air dry for 2 h in a sterile hood prior to seeding with cells.
2. Buffers: All antibody solutions, fixatives, permeabilization solutions, and blocking solutions, should be prepared in an isotonic saline solution (PBS or Tris-buffered saline). Stock solutions of PBS or TBS should be prepared with high-quality, glass-distilled deionized water (preferably 18 mΩ resistivity) and

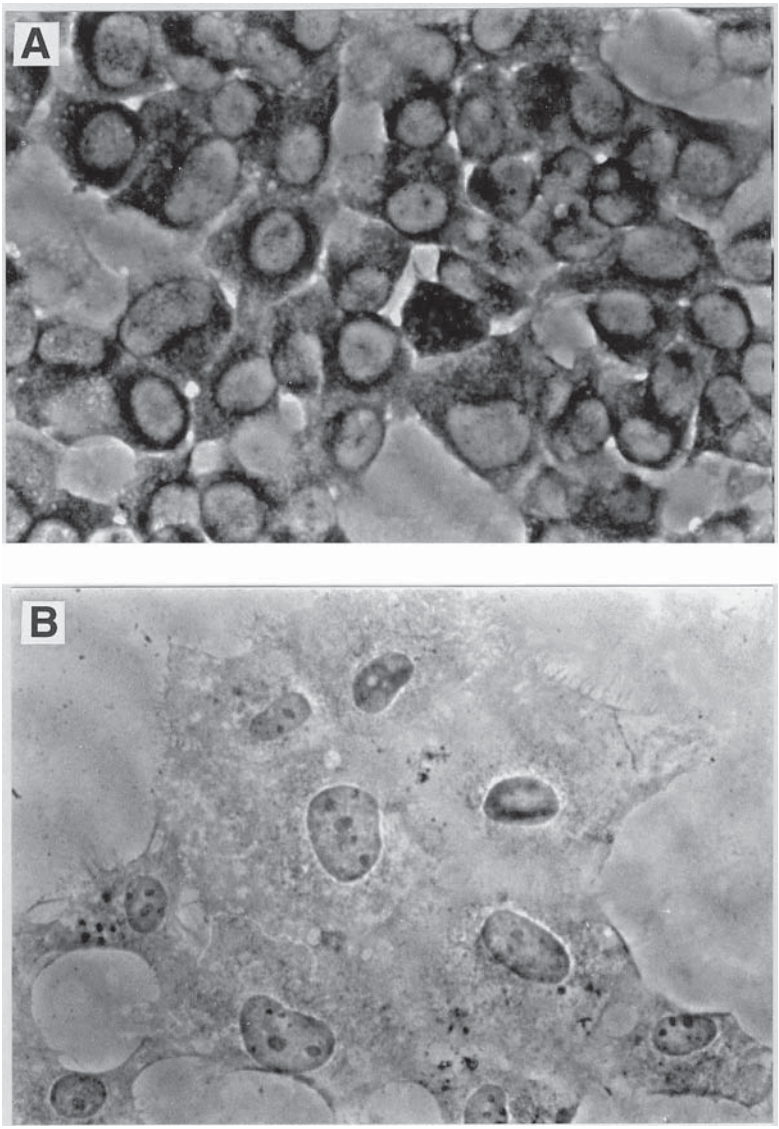

Fig. 4. Demonstration of antibody specificity; effects of membrane permeabilization. **(A,B)** HOG cells immunocytochemically labeled with (A) P_2Ab (1:100) that had been preabsorbed with the cognate peptide. Metal-enhanced DAB was used as the chromogen. Reprinted with permission of **ref.** *7*.

filtered at 5 µm to remove any undissolved solids. Nondistilled water (even with low conductivities) may contain inhibitors of peroxidase and can reduce sensitivity when using this enzyme in the detection method. Sodium azide should not be added PBS or TBS stock solutions because of its ability to inhibit

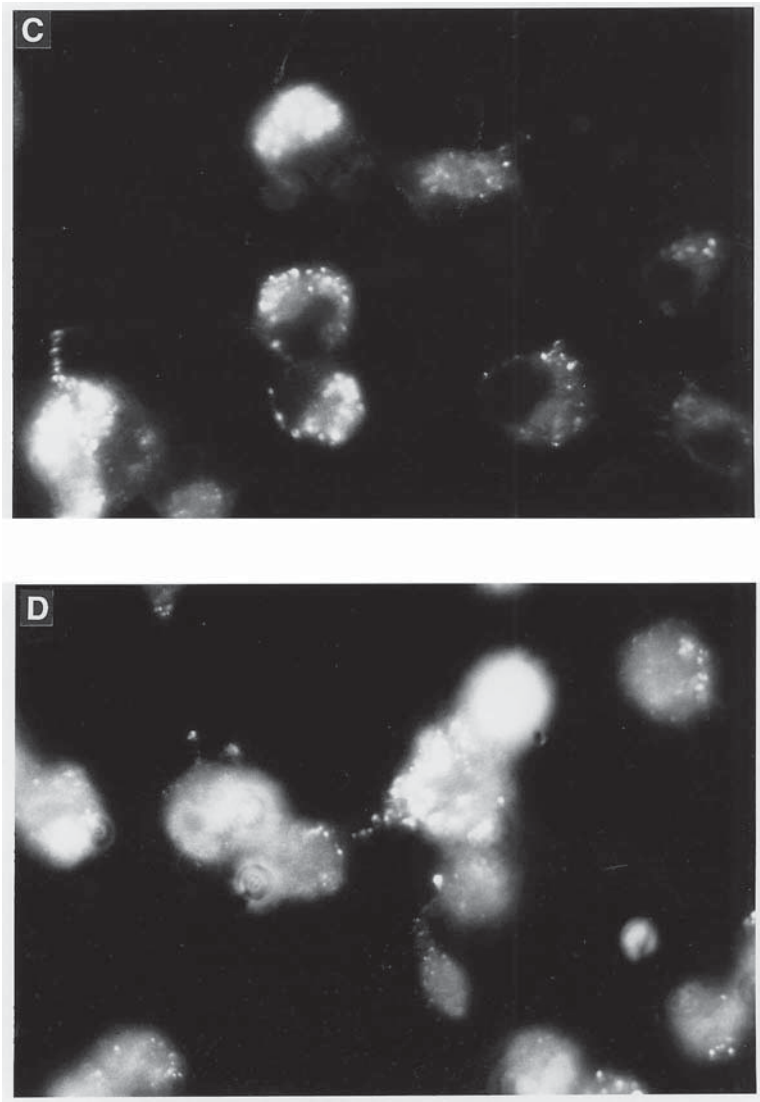

Fig. 4. *(continued)* Demonstration of antibody specificity; effects of membrane permeabilization. **(C,D)** Nonpermeabilized (C) and Triton X-100 permeabilized (D) HOG cells were labeled with TxAb (1:100). Immunoreactivity was visualized using a FITC-conjugated secondary antibody (×1000 magnification).

HRP activity. Sodium azide can be added, however, directly to primary antibody solutions and blocking buffers at a concentration of 0.02% (2 μL/mL of a 10% stock solution).

3. Fixation, permeabilization, endogenous peroxidase, and blocking:
   a. Fixation: Typically, immunocytochemical labeling studies are performed following a primary fixation step. An exception would be the labeling of living cells, which are fixed after the antibody incubations to preserve them for mounting under a coverslip. Fixation of cells or intact tissues must maintain cellular morphology, maintain the immunoreactivity of antigenic epitopes, and permeabilize the cell to allow access of the antibody, if so desired. Two main methods have been tested: fixation by aldehyde crosslinking reagents and fixation by organic solvent.
      i. Crosslinking reagent method: Fixation in protein crosslinking reagents, such as paraformaldehyde (PFA) or glutaraldehyde (GA), preserves cell structure better than organic solvents, however the antigenicity of some epitopes may be reduced. In addition, simple fixation with PFA or GA does not allow full access of the antibody to the specimen, and therefore is often followed by a permeabilization step involving an organic solvent or nonionic detergent. Cultured cells are immediately accessible to the fixative solution, so that an extended fixation is not necessary; 10–15 min is sufficient. For convenience, fixation is performed at room temperature. Stock solutions of PFA should be prepared fresh. To prepare a 4% solution, dissolve 8 g in 100 mL dH_2O. Heat to 60°C in a fume hood, stirring constantly. Add a few drops of 1 *N* NaOH until the solution clears. Do not use NaOH in excess. When the solid has completely dissolved, cool the solution to room temperature, filter with a 5 μ*M* membrane filter under vacuum, and add 100 mL of 2X PBS. This solution is stable for several weeks at 4°C.

         For glutaraldehyde fixation, fresh 1% solutions of GA (electron microscopic grade) should be prepared in PBS, in a fume hood. The free reactive aldehyde formed following GA fixation may be blocked by incubating the fixed specimens with 0.2 *M* ethanolamine, pH 7.5, for 2 h at room temperature, or by incubating with three changes of 5 min each with 0.5 mg/mL sodium borohydride in PBS. In some cases this might also help PFA-fixed cells, but in general is not necessary. It should be noted that sodium borohydride is a strong reducing agent and may change the antigenicity of the sample.
      ii. Organic solvent method: This method of fixation removes lipids, dehydrates the cell, and precipitates the proteins on the cellular architecture. It should be noted that cellular morphology is not as well preserved as with an aldehyde fixative. Prior to fixation by organic solvent, coverslips or slides should be rinsed once with PBS. The specimen is allowed drain well, but should not be allowed to dry. For glass coverslips or tissue sections mounted on glass slides, plunge into a large volume of solvent (either anhydrous methanol (MeOH), acetone, or a freshly prepared mixture of 50% MeOH/50% acetone). Solvents should be at room temperature. For fixation within a tissue culture plate, simply fill the well with a freshly prepared mixture of 50% MeOH/50% acetone at room temperature. Agi-

tate gently and incubate for 2 min, drain off the solvent and rinse in PBS.

Specimens fixed in organic solvents can be stored at –70°C. Simply air-dry after organic solvent incubation, but do not wash in PBS. Store the samples (e.g., coverslips in a multiwell plate) in a sealed container. For use, thaw carefully to avoid damage by immediately incubating the specimens in a 1:1 MeOH/acetone solution that was previously cooled on dry ice. Following this incubation, allow the solution to equilibrate to room temperature. Once it has done so, wash the specimens in PBS.

b. Permeabilization: In most cases, following aldehyde fixation, it is necessary to permeabilize cells in order to allow the antibody access to intracellular epitopes. This can be accomplished by using either organic solvents or detergents. Organic solvent permeabilization may be performed by treating the cells with methanol for 2 min or acetone for 30 s, at room temperature. For cells grown on tissue culture plates, 50% acetone/50% MeOH should be used to prevent dissolution of the polystyrene plates. Although the use of an organic solvent for permeabilization is easy, it can destroy certain elements of the cell's architecture. If preservation of structure is important, the best first choice would be a nonionic detergent.

   The most commonly used detergents are Triton X-100 (0.1–0.5%) and Igepal CA-630 (0.2%) (formerly Nonidet P-40). The final concentration of detergent and the length of exposure to the specimen will depend on the experimental system and should be refined to prevent damage to, or loss of the specimen. The investigator should seek the optimal balance between antibody penetration and preservation of cell structure.

c. Endogenous peroxidase: If HRP-based detection methods are to be used, it may be necessary to block endogenous enzyme activity within the specimens before the application of antibody solutions. It should be noted that inhibition of endogenous peroxidase activity may harm or destroy some antigens, therefore it may be easier to use an alternative detection method (i.e., immunofluorescence).

   To block endogenous peroxidase activity, incubate the specimen with a solution of 0.3% hydrogen peroxide in PBS for 10–20 min at room temperature. H_2O_2 is available as a 30% solution, and should be stored at 4°C until the expiration date indicated on the container. Solutions at a concentration of 0.3% should be prepared immediately prior to use. Higher concentrations of peroxide (up to 1%) can be used in an attempt to completely eliminate endogenous peroxidase activity, but at the risk of reducing or abolishing antigenicity in certain cases. Application of 3% solutions of H_2O_2 should be avoided as violent oxidative reactions and bubble formation can obliterate the specimen.

d. Blocking nonspecific sites: Several protein blocking agents are available for use in immunocytochemical protocols, including gelatin (0.3–1%), nonfat dry milk (5–10%), bovine serum albumin (BSA) (1–3%), and nonimmune ("normal") serum (1–10%) from the same species as the secondary antibody. The addition of normal serum lowers background staining by blocking immunoglobulin and nonspecific binding sites, prior to primary antibody application.

4. Primary and secondary antibody solutions:
   a. Primary antibody: Primary antibodies should always be prepared in a blocking solution that contains a carrier protein, and a salt concentration sufficient for reducing nonspecific binding. This is particularly important when using a PAb.

      If the primary antibody to be employed is scarce, expensive or requires a very low dilution, it is possible to incubate 12 mm coverslips in a volume as small as 50–100 µL. Briefly, a strip of Parafilm is taped to the lab surface, paper side down, the antibody is spotted onto the Parafilm, and the coverslip is placed on the drop of primary antibody, face-down. Antibody incubations must be shortened to avoid significant evaporation (20–30 min). Alternatively, this procedure can be performed inside of a large plastic container with a tight fitting lid. The Parafilm is placed on top of a flattened layer of moist paper towels, and the incubation chamber sealed during the incubation to help maintain a humid environment.
   b. Secondary antibody: Most commercially available secondary antibodies are extremely reliable, and have been well characterized. A good starting dilution for a new series of immunocytochemistry experiments would be the recommended titer provided by manufacturer. An alternative approach would be the paired testing of varying concentrations of primary and secondary antibodies as outlined in **Note 9** and **Table 1**.

      An important concern when using secondary antibodies in an indirect labeling system is the ability of these antibodies to bind nonspecifically to the specimen. Preabsorption of the secondary antibody with an acetone powder extract from a matching tissue (e.g., rat brain acetone powder) can significantly decrease nonspecific binding. This can be accomplished by diluting the secondary antibody to its final concentration in PBS + T in a 50-mL conical centrifuge tube. A higher concentration may be used in anticipation of antibody loss during preabsorption. Also, it is important to not use the same-species normal serum in the preparation of the secondary antibody (*see* **Note 9**). Ten milligrams of rat brain acetone powder is added for each milliliter of secondary antibody solution, the mixture is vortexed and allowed to incubate for 30 min at 37°C. The solution should be agitated every 10 min. Following incubation, the solution is centrifuged at 3000*g* for 20 min, and supernatant filtered through a 0.22-µm membrane filter. Occasionally, prefiltering through a 0.45 µm filter is necessary to remove large debris. The final filtrate is then used for immunostaining. Alternatively, several manufacturers are now offering secondary antibodies preabsorbed to a variety of animal and human proteins, however the choice of markers and target species is not as broad.
5. Washing: Adequate washing of specimens following incubation with antibodies or avidin conjugates is essential for the reduction of nonspecific staining. If preliminary results are complicated by high backgrounds, increasing the length and number of washes may help resolve this problem. Increased washing, using a

Table 1
Troubleshooting Chart for Immunocytochemistry

Component	Problem	Potential diagnosis	Remedy
Primary antibody	Weak or absent staining	Reduced potency caused by inactivation, denaturation, or degradation	Use a higher concentration of primary antibody, or prepare a fresh antibody solution. Store antibody stock in small aliquots and only thaw once. Store diluted antibodies at 4°C in the presence of 0.02% sodium azide.
	High background staining	Incorrect pH of diluent is inhibiting binding	Check the pH of the diluent PBS or TBS, pH 7.0–8.2. is recommended.
		Diluent contains an antigen that binds to the primary antibody	Ensure that the diluent for the antibody does not crossreact with the primary antibody (i.e., by immunoblot analysis). Change the blocking agent used in the antibody solutions (e.g., normal serum, BSA, nonfat dry milk).
	Small, amorphous, punctate staining	Too much primary antibody	Reduce the primary antibody concentration. Optimize by testing serial dilutions of the primary antibody.
		Cross-reactivity with other cellular antigens, or nonspecific binding	Add an appropriate blocking agent and/or detergent to the antibody diluent.
		The diluent contains little or no NaCl	Ensure that the diluent for the primary antibody has sufficient sodium chloride to block nonspecific binding (0.15–0.6 *M*)
		Specimen dried out during procedure	Take care to ensure that specimens remain moist during all steps of the procedure.
		Precipitation of denatured immunoglobulin	Centrifuge primary antibody (10,000 rpm in a microfuge) and use the supernatant.

(continued)

Table 1 *(continued)*

Component	Problem	Potential diagnosis	Remedy
Secondary antibody	Weak or absent staining	Inappropriate concentration of secondary antibody	Generally a 1:200 to 1:500 dilution of a biotinylated secondary antibody will give optimal staining (when used in an avidin/SA enzyme conjugate). High dilutions can result in diminished staining. Fluorescent-conjugated secondary antibodies should be used at a higher titer (1:50 to 1:100).
		Antibody diluent contains neutralizing antibodies (e.g., antimouse IgG diluted with mouse serum)	Neutralizing antibodies can bind to the secondary antibody an prevent it from binding to the primary antibody. Remove the source of neutralizing antibodies.
		Wrong secondary antibody	Ensure that the target species of the secondary antibody matches the host species of the primary antibody.
	High background staining	Crossreactivity between the secondary antibody and endogenous immunoglobulins or other cellular proteins	Add 2% or more normal serum from the same species as the cell/tissue preparation to the secondary antibody solution and/or reduce the secondary antibody concentration.
		Nonspecific binding	Add additional blocking agents (e.g., 2% BSA, nonfat dry milk, gelatin) and/or 0.1% Tween-20.
		Wrong species of blocking serum	When preparing the blocking solution, make sure that the normal serum is from the same species in which the secondary antibody was produced.
Enzyme/ substrate	Weak or absent staining	Inhibition of peroxidase reaction	Make sure that glass-distilled water was used to prepare the substrate solution. In addition, make sure there is no sodium azide present in the wash buffer, secondary antibody diluent, avidin/SA-enzyme conjugate, or substrate solution.

	Wrong pH	Buffers of different pH values are recommended for different enzyme substrates. Prepare according to manufacturer's instructions.
	Old hydrogen peroxide	Use freshly diluted H_2O_2 to prepare the substrate solution. The final concentration should be about 0.01%. In addition, use clean glassware; traces of cleaning solutions or chlorine may inhibit the peroxidase reaction.
High background staining	Endogenous enzyme	Block endogenous peroxidase by treating cell/tissue specimen with 0.3% H_2O_2 for 5–10 min at room temperature. Be certain to wash thoroughly with PBS/TBS before adding primary antibody. Endogenous alkaline phosphatase activity can be blocked with levamisole.

buffer supplemented with a nonionic detergent, such as Triton (0.1–0.5%) or Tween-20 (0.05–0.2%), should selectively dissociate weakly bound antibodies. It is important to note that high concentrations of detergents may degrade specimens, particularly cell monolayers, therefore it is important to occasionally assess the specimen integrity with an inverted microscope.

6. Mounting and photobleaching: Mounting media must be compatible with the detection method used. A suitable aqueous media can be made from Mowiol (Calbiochem, San Diego, CA). If this reagent is not available, glycerol can be substituted, but this is not a permanent mount. Conveniently, several high-quality commercial mounting preparations are available for both chromogenic and fluorescent (VECTASHIELD® Mounting Medium, Cat.#H-1000, Vector Laboratories, Burlingame, CA) applications. A suitable nonaqueous mounting is Permount (Fisher Scientific, Fair Lawn, NJ). DPX, a mixture of distrene-80, dibutyl phthalate, and xylene, is a nonaqueous medium for fluorescence applications (Cat. #13510, Electron Microscopy Sciences, Fort Washington, PA).

   For mounting media prepared in the laboratory for fluorescent applications, it is important to add an antifade agent to help prevent photobleaching ***(16)***. P-phenylenediamine (PPD, Cat. #P-6001, Sigma) appears to be the best antifade agent. It works well with fluoroscein but has a peculiar effect on the fluorochrome DAPI, in which it induces a dim red fluorescence. It is used at a concentration of 0.1% in a mountant containing 10% phosphate-buffered saline and 90% glycerol. This mountant should be stored at –20°C or preferably, –70°C. It may turn brown but can still be used until dark brown. It should be noted that this agent has carcinogenic properties and should be handled with great care. Diazabicyclo-octane or DABCO (0.1%) (Aldrich # D27802) is less effective than PPD, but has the advantage of lower toxicity and easier storage, being stable at room temperature.

   Mowiol is prepared by combining 6 g glycerol with 2.4 g Mowiol 4–88 in 6 mL distilled water. Twelve milliliters of 0.2 *M* Tris buffer, pH 8.5, is added and the entire mixture is incubated for approx 6 h on a shaker. Following this incubation, the mixture is allowed to stand for 2 h (the Mowiol will not dissolve completely). The mixture is then incubated at 50°C for 10 min and centrifuged at 5000*g* for 15 min. 0.1% PPD or 0.1% DABCO, along with 230 µL 1.0% thimerosal (w/v in water), are added and the mounting media is then aliquotted and frozen at –20°C until use. When thawing, it is important to ensure that the Mowiol solution has come to room temperature before mounting the specimen, otherwise small air bubbles will form under the coverslip. After the Mowiol solution has been used, it can be stored at 4°C for approx 1 mo, however it should be discarded if "crystalline" deposits are seen in the slides.

7. Photomicrographs: Imaging of fluorescently-labeled cells or tissues can provide dramatic color documentation of receptor localization. However, for single-label preparations, any visualized antigen is of the same color, thus color film photography is not particularly necessary. In addition, color film can prove to be difficult for capturing imunofluorescence images. Color films are generally less sensitive than black and white films, thus requiring longer exposure times and a

higher risk of photobleaching of the fluorochrome. Slides made from color film are very dark and are often not as practical for projection. Therefore, we choose to use only black and white film for recording single-label immunocytochemical experiments. Kodak Tri-X 400 ASA film is convenient for routine use, providing good sensitivity and a sharp image definition.

8. Handling antibody solutions: Antibodies are often the most expensive component of an immunocytochemical experiment. Unfortunately, they are also the most labile, and require proper storage, handling, and testing. Taking the proper steps will help produce a strong signal and reduce background reactivity. Unconjugated PAbs and MAbs should be stored undiluted and frozen (–20 or –70°C if possible) in aliquots sufficient for one experiment at a time. It is important to avoid repeated freeze/thaw cycles, which can cause aggregation of antibodies and loss of activity. Conjugated antibodies should be kept undiluted and frozen, or at 4°C, according to the manufacturer's instructions. Concentrated antibody solutions can be stored for 1–2 mo at 4°C provided that a preservative is added to prevent bacterial growth (e.g., 0.01% thimerosal or 0.02% sodium azide). If antibodies are to be kept at low concentrations, the addition of a carrier protein (e.g., 1% BSA) is recommended to prevent adhesion of the antibody molecules to the container walls.

   Often it is economical to save and reuse primary antibody solutions. In our hands, primary antibody solutions have been reused up to four times, however it is recommended that they be reused only twice, in order to maintain consistent immunoreactivity. It is important to indicate on the container label how many times the antibody solution was used, and when. Reused antibody solutions should not be employed in critical experiments. Furthermore, conjugated secondary antibodies are relatively inexpensive and need not be reused. Reused antibody solutions need to be stored at their working dilutions and should be treated with both an antibacterial agent as well as a carrier protein. These solutions should not be frozen, but instead stored at 4°C. Finally, antibody solutions should be cleared of any debris or turbidity, resulting from their prior use, by centrifugation.

   Optimizing immunocytochemistry protocols is important for achieving superior results. In this regard, the specific aim of antibody testing is to determine the ideal working concentration and quality of a specific lot or batch of antibody. This is especially crucial when characterizing a MAb or PAb that was developed in the laboratory. It is important to use a tissue or cell preparation that contains sufficient quantities of the target antigen. We recommend performing these tests using the coverslip/24-well format detailed in this chapter. All blocking, washing, permeabilizing, developing, and incubating conditions should be kept constant, and noted in detail for later reference. We suggest the following protocol for the simultaneous testing of both a primary and secondary antibody: Working dilutions of the primary antibody should be made along the horizontal rows of the 24-well plate, varying by a factor of 4× or 5× starting with the undiluted stock (serum, supernatant, or IgG). If a commercial purified IgG is being tested, start the serial dilutions at 1:16 or 1:64 and vary by a factor of 3–5× (e.g., 1:16, 1:64,

1:256, 1:1024, and so on). It is important to note that for MAbs, the optimal working dilution is more a function of antibody preparation type (i.e., supernatant, ascites, or purified immunoglobulin) than of antibody affinity. Therefore, antibodies in supernatants will often be used at low dilutions, whereas purified IgG fractions will often be used at very high dilutions. Secondary antibody solutions should vary in concentration along the vertical axis of the 24-well plate. Effective dilutions usually range from 1:10 to 1:200, therefore lower dilution factors should be used (2 or 3×) beginning at 1:10 (e.g., 1:10, 1:30, 1:90, 1:270). It is not necessary to test high dilutions of secondary antibodies since they must be used in excess. When choosing the optimal dilutions, look for the combination of primary and secondary antibody titers that produce the best signal-to-noise ratio.

9. Troubleshooting: Immunocytochemical techniques require a large number of steps, accurate timing of incubations, and high-quality reagents. Given these stringent criteria, problems often arise. Tissue sections may have a high level of background stain, the desired staining may be absent or diminished, or color/fluorescence may occur in inappropriate locations. Because troubleshooting of immunocytochemical experiments can be time-consuming and frustrating, it is important to run multiple controls that account for the major variables in the experiment: the primary antibody, the secondary antibody, and the enzyme/substrate (if a chromogenic stain is used). Problems involving the primary antibody solution can result from excessive antibody concentrations, crossreactivity with other tissue epitopes, nonspecific binding, or antibody denaturation/degradation (*see* **Note 8**). Secondary antibody difficulties often result from inappropriate dilution with a buffer that contains neutralizing antibodies, incorrect blocking solutions, or inappropriately high dilutions. Enzyme and substrate problems tend to be related to the manner in which the buffers and substrate solutions are prepared, or the presence of high levels of endogenous enzyme activity in the cell/tissue preparation. The major troubleshooting points for these three variables are outlined in **Table 1**.

   Problems involving crossreactivity between the blocking solution and the primary or secondary antibodies can be experimentally determined by performing an immunoblot analysis of the blocking agent in question and looking for presence of a strong signal when probing with the primary and/or secondary antibody used for immunocytochemistry. Resolution of these problems can be accomplished by experimenting with different blocking proteins, such as an immunohistochemical grade of BSA, gelatin, nonfat dry milk, or normal serum. The addition of a detergent such as Tween-20, Triton X-100, or Igepal CA-630 may alleviate high background staining problems.

   Destruction of antigen during the fixation process may be the source of weak or absent staining in specimens known to possess the epitope of interest. If possible, ensure that the method employed for preparing the specimen is appropriate to preserve the antigen and provide access to antibodies and detection reagents. Addition or removal of permeabilizing agents, alterations in the concentration of fixative, or the use of antigen unmasking techniques (e.g., heat or enzymatic digestion) may be necessary to unmask or recover antigen.

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Epoxyeicosatrienoic Acids

Biosynthesis, Regulation and Actions

John D. Imig

1. Introduction

Arachidonic acid is a polyunsaturated fatty acid that is esterified at the *sn*-2 position of the glycerol backbone of membrane phospholipids. Hormonal and paracrine stimuli can activate phospholipases that cleave arachidonic acid from membrane phospholipids. The free intracellular arachidonic acid is then available for metabolism via either the cyclooxygenase, lipoxygenase, or cytochrome P450 monooxygenase pathway. The biological importance of cyclooxygenase and lipoxygenase metabolites of arachidonic acid has been clearly established. In recent years studies have focused on the biological regulation and actions of arachidonic acid metabolites of the cytochrome P450 enzymes. Mammalian cytochrome P450 monooxygenase enzymes catalyze three types of chemical reactions; the hydroxylation of fatty acid carbons near the methyl terminus generating hydroxyeicosatetraenoic acids (HETEs), allylic oxidation generating regioisomeric HETEs, and olefin bond epoxidation to generate regioisomeric epoxyeicosatrienoic acids (EETs; **Fig. 1**). Once formed the cytochrome P450 metabolites possess a wide array of potent biological actions that are not well understood. This chapter will focus on the localization and array of cytochrome P450 enzymes that generate EETs, the regulation of cytochrome P450 enzymes and EETs formation, and the biological actions of EETs.

2. Biosynthesis

Until the 1980s the role of cytochrome P450 enzymes was thought to mainly entail the detoxification of xenobiotics that occurs primarily in the liver ***(1,2)***. The beginning of a new era of cytochrome P450 research started with the dis-

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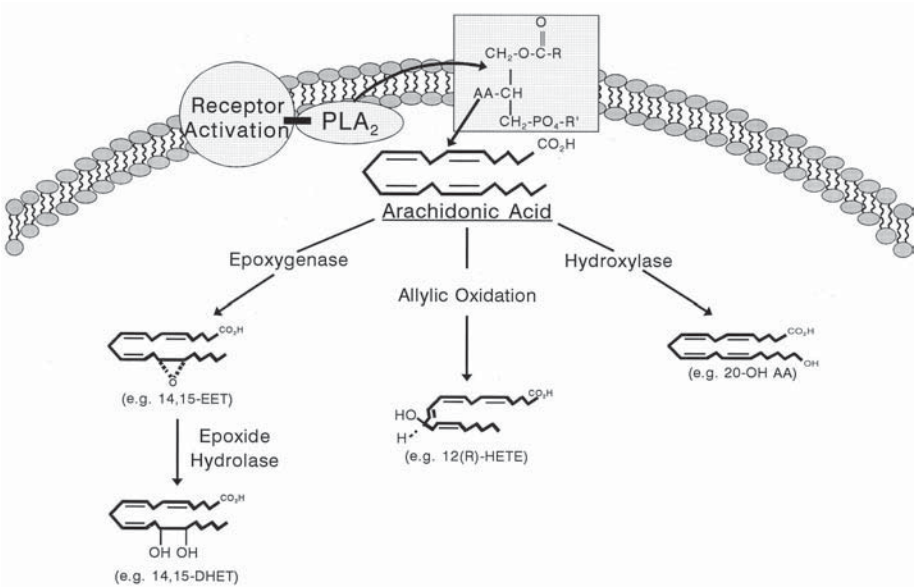

Fig. 1. Arachidonic acid metabolic pathways for mammalian cytochrome P450 monooxygenase enzymes.

covery that cytochrome P450 enzymes could metabolize arachidonic acid. One of the reactions catalyzed by cytochrome P450 enzymes is the epoxidation of olefin bonds in arachidonic acid to form four regioisomeric EETs (5,6-; 8,9-; 11,12-; 14,15-). Although production of all four regioisomers has been demonstrated, studies have only been able to quantify the production of 8,9-; 11,12-; and 14,15-EET because 5,6-EET is labile and suffers from extensive decomposition during the extraction and purification processes ***(1,2)***. The epoxidation of arachidonic acid by cytochrome P450 enzymes requires the presence of NADPH and molecular oxygen ***(3)***. The demonstration of stereoselective formation of EETs by cytochrome P450 enzymes clearly established these metabolites as members of the arachidonic acid metabolic cascade ***(4,5)***. Stereoselective formation of EETs has now been demonstrated for a number of mammalian cytochrome P450 enzymes. Once formed, EETs are hydrated to dihydroxyeicosatrienoic acids (DHETs) by cytosolic or microsomal epoxide hydrolases (**Fig. 1**). Cytochrome P450 enzymes demonstrate tissue specific expression and each cytochrome P450 enzyme isoform produces a distinct pattern of EETs.

The liver contains numerous cytochrome P450 enzymes that were thought to only carry out detoxification of xenobiotics. In recent years the ability of

human, rat, and rabbit liver microsomes to metabolize arachidonic acid to HETEs, EETs, and DHETs has clearly been demonstrated. Human liver microsomal fractions metabolize arachidonic acid via the cytochrome P450 enzymes to hydroxylase and epoxygenase products ***(6,7)***. Epoxidation of arachidonic acid by human liver microsomes results in the production of 8,9-; 11,12-; and 14,15-EET with the major metabolite being 14,15-EET ***(6,7)***. Using a combination of high performance liquid chromatography (HPLC) and gas chromatography mass spectrometry (GC/MS), substantial amounts of EETs and DHETs (380 ng/g tissue) have been detected in human liver tissue ***(8)***. The major epoxygenase metabolite is 14,15-EET accounting for 50% of the total EETs and chiral analysis has revealed that 14(R),15(S)-; 11(R),12(S)-; and 8(S),9(R)-EET are the predominant antipodes ***(8)***. Although the presence of epoxide hydrolase has been demonstrated in both the microsomal and cytosolic fractions ***(7,8)***, the formation of DHETs by the human liver is mainly a consequence of cytosolic epoxide hydrolases ***(8)***.

The predominant cytochrome P450 isoforms responsible for the epoxidation of arachidonic acid by the liver belong to the CYP2C gene family. The majority of epoxygenase production by cultured liver hepatic cells occurs via the CYP2C8 and CYP2C9 enzymes with some contribution by the CYP1A2 enzyme ***(7)***. Although human liver constitutively expresses a number of CYP2C isoforms including CYP2C8, CYP2C9, CYP2C10, CYP2C18, and CYP2C19, the recombinant CYP2C8 demonstrates an enantioselective EET production profile that is similar to that of liver tissue and microsomes ***(6)***. Both protein and mRNA expression of the 2C8 enzyme have been demonstrated in the human liver whereas extrahepatic tissue levels were below Northern analysis detection limits ***(6)***. Thus, the constitutively expressed CYP2C isoforms are responsible for the formation of EETs in the liver.

Unlike the liver, the predominant cytochrome P450 isoforms responsible for the formation of EETs in the heart appear to be of the CYP2J subfamily. The human heart constitutively expresses the CYP2J2 and purification of the enzymatic protein demonstrated the formation of the four regioisomeric EETs ***(9)***. 14,15-EET and 8,9-EET were the primary metabolites of human heart microsomal fractions and epoxidation of arachidonic acid at the 14,15-olefin bond was highly enantioselective for 14(R),15(S)-EET ***(9)***. Rat atrial and ventricular myocytes contain the CYP2J3 isoform which has 70% sequence homology with the CYP2J2 isoform ***(10)***. Recombinant CYP2J3 produces all four EETs with 14,15-EET being the major metabolite formed ***(10)***. In addition to the ventricular and atrial myocytes, coronary endothelial cells also demonstrate epoxygenase activity ***(11)***.

The ability of the brain to generate EETs was first demonstrated in mouse brain slices by Amruthesh et al. ***(12)***. The cerebral vasculature ***(13)*** and cul-

tured astrocytes ***(14,15)*** are two cell types within the brain that have the capacity to generate EETs. 14,15-EET is the major epoxygenase metabolite formed in the brain and by astroglial cells. Recently a cytochrome P450 enzyme has been cloned and sequenced from cortical astrocytes and is homologous to the sequenced 2C11 cDNA from rat liver ***(15)***. The brain contains high levels of epoxide hydrolase activity that acts upon EETs to produce the corresponding diol, DHETs. Epoxide hydrolase activity of the rat and human brain demonstrates regiospecific tissue expression ***(16,17)***. Many neurons and astrocytes express microsomal epoxide hydrolase and suggest that epoxide hydrolase activity may regulate the actions of EETs formed in the central nervous system.

The production of EETs by the kidney has been recognized for several years and like other organ systems demonstrates high enantiofacial selectivity. The cDNAs for the CYP2C11, 2C23, and 2C24 have been cloned from the rat kidney ***(18)***. Rat kidney microsomal epoxygenases catalyze the asymmetric epoxidation of arachidonic acid to produce 8(R),9(S)-; 11(R),12(S)-; and 14(S),15(R)-EETs with optical purities of 97, 88, and 70%, respectively ***(19)***. In the human kidney, the predominant epoxide formed is 14,15-EET and 66% of this regioisomer is in the (R,S) configuration ***(20)***, whereas 14(S),15(R)-EET is the predominant stereoisomer and 11(R),12(S)-EET is the major epoxide produced in the rat kidney ***(19)***. Additionally, renal microvessels and microsomes prepared from these vessels metabolize arachidonic acid to two major metabolites, 20-HETE and 11,12-DHET, the hydrolytic metabolite of 11,12-EET ***(21–23)***. Renal microvessels selectively express CYP4A2 protein and expression of CYP4A2 using baculovirus and Sf9 insect cells demonstrates that the major metabolite is 20-HETE but 11,12-EET is also produced ***(22)***. Thus, the kidney vasculature and nephron population may contain distinct CYP isoforms that may allow for specific regulation of vascular EET production.

The aorta and blood vessels isolated from many organs have demonstrated the ability to produce EETs and their corresponding diols, DHETs. Coronary artery production of all four regioisomeric EETs and their corresponding DHETs have been detected by HPLC and confirmed by GC/MC ***(24)***. Resistance-sized blood vessels isolated from the cerebral ***(13)*** and renal ***(21)*** vasculature produce both 20-HETE and EETs. The two components of the vasculature, vascular smooth muscle cells and endothelial cells, have the capacity to produce EETs. Hasunuma et al. ***(25)*** showed that microsomes of rat cultured aortic smooth muscle cells incubated with arachidonic acid generate 5,6– and 14,15-EET. The ability of coronary artery endothelial cells to produce EETs was demonstrated by Rosolowsky and Campbell ***(11)***. The specific cytochrome P450 isoforms responsible for EET production by the vascular smooth muscle and endothelial cells have yet to be identified. Additionally, aortic cultured endothelial ***(26,27)*** and vascular smooth muscle cells ***(28)*** can incorporate

EETs and DHETs in the phospholipid membrane and raises the possibility that these metabolites may have long lasting vascular effects.

Epoxygenase production has been demonstrated for other organ systems but the isoforms responsible and the biological actions in these organ systems are not as well understood. The pancreas, reproductive tissues, intestine, and lung are among some of the other tissues that produce EETs. EET production by the human and rat pancreas is localized to the islets of Langerhans and the cytochrome P450 enzyme responsible for EET production belongs to the CYP2J subfamily ***(29)***. Tissues related to reproduction including the amnion, trophoblast, and umbilical vein produce EETs with 14,15-EET being the major metabolite produced ***(30)***. Human and rat intestinal tissue also contains the CYP 2J enzymes, and the CYP2J2 and CYP2J3 are mainly expressed in the small intestine and colon ***(31)***. CYP2J proteins are expressed in many cell types along the gastrointestinal tract and are present in high amounts in the nerve cells of autonomic ganglia, epithelial cells, intestinal and vascular smooth muscle cells ***(31)***. 8,9-EET is the major metabolite of guinea pig lungs ***(32)***, whereas 14,15-EET is the major metabolite of rat and human lungs with 14(R),15(S)-EET as the predominant antipode ***(33)***. This difference in lung EET metabolites may be related to the isoforms responsible for metabolism in these species. The lung EET production of the rabbit and guinea pig appears to be mediated by the CYP2B subfamily ***(32,34)*** and the rat and human appears to be via the CYP2J subfamily. Zeldin et al. ***(33)*** demonstrated in vivo production of EETs by the rat and human lung and localization of the CYP2J isoforms primarily in ciliated epithelial linings. Other areas of the lung that stained positive by immunohistochemical analysis included the nonciliated airway epithelial cells, bronchial and pulmonary vascular smooth muscle cells, and alveolar macrophages ***(33)***.

The number of cytochrome P450 enzymes with the demonstrated ability to convert arachidonic acid to EETs is steadily increasing. The localization of each of the epoxygenase enzymes and the cell types within an organ capable of EET production are continuously being described. The lability of epoxygenase metabolites and their metabolism by cyclooxygenase enzymes complicates the ability of investigators to examine the regulation and biological actions of EETs. Even with these limitations, investigations are beginning to describe some of the biological actions of the EETs in these tissues and regulation of these epoxygenase enzymes under normal physiological and pathophysiological conditions.

3. Regulation

Epoxygenase production by many organs and cell types has been clearly established and an ever increasing number of P450 enzymatic isoforms have been

identified that can produce these metabolites. The specialized localization of the many cytochrome P450 enzymes capable of producing epoxygenase metabolites raises the possibility that the level of EET production may be tightly controlled at the organ and cellular level. Although there has been a number of recent studies identifying EET production by specific isoforms, little is known about the regulation of the enzymatic isoforms and EET production. As an example, investigations have demonstrate that human chorionic gonadotropin stimulates the production of EETs in human luteinized granulosa cells ***(35)*** and EETs induce rat uterine contraction ***(36)*** but the potential role of EETs in this area awaits investigation. Regulation of renal, cardiovascular and neural epoxygenase production are the three main areas that have received most of the attention.

Alterations in kidney EET production during various pathophysiological states has received considerable attention. Following uninephrectomy there is an increased epoxygenase production that may play a role in maintaining renal physiological function ***(37)***. Administration of ketoconazole, an inhibitor of renal epoxygenase activity, results in an acute increase in glomerular filtration rate and renal plasma flow in uninephrectomized rats ***(37)***. In contrast, a decrease in renal microsomal EET production may contribute to the decrease in glomerular filtration rate and renal plasma flow that occurs during bilateral uretral obstruction ***(38)***. Renal ischemia of 60 min is associated with a decrease in cytochrome P450 2C23 protein ***(39)*** but this study did not determine if this enzymatic decrease was associated with an alteration in EET production.

Kidney EET production is enhanced by increases in dietary salt intake and may act to regulate renal sodium excretion. Sprague-Dawley rats increase urinary excretion and renal microsomal production of 11,12-, 14,15-, 8,9-EET and 5,6-DHET in response to excess dietary salt ***(18)***. In addition, immunological studies indicate that high salt diet induces the 2C epoxygenase isoforms but does not affect cytochrome P450 ω-hydroxylase isoforms of HETE production ***(18)***. Examination of the regulation of kidney microsomal epoxygenase production in the Dahl salt-resistant (DR) and salt-sensitive (DS) rat model of hypertension has demonstrated that an inability to increase EET production is associated with the development of salt-sensitive hypertension. Excess dietary salt intake results in a threefold increase in renal microsomal EET production in the DR rat whereas in the DS rat EET production is unaffected and blood pressure increases ***(40)***. Besides the direct action of EETs on the vasculature and tubules to promote sodium excretion, part of the excretory response may be owing to the ability of 14,15-EET to inhibit kidney renin production ***(41)***. Inhibition of renin release would decrease angiotensin II and aldosterone levels resulting in a net increase in urinary sodium excretion. Thus, regulation of epoxygenase production may importantly act to maintain renal hemodynamic and sodium balance during various pathophysiological states.

Regulation of the epoxygenase pathway has been investigated as an important factor contributing to the control of heart contractility, and blood flow to the heart and brain. An increase in canine coronary artery epoxygenase production occurs in the stenosed endothelial injured artery and the actions of EETs are consistent with the possibility that these metabolites act to counteract the deleterious vascular effects of thromboxane ***(24)***. Rabbit aorta endothelium does not normally produce epoxygenase metabolites, but with high cholesterol feeding the endothelium starts to produce measurable amounts of EETs ***(42)***. Human atherosclerotic vessels produce EETs and an increase in EET production may contribute to vascular hypertrophy associated with hypertension ***(43)***. The epoxygenase enzyme CYP 2J3 has been found to be highly expressed in the atrial and ventricular myocytes and production of EETs by these cells may have beneficial actions ***(10)***. In isolated ventricular myocytes, both 11,12-EET and 5,6-EET increase cell shortening as well as intracellular calcium concentrations ***(44)***. Wu et al. ***(10)*** demonstrated that one of the CYP 2J3 products 11,12-EET improves postischemic recovery of cardiac contractile function in an isolated perfused rat heart model. This beneficial effect of 11,12-EET on the heart is limited to this epoxygenase metabolite since 14,15-EET did not improve heart contractility ***(10,44)***. In the brain, regulation of EET production by astrocytes may act to increase cerebral blood flow to areas of the brain with high neural activity. Alkayed et al. have recently demonstrated that the excitatory amino acid neurotransmitter, glutamate, increases the expression of CYP 2C11 protein and EET production by cultured astrocytes ***(45)***. Additionally, these investigators demonstrated that the increase in cerebral blood flow to glutamate was attenuated by the epoxygenase inhibitor, miconazole ***(45)***. In general, an increase in the production of epoxygenase metabolites is beneficial to coronary and cerebral blood flow.

4. Actions

There have been an array of biological actions attributed to EETs and DHETs, and the physiological significance of these actions is beginning to emerge. Some of these biological effects of epoxygenase metabolites appear to be regioisomeric and enantioselective. Most of the biological actions of EETs and DHETs occur between nanomolar and micromolar concentrations. Epoxygenase metabolites affect the flux of ions across cell membranes, affect cell proliferation, possess vasoactive properties, stimulate hormone release and modulate responses to paracrine and hormonal agents.

The biological actions of epoxygenase metabolites on epithelial cell ion fluxes has been demonstrated in the lung and kidney. In the kidney, epoxygenase metabolites inhibit sodium reabsorption and potassium secretion in the collecting tubule ***(46)*** (**Fig. 2**). 5,6-EET is the only regioisomer to inhibit

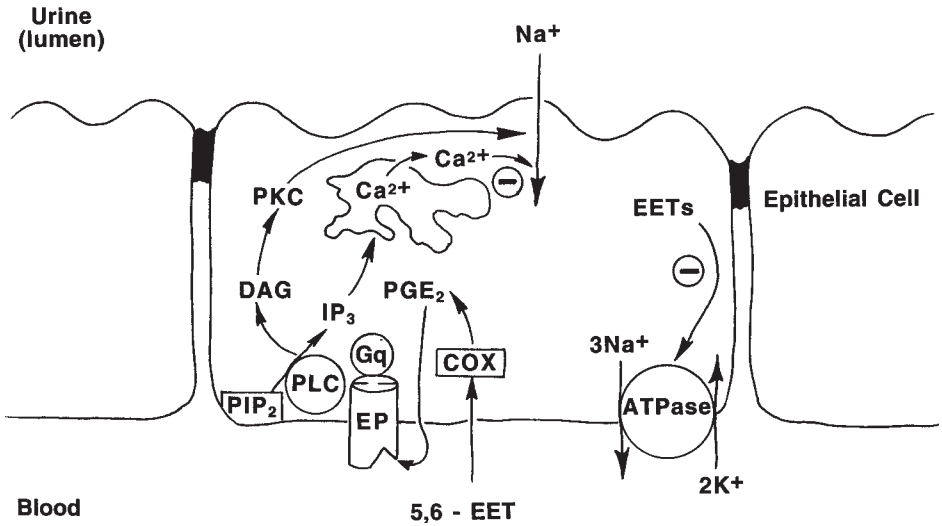

Fig. 2. Action of EETs on renal epithelial cells. EETs inhibit sodium reabsorption and potassium secretion by inhibiting Na^+, K^+ ATPase. The action of 5,6-EET to inhibit cortical collecting duct sodium reabsorption is the result of stimulation of prostaglandin (PG) production.

sodium and depolarize the transepithelial voltage in rabbit cortical collecting duct ***(46)***. The actions of 5,6-EET to inhibit sodium reabsorption in the cortical collecting duct are also the result of cyclooxygenase activity ***(47)***. In this renal cell type 5,6-EET stimulates PGE_2 synthesis and it was demonstrated that PGE_2 had similar actions as 5,6-EET on cortical collecting duct transepithelial voltage and intracellular calcium levels, but the cyclooxygenase metabolites of 5,6-EET, 5,6-epoxy-PGE_1, and 5-hydroxy-PGI_1 were without affect ***(47)***. Epoxygenase metabolites have also been shown to be potent inhibitors of Na^+,K^+-ATPase in renal cells ***(48)***. In the lung, EETs have been demonstrated to cause a relaxation of the airway smooth muscle ***(34)***. 11,12-EET increases the tracheal transepithelial voltage variation, decreases the transepithelial short circuit current variation and these changes are likely mediated by inhibition of a conductive chloride pathway ***(49)***.

In addition to the direct actions of epoxygenase metabolites on epithelial transport, EETs may act to modulate hormonal responses. Vasopressin stimulated osmotic water flow across the toad urinary bladder was inhibited by 5,6-, 11,12- and 14,15-EET with 5,6- and 11,12-EET being the most potent ***(50)***. The action of the EETs on the vasopressin-induced hydroosmotic effect most probably is owing to hydrolysis of EETs to their respective vicinal diols since

the DHETs actions paralleled that of the EETs ***(50)***. Likewise, in the rabbit cortical collecting duct, 14,15-DHET inhibited the hydroosmotic effect of vasopressin ***(51)***. In cultured rabbit proximal tubule cells, 5,6-EET inhibits sodium transport and may mediate the inhibitory effects of angiotensin II on the sodium-hydrogen cotransporter ***(52,53)***. Additionally, angiotensin II stimulates the formation of 5,6-EET and 5,6-EET can cause influx of calcium through voltage-sensitive channels of proximal tubular epithelial cells ***(54)***. EETs also have effects on the regulation of cell calcium mobilization in liver cells. In liver microsomes, 14,15-EET affected the flux of calcium and metabolism ***(55)***. EET metabolites have been shown to increase phosphorylase a activity, intracellular calcium, and are involved in vasopressin-induced glycogenolysis in isolated rat hepatocytes ***(56)***.

Epoxygenase metabolites also appear to play a role in the mediation of cell growth and proliferation. In cultured rat glomerular mesangial cells, 14,15-EET stimulates proliferation and activates Na^+-H^+ exchange ***(57)*** and EETs may modulate agonist induced stimulation of mesangial cell immediate-early genes c-*fos* and *Egr-1* ***(58)***. EETs have also been shown to induce recovery of cultured vas deferens smooth muscle cells from thapsigargin-induced intracellular Ca^{2+} store depletion and growth arrest ***(59)*** and may stimulate growth in vascular smooth muscle cells ***(60)***. In renal proximal tubule epithelial cells epoxygenase inhibition with ketoconazole or clotrimazole inhibits epidermal growth factor stimulated thymidine incorporation ***(61)***. Along these lines, 14,15-EET induces the synthesis of cyclooxygenase-2 mRNA in intestinal epithelial cells and may contribute to the antiapoptotic role of prostaglandins in this cell type ***(62)***. The exact mechanisms by which epoxygenase metabolites affect cell growth and proliferation remains to be determined.

The vascular actions of epoxygenase metabolites have been described for a number of organs. In the kidney, epoxygenase metabolites have been found to be either renal vasoconstrictive or vasodilatory depending on the experimental conditions and species studied ***(63–65)***. Infusion of 5,6-EET or 8,9-EET into the renal artery of the rat results in an increase in renal vascular resistance, a decrease in glomerular filtration rate and no change in sodium excretion ***(64)***. The observed vasoconstriction was found to be cyclooxygenase dependent because 5,6-EET increased renal blood flow during administration of indomethacin ***(64)***. In contrast, 5,6-EET, 8,9-EET, and 11,12-EET vasodilated the isolated perfused rabbit kidney preconstricted with phenylephrine and the vasodilation to 5,6-EET was cyclooxygenase dependent ***(65)***. Analysis of renal venous effluent material revealed that the cyclooxygenase-dependent vasodilator activity of 5,6-EET is caused by release of prostaglandins PGE_2 and PGI_2 and metabolism of 5,6-EET to 5,6-epoxy-PGE_1 ***(66)***.

In a recent study that directly examined the responses of the preglomerular vasculature to all four regioisomeric EETs it was demonstrated that 11,12-EET and 14,15-EET vasodilated and 5,6-EET resulted in vasoconstriction of the interlobular and afferent arterioles ***(67)***. The preglomerular vasoconstriction to 5,6-EET was cyclooxygenase-dependent, required activation of the thromboxane receptor and an intact endothelium. Elimination of the renal vasoconstriction to 5,6-EET by endothelial removal provides evidence that the endothelium metabolizes 5,6-EET via the cyclooxygenase pathway and/or is stimulated to produce a cyclooxygenase vasoconstrictor metabolite ***(67)***. Additionally, Fulton et al. ***(68)*** have demonstrated that incubation of 5,6-EET with washed rat platelets yields cyclooxygenase dependent products that vasoconstrict the isolated perfused rat kidney. In contrast, adventitial administration of 11,12-EET and 14,15-EET elicited endothelium- and cyclooxygenase-independent vasodilation of the renal preglomerular vasculature, whereas the predominant epoxide hydrolase metabolite, 11,12-DIHET, did not have a significant effect on the preglomerular vascular tone ***(67)***. These results suggest that 11,12-EET acts directly on vascular smooth muscle cells and does not act via stimulation of cyclooxygenase metabolites nor is it metabolized to a vasoactive product via the cyclooxygenase pathway. In the dog, 11,12-EET has been shown to dilate renal arteries and activate Ca^{2+}-activated K^+ channels ***(69)***. Furthermore, the action of EET on renal vessels and Ca^{2+}-activated K^+ channels are consistent with the possibility that these metabolites are endothelium-derived hyperpolarizing factors (EDHF). Epoxygenase metabolites have recently been putatively identified to be EDHF ***(70,71)*** and EDHF appears to mediate a large portion of the vasodilatory response to bradykinin in the isolated perfused kidneys ***(72)*** (**Fig. 3**).

Coronary arteries have the capacity to generate EETs and these epoxygenase metabolites vasodilate the heart vasculature ***(24,73,74)***. In freshly isolated coronary artery smooth muscle cells, 11,12-EET causes membrane hyperpolarization and in the cell-attached mode of patch clamp both 14,15-EET and 11,12-EET activated Ca^{2+}-activated K^+ channels ***(70)***. 11,12-EET activates the Ca^{2+}-activated K^+ channels by a G_s protein-mediated mechanism and is independent of an increase in cAMP or cGMP tissue content ***(75)***. The effect of 11,12-EET on K^+ channels appears to be selective for the Ca^{2+}-activated K^+ channel since the delayed rectifier K^+ channels were unaffected by 11,12-EET ***(76)***.

The contribution of EETs to the control of cerebral blood flow has also been investigated. As mentioned earlier both the cerebral vasculature and surrounding tissue have the capacity to generate epoxygenase metabolites ***(13–15)*** and these EETs vasodilate the cerebral vasculature ***(14,15,77)***. Likewise, inhibition of EET production by administration of miconazole into the subdural space decreases cerebral blood flow ***(78)***. All EET regioisomers have been shown to

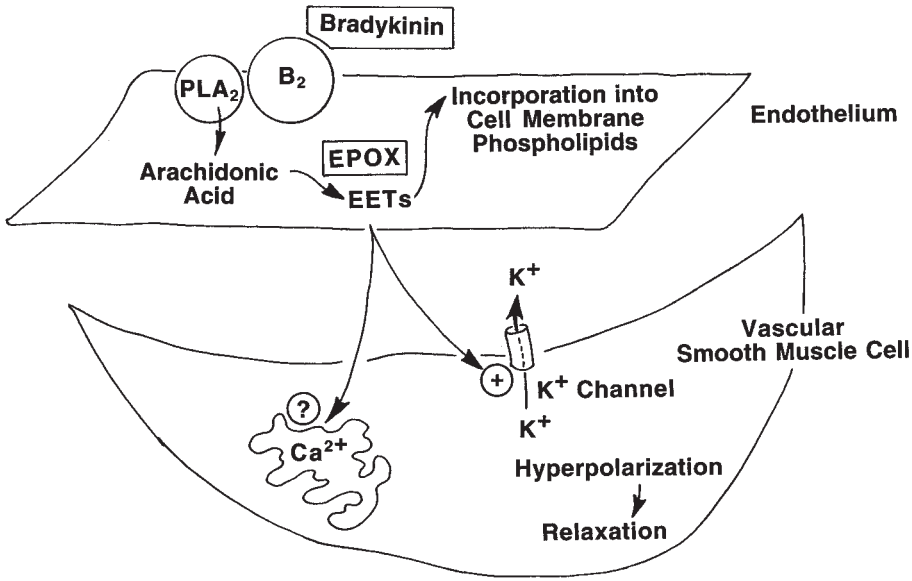

Fig. 3. Action of EETs on vascular smooth muscle tone. Vascular smooth muscle activation of K^+ channels, hyperpolarization and relaxation by endothelial-derived EETs in response to bradykinin is consistent with the indentification of EETs as endothelium-derived hyperpolarizing factors (EDHF).

dilate cerebral arteries of a number of species. Similar to the renal and coronary circulation, the vasodilatory action of EETs on the cat cerebral vasculature is associated with activation of Ca^{2+}-activated K^+ channel activation ***(15,79)***. The epoxygenase metabolite 5,6-EET elicits vasodilation of pial arterioles via cyclooxygenase-dependent oxygen radicals ***(77)***. The cerebral microvasculature EET-induced vasodilation of the newborn pig requires an intact prostanoid system and prostacyclin receptor activation ***(80)***. The specific roles of the EETs produced by astrocytes and the cerebral vasculature on the control of regional cerebral blood flow remain unknown.

The contribution of epoxygenase metabolites to other vascular beds has also been investigated but the mechanisms of EET actions have not been characterized. Although the vasodilatory actions of EETs was first described in the intestinal vasculature ***(81)***, only recently has a cytochrome P450 metabolite been suggested to contribute to the acetylcholine hyperpolarization and vasodilation of the mesenteric artery ***(82)***. Even though 11,12-EET caused membrane hyperpolarization, the acetylcholine-induced hyperpolarization of the rat mesenteric artery is not affected by cytochrome P450 inhibition ***(83)***. The actions of EETs on the rat hepatic artery ***(84)*** are endothelium-dependent and together with the

effect of cytochrome P450 inhibitors on the rat portal vein ***(85)*** do not support the concept that EETs are EDHF. In contrast, a recent study in the rabbit carotid artery showed that cytochrome P450 inhibition attenuated the NO/PGI_2-independent vasodilation to acetylcholine ***(86)***. EETs have also been shown to be activated by depletion of intracellular cell Ca^{2+} pools and mediate hyperpolarization of cultured endothelial cells ***(87)***. Further investigations will be needed to clearly determine whether or not EETs are indeed EDHF.

Even though previous studies have provided evidence that cytochrome P450 metabolites may modulate responses to hormonal and paracrine agents, little is known about the vascular interactions between these vasoactive compounds and metabolites of the cytochrome P450 pathway. Vasopressin has been demonstrated to increase renal effluent cytochrome P450 metabolites ***(88)*** and in renal mesangial cells the vasopressin-induced increases in cytosolic calcium are amplified by epoxygenase metabolites ***(89)***. Additionally, cytochrome P450 inhibition reduced the magnitude of the mesangial cell ***(90)*** and vascular smooth muscle cell ***(91)*** increase in calcium in response to vasopressin. In the isolated perfused rat kidney, the renal vasoconstrictor response to vasopressin was attenuated by the cytochrome P450 inhibitor, 7-ethoxyresorufin ***(88)***. Using the in vitro juxtamedullary nephron preparation, Imig and Deichmann ***(92)*** demonstrated that inhibition of the cytochrome P450 pathway with either miconazole or 17-ODYA enhanced the afferent arteriolar response to angiotensin II but was without affect on the vascular response to norepinephrine. Since miconazole selectively inhibits the epoxygenase pathway, the results of this study suggested that EETs may counteract the vasoconstrictor actions of angiotensin II. Likewise, the epoxygenase metabolites, 11,12-EET and 14,15-DHET, have been demonstrated to potentiate the bradykinin-induced vasodilation of porcine coronary arteries ***(74)***. Thus, the exact roles of specific cytochrome P450 metabolites involved in the vascular responses to vasoactive agents remains unclear.

5. Future Directions

Evidence is emerging that P450 epoxygenase metabolites modulate responses to hormonal and paracrine agents. The diverse actions of epoxygenase metabolites that include effects on calcium mobilization from intracellular stores and modulation of plasma membrane ion channels allow for interaction with other signaling components. EETs and DHETs diffuse easily across cell membranes and may function in an autocrine or paracrine fashion. The onset of hormonal-induced arachidonic acid release and EET production lags behind biosynthesis of short-term second messengers, such as phospholipase C, but precedes long-term signaling events, such as protein kinases. The incorporation of EETs and DHETs into the phospholipid of cell membranes of endothelial cells ***(26,27)***,

vascular smooth muscle cells ***(28)*** and astrocytes has recently been demonstrated ***(93)*** suggesting that epoxygenase metabolites may have long lasting membrane effects or can be released following hormonal activation. Thus, epoxygenase metabolites may act as cell signaling messengers which modulate the activity of sustained long-term protein kinase signaling.

The study of the biological actions of cytochrome P450 enzymes and epoxygenase metabolites has been difficult to determine experimentally. Because of the multitude of cytochrome P450 enzyme isoforms capable of producing EETs it has been difficult to develop very selective inhibitors. Additionally, blockade of one arachidonic acid metabolic pathway may alter the amount of arachidonic acid available for utilization by other pathways. Thus, one cannot exclude the possibility that responses to epoxygenase inhibition are due to increased production of other arachidonic acid pathways. Another problem is that EETs can be metabolized and it is difficult to determine whether the responses are due to the action of the administered EETs. Indeed, dihydroxy-hexadecadienoic acids (DHHD) have recently been identified as an epoxygenase metabolite that may contribute to the 11,12-DHET-induced coronary artery vasodilation ***(94)***. The development of stable unmetabolizable analogues will allow for a better assessment of the biological actions of the different epoxygenase metabolites.

In the years to come, various cytochrome P450 enzymes capable of generating epoxygenase metabolites will be identified and their organ localization established. This will undoubtedly lead to numerous studies of the regulation of these enzymes during disease and pathophysiological states. Currently the quantitative measurement of EETs and DHETs requires a combination of HPLC and GC/MS methodology. If immunological assay techniques can be developed to measure EETs and DHETs it would greatly increase investigators ability to easily measure these metabolites. Development of mice with specific cytochrome P450 epoxygenase enzymes knocked out will be another area of investigation that should greatly expand in the next five years. Lastly, the ability to design a cytochrome P450 enzyme to selectively produce a regio- and stereo-specific EET has recently been demonstrated for the P450BM-3 enzyme ***(95)***. This technology can be used to selectively overexpress various EETs in cell culture systems and possible in vivo. The incorporation of these exciting novel technologies will enable investigators to better understand the many biological roles of epoxygenase metabolites.

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14

Assessment of the Expression of Prostaglandin Synthase-2 in Swiss 3T3 Fibroblasts

Srinivasa T. Reddy and Harvey R. Herschman

1. Introduction

Prostaglandin synthases are key enzymes in the prostanoid biosynthetic pathway. Arachidonic acid released from membrane phospholipid stores by the activity of phospholipases is converted to PGH2, a common precursor of prostaglandins, by the enzymatic activity of prostaglandin synthase (PGS). There are two forms of PGS enzymes; PGS1 and PGS2. PGS1 is constitutively expressed in almost all cell types, whereas PGS2 is induced in a number of cell types by appropriate stimuli. This chapter describes methods used for the detection of PGS2 expression by western and northern analyses in murine Swiss 3T3 fibroblasts. These methods have also been used successfully to detect PGS2 expression in a number of other murine cell lines (**Table 1**).

2. Materials

2.1. Western Analysis

1. Swiss 3T3 fibroblasts or other murine cell line (*see* **Table 1**).
2. TPA (Chemsyn Science Laboratories, Lenexa, KS) or other inducers appropriate for the cell type being examined.
3. Protein electrophoresis and transfer apparatus, supplied by Bio-Rad Laboratories (Hercules, CA).
4. Nitrocellulose paper, 0.45 µm pore size (Intermountain Scientific Corporation, Keene, NH).
5. Phosphate-buffered saline (PBS); Make 1 L containing 10 m*M* NaH_2PO_4, 150 m*M* NaCl adjusted to pH 7.4 using NaOH.
6. PGS2 polyclonal antibody (Cayman Chemicals, [Ann Arbor, MI] cat. no. 160106).
7. Goat antirabbit IgG conjugated to horseradish peroxidase (Sigma, St. Louis, MO).
8. Chemiluminescence reagents for western blot detection (ECL kit) (Amersham, Arlington Heights, IL).

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Table 1
Induction of Murine Cell Lines

Murine cells in which PGS2 is induced	Inducer	Reference
RAW 264.7 (macrophages)	LPS	***1***
MMC-34 (mast cells)	IgE-anti IgE	***2***
Bone marrow derived mast cells	IgE-anti IgE	***3***
Peritoneal macrophages	LPS, Adrenelectomy	***4***
Primary embryonic neuronal cultures	BDNF, NT-3	***5***
Murine embryonic fibroblasts	TPA, TGF-beta	***6***
3T3 Fibroblasts	TPA, Serum	***7***

9. PBS/10% nonfat dried milk; make 50 mL using PBS and 5 g of nonfat dried milk.
10. PBS/1% nonfat dried milk; make 500 mL using PBS and 5 g of nonfat dried milk.
11. SDS gel loading buffer: 50 m*M* Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 m*M* dithiothreitol, and 0.2% bromophenol blue. The buffer without DTT can be stored at room temperature. DTT is added just before use from a 1 *M* stock.
12. Protein gels: Precast gels can be obtained from Bio-Rad (cat. no. 161-0907). These will readily fit the Bio-Rad minigel apparatus. Alternatively, protein gels should be prepared.
13. 10% resolving gel: In order to pour two 0.75-mm thick gels prepare 10 mL of 10% resolving gel; mix 4.0 mL of water, 3.3 mL of 30% acrylamide mix, 2.5 mL of 1.5 *M* Tris, pH 8.8, 0.1 mL of 10% SDS, 0.1 mL of 10% ammonium persulfate, and 0.004 mL of TEMED (TEMED should be added just before pouring the gel, since this catalyzes the polymerization of the polyacrylamide. The gel will solidify if not poured immediately).
14. 5% stacking gel: When the resolving gel has solidified, overlay stacking gel on top of it. Prepare 2 mL of stacking gel; mix 1.4 mL of water, 0.33 mL of 30% acrylamide mix, 0.25 mL of 1 *M* Tris, pH 6.8, 0.02 mL of 10% SDS, 0.02 mL of 10% ammonium persulfate, and 0.002 mL of TEMED. Place a 0.75 mm comb (for wells) on the gel before pouring the stacking gel.
15. Tris-Glycine buffer (5X): Mix 15 g of Tris-HCl and 72 g and make up to 1 L.
16. Running buffer: 1X Tris-glycine buffer and 0.1% SDS. Prepare 1 L.
17. Transfer buffer: 1X Tris-glycine buffer and 20% methanol. Prepare 1 L.
18. Kodak scientific imaging film from Eastman Kodak Company.
19. Protein molecular weight markers (Amersham, RPN 756).
20. Power supply. Model EC105 from Bio-Rad is suitable or any power supply used in the laboratory that has a capacity of 200 V and 500 mA.

2.2. Northern Analysis

1. Swiss 3T3 fibroblasts or other murine cell lines (*see* **Table 1**).
2. TPA, from Chemsyn Science Laboratories (Lenexa, KS) or other inducers appropriate for the cell type being examined.

3. RNA isolation kit (Qiagen, Santa Clarita, CA, cat. no. 74104).
4. Gel electrophoresis apparatus supplied by Bio-Rad Laboratories.
5. SDS: Add 50 g of SDS to 450 mL of water, mix (heat a little if necessary, to dissolve), and make up to a final volume of 1L. This can be stored as is at room temperature.
6. 10X MOPS Buffer: Mix 4.1 g of sodium acetate, 46.25 g of Na^+ MOPS (Sigma), 3.36 g of EDTA, and 1.2 g of NaOH, and make up to 1 L. Adjust the pH to 7.0 with NaOH. This buffer is light sensitive and should be stored in a brown bottle, at room temperature.
7. Oligolabeling kit from Pharmacia Biotech (Alameda, CA), cat. no. 27-9250-01. This kit contains all the reagents necessary for labeling cDNA, except for the radioactive [α-^{32}P] dCTP (3000 Ci/mmol), which can be obtained from NEN (Boston, MA). Each labeling reaction uses 50 µCi of radiolabeled dCTP.
8. DEPC-treated water: DEPC can be obtained from Sigma (D-5758) in liquid form. Add 0.5 mL of DEPC to 500 mL of water and autoclave.
9. 20X SSC: 3 *M* sodium chloride and 0.3 *M* sodium citrate. Make 2 L.
10. Salmon sperm DNA (SS DNA): Make a 10 mg/mL final stock solution in water. Before making the stock, sonicate the SS DNA for 4 min at 50% duty cycle. The SS DNA should then be run on a gel and tested for size, which should be between 200–600 bp. At this point dilute to 10 mg/mL stock, aliquot, and freeze at –20°C.
11. Nylon membrane: Hybond™-N, from Amersham.
12. Agarose gel: Add 1.65 g of agarose (Sigma) to 110 mL of DEPC-treated water and heat in a microwave until the agarose is melted uniformly. Let the contents cool to approx 50°C and add 15 mL of 10X MOPS and 25 mL of formaldehyde. Mix immediately and pour the gel. This is enough to pour a 15 cm/20 cm gel or two 7.5 cm/10 cm gels.
13. PGS2 cDNA: can be obtained from Oxford Biomedical Research (Oxford, MI).
14. RNA molecular weight markers, can be obtained from Gibco-BRL (Long Island, NY).
15. Hybridization oven from Labnet (Woodbridge, NJ).
16. RNA sample loading buffer: To make a total of 1 mL add the following: 1 µL of ethidium bromide, 670 µL of DEPC-treated H_2O, 100 µL of 10X MOPS, 150 µL of formaldehyde, 80 µL of gel-loading dye. Gel-loading dye contains 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in water.
17. Hybridization mix: To make 20 mL of hybridization mix add the following; 10 mL of deionized formamide, 1 mL of 5 *M* NaCl, 2.8 mL of 10% SDS, 5 mL of 1 *M* sodium phosphate buffer, pH 6.8, and 0.8 mL of DEPC-treated water. In a separate Eppendorf tube boil 0.4 mL of 10 mg/mL SS DNA (*see* **item 10**) for 5 min and immediately chill on ice for 5 min. Add this 0.4 mL of SS DNA to the hybridization mix just before starting the prehybridization step.
18. Deionized formamide is prepared by adding Dowex XG8 (Bio-Rad) mixed-bed resin and stirring on a magnetic stirrer for 1 h. The solution is then filtered twice through Whatman No. 1 filter paper. Deionized formamide can be stored in small aliquots at –20°C.

19. Wash solutions: 2X SSC, 0.5% SDS; make 500 mL, and 0.1 X SSC, 0.1% SDS; make 100 mL.
20. Power supply: Model EC105 from Bio-Rad is suitable.

3. Methods

3.1. Western Analysis

1. Plate Swiss 3T3 cells (1×10^6 cells/10 cm plate) in DMEM/10% FBS medium and grow to confluence (usually takes 2 d). Change the medium to DMEM/0.5% FBS the day before the experiment (overnight).
2. On the day of the experiment, wash cells with 1X PBS once and add fresh DMEM/0.5% FBS containing 50 ng/mL of TPA. One plate is treated with vehicle alone as a negative control (uninduced).
3. At a predetermined time point, anywhere between 0–24 h after TPA induction, wash the cells twice with 1X PBS and carefully aspirate any remaining PBS on the plate. Add 200 µL of the SDS gel loading buffer, and scrape the cells off the plate. Collect the cell lysate into an Eppendorf tube and boil for 10 min by placing in a boiling water bath.
4. Prepare 10% polyacrylamide gel (as described in **Subheading 2.1.**) and set up in the electrophoresis apparatus. Fill the electrophoresis tanks with 1X running buffer. Load approx 50 µg of protein sample per lane. Molecular weight markers should be loaded in one lane. Rainbow markers (Amersham, RPN 756) will be ideal for this experiment.
5. Apply a voltage of 8 V/cm until the protein band moves into the resolving gel. Then increase the voltage to 15 V/cm. Run the gel for approx 2 h or until the blue dye just crosses the bottom of the resolving gel.
6. Prepare 1X transfer buffer and cut nitrocellulose membranes to the required size. Once the electrophoresis is completed, remove the gels carefully from the glass plates, cut off the stacking gel, and soak the resolving gel and the nitrocellulose membranes in 1X transfer buffer for 10 min.
7. Set up the transfer using the Bio-Rad apparatus.
8. Remove the membranes after the transfer, and put them into a container containing the blocking solution (PBS/10% nonfat dried milk), for 1 h.
9. After blocking, wash the membranes three times (5 min each), in PBS/1% milk.
10. Incubate the membrane for 2–3 h (with constant shaking) with primary antibody (anti-PGS2). Dilute the primary antibody in PBS/1% milk. The antibody from Cayman Chemicals works well at 1:100 dilution.
11. Wash the membrane three times (5 min each) in PBS/1% milk.
12. Incubate the membrane for 1 h with secondary antibody, antirabbit IgG conjugated to horseradish peroxidase. Dilute the secondary antibody in PBS/1% milk. The dilution is based on the manufacturers suggestions, is usually between 1:4000–1: 8000.
13. Wash the membrane three times, 10 min each, in PBS/1% milk.
14. Blot the membrane on a filter paper. Make sure it is not completely dry. Place the membrane on a smooth surface (Saran Wrap) protein face up. Mix the substrate

reagents supplied with the ECL kit (1:1). Add the cocktail to the top of the membrane and incubate for 1 min.

15. Blot the membranes on to a filter paper in order to get rid of excess substrate mix. Cover the membrane in a Saran Wrap making sure there are no air bubbles.
16. Expose the membrane to X-ray film and develop. Different exposures can be done on the same film (10 s, 30 s, and 1 min).
17. Analyze the result by comparing the bands (induced vs uninduced) and checking the molecular weight markers.

3.2. Northern Analysis

1. Start a culture of Swiss 3T3 cells (1×10^6 cells/10 cm plate) in DMEM/10% FBS medium and grow to confluence (usually takes 2 d). Change the medium to DMEM/0.5% FBS the day before the experiment (overnight).
2. On the day of the experiment, wash cells with 1X PBS once and add fresh DMEM/0.5% FBS containing 50 ng/mL of TPA. One plate is treated with vehicle (acetone) alone as a negative control (uninduced).
3. At a predetermined time point, anywhere between 1–8 h after TPA induction, wash the cells twice with 1X PBS and carefully aspirate any remaining PBS on the plate. Add 250 µL of the RLT buffer supplied with Qiagen total RNA isolation kit. Scrape the lysates into sterile (RNase free) 1.7-mL Eppendorf tubes.
4. Follow the simple instructions of the RNEasy kit (Qiagen) and isolate total RNA. This procedure can be performed in <1 h. Quantify the RNA on a spectrophotometer, by obtaining the optical density (OD) at 260 nm. Usually, the purity of the RNA is also checked by looking at the OD_{260}/OD_{280} ratio. A ratio of 1.8–2.0 is desirable.
5. Take appropriate volume of RNA (10 µg of each sample) and add an equal volume of RNA loading buffer (*see* **Subheading 2.2.**) to it. The total volume should be kept under 20 µL. If the initial volume of RNA sample is more, it should be either precipitated in ethanol and resuspended in a smaller volume or alternatively the sample could be lyophilized in a Speed-Vac.
6. Prepare the agarose gel as described in **Subheading 2.2.**, and load the samples. Use RNA molecular weight markers for size determination.
7. Perform electrophoresis at 10 V/cm. Stop the electrophoresis when the faster migrating dye has reached the bottom of the gel (usually takes <3 h).
8. Remove the gel and take a photograph under UV illumination. The 28S and 18S RNA species should be clearly visible at this time. Intact total RNA preparations have 28S/18S ratio of 2. This can be checked visually from the photograph.
9. Prepare 1 L of 10X SSC and pour into a horizontal tray. Soak the gel and the nylon membranes (for transfer) in 10X SSC for approx 10 min. This step ensures equilibration of the gel and the membrane to the buffer (10X SSC).
10. Set up capillary transfer of RNA to the nylon membranes. Transfer can be done anywhere from 4 h to overnight.
11. Remove the membrane after the transfer and place it in a Saran Wrap or between two 3 *M* Whatman papers. RNA is then immobilized either by UV-crosslinking

or by baking the membrane for 2 h at 80°C. Once the RNA is immobilized, the membranes can be wrapped in Saran Wrap and stored at –20°C.

12. From now on, extreme caution and care has to be taken since the following steps involve use of radioactivity. It is important to be trained in handling radioactivity before starting the next part of the experiment.
13. Prepare the hybridization mix as described in **Subheading 2.2.** Place the membrane in a bottle (supplied with the hybridization oven), add 10 mL of the hybridization mix, and incubate in the hybridization oven (with shaker on) at 42°C for 2–4 h. For alternative methods, *see* **Note X**.
14. While the prehybridization is going on, prepare the ^{32}P labeled PGS2 cDNA probe. Follow instructions from the oligolabeling kit for making the probe. Briefly, the PGS2 cDNA is denatured by boiling for 5 min and immediately cooled (in order to prevent reannealing of the denatured DNA) on ice for an additional 5 min. Set up a 50-µL reaction containing 20–50 ng of PGS-2 denatured (single-stranded) cDNA, 10 µL of reaction mixture supplied with the kit, 50 µCi of ^{32}P-labeled dCTP (NEN) and 1 µL of the Klenow fragment (supplied with the kit) and incubate at 37°C for 1 h.
15. After the incubation period, purify the labeled cDNA from the incorporated radioactive nucleotides (optional, please *see* **Note X**), boil the labeled probe for 5 min and immediately cool on ice for 5 min. This facilitates converting the labeled probe into a single stranded form. Add the labelled probe to the bottle containing the membrane and hybridization mix, and incubate in the oven for another 12 h (usually over night).
16. At the end of hybridization, remove the membrane and wash it three times, 10 min each, in 2X SSC, 0.5% SDS, at room temperature. Wash the membrane for a further 30 min in a second wash solution (0.1X SSC, 0.1%SDS) at 65°C.
17. Remove the membrane, dry it briefly on a 3 *M* Whatman paper (do not dry completely). Put the membrane in a saran wrap, and place it in an autoradiography cassette and expose it to X-ray film.
18. Develop the film after 12 h or more and analyze the result.

4. Notes

4.1. Western

1. A number of reagents can be used for blocking of nonspecific binding sites on nitrocellulose membranes. BSA, fetal bovine serum, hemoglobin, gelatin and nonfat dried milk are some of the commonly used. Nonfat dried milk is the most economical and works quite well. However, if a problem with background arises, it is helpful to include a nonionic detergent in the blocking as well as all the other washing solutions. Tween-20, at a final concentration of 0.2%, works well for this purpose. Some of the other nonionic detergents that can be used are Triton X-100 and Nonidet P-40.
2. The diluted primary antibody can be reused 2–3 times. After the incubation with primary antibody, before the washing steps, remove antibody-containing buffer and freeze at –20°C. This buffer can then be reused for another primary antibody incubation step.

3. It is important to mark the protein-side of the nitrocellulose membrane either before or just after the transfer. This is usually done by clipping a corner or marking with a pencil. In order to get consistent results, it is necessary to perform all the incubations and washes of the nitrocellulose membrane with the protein side facing up.
4. Using the Bio-Rad Mini-Protean gel apparatus, protein transfer can be set up at room temperature or at 4°C. It normally takes up to 3 h at room temperature, and over night at 4°C. In order to verify efficient transfer of proteins, stain the gel after transfer using Coomassie Blue or stain the membrane using Amido Black. Bio-Rad also supplies a "Transblot" cell, semidry transfer apparatus. This system is easier to set up and also requires much less time (30 min) for efficient transfer.

4.2. Northern

5. Extreme caution and appropriate precautions should be taken while handling the following chemicals used in northern analysis: Formaldehyde, ethidium bromide, Formamide, DEPC, and 2-mercaptoethanol. The user should consult the material safety data sheets before handling these chemicals.
6. Formaldehyde generates highly toxic vapors, therefore the RNA electrophoresis should be performed in a chemical fume hood. Although constant circulation of the running buffer is not necessary for formaldehyde-based RNA gel electrophoresis.
7. Hybridization step can also be performed in a regular shaking waterbath. The membranes are placed inside a Seal-a-Meal bag, with sufficient hybridization buffer and sealed. This bag is then placed between two glass plates and incubated in the shaking water bath, at 42°C. After the prehybridization, the membrane is removed and single-stranded labeled cDNA is added to the Seal-a-Meal bag through a small cut made in one corner. The bag is resealed, checked for leaks, and placed back in the shaking waterbath for further incubation. It is very important to remove all air bubbles from the bag before sealing.
8. After the oligolabeling, the reaction mixture contains ^{32}P-labeled cDNA as well as some unincorporated labeled nucleotides. The incorporated labeled nucleotides or oligonucleotides could lead to background signals. In order to avoid these background signals it is useful to purify the labeled cDNA away from the unincorporated nucleotides. This can be accomplished by passing the contents of the oligolabeling reaction mixture through a G-50 Sephadex column (equilibrated with water). All the unincorporated nucleotides and oligonucleotides are retained on the column while the cDNA molecules come off the column in the flow-through. Alternatively, Chroma Spin-30 columns supplied by Clontech (Palo Alto, CA) can be used to purify the labeled cDNA.
9. The nonspecific blocking of nylon membranes is critical in order to avoid background signals. The salmon sperm DNA should be sonicated and sized on a gel (200–600 bp). Moreover, salmon sperm DNA should be denatured (boiling and cooling steps) before adding to the prehybridization mix.
10. The stringency of the washes described here is quite adequate for detecting a clean PGS2 signal. However, in case of unexpected background bands the mem-

branes should be washed longer (usually an additional 30 min) in the second wash solution (0.1X SSC, 0.1% SDS) at 65°C.

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15

Assessment of 5-Lipoxygenase Activity and Cellular Distribution

Robert A. Lepley

1. Introduction

5-Lipoxygenase (5-LO) catalyzes the conversion of arachidonic acid to leukotriene mediators of inflammation ***(1,2)***. Leukotriene biosynthesis in intact cells involves redistribution of 5-LO from a soluble cellular compartment where it is inactive to the nuclear envelope where its association with an activating protein, termed FLAP, is required for activation and optimal catalysis ***(3)***. The mechanism and extent of this redistribution and activation varies among different leukocytes and the choice of agonist ***(4)***. Although the enzymatic importance of 5-LO in leukotriene biosynthesis is well documented, nonenzymatic properties of 5-LO that govern its cellular distribution ***(4)*** and participation in signal transduction cascades ***(5–7)*** have only recently been investigated. This combination of enzymatic and nonenzymatic cellular biology makes 5-LO a complex macromolecule to study in terms of its cellular distribution and activation. The following series of protocols provides a means for determining the distribution/redistribution and activity profile of 5-LO in resting and activated human polymorphonuclear cells ***(8)*** and granulocytic HL-60 cells ***(9)***. The protocols that are presented provide methodology pertinent to cellular fractionation to generate a cytosolic and membrane fraction; cellular fractionation to generate cellular and nuclear soluble and membrane fractions; determination of 5-LO activity by reversed phase HPLC; and determination of 5-LO cellular distribution by Western analysis.

2. Materials

2.1. Cellular Fractionation to Generate Cytosol and Membrane Fractions

1. Dulbecco's phosphate-buffered saline (DPBS): 0.5 m*M* $MgCl_2$, 2.7 m*M* KCl, 1.2 m*M* KH_2PO_4, 138 m*M* NaCl, 8.1 m*M* Na_2HPO_4. Prepare Ca^{2+}

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DPBS by adding 0.100 g/L anhydrous $CaCl_2$ to generate a final 0.9 m*M* Ca^{2+} concentration.

2. The calcium ionophor A23187, is an effective means of inducing 5-LO redistribution and activation in granulocytic cells. A 100 μ*M* working dilution of A23187 is prepared by adding 5 μL of a 20-m*M* A23187 DMSO stock (Sigma, C-7522, MW = 523.6) to 95 μL of DMSO. Immediately prior to the cell assay, this is brought to 100 μ*M* by adding 900 μL of Ca^{2+} DPBS. Add 25 μL of the 100-μ*M* stock to each assay tube to yield 2.5 μ*M* A23187 in the cell preparation.
3. Preparation of membrane buffer: 50 m*M* potassium phosphate, pH 7.1, 100 m*M* NaCl, 2 m*M* EDTA, 5 m*M* benzamidine.

   In the preparation of this reagent, benzamidine has been used in place of phenylmethylsulfonyl fluoride as a serine proteinase inhibitor in the 5-LO buffers (*see* **Note 1**).
4. Prepare 5X tracking dye: 5% (w/v) SDS, 250 m*M* Tris-HCl, pH 6.8, 50% (v/v) Glycerol, 10% (w/v) dithiothreitol, bromophenol blue (trace).

2.2. Cellular Fractionation to Generate Cellular Soluble/ Membrane and Nuclear Soluble/Membrane Fractions

1. Preparation of sucrose-TKM buffer: 250 m*M* sucrose, 50 m*M* Tris-HCl, pH 7.4, 25 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* EGTA, 1 m*M* dithiotheritol, 5 m*M* benzamidine, 1 μg/mL leupeptin.

2.3. Determination of 5-LO Activity by Reversed-Phase HPLC

1. Preparation of 5-LO assay buffer: 25 m*M* potassium phosphate, pH 7.3, 0.5 m*M* $CaCl_2$, 24 mg/mL L-α-phosphatidylcholine, 1 m*M* ATP. The potassium phosphate component of this buffer can be made from 25-m*M* solutions of mono- and dibasic solutions that are mixed to prepare a stock at pH 7.3. L-α-phosphatidylcholine (Sigma, #P-4279: 100 μg/μL in ethanol) should be pipetted into a sterile 50-mL disposable tube and the solvent removed under nitrogen. Once dried, add the phosphate buffer stock and shake vigorously to suspend the phosphatidylcholine. Add $CaCl_2$ and ATP (Sigma #A-2383) last. The 5-LO assay buffer should be prepared shortly before the assay and kept on ice until it is used.
2. The 5-LO substrate stock is prepared by addition of 5 μL arachidonic acid (Nu Chek Prep # U-72A) into 297 μL absolute ethanol. This stock is 50 nmoles/μL. Dilute the 50 nmole/μL stock 1:5 in absolute ethanol to generate a 10 nmole/μL 5-LO substrate stock (*see* **Note 2**).
3. Lipid standards for RP-HPLC analysis standards should be prepared just prior to analysis. The mixture should contain 1 nmole PGB_1, 1 nmole LTB_4, and 1 nmole 5-HETE. Standards can be prepared in 100 μL HPLC grade methanol, 100 μL HPLC grade water, and 10 μL 10% (v/v) acetic acid. Standards can be purchased from established lipids suppliers such as Cayman, Oxford or BioMol.

2.4. Determination of 5-LO Cellular Distribution by Western Analysis

1. Preparation of Towbin Transfer Buffer (TTB): 25 m*M* Tris, 192 m*M* glycine, 10% (v/v) methanol. The Tris buffer component should be made by adding

3.03 g/L Tris base (TRIZMA Base, Sigma T-8404). Proteins with molecular weights >50 kDa may not transfer efficiently in the Towbin Tris/glycine buffer at 20% methanol. The concentration of methanol in the 5-LO transfer buffer has been reduced to 10% (v/v) to improve the transfer efficiency of the 78 kDa 5-LO protein. The buffer should be prepared well ahead of time so that it is equilibrated at 4°C for the transfer. Transfer buffer can be made in large quantities and stored at 4°C for several months (*see* **Note 3**).

2. The nitrocellulose membrane used in the 5-LO transfer is Schleicher and Schuell OPTITRAN BA-S 83 supplied as a 30 cm × 3 m roll and cut to fit the dimensions of the SDS-PAGE separating gel. Gloves should be worn at all times while handling the membrane to prevent the transfer of proteins to the membrane. Similarly, gloves should be worn during the preparation of Whatman 3MM (or equivalent) filter paper used in the 5-LO transfer.
3. Prepare Tween based TBS (TTBS): 50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 0.1% (v/v) FSG, 0.05% (v/v) Tween-20. Fish Skin Gelatin (FSG) is available as HIPURE LIQUID GELATIN (Norland Products, New Brunswick, NJ). Fish skin gelatin is an effective and economical blocking agent that is supplied in liquid form by the manufacturer. It does not solidify at reduced temperatures and does not require heating to solubilize the reagent in preparation of TTBS. In the preparation of 0.1% (v/v) Triton based TBS (TxTBS) add 1 mL/L Triton X-100 to TTBS.
4. If the 5-LO nitrocellulose filter is to be probed with a second primary antiserum, the filter can be washed with membrane stripping buffer to remove the 5-LO primary and secondary antibodies. Membrane Stripping Buffer, pH 2.2: 200 m*M* Glycine, 0.1% (w/v) SDS, 1% (v/v) Tween-20. To prepare this reagent, add the glycine to 200 mL water and adjust the pH to 2.2 with HCl. Add the remaining components and adjust the volume to 250 mL with water.

3. Methods

3.1. Cellular Fractionation to Generate Cytosol and Membrane Fractions

When granulocytic cells are activated, appreciable quantities of 5-lipoxygenase redistribute to the nuclear envelope ***(10)***. A straightforward means of following 5-LO redistribution is to prepare a 100,000g_{ave} soluble/microsomal fraction from a cell homogenate. The following section provides a basic protocol for this analysis. **Steps 1–7** deal with the preparation and assay of either PMN or granulocytic HL-60 cells (American Type Culture Collection #CCL-240) and **steps 8–16** deal with the preparation of the cytosol and membrane fractions. Processing human PMN and HL-60 cells presents a possible source of pathogen exposure to the investigator. Appropriate protective clothing (lab coat, gloves, facemask, or shield) should be used. Samples should be capped or sealed to prevent aerosol formation during centrifugation and sonication. Proper decontamination and disposal procedures should be followed.

1. Recover the cultured cells from T-flasks by pooling them in disposable, sterile 50-mL conical centrifuge tubes.
2. Pellet the cell preparation at $300g_{ave}$ for 10 min at ambient temperature.
3. Discard the resulting supernatant and suspend the cell pellet in Ca^{2+} free Dulbecco's phosphate-buffered saline (DPBS), pH 7.4. Pellet the cell suspension at $300g_{ave}$ for 10 min at ambient temperature.
4. Discard the resulting supernatant and resuspend the cell pellet in DPBS, pH 7.4, containing 0.9 m*M* Ca^{2+}. Pellet the cell suspension at $300g_{ave}$ for 10 min at ambient temperature (*see* **Note 4**).
5. Count the cells and adjust the volume with 0.9 m*M* Ca^{2+} DPBS to achieve a final cell concentration of 2×10^7 cells/mL.
6. Carry out the cell assay according to the experimental design using the cell assay protocol outlined below.
   a. The cell assay is carried out in sealable glass tubes. Either 13 × 100 mm, plug seal tubes (Fisher #14-962-10C) or screw cap culture tubes (Fisher #14-962-26D) work well. It is important to use sealable glass tubes to permit solvent extraction of the 5-LO assay products after the assay has been completed.
   b. If the protocol requires that a specific drug treatment is to be used, add the treatment prior to addition of cells using a Hamilton type glass syringe.
   c. Cells (2×10^7/mL) are added to the assay tube using a Gilford pipet. Cut approx 1–2 mm off of end of the blue tip to facilitate cell delivery and to avoid injury to the cells.
   d. Gently vortex the cells for 10 s to disperse the cells and any treatment added as specified by the protocol.
   e. Incubate the assay tubes at 37°C.
   f. Following any required drug preincubation period, stimulate the cells with 2.5 μ*M* A23187 for 10 min. Gently vortex the samples at 2–3 min intervals to maintain a uniform cell suspension.
   g. When the assay is completed, quench each assay tube by addition of EDTA to chelate the available Ca^{2+}. Add 50 μL/mL of a 100 m*M* EDTA, pH 7.4, stock to generate a final concentration of 5 mM EDTA.
7. At this point the samples are independently processed for cytosol/membrane fractionation or for reversed-phase HPLC analysis (*see* **Note 5**).
8. Immediately following the assay, pellet the cells at $1000g_{ave}$ for 10 min at 4°C.
9. Discard the supernatant. Immediately freeze the cell pellet on dry ice.
10. Resuspend the cells in one-fifth of the original cell volume using ice-cold membrane buffer. From this point on, work as quickly as possible and keep the preparation ice cold. The cell suspension can be transferred to a 1.8-mL microfuge tube at this point.
11. Sonicate the thawed resuspended cells using a Branson 450 Sonicator equipped with a microprobe. A 20-s sonication should be repeated three times for a total of 60 s. The sonicator should be set at 75% duty cycle and a power setting of 3. Perform the sonication on ice and allow 30 s between pulses to prevent sample heating (*see* **Note 6**).

12. Pellet the sonicated samples at 12,000g_{ave} for 12 min at 4°C in a microfuge.
13. Recover the supernatant and centrifuge at 100,000g_{ave} for 60 min at 4°C to generate a microsomal and cytosolic fraction.
14. Carefully recover the supernatant and designate it "CYTOSOL". Wash the pellet once with 500 µL of ice-cold membrane buffer. Suspend the membrane pellet in 250 µL membrane buffer and transfer the fraction to a Potter-Elvejem micro tissue homogenizer that has been rinsed with cold membrane buffer and chilled on ice (0.5 mL Potter-Elvehjem type: Kontes KT886000-0018 or similar). Thoroughly homogenize the cell pellets using 10–20 strokes with the pestle. Designate this fraction "MEMBRANE" (*see* **Note 7**).
15. Determine the protein concentration of each sample by BCA assay (*see* **Note 8**).
16. Once the SDS-PAGE samples have been prepared and the protein concentrations determined, the samples can be fractionated by SDS-PAGE and the presence of 5-LO determined by Western analysis as described in **Subheading 3.4.** (*see* **Note 9**).

3.2. Cellular Fractionation to Generate Cellular Soluble/ Membrane and Nuclear Soluble/Membrane Fractions

The cellular 5-LO activity and redistribution profile of 5-lipoxygenase can be determined by refining the protocol presented in Subheading 3.1 to produce four cellular fractions: cell membrane, cell cytosol, nuclear membrane, and nuclear soluble fractions using an adaptation of the methodology presented in **ref. *3***.

1. Follow **Subheading 3.1.** through **step 7** to complete the desired cell assay. The cell assay conducted at this point provides an index of the intact cell 5-LO activity. Following the preparation of the individual cellular and nuclear fractions, each will be assayed for 5-LO activity (**step 9**) to determine the redistribution pattern of active 5-LO enzyme.
2. Pellet the cells at 300g_{ave} for 10 min. Based on the assay cell count, suspend the cell pellet at 2×10^7 mL in Sucrose-TKM buffer.
3. Disrupt cell preparation by nitrogen cavitation at 400 psi for 5 min at 4°C. Inspect the cavitate for complete disruption using a phase microscope with a 40X oil immersion objective lens. The nuclei should appear as phase dark spheres. If there are a substantial number of phase bright, intact cells, repeat the nitrogen cavitation holding the preparation at 400 psi for 2 min.
4. Pellet the nuclei at 1000g_{ave} for 30 min at 4°C.
5. Remove the supernatant fraction and retain the sample at 4°C for subsequent processing.
6. Suspend the nuclear pellet in ice-cold Sucrose-TKM at a cellular equivalent of 1×10^8 cells/mL based on the original assay volume. Sonicate the suspension of nuclei with a Branson model 450 sonicator equipped with a microprobe at a Power setting of 2 and 20% duty cycle. Hold the samples on ice during the sonication procedure. Subject each sample to 10 sonicator bursts (*see* **Note 6**).
7. Pellet the sonicated nuclei fractions and the supernatant fraction recovered in **step 5**. Centrifuge each fraction at 100,000g_{ave} for 30 min at 4°C.

8. Recover the following fractions: cellular soluble, C_s; cellular particulate, C_p; nuclear soluble, N_s; and nuclear particulate, N_p. Suspend the particulate fractions in 250 µL ice-cold Sucrose-TKM using microtissue homogenizer with 10–20 strokes (0.5 mL Potter-Elvehjem type: Kontes KT886000–0018 or similar). Perform all homogenizations on ice. As each fraction is recovered, prepare the SDS-PAGE samples using 5X Tracking Dye and boil 5 min (*see* **Note 7**). The quantity used to prepare the SDS/PAGE sample should be determined based on retention of 50 µL for 5-LO activity assay and 40 µL for a microprotein assay (*see* **Note 10**).
9. At this point the SDS-PAGE samples can be held on ice while the protein assay and enzyme assay are completed. The 5-LO enzyme activity of each fraction can be determined using the procedure presented in **Subheading 3.3.** Western analysis is carried out using the procedure presented in **Subheading 3.4.** (*see* **Note 9**).

3.3. Determination of 5-LO Activity by Reversed-Phase HPLC

1. 5-lipoxygenase enzyme assays are preformed in 1 mL of 5-LO assay buffer. Add 5-LO Assay buffer to 13 × 100 mm glass tubes and preincubate at 25°C. Add the 5-LO enzyme test fraction. Finally, add 2 µL arachidonic acid substrate (10 nmoles/µL) and vortex gently to start the reaction. The assay should be conducted in glass tubes that can be sealed. Threaded screw capped 13 × 100 mm culture tubes (Fisher #14-962-26D) or standard 13 × 100 mm glass tubes with plug seals work well (Fisher #14-962-10C).
2. After a two-minute incubation at 25°C, stop the reaction by the addition of 40 µL 1 *N* citric acid followed by 2 mL hexane/ethyl acetate (1:1). Add 1 nmole of PGB_1 standard. Extract the sample by shaking or vortexing vigorously for 60–90 s. Centrifuge the samples at $3000g_{ave}$ for 5 min to separate the aqueous and organic phases. Transfer the upper organic phase into a clean glass tube. Repeat the extraction two times, pooling the organic phases (*see* **Note 11**).
3. Dry the pooled extract under nitrogen. Add 100 µL HPLC grade methanol, 100 µL HPLC grade H_2O, and 10 µL acetic acid. Transfer this to a sterile 500 µL microfuge tube. Hold the samples at –20°C and centrifuge in a microfuge at full speed for 5 min immediately prior to injection to remove all debris (*see* **Note 12**).
4. Apply the fraction to a C_{18} column (Beckman Ultrasphere ODS, 250 × 4 mm). The HPLC system pump program is based on a flow rate of 1 mL/min and a binary solvent delivery module using as solvent A, water/0.1% (v/v) trifluoroacetic acid (TFA) and solvent B, 60%/40% (v/v) acetonitrile/methanol, 0.1% TFA. The pump program is 38% A/72% B for 18 min increasing to 76% B and continued for 12 min (30 min run time). Product elution is monitored at 280 nm to detect PGB_1 (11–12 min), nonenzymatic 5,12-diHETE products (13 min), and LTB_4 (14 min) followed by monitoring at 234 nm to detect elution of 5(S)-HETE at 25–27 min (*see* **Note 13**).
5. Based upon the standard sets, the integrated peak areas per nmole of each standard can be determined. Within each 5-LO assay fraction, by comparison of the PGB_1 internal standard integrated peak area to that of the PBG_1 standard set, the extraction efficiency for that sample can be calculated and used to correct the

integrated peak areas for the observed 5-LO products. The corrected 5-LO integrated peak areas can then be compared to their respective standard set and used to calculate the nmoles of 5-LO product formed. The 5-LO specific activity, nmoles/µg/min, can be calculated from the cell assay time and protein assay value for each fraction (*see* **Note 14**).

3.4. Determination of 5-LO Cellular Distribution by Western Analysis

The following section of this chapter deals with the transfer of proteins from a Laemmli gel to nitrocellulose filters. It is based on the procedure of Towbin et al. ***(11)*** and assumes that the 5-LO has been fractionated using the denaturing SDS-PAGE system of Laemmli ***(12)***. A 10% separating, and 4% stacking gel resolve the 78 kDa 5-LO into the middle portion of the gel when the tracking dye front is run to within 2–3 mm of the bottom of the separating gel. Load the gel samples into the stacking gel wells as quickly as possible after boiling. Load and run equal quantities of protein in each gel lane. A protein load of 5–20 µg/lane has been found to work well when probing for 5-LO using the LO-32, 5-LO antiserum (**step 6**).

1. Carefully disassemble the SDS-PAGE gel cassette. Place the gel in sufficient Towbin Transfer Buffer (TTB) to completely cover the gel and gently agitate for 30 min. After 15 min, replace the TTB with fresh buffer. Place a nitrocellulose membrane cut to the size of the gel in water for 5 min to thoroughly wet the filter and then replace the water with TTB (*see* **Note 3**).
2. Prepare the gel transfer sandwich by laying one Whatman 3MM filter paper moistened with TTB on a wetted scrubber supplied with the transfer apparatus. Be careful to exclude air bubbles from the layered system at all times. A convenient means of doing this is to flood the bottom of a Pyrex baking dish with TTB to a depth that will cover the sandwich. Carryout the assembly with each element submerged under TTB. Carefully place the SDS-PAGE gel over the 3MM paper, again excluding air bubbles. Overlay the gel with a precut sheet of nitrocellulose membrane excluding air bubbles. Overlay the existing sandwich with another sheet of moistened 3MM paper and finally, a scrubber pad.
3. Once the transfer sandwich is complete, use a transfer cassette as a spatula to lift the sandwich out of the assembly dish. Quickly place the assembled sandwich in the buffer reservoir so that the nitrocellulose filter faces the anode (usually marked as the RED electrode). Proteins will migrate out of the gel toward the anode during the transfer.
4. Apply current to the system. A minigel with "ice block" cooling can be transferred at 125–175 mA for 1–2 h. A larger tank system can be run at 40–50 V, 100 mA for 4–6 h or overnight at 4°C.
5. Following the transfer remove the nitrocellulose filter from the cassette and rinse it with water prior to blocking. Block the filter with 5% (w/v) nonfat dried milk for 2–12 h (*see* **Note 15**).

6. Wash the filter 1X for 15 min in TTBS at room temperature to remove excess blocking buffer. Incubate the blocked filter with the LO-32 5-LO specific primary antibody (*see* **Note 16**). The primary antibody is prepared at a dilution of 1:400 in 1% FSG TTBS. Incubate one hour at room temperature with gentle agitation. Following this incubation, do not discard the primary antiserum. The preparation can be saved and reused many times if the solution is brought to 1 m*M* NaN_3 and sterile filtered through a 0.2-µm syringe filter. Store the reagent at 4°C between uses (*see* **Note 17**).
7. Wash the filter three times, 20 min each with TTBS at room temperature.
8. Incubate for 1 h at room temperature with secondary antibody. A goat antirabbit secondary antibody conjugated to horseradish peroxidase (Bio-Rad 170–6515) at a 1:100,000 dilution (1 µL/100 mL) in TTBS 1% FSG provides a sensitive 5-LO detection system when used in conjunction with a chemiluminescent horseradish peroxidase substrate.
9. Wash the filter with TxTBS to remove nonspecific binding secondary probes. Wash 4 × 250 mL, 15 min each, and drain excess buffer. Incubate for 60 s in complete chemiluminescent reagent (Amersham ECL or Renaissance by NEN work well). After 60 s, place the wet filter on 3MM filter paper backing and wrap with Saran Wrap film or place between leaves of a 8.5 × 11 in. clear sheet protector on a sheet of 3MM filter paper backing. Load the wrapped filter into a suitable X-ray film cassette and expose the filter to X-ray film. Producing a desirable image may require exposure times as short as 10–15 s or as long as 30 min.
10. It is possible to re-probe the filter by stripping the initial antibody probes from the blot. Wash the blot 2 × 60 min in membrane stripping buffer. Equilibrate in TTBS and proceed with the Western protocol at the point of primary antibody addition. Optimization with respect to the initial blocking agent should be considered (*see* **Note 15**).

4. Notes

1. Although phenylmethylsulfonyl fluoride (PMSF) is an effective serine proteinase inhibitor, its effectiveness suffers from the fact that it is neither stable nor soluble in aqueous solutions. Benzamidine is also effective as a serine proteinase inhibitor. Unlike PMSF, benzamidine is both stable and readily solubilized in aqueous buffer systems. If the investigator feels that the inclusion of PMSF is desirable, it may be made as a 100 m*M* stock in ethanol and stored at –20°C. It should be added immediately prior to use because of its instability in aqueous solutions.
2. Arachidonic acid has a molecular weight of 304.2 g/mole and a density of 0.92 g/mL. The neat arachidonic acid stock is 3020 nmoles/µL. The manufacturer's vial of stock arachidonic acid should be opened and aliquoted into small glass vessels. The vessels should be flooded with nitrogen, tightly sealed, wrapped with teflon tape, and stored at –80°C. HPLC autosampler vials with microglass inserts and PTFE/silicon seal caps work well for this purpose.
3. Methanol is a toxic substance. Owing to the fact that transfer reagent is prepared in relatively large quantities and that the actual transfer methodology requires

frequent hand contact with this reagent, care should be exercised to protect the investigator's hands and eyes in addition to wearing a standard laboratory coat. In addition, federal and state regulations may exist that apply to the safe storage and disposal of this reagent.

4. If DMSO differentiated HL-60 cells are used, suspend the cell pellet in approx 1/25 of the original culture volume assuming that the original cell culture population was approx 1×10^6 cells/mL.
5. In determining the extent of 5-LO redistribution in cells, it is desirable to assay duplicate samples for each treatment group. This insures that 5-LO enzymatic activity in samples processed for reversed-phase HPLC can be matched to samples processed for Western analysis.
6. By starting each sonication cycle at a Power setting of 0 and quickly increasing to the desired setting, cavitation, and foaming of the sample can be avoided. If foaming occurs return the sample to the ice bucket and process the remaining samples. The foam will diminish and sonication can be completed. Prior to initiating the sonication the sonicator probe should be immersed in ice to ensure that it is at 4°C. Rinse the probe with ice cold buffer between each sample.
7. When the "Cytosolic" fractions are recovered, immediately mix a portion with 5X Tracking Dye and boil for 5 min. Similarly, as each "Membrane" fraction is completed, mix a portion with 5X tracking dye and boil for 5 min. By preparing the samples for SDS-PAGE in this manner, any proteolytic degradation of the sample is avoided. Hold the samples at 4°C while the protein assay is completed.
8. By performing a microtiter plate assay only small quantities of each sample are required. This is particularly important for determining the "Membrane" fraction protein concentration. Typically, the "Cytosolic" fraction is assayed without dilution and the "Membrane" fraction is diluted 5 to 10-fold to fall within the linear range of a 0–1000 μg BSA standard curve.
9. Prior to gel fractionation, boiled SDS-PAGE samples can be stored at –80°C for a short period of time. The 5-LO signal produced from samples that have been stored does deteriorate. After several weeks of storage the 5-LO signal may have deteriorated to levels that prevent detection.
10. The Sucrose-TKM buffer contains dithiothreitol that interferes with the BCA type protein assay system. A coomassie based assay (Coomassie Plus, Pierce Chemicals) works well in a microtiter plate format using 20-μL sample/well and 250 μL of coomassie reagent.
11. It is very important to add citric acid to the samples. This reagent serves to chelate calcium, thus stopping the 5-LO enzyme activity and to acidify the reaction mixture that protonates 5-LO reaction products. Protonation allows the 5-LO reaction products to be efficiently extracted from the aqueous phase. Occasionally a sharp interface fails to form between the aqueous and organic phases. This problem can be corrected by adding 1 mL of a 0.9% (w/v) NaCl solution and re-extracting the sample.
12. If it is not possible to process samples for RP-HPLC immediately, they can be stored for several weeks at –20°C as the extracted, pooled organic phase. Simi-

larly, samples prepared for RP-HPLC can be stored at –20°C for several days prior to RP-HPLC.

13. The HPLC program provided is designed to function with a very basic pump/detector system. Although elution times can be expected to vary based on column choice, column use and guard column use, the order of elution will remain unchanged.
14. Owing to the importance of the assay standard sets and the PGB_1 internal standard, these should be prepared very accurately. It is recommended that the concentration of the stock be calculated using the extinction coefficient for each lipid. Extinction coefficients are: PGB_1 λ_{max} 278 nm ε= 30,000; LTB_4 λ_{max} 236 nm ε = 27,000; 5(S)-HETE λ_{max} 278 nm ε= 50,000. All liquid transfers should be made using clean Hamilton glass syringes.
15. The efficiency of the transfer can be monitored if colored molecular weight markers were used during the SDS-PAGE. Alternatively, the spent gel can be silver-stained following the transfer. If the filter can not be probed immediately, it can be stored in water containing 1 m*M* NaN_3 at 4°C for several days. Although the use of nonfat dried milk as a blocking agent works well in the protocol presented in this chapter, if variations to the protocol are introduced other agents, such as BSA (fraction V), bovine gelatin, fetal bovine serum or Tween-20 alone, should be considered. Specific problems may be encountered if the 5-LO membrane is stripped and reprobed with a second primary antibody. If the membrane is to be reprobed with an antiphosphoamino acid antibody it should be kept in mind that certain nonfat dried milk formulation contain levels of phosphate that produce significant background. The supplier of the antibody should be consulted for specific recommendations regarding blocking agent selection.
16. The LO-32 anti 5-LO antiserum is a rabbit polyclonal antibody that has been secured from Merck-Frosst, Pointe Claire-Dorval, Quebec, Canada. The resolution of 5-LO using a 4% stacking gel/10% separating gel system has been found to be satisfactory with both standard and mini gel systems.
17. The addition of NaN_3 to the primary antibody aids in the suppression of microbial growth during storage. It should be noted that azide inhibits horseradish peroxidase. Inhibition of the secondary antibody horseradish peroxidase conjugate has not presented a problem when following the western protocol described for 5-LO detection.

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Leukotrienes, Lipoxins, and Hydroxyeicosatetraenoic Acids

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1. Introduction

Extravasation of neutrophils is an early and critical event during an acute inflammatory response. The process of emigration of leukocytes through the wall of an intact capillary has been described as a "flowing" between endothelial cells ***(1)***; i.e., the leukocyte reaches the perivascular space by ameboid movements. With an improved understanding of adhesion molecules and the cytoskeleton, the molecular basis for diapedesis is becoming clear. Furthermore, since diverse forms of tissue injury are to an extent neutrophil-dependent; e.g., ischemia-reperfusion injury, immune complex glomerulonephritis, Arthus pleurisy, and flares of colitis; research aimed at the molecular attachments between vascular endothelial cells ***(2)*** and transendothelial leukocyte migration ***(3–6)*** is a promising strategy for discovery of anti-inflammatory medications.

Eicosanoids contribute importantly to the regulation of endothelial junctions, and endothelial mechanisms will be a major focus of this chapter, particularly as it relates to the ability of lipoxygenase (LO) metabolites of arachidonic acid (AA) to influence neutrophil traffic across endothelium. The objective is to present a selective overview of an extensive literature on the biology and biochemistry of LO products. Although the discussion touches on various immunoinflammatory states, particular attention is given to consideration of how LO products may underlie leukocyte-mediated tissue damage in asthma and nephritis.

Lipoxygenase enzymes are ancient enzymes in an evolutionary sense, carrying out similar reactions in the plant and animal kingdoms. Indeed, a good deal of the fundamental information on LO biochemistry was acquired from studies conducted with plant enzymes, predating, in some instances, the work

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on mammalian lipoxygenases, e.g.; the finding that 5-LO activity and LTA_4 synthase activity resided in a single enzyme was based on work with potato 5-LO *(7)*, and detailed mechanistic studies of substrate/inhibitor binding to the 15-LO *(8,9)* were originally conducted with soybean 15-LO. By definition, LOs catalyze the oxygenation of polyunsaturated fatty acids (PUFA) containing a 1,4-cis, cis-pentadiene system; i.e., 2 cis double bonds separated by a methylene group. The primary products are monohydroperoxides, but the different LOs differ with respect to the specificity (regiospecificity) in directing the oxidation reaction, although this regiospecificity may not be absolute. Three mammalian LOs have been purified, cloned, and expressed. All are capable of the oxygenation of AA and other PUFA with the requisite 5 carbon unit. The initial oxygenation of AA leads to formation of either 5-S-HPETE (as determined with purified 5-LO from human leukocytes *[10]*; HPETE, abbreviation for hydroperoxyeicosatetraenoic acid), 12-S-HPETE (as determined with purified 12-LO from human blood platelets *[11]*), or 15-S-HPETE (as determined with purified 15-LO from human eosinophils *[12]* or platelets *[13]*). These HPETEs are highly reactive, short-lived intermediates, which are converted to monohydroxy eicosatetraenoic acids (HETEs) by cellular peroxidases, such as glutathione peroxidase.

Nearly 20 years after the initial structural elucidation of the 5-LO product leukotriene B_4 (LTB_4) *(10)*, leukotriene (LT) receptor antagonists and 5-LO inhibitors are entering the clinical arena as important therapeutic agents for the pharmacotherapy of asthma. Before elaborating on this clinical application, the biochemical work that established the proinflammatory nature of LTs will be considered.

2. The 5-LO Pathway and Leukotrienes

Shortly after the structure of LTB_4 was published, Murphy, Hammarstrom, and Samuelsson *(14)* proposed that the unstable 5,6-epoxide LTA_4 was an intermediate in the formation of LTB_4 as well as LTC_4. Further work by the Karolinska group *(15)* and by Austen and colleagues *(16)* established that LTC_4 was metabolized to LTD_4 by elimination of glutamic acid, and then to LTE_4 by elimination of glycine. Decades prior to clarification of this reaction sequence, investigators studying the immunopathogenesis of asthma had recognized that a lipid material of uncertain composition, "slow reacting substance of anaphylaxis (SRSA)", was released after antigen challenge of a sensitized lung. Because it was potent in causing bronchospasm, efforts began in many laboratories to determine the identity of SRSA. The laboratories of Samuelsson *(14)* and of Austen *(16)*, showed unambiguously that SRSA was a variable mixture of LTC_4, LTD_4, and LTE_4. These LTs are often referred to as sulfidopeptide leukotrienes

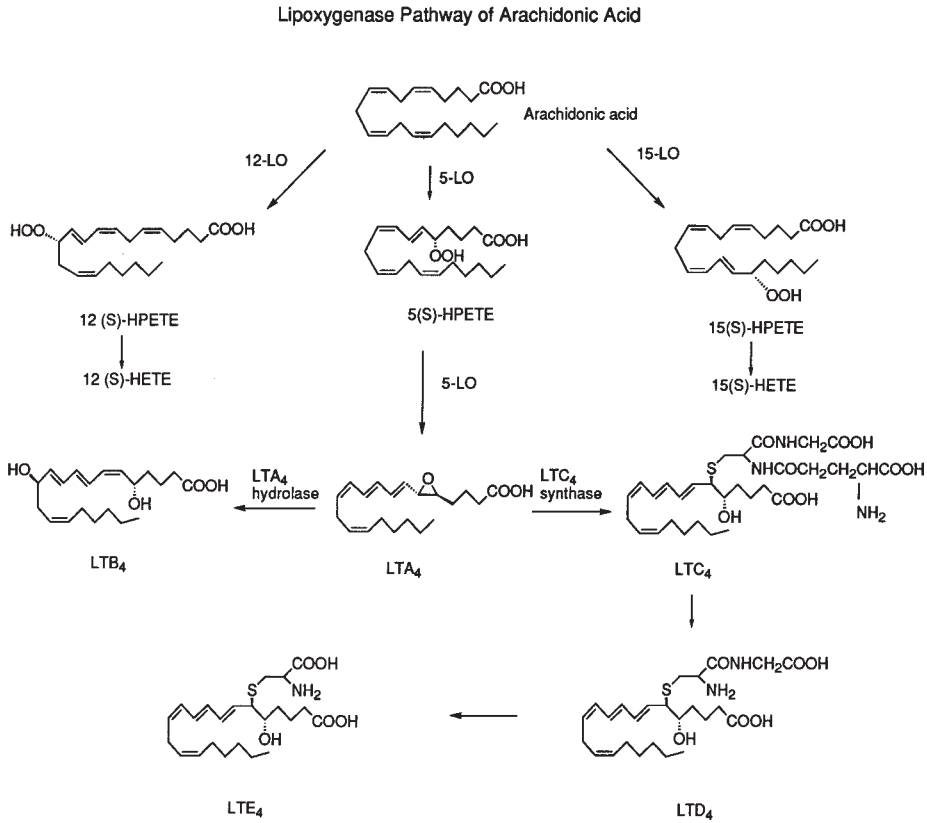

Fig. 1. Pathways for the metabolism of arachidonic acid (AA) via lipoxygenase (LO) enzymes. Leukotriene (LT) biosynthesis is dependent on initial oxidation catalyzed by a 5-LO (5-lipoxygenase). Enzymatic transformations dependent on the two other mammalian LOs are also depicted. The hydroxyacids, 12-S-HETE and 15-S-HETE, are produced over the 12-LO and 15-LO pathways, respectively, through a hydroperoxy eicosatetraenoic acid (HPETE) intermediate.

(sp-LTs) since all possess a cysteinyl residue at carbon 6. Biosynthetic pathways leading to formation of the sp-LTs are summarized in **Fig. 1**.

Much of the original work on LT production was based on the analysis of incubates of human polymorphonuclear leukocytes (PMNs) and other human leukocyte preparations. In fact, LTA_4 was first identified as a labile intermediate produced during stimulation of human neutrophils. As indicated, the activities of 5-LO and LTA_4 synthetase are inseparable; the latter activity depends on the stereoselective abstraction of hydrogen from C-10 of the 5-hydroperoxide, followed by double bond rearrangement to form a conjugated triene. Yamamoto and colleagues *(7)*, using porcine leukocytes, demonstrated a par-

allel inactivation of 5-LO and LTA_4 synthase with increasing temperature and similar responses to calcium, ATP, and 5-LO inhibitors. Like the 5-LO, the LTA_4 hydrolase activity showed "suicide-type" inactivation, presumably related to autodestruction of the enzyme protein by the reactive hydroperoxide.

Conversion of the epoxide LTA_4 to the potent chemotactic agent LTB_4 (the diol 5,12-Di-HETE) is carried out by a specific LTA_4 hydrolase, a zinc metalloenzyme localized chiefly in the cytosol ***(17)***. Found in human leukocytes, as well as in endothelial cells, the LTA_4 hydrolase is responsible for stereospecific opening of the 5,6-epoxide (5S) and directed hydroxylation at C12 (12R). Work is underway at a number of laboratories, aimed at developing selective LTA_4 hydrolase inhibitors, to interrupt the process of neutrophil transendothelial migration, and perhaps curtail inflammation at a critical stage.

Another important protein in the LT biosynthetic pathway is the five lipoxygenase-activating protein (FLAP), which is membrane-associated (unlike the 5-LO) and facilitates the delivery of PUFA substrate to the 5-LO through translocation of the cytosolic 5-LO to the cell membrane ***(18)***. In order to generate LTs in high yield, when incubating leukocytes or isolated tissue, a commonly employed approach is to add calcium ionophore, A23187. Whereas addition of A23187 was originally an empirical practice, it subsequently it became clear that A23187 coupled the FLAP to the 5-LO, through work conducted by Rouser and Dixon and coworkers ***(19)*** at the Merck Frosst laboratories. This pharmaceutical effort, which involved isolation and purification of the FLAP, led to the introduction of MK886, a novel inhibitor of LT biosynthesis and 5-LO translocation ***(20)***. Using intact, activated leukocytes from either rats or humans, the Merck-Frosst group found that low concentrations of MK-886 inhibited LTB_4 biosynthesis in vitro (IC_{50} = 2.5 n*M*, for inhibition of A23187-induced LTB_4 formation by human PMN). When it became clear that MK-886 lacked a direct inhibitory action on 5-LO in cell-free systems, these workers synthesized several photoaffinity labels based on the MK-886 structure in an effort to monitor the purification of FLAP. The selection of the ^{125}I-radiolabeled analog L669083 was based on effective inhibition of LTB_4 biosynthesis in suspensions of intact leukocytes (IC_{50} = 100 n*M*). Nonspecific binding was largely eliminated by carrying out the incubation in the presence of 100 n*M* cold MK-886. Using human or rat neutrophil microsomal proteins, the relative molecular mass of FLAP was determined to be 18,000 by SDS-PAGE.

3. The 12-LO Pathway and Generation of 12-HETE Enantiomers

Another well characterized mammalian lipoxygenase (LO) is the 12-LO found in human blood platelets and leukocytes. As with the 5-LO and the LTA_4 hydrolase activities, this enzyme is found predominantly in the high-speed supernatant fraction. It is not clear, however, whether an activating protein

similar to the FLAP participates in the synthesis of 12-LO products. The reactivity of the purified 12-LO with AA and other substrates was evaluated by Yamamoto and coworkers ***(21)*** using an immunopurified 12-LO from porcine leukocytes. When 15-S-HPETE was incubated with the purified 12-LO, the rate of conversion was comparable to that obtained with AA substrate, and a major reaction product derived from 15-S-HPETE was a 14, 15 epoxide; viz., 14, 15-LTA_4. The recovery of this LTA-type compound also supports the argument that LO enzymes serve a dual role; namely that of a dioxygenase and an LTA synthetase.

Uncertainty exists concerning the enantiomeric form of the 12-HETE produced in various tissues. Early reports indicated that a 12-HETE was generated in a variety of tissue types, including skin ***(22)***, vascular smooth muscle ***(23)***, endothelium ***(24)***, colonic tissue ***(25)***, kidney ***(26)***, macrophages ***(27)***, and lung ***(28)***. In general, these studies did not address the stereochemical configuration at C12 or the enzyme responsible for the C12 oxidation. From the published literature at the time, it seemed that 12-S-HETE would be the stereoisomer likely to be formed by mammalian tissues. This preconception vanished with the report ***(29)*** that high concentrations of 12-R-HETE existed in human psoriatic scale (levels 1000-fold greater than that of LTB_4). The question then arose regarding which was the biologically important enantiomer. Neither 12-R-HETE nor 12-S-HETE exhibited impressive activity as regards effects on leukocyte chemotaxis. Based on in vitro studies of human leukocyte chemokinesis, as well as in vivo studies involving intradermal injection, 12-R-HETE appeared to have roughly 1/1000 the neutrophil chemoattractant activity of LTB_4 ***(30)***. However, 12-R-HETE was about 20-fold more potent than 12-S-HETE in these experiments. Also, 12-R-HETE is much more active in terms of reported effects on epithelial ion transport (**Table 1**).

Besides arising from LO-catalysis, mono-HETEs may be produced through cytochrome P450-dependent pathways. It is clear that the microsomal P450 system of liver and other cells is capable of catalyzing the omega oxidation of various straight-chain fatty acids (e.g., cytochrome P452, "lauric acid hydroxylase") and prostaglandins as well as LTs. Several groups have demonstrated that human neutrophils contain a LTB_4 20-hydroxylase, which is a member of the P-450 family of mixed function oxidases. This omega oxidation step results in the inactivation of LTB_4, as judged by chemotactic assay ***(31)***. However, certain P450-derived mono- and di-HETEs appear to possess significant biological activity; in particular, those produced by the P450 arachidonate epoxygenase pathway seem to function in the physiological control of electrolyte and fluid balance ***(32)***. Interest in P450-related epithelial ion transport was stimulated by a report ***(33)*** that a mono-HETE, 12-R-HETE, exerted a potent inhibitory effect on the sodium potassium ATPase of corneal epithelium. Rat

Table 1
Epithelial Ion Translocation Processes Affected by Eicosanoids Derived from Cytochrome P450 or Lipoxygenase(s)

Enzyme and mediator	Effect	Cell type	Species	Ref.
P450 11,12-dihydroxy eicosatrienoic acid (DHT)	Inhibition of NaK-ATPase	Renal epithelium (mTALH)	Rabbit	***138***
P450 5,6-EET[a]	Inhibition of electrogenic ion transport	Coritical collecting duct	Rabbit	***139***
P450 5,6-EET	AngII-induced natriuresis	Proximal tubule	Rabbit	***140***
P450 5,6-EET	Inhibition of ion transport	Renal collecting tubules	Rabbit	***141***
P450 11,12-DHT	Inhibition of AVP-stimulated water transport	Bladder epithelium	Toad	***142***
P450 19S-HETE	Stimulation of NaK-ATPase	Kidney cortex	Rat	***143***
P450 or LO; 12-R-HETE	Inhibition of NaK-ATP-ase	Kidney cortex	Rat	***144***
P450 12-R-HETE	Inhibition of NaK-ATP-ase	Corneal epithelium	Bovine, rat, and human	***144***
P450 20-HETE	Ang II-block of 70 pS K^+ channel	Renal TAL	Rat	***145***

[a]Epoxylicosatrienoic acid.

liver microsomal P450 MFO is known to catalyze the NADPH-dependent metabolism of AA to six regioisomeric cis, trans-dienols, and also gives rise to 12-R-HETE with a high degree of enantioselectivity ***(34)***. The epoxidation at carbons 11 and 12 is of special interest in light of its reported enantioselectivity (97 vs 3%, 11-S, 12-R vs 11-R, 12-S) and because the 11-S, 12-R-epoxide may be the precursor of 12-R-HETE.

Other enzymatic routes for the generation of 12-R-HETE may exist from a theoretical standpoint. The hydroxy acid could arise from a 12-R-HPETE through catalysis by a 12-R-LO, similar to the 11-R and 12-R LOs found in marine invertebrates ***(35)***. In addition, there has been speculation that 12-R-HETE might arise from a "lipoxygenase-like" reaction catalyzed by a microsomal P450 enzyme ***(36)***. Another possibility suggested by the work of Rokach

and colleagues ***(37)*** is that 12-keto-5, 8, 10, 14 eicosatetraenoic acid (12-KETE) can be converted to 12-R-HETE by the action of a 12-ketoreductase. The 12-keto-intermediate itself can be produced by hemoproteins acting on 12-S-HPETE ***(38)***, or, alternatively, by an action of a dehydrogenase acting on 12-HETE ***(39)***. At the present time, it cannot be unequivocally stated whether the 12-R-HETE found in the psoriatic lesion is a product of epoxygenase catalysis; e.g., an arachidonate-specific P450 epoxygenase, such as P450 2C23 ***(40)***, or whether 12-R-HETE formation depends on other enzymatic or nonenzymatic routes.

Evidence has been obtained for the production of 12-R-HETE in human corneal epithelium ***(33)***. The 12-R-HETE enantiomer, but not the 12-S isomer, was an effective inhibitor of corneal epithelial ATPase. This group ***(41)*** also identified a 10,11-dihydro-derivative of 12-R-HETE (12-R-dihydro-HETrE), which they tested for biological activity in the rabbit eye. The 12-R-dihydro-HETrE exhibited angiogenic activity, caused ocular vasodilatation, and increased the permeability of the blood aqueous humor barrier. It seems reasonable to suspect that 12-R-HETE and 12-R-dihydro-HETrE arise from a common intermediate. A plausible biosynthetic scheme for production of 12-R-HETE and 12-R-HETrE is given in **Fig. 2**. Schwartzman and colleagues speculated ***(41)*** that the common intermediate may be 11S, 12-R-epoxyeicosatrienoic acid formed by an arachidonate epoxygenase.

Nucleophilic attack resulting in epoxide opening may occur nonenzymatically in aqueous media, from above or below the plane of the oxygen ring, so that from one epoxide intermediate three stereochemically distinct dihydroxy eicosatrienoic acids are possible. Clearly, a detailed knowledge of the mechanism for hydrolytic cleavage of the AA epoxides, and of the factors involved in the control of P450-dependent mono-HETE formation, would be useful, since substantial evidence now supports the idea that epoxyeicosatrienoic acids (EETs) or omega hydroxylated eicosanoids (e.g., 20-hydroxyeicosatetraenoic acid, 20-HETE) are endogenous modulators of ion and water transport across epithelial sheets. This concept has developed from work with a variety of epithelial preparations (**Table 1**) and suggests that regulation of ion transport by oxy-eicosanoids may represent a general mechanism operating in specialized epithelia throughout the vertebrate classes.

4. The 15-LO Pathway

Although 15-LO is widely distributed in mammalian cells, the work of many groups indicates that the background activity of 15-LO is quite low. The enzyme, as it exists in unactivated platelets and neutrophils, appears to be present in a cryptic state. Perhaps the low basal activity in these blood elements may imply a high degree of compartmentalization and tight regulation until 15-LO prod-

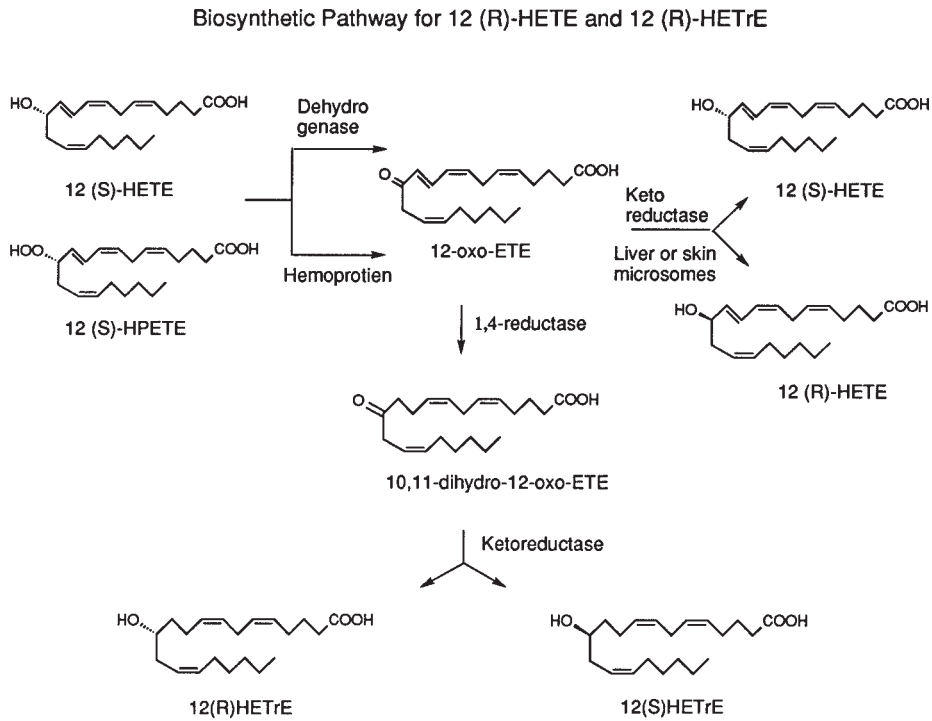

Fig. 2. Theoretical routes for generation of 12-R-hydroxy eicosatetraenoic acid (12-R-HETE) and 12-R-hydroxy eicosatrienoic acid (12-R-HETrE). Possible routes are shown for generation of 12-R-HETE and 12-R-HETrE from a common intermediate, 12-keto-5, 8, 10, 14-eicosatetraenoic acid (12-oxo-ETE).

ucts are needed for a specific purpose. Moreover, the work of Yamamoto and coworkers ***(42)*** indicates that a high degree of sequence homology exists between the 15-LO and 12-LO enzyme proteins. A great variety of human cell types (e.g., keratinocytes, eosinophils, endothelial cells, and epithelial cells from human trachea) produce this mono-HETE as a major product ***(43–46)***. The formation of 15-HETE has also been reported with various human tissues and fluids, including psoriatic skin ***(47)***, asthmatic lung homogenates ***(48)***, and synovial fluid from arthritic patients ***(49)***.

Substantial evidence indicates that 15-HETE exerts immunomodulatory effects, including prominent actions on T and B lymphocytes ***(50)***. However, limited information is available regarding the existence of 15-HETE receptors. Vanderhoek and colleagues ***(51)*** have initiated studies to characterize 15-HETE binding sites in a mast/basophil PT18 cell line; activation of specific receptors for 15-HETE resulted in stimulation of a latent 5-LO activity in the PT18 cells.

Also, it seems clear that 15-HETE can be esterified into membrane phospholipids, and this membrane alteration could have important ramifications. If a significant portion of phospholipids bordering key membrane-embedded proteins (e.g., adhesion molecules) were oxidatively modified, changes in leukocyte chemotaxis/diapedesis could well result because of effects on "membrane order" or fluidity. That appreciable segments of the membrane may be affected is supported by the work of Nakao and coworkers ***(52)***. In this study ***(52)***, conducted with a cell-free human PMN preparation, soybean 15-LO exhibited a remarkably high capacity to oxygenate AA esterified to membrane phospholipids, relative to other LOs or P450.

Recent studies with isolated leukocyte preparations indicate that certain cytokines, such as IL-4 and IL-13, are capable of "turning on" the expression of 15-LO in inflammatory cells. These in vitro observations are likely to have in vivo pathophysiological relevance, in view of findings by a group using a model of acute nephritis; i.e., Badr et al. ***(53)*** have presented strong evidence for induction of macrophage 15-LO /12-LO in vivo at a discrete phase during the progression of acute inflammation. This issue will be revisited in **Subheading 8.**

5. Contribution of LTs and HETEs to Inflammation and Cell Proliferation

After the structures of the components of SRSA were determined, a major effort was launched to determine the medical significance of the LTs. An extensive literature has since accumulated, and the picture that emerges is that LTs are proinflammatory mediators participating in various forms of acute and subacute inflammation. In addition to human allergic asthma, considerable evidence suggests that LTs contribute to the pathologic changes during colitis ***(54)***, psoriasis ***(55)***, glomerulonephritis ***(56)***, and endotoxemia ***(57)***. The influence of LTB_4 and the sp-LTs on inflammatory events is quite different. Whereas LTC_4, LTD_4, and LTE_4 have potent contractile actions on bronchial and vascular smooth muscle, LTB_4 has relatively little musculotropic activity ***(58)***. On the other hand, LTB_4 is a potent aggregatory, chemotactic, and adhesive agent for neutrophils, whereas the sp-LTs are devoid of these effects on neutrophils. With regard to the chemotaxis of human PMNs, LTB_4 is effective at a concentration of 1 n*M*. The sp-LTs promote capillary leakage ***(59)***, tissue edema, mucous secretion ***(60)***, and also appear to affect intestinal electrolyte transport ***(61)***. The potencies of the different sp-LTs in causing biological responses are not equivalent; LTE_4 tends to exert weaker effects than LTC_4 and LTD_4 and the dipeptidase step, which leads to LTE_4 production, is generally considered an inactivation route ***(58,62)***.

A substantial body of evidence suggests that the LTs (both LTB_4 and the sp-LTs) may exert a stimulatory effect on the proliferation of various cell types. The initial reports concerned the ability of LTs to enhance epidermal proliferation, observations that focused attention on a possible role for 5-LO products in the epidermal hyperplasia of psoriasis. Proliferation was stimulated in vitro ***(63)*** when cultured human epidermal keratinocytes were exposed to LTB_4, LTC_4, and LTD_4, and also in vivo ***(64)*** when LTB_4 was applied topically to the skin of healthy and psoriatic patients. When applied topically to the skin of the guinea pig ear, LTB_4 as well as 12-HETE (both at 3–30 n*M*), stimulated proliferation in a dose-dependent fashion ***(65)***. A role for LO products as positive modulators of cell proliferation has also been suggested based on studies of glioma cell division ***(66)*** and of the growth of lymphomas ***(67)***, nasal polyps, and mammary cancers ***(68)***. In addition, in a study of large bowel tumors ***(69)***, chemically induced in rats by methylazoxymethanol, tumor formation was antagonized by the LO inhibitor nordihydroguaiaretic acid (NDGA). The latter inhibitor is relatively nonspecific (unpublished results of J. R.) and would be expected to block formation of arachidonate products formed through both the 5-LO and 15-LO pathways.

One of the first reports that LO products may affect cell–cell adhesion came from the laboratory of Buchanan ***(70)***, who showed that 13-S-hydroxy octadecadienoic acid (13-HODE), a lipoxygenation product of linoleic acid, exerted a vessel wall "chemorepellant effect"; i.e., 13-HODE reduced the adhesion of platelets to the surface of endothelial cells. Subsequently, Honn and coworkers ***(71)*** studied the influence of LO products on the metastatic potential of tumors. These investigators reported that 12-S-HETE, but not 12-R-HETE, stimulated the adhesion of 3LL cells (Lewis lung carcinoma) to endothelial cells, subendothelial matrix, and fibronectin. The proadhesive effects of 12-S-HETE were dose-dependent, could not be reproduced with 5-HETE or 15-HETE, and seemed to be caused in part by the expression of the integrin receptor glycoprotein Gp2b/3a on tumor cells.

6. Leukotrienes and Bronchial Asthma

Consistent with the animal studies, the early clinical trials with LTD_4 receptor antagonists showed that LTD_4 receptors mediate pathophysiologic responses within the lung. Furthermore, these clinical studies also advanced our understanding of the basis for variations in allergen-induced bronchoconstrictor responses between individuals. It is evident that, in a subset of patients with allergic asthma, the sp-LTs are one of the predominant mediators responsible for pulmonary injury associated with allergen inhalation. In a clinical investigation of the antiasthmatic effectiveness of a prototype LTD_4 antagonist L648051, Rokach and coworkers ***(72)*** observed borderline efficacy with this

lead compound as determined by effects on FEV_1 following antigen challenge. However, the extent of variation of protection against antigen-induced bronchoconstriction warrants comment. The results of individual volunteers ranged from complete protection in some subjects to no or minimal protection in others. These FEV_1 data are in accord with a widely adopted view that, although allergic bronchoconstriction often reflects the combined influence of multiple mediators (histamine, serotonin, thromboxane A_2 [TXA_2], $PGF_{2\alpha}$, platelet-activating factor [PAF], neuropeptides, and sp-LTs), in selected patients the hyperreactive condition may be dominated by a single mediator. It is clear that up to 5% of asthmatics respond to antihistamines, suggesting that in a portion of the patients in the general population histamine could be the primary mediator.

Although the clinical experience with LTD_4 antagonists is rather limited, a growing number of investigators feel that such compounds are particularly effective in asthma induced by aspirin (vide infra). Also, a recent clinical trial ***(73)*** conducted with the 5-LO inhibitor zileuton suggests that LTs contribute in a major way to bronchospasm associated with exercise-induced asthma. Urinary assay of LTs is under consideration at some centers as a strategy for rational selection of patients; i.e., identifying in advance those likely to respond favorably to the LTD_4 antagonist or 5-LO inhibitor. Patients may be preselected through analysis of urine for an increased level of LT metabolites following antigen challenge. Considerable progress has been achieved in the development of reliable, simple assays for measurement of LTE_4 in human urine ***(74,75)***. In a study involving reverse-phase high performance liquid chromatography (RP-HPLC) and RIA analysis ***(74)***, a statistically significant correlation was found between the early-phase bronchoconstrictor response of asthmatic volunteers and urinary LTE_4 concentration measured 2–3 h after inhalation of antigen or occupational sensititizer.

The following pulmonary responses (**Fig. 3**) were generally observed:

1. Patients who exhibited early, severe bronchoconstriction (depressed FEV_1); this transient response was accompanied by a distinct rise in urinary LTE_4 excretion;
2. Patients who exhibited delayed (6–8 h), progressive, and equally marked bronchoconstriction that occurred without significant alterations in urinary LTE_4; and
3. Patients who exhibited both early and late responses.

Patients in the third category showed a significant increase of LTE_4 in the early phase only. It is possible that the lack of a measurable rise of urinary LTE_4 during the late phase in this pilot clinical investigation was related to rapid metabolism by ω-oxidation. Urinary concentrations of ω-oxidized forms of LTE_4 were not measured.

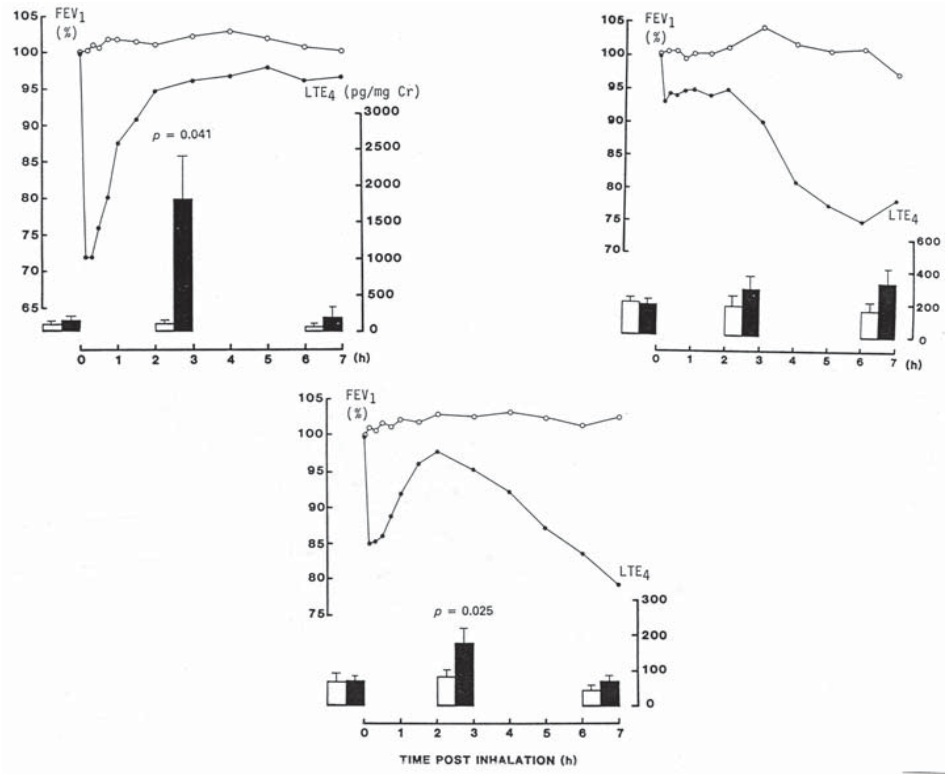

Fig. 3. Effects of antigen challenge on forced expiratory volume (FEV_1, closed circles) and urinary LTE_4 concentrations in asthmatic patients. Three categories of responses are shown; i.e., patients who exhibited early bronchoconstriction (5 patients, **A**), delayed bronchoconstriction (5 patients, **B**), or a dual asthmatic response (8 patients, **C**). The time post-inhalation is indicated in each panel. The closed bars show the level of urinary LTE_4 (pg LTE_4/mg creatinine, mean ± SEM) measured before inhalation of the allergen, between 2 and 3 h and between 6 and 7 h after inhalation of the allergen. Note the difference in scales for urinary LTE_4.

Since the fall of 1996, two sp-LT antagonists as well as the 5-LO inhibitor zileuton have been approved by the US FDA for the prophylaxis and chronic treatment of asthma, a disorder affecting up to 15 million Americans. Many "anti-LT" medications will undoubtedly follow. In a relatively short period of time, a considerable clinical literature ***(76)*** has accumulated, providing a knowledge base for effective dosing protocols and for managing specific subsets of patients; e.g., asthmatics with bronchoconstriction induced by exercise, aspirin, cold air, and sulfur dioxide.

Clinical experience with these agents reinforces the idea that chronic asthma is a multifaceted disease, involving not only the airway smooth muscle, but also persistent airway obstruction associated with edematous thickening and mucus secretion. The disease is characterized by acute exacerbations precipitated by various causes, often during the night. Of interest, in adult asthmatics, the LTD_4 antagonist montelukast sodium (Singulair, MK 0476) is reported to alleviate asthma exacerbations when given orally as a single dose at bedtime; this antagonist would appear to be long-acting ***(77)***, although the estimated plasma half-life is <6 h. In addition, it seems clear that such treatment is effective in preventing both early- and late-phase bronchoconstriction in asthmatic patients.

One of the early multicenter trials ***(78)*** was conducted with zafirlukast (Accolate, ICI204219), a selective LTD_4 receptor antagonist. Efficacy was evaluated over the course of 6 wk of therapy, by pulmonary function testing and recording nocturnal awakenings and beta agonist rescue inhaler use. Oral administration of zafirlukast resulted in significant elevations of evening peak expiratory flow rate (+11%) and alleviated day and night symptoms, significantly decreasing nighttime awakenings (–46%) and albuterol use (–30%).

Initial indications that sp-LT antagonists could suppress both early and late bronchoconstrictor responses to inhaled allergen arose from work with whole animal models, particularly through the use of the allergic sheep models. The first demonstration of the effectiveness of sp-LT antagonists against early- and late-phase responses was carried out in allergic sheep with the prototype LTD_4 receptor antagonist MK-571 (L660711) (unpublished collaborative studies between Dr. W. M. Abraham, Mt. Sinai Hospital, Miami, FL, and the Merck Frosst laboratory). Abraham and coworkers in 1988 ***(79)*** convincingly demonstrated suppression of the early response (percent increase of specific lung resistance in allergic sheep) and elimination of the late response to Ascaris antigen with the orally effective sp-LT receptor antagonist LY171883. Jones and colleagues ***(80)*** at Merck Frosst reported that an iv infusion of montelukast sodium decreased the peak early response (–70%) as well as the late response (–75%) to Ascaris aerosol in a similar experimental preparation.

The above experimental animal work was soon followed by studies of patients having allergic asthma. These investigations established the value of sp-LT

antagonists in the human for inhibition of both early and late-phase bronchoconstriction induced by antigen challenge. In a placebo-controlled crossover study with montelukast sodium (MK-0476, Singulair), the early- and late-phase bronchoconstrictor responses were suppressed by 75 and 57%, respectively. Also, in a study ***(81)*** involving randomized pretreatment with the LTD_4-receptor antagonist zafirlukast (Accolate), comparable reductions in the early- and late-phase responses to allergen bronchoprovocation were noted. As indicated, certain of these agents can impressively reduce the bronchoconstrictor response to aspirin challenge in aspirin-sensitive asthmatics. In a small scale study ($n = 8$) of adults with known aspirin sensitivity ***(82)***, the sp-LT antagonist MK0679 significantly attenuated the airway obstruction produced by inhaled lysine-aspirin.

Beside neutrophils which are recruited into the airways during both early and late bronchial responses, eosinophils, which tend to appear late after antigen inhalation, are also believed to contribute mediators that promote bronchoconstriction and/or plasma extravasation ***(75)*** (coincubations of eosinophils and neutrophils show a particularly high capacity for sp-LT generation; ***83***). Of interest in this regard are the results of Sun and coworkers ***(84)*** at Upjohn, who recently reported that the increased vascular permeability and plasma leakage, produced in the lungs of sensitized Brown Norway rats in response to ovalbumin aerosols, were independent of eosinophil infiltration. Through appropriately timed injections of a selective monoclonal antibody (MAb) against ICAM-1 (administering the MAb, 1A29, before and after ovalbumin challenge), they prevented in large measure eosinophil infiltration into the alveolar space without suppression of vascular leakage and lung edema. The Upjohn group concluded that the pulmonary vascular permeability changes in the allergic rat model may depend on mediators derived from other inflammatory or structural cells or from plasma itself. Certainly, the consequences of possible activation of the plasma kallikrein-kinin system should not be overlooked when considering mechanisms for vascular permeability changes.

7. Lipoxins and Transcellular Metabolism

The concept of transcellular metabolism of eicosanoids developed as an outgrowth of investigations into the release of prostacyclin (PGI_2) during the interaction between blood platelets and the vascular wall. A germinal finding was that platelets participated in the biosynthesis of PGI_2 by donating the unstable prostaglandin (PG) endoperoxides, generated by platelet cyclo-oxygenase, to the prostacyclin synthase-containing cells of the intima. With this initial report of a biochemical "co-operation" between the platelet and the endothelial cell ***(85)***, research interest in cell–cell interactions was immediately stimulated, and investigators turned their attention to other cell–cell interactions critical for biosynthesis of eicosanoid mediators. Numerous studies have now shown that

the unstable epoxide LTA_4, generated by the 5-LO in neutrophils (also in basophils, eosinophils, mast cells, and monocytes/macrophages), can undergo transformation to other LTs. For example, if a neutrophil makes contact with an endothelial cell (**Fig. 4**), a cell known to contain LTB_4 synthase, the neutrophil can donate LTA_4 for conversion to LTC_4 as well as to LTB_4 via endothelial LTA_4 hydrolase activity ***(86)***. Transcellular metabolism is crucial for the synthesis of lipoxins (LXs), an acronym for lipoxygenase interaction products. As with the LTs, the biosynthesis of LXs can be initiated by multiple cell types (**Fig. 4**) after activation by an array of immunological stimuli. An important distinction is that LX biosynthesis requires the sequential operation of two LO enzymes ***(87)***; i.e., the 5-LO and the 15-LO; or, alternatively, the 5-LO and 12-LO. (**Fig. 5**) The initial LO step may be catalyzed by a neutrophil 5-LO, a macrophage 15-LO or platelet 12-LO (the platelet enzyme forms a 15-hydroperoxy 5,6-epoxytetraene from LTA_4 via n-6 oxygenase activity). Cytokines may influence LX synthesis by enhanced AA release or through effects on LO gene expression (*see* **Subheading 8.**, **Fig. 4**).

From the first report of the natural formation of LXs ***(88)***, which was an analysis of the products formed during incubation of 15-S-HPETE with human leukocytes, it was clear that LXs produced from AA contain a fully conjugated tetraene system, and are hydroxylated either at carbons 5, 6, and 15 (LXA_4) or 5, 14, and 15 (LXB_4). The biochemical mechanism for synthesis has been determined using various approaches, including the use of isotopic oxygen, various chiral substrates, and purified LO enzymes. The pioneering work with human leukocytes showed that 15-S-HPETE was converted to a mixture of isomeric compounds; the Karolinska and Merck Frosst ***(89)*** groups separated 4 isomers of LXA and 3 isomers of LXB. Shortly after the initial finding ***(88)*** that LXs were endogenously produced via the AA cascade, Wong and colleagues ***(90)*** demonstrated that leukocytes are capable of generating analogous trihydroxy-pentaenes from eicosapentaenoic acid (20:5). The same laboratory, has also elicited the biosynthesis of LXs from endogenous AA, using a phospholipase A_2 isoenzyme. In this study ***(91)***, conducted with porcine leukocytes and snake venom phospholipase A_2, LXB along with two of its structural isomers (8-trans-LXB and 14-S-8-trans-LXB) were identified.

Additional consideration should be given to the origin of the LX isomers found in the initial report of Serhan et al. ***(92,93)***, who incubated 15-HPETE with human leukocytes. These investigators described two general classes of LXs derived from AA; i.e., LXs bearing hydroxyl groups on 5, 6, and 15 (LX "A" compounds), and LXs with hydroxyls on 5, 14, and 15 (LX "B" compounds). In the mid-1980s, Serhan and colleagues, as well as the group of Rokach ***(94)***, actually found that the leukocytes converted 15-HPETE to a complex mixture of LXA and LXB isomers with different cis-trans arrangements

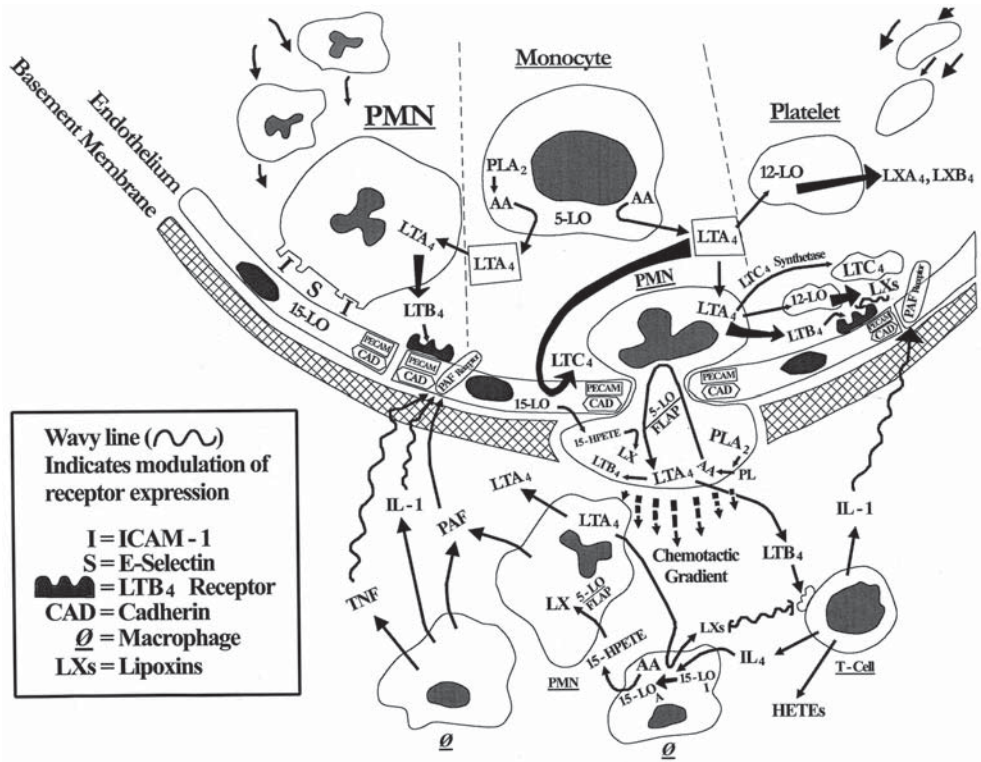

Fig. 4. Overview of the transcellular metabolism of arachidonic acid (AA) in the microcirculation during inflammation. This theoretical scheme illustrates possible routes leading to biosynthesis of leukotrienes (LTs) and lipoxins (LXs), and how lipid mediators and cytokines may contribute to the regulation of leukocyte diapedesis and permeability of capillary vessels.

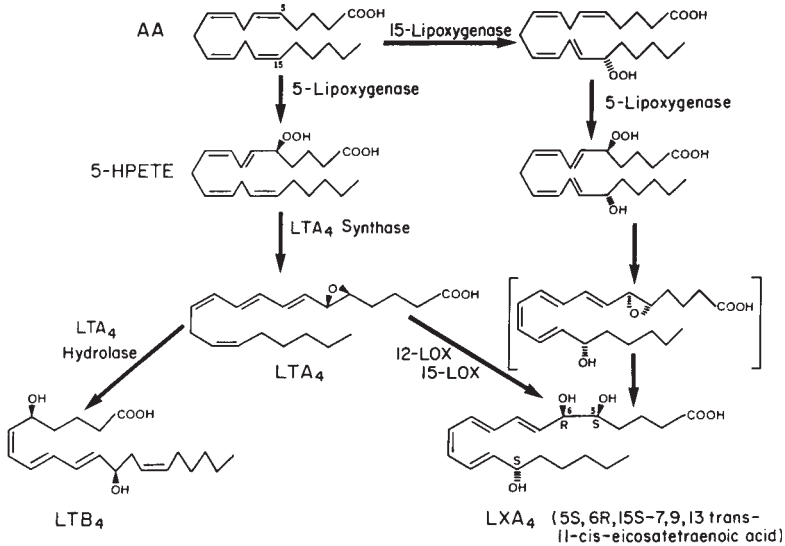

Fig. 5. Chemical structure of lipoxin A_4 and enzymatic pathways for lipoxin biosynthesis. Lipoxygenase = LOX.

in the conjugated tetraene. In order to establish the stereochemistry and origins of the hydroxyl oxygens, the laboratory of Rokach, then at Merck Frosst, prepared the authentic LXA and LXB standards by total synthesis, and used them as reference compounds in studies of the enzymatic and nonenzymatic conversion of intermediates, notably the 14,15-tetraene epoxide, which, in theory, could account for the formation of most of the isomers found in the studies with intact cells.

These investigations at Merck Frosst led to the proposal that arachidonic acid liberated from cell membranes was subject to "triple lipoxygenation"—oxygenation reactions that require three sequential lipoxygenase activities. The Merck Frosst workers synthesized the tetraene epoxide ***(95)*** as well as all seven LX isomers ***(96–99)***, and investigated the mechanism of the oxygenation reactions with cell-free systems. Triple lipoxygenation was demonstrated in a series of experiments that provided evidence for the biochemical formation of LXB, via catalysis by a single enzyme or two or three enzymes. The first study ***(90)*** utilized soybean 15-lipoxygenase to demonstrate a pathway for LXB synthesis that proceeds from arachidonic acid through the intermediacy of 5, 15-diHPETE; i.e., in this cell-free system, substantial LXB formation was found using either AA or 5, 15-diHPETE as substrate.

In a subsequent study ***(89)***, purified porcine leukocyte 12-LO was used as the enzyme with 5,15-diHPETE as the substrate, since the 5, 15-dihydroxy acid was

known to be a product formed via double lipoxygenation in arachidonate-stimulated cell preparations. Again, a mixture of LXA and LXB isomers were formed, although one 8-cis-LXB isomer was the predominant product. Formation of this LXB isomer was dramatically suppressed when oxygen was excluded from the incubation, indicating that the purified 12-LO was needed for incorporation of the molecule of oxygen at C14, a reaction involving abstraction of H from the 10-carbon. This conclusion, that the 12-LO will catalyze a stereoselective direct 14-lipoxygenation of 5,15-diHPETE, is consistent with studies ***(100)*** of the active site binding conformation of synthetic AA analogs using purified 12-LO.

For the third demonstration of a triple lipoxygenation mechanism in LXB formation, Rokach and coworkers ***(101)*** relied on a purified rabbit reticulocyte enzyme primarily exhibiting 15-LO activity as demonstrated with arachidonate methyl ester. When 15-S-HETE methyl ester was incubated with this enzyme, lipoxin B methyl ester (5S, 14R, 15S-trihydroxy-6E, 8Z, 10E, 12E eicosatetraenoate methyl ester) was the chief product. The kinetics of LXB methyl ester formation was determined in this experimental series; analysis of product-precursor relationships clearly indicated that 5S, 15S-diHETE methyl ester was an intermediate in the formation of LXB methyl ester. Incubations carried out under an atmosphere of $^{17}O_2$ showed that formation of LXB methyl ester from 15-S-HETE methyl ester occurred via sequential oxygenation of the substrate and not via hydrolysis of an epoxide intermediate.

Many studies of high- and low-affinity binding sites for LTs and LXs have been carried out. The reader is referred to several excellent reviews ***(87,102,103)*** that cover this material in depth. A related issue that has received less attention is the modulation of LTB_4 receptors by LXs. Various myeloid cells exhibit high affinity LTB_4 receptors, notably eosinophils and macrophages; recent work indicates that T-cells also contain such receptors. Stereospecific LTB_4 receptors have been identified on T-cells from human peripheral blood ***(104)*** and from guinea pig peritoneal exudate ***(105)***. Expression of LTB_4 receptors on T-cells and other cells may be modulated by LXs released from adjacent cells, a possibility in line with recent work by Wong and coworkers ***(105)***, who observed a reduction in the density of LTB_4 receptors after exposure of guinea pig T-lymphocyes to LXA_4 (**Fig. 6**). This receptor down modulation is likely an effect of LXA_4 on the protein kinase C signal transduction mechanism, since LXA_4 does not compete for LTB_4 binding sites in T-cells (**Fig. 7**) ***(105)*** or granulocytes ***(106)***, and because LTB_4 receptor downmodulation can be reversed by the protein kinase C inhibitor K252a ***(105)***. Low concentrations (nanomolar range) of LXA_4 are reportedly effective in antagonizing the chemotactic response of human PMNs to LTB_4 ***(107)***, and the indirect effect of the LX on PKC signal transduction would seem a reasonable explanation for the inhibition of

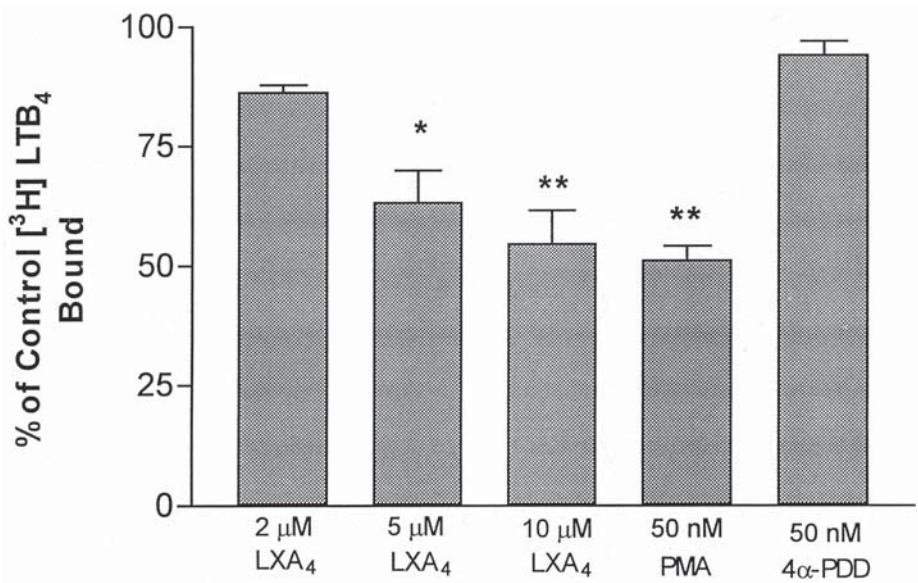

Fig. 6. Effects of lipoxin A_4 (LXA_4), phorbol myristyl acetate (PMA), and 4α-PDD on B_{max} of guinea pig T-cell LTB_4 receptors. Cells were treated with LXA_4, PMA (an activator of protein kinase C), or 4α-PDD (an inactive structural analog of PMA) for 30 min at 37°C; binding studies were performed at 4°C for 1 h. Results are shown as percent decrease in B_{max} from control and represent the mean ± SEM of three experiments. $*P < 0.05$ vs control; $**P < 0.01$ vs control.

LTB_4-induced chemotaxis. A PKC-related mechanism has also been invoked to explain the ability of intrarenal arterial infusions of LXA_4 to reduce glomerular capillary ultrafiltration coefficient in euvolemic rats; i.e., "downregulation" of LTD_4 receptors on mesangial cells secondary to PKC activation ***(108)***.

Support for in vivo LX synthesis initially arose from studies of the pulmonary system ***(109)***. A definitive study ***(109)*** showing that LXA_4 could be produced by human lung was conducted using GC-MS with selected ion monitoring for quantitative analysis of LXA_4 in bronchoalveolar lavage (BAL) fluid. The laboratory of Lee in collaboration with Spur measured rather high concentrations (ng/mL levels) of LXA_4 in BAL fluid from 9 of 12 patients with pulmonary disease (conditions included asthma, pneumonia, sarcoid, and undefined alveolitis) but were unable to detect LXA_4 in BAL from normal subjects ($n = 6$). Levels of sp-LTs (LTC_4, LTD_4, and LTE_4) as determined by RIA after RP-HPLC were roughly an order of magnitude lower and did not differ between patients with pulmonary disease and normal subjects. Subsequently, Serhan and coworkers ***(110)*** demonstrated pulmonary release of LXA_4 in aspirin-sensitive asthmatics administered aspirin. This influence of aspirin is a provoca-

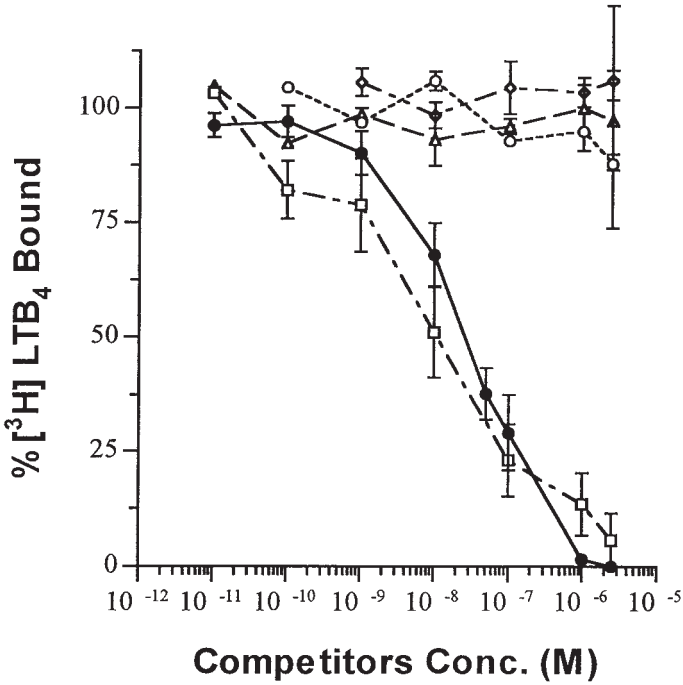

Fig. 7. Effects of lipoxins on LTB_4 binding. T-cells were incubated at 4°C for 60 min with [^{3}H]-LTB_4 (1 n*M*) in the absence or presence of LXA_4 (○), lipoxin B_4 (◇), the selective LTB_4 receptor antagonist SC-41930 (□), the selective LTD_4 receptor antagonist LY171883 (△), or unlabeled LTB_4 (●) at various concentrations. Results are the mean ± SEM of three experiments.

tive result considering the evidence that aspirin-acetylated type 2 prostaglandin G/H synthase converts AA to 15-HETE instead of prostaglandin G_2 ***(111)***.

8. Lipoxins and Renal Inflammation

The question of whether LXs are produced within the mammalian kidney has been addressed in recent studies with animal models. A recent report by Brady, Papayianni, and Serhan ***(112)*** provides evidence for renal biosynthesis of LXA_4 during renal inflammation. These workers relied on two forms of experimental nephritis, both known to be associated with significant PMN-dependent glomerular injury: experimental immune complex nephritis produced in rats by intrarenal infusion of concanavalin A (con A) conjugated with ferritin followed by iv injection of antiferritin antibody ***(112)*** and acute GBM nephritis in mice ***(113)***. In the case of immune complex GN in rats ***(112)***, renal production of LXA_4 was markedly stimulated as determined by ELISA in renal

homogenates prepared 15 min after induction of nephritis. Very low levels of renal LXA_4 production were measured in normals and in sham controls. Because animals depleted of neutrophils or platelets with anti-PMN or antithrombocyte serum prior to challenge with antiferritin antibody showed greatly reduced levels of renal LXA_4 production, transcellular metabolism related to platelet-neutrophil interaction was probably responsible for LXA_4 generation by the kidney. Furthermore, these investigators observed a striking inhibition of LXA_4 production (about 50%) after in vivo treatment of the rats with a MAb (MAb CY 1747) against P-selectin prior to induction of nephritis. The MAb CY1747, which did not block leukocyte recruitment, was also effective in blocking renal LXA_4 production when tested in vitro using renal homogenates from the nephritic animals.

Very recently, the laboratory of Brady ***(113)*** extended this work on P-selectin-facilitated PMN-platelet interaction in comparative studies of renal inflammatory responses of P-selectin-deficient and wild-type mice. Acute nephritis was induced in both strains by iv injection of serum containing rabbit anti-GBM antibody. The P-selectin deficiency was associated with prominent glomerular PMN infiltration and a marked rise of urinary albumin excretion as compared to wild-type mice. Renal LXA_4 biosynthesis was apparently dependent on transcellular metabolism, since generation of LXA_4 (ELISA method) by renal homogenates of the mutant mice was only about 60% of that of wild-type mice, and could be increased to the levels found in wild-type homogenates by administration of wild-type platelets to the P-selectin-deficient mice. These findings highlight the importance of P-selectin and cell–cell interaction for LXA_4 biosynthesis in the two animal models, but may not apply in a direct way to results obtained with other models of experimental nephritis, such as macrophage-dependent accelerated nephrotoxic serum nephritis (NSN) ***(114)***.

Considerable progress has been made in defining the function of adhesion molecules on the surface of leukocytes and endothelial cells during renal inflammatory diseases ***(115)***. A number of groups using different models of experimental nephritis (immune glomerulonephritis, tubulointerstitial nephritis), have concluded that requirements exist for certain specific adhesion molecules ***(116–118)***. For the most part these studies have evaluated the effects of specific antibodies against different classes of adhesion molecules on functional responses (usually proteinuria), or have examined adhesion molecule upregulation by immunohistochemical means. In both the rat ***(119)*** and murine ***(120)*** models of nephritis, the upregulation of ICAM on endothelial cells has been a consistent result, and appears essential for facilitating neutrophil emigration to extravascular sites, and indeed for reaching their destination; e.g., an immune complex located in the subendothelial space outside the glomerular capillary. Also, there is general agreement that the appearance of the CD11/

CD18 family of adhesion molecules on activated PMNs is a critical, obligatory step that allows the cells to emigrate from the vascular compartment. Using a rat model of immune complex nephritis (con A/anti-con A), Zachem et al. ***(118)*** administered anti-P-selectin and observed reductions of glomerular platelet and PMN accumulation in the absence of a significant effect on the proteinuria. As mentioned, Brady and colleagues ***(112)*** have presented evidence for an anti-inflammatory action of endogenous LXs, based on work with an immune complex model of nephritis that is similar in some respects to that employed by Zachem et al. ***(118)***.

The rat model of con A/anti-con A immune complex nephritis is widely regarded as a clinically relevant preparation, and a thorough description of the time-course for the appearance of both neutrophils and platelets has been presented ***(121)***. Proteinuria in this model can be reduced by depletion of complement, as well as by induction of neutropenia with anti-PMN serum, and the glomerular platelet accumulation is complement-dependent. In the above cited study of Zachem et al. ***(118)***, one may have anticipated exacerbation of the glomerular injury after elimination of endogenous LXs with the anti-P-selectin antibody. The basis for the seemingly disparate results of the two studies is not clear—glomerular injury in both preparations stems from a planted antigen, and inflammation triggered by subendothelial immune complexes. However, the extent of glomerular injury may not have been comparable, and if this were the case, structural changes may have affected the ability of cells to pair and elaborate LXs. Fibrin deposition associated with severe endothelial injury was noted in some of the rats with immune complex glomerulonephritis studied by Zachem and coworkers ***(118)***. Tissue-factor, perhaps of subendothelial origin ***(122)*** or from resident glomerular cells ***(123)***, may have activated the extrinsic coagulation pathway to the extent that cell-pairing was obstructed. Unfortunately, renal lipoxin production was not assessed in the studies by Zachem and colleagues.

Lipoxins are considered autacoids since these LO products are formed locally, in a restricted microenvironment, and are believed to act near their sites of synthesis. More needs to be learned about inactivation routes for the LXs. Studies of the metabolic transformations of conjugated tetraenes are challenging and the extracted products may not be the actual intermediates, because of the tendency of such structures to undergo intramolecular rearrangement, epimerization, or auto-oxidation. Indeed, the exact LX isomers formed by various tissues, in the setting of inflammation or immunological challenge, are not known with certainty. Chemical syntheses and analyses are complicated by the presence of three chiral centers, and the cis- or trans stereochemistry; e.g., some isomers found in biological systems, such as the all-trans isomer of LXB_4, may have been artifactually created during the extraction process.

Relatively little work has been directed at the oxidative metabolism of LXs, and basic pharmacokinetic data are lacking. However, it seems likely that the initial degradation step relies on the catalytic activity of 15-hydroxy prostaglandin dehydrogenase (15-OH PGDH), the same enzyme responsible for initiating degradation of the primary PGs, PGE_2 and PGF_{2a} ***(124)***. Evidence for metabolism of LXA_4 by the 15-OH PGDH has been obtained by incubating recombinant human PGDH or differentiated HL-60 cells ***(125)*** with LXA_4 or various synthetic analogs of LXA_4. Some analogs with C15 and C16 modifications appeared resistant to oxidation in these in vitro studies. Work with human leukocytes and rat liver microsomes ***(126)*** has emphasized the importance of the stereochemistry of the delta 11 double bond as a determinant of the pathway for biotransformation. Another likely metabolic pathway for LX metabolism is by a cytochrome P450 isoenzyme(s) similar to the LTE ω-hydroxylase ***(127)***. Human leukocytes as well as rat liver microsomes ***(126)*** are capable of oxidizing LXA_4 and LXB_4 to ω and ω-1 oxidation products, in a manner similar to LTB_4, 12-monohydroxy eicosatetraenoic acid (12-HETE) ***(128)*** and LTC_4 ***(129)***. However, major species differences in the metabolism of LXs could well exist, because such differences were conspicuous in studies by Rokach and coworkers ***(129,130)*** who compared omega oxidation pathways for transformation of sp-LTs in the monkey and rat.

An area of intense research activity is the connection between LXA_4 generation and the extravasation of leukocytes and erythrocytes. Hedquist et al. ***(131)*** first proposed that LXA_4 may serve as "an endogenous inhibitor of inflammatory reactions," based on their videomicroscopy study of plasma leakage and leukocyte emigration in the hamster cheek pouch microcirculation. Striking inhibition of LTB_4-induced plasma leakage from venules was observed in the presence of 3 μ*M* LXA_4 whereas histamine-induced plasma leakage was unaffected by the lipoxin. More recently, Spur and coworkers ***(132)*** demonstrated that LXA_4 caused concentration-dependent inhibition of LTB_4-induced neutrophil chemotaxis. Moreover, they synthesized various LXA_4 analogs that proved useful for determining the structural requirements for LXA_4 blockade of LTB_4 chemotactic responses. Some of these LX analogs were effective inhibitors of LTB_4-induced chemotaxis at picomolar concentrations. A similar approach was recently taken by another group ***(133)*** who reported concentration-dependent inhibition of PMN transmigration and endothelial adhesion in response to LXA_4 and various analogs using monolayer assay systems. Taken together, these results suggest a possible anti-inflammatory role for LXA_4, an influence that maintains normal barrier function of endothelium and opposes the pro-inflammatory effects of the LTs.

One could argue that the concept that "LXs are anti-inflammatory" by opposing leukocyte extravasation is somewhat simplistic, and at odds with results

(134) obtained with human neutrophils and HUVECs, which suggest that LXA_4 may stimulate neutrophil-dependent endothelial cytotoxicity. Such conflicting reports highlight the need for systematic studies, particularly experiments conducted with intact, blood-perfused microcirculations, of the effects of LXs on endothelial cells from different regional beds and specialized capillary networks. Additional information is needed concerning cytokine modulation of the "gatekeeper" function of LO products; i.e., influences that would restrict or facilitate leukocyte extravasation in a manner appropriate to the stage of inflammation. At present, it seems fair to state that tumor necrosis factor (TNF) probably is a pivotal cytokine as regards the regulation of the expression of endothelial adhesion molecules ***(135,136)***, and other endothelial proteins critical for circulatory homeostasis. In a recent study with a model of anti-GBM-induced acute nephritis ***(116)***, TNF_α seemed to play a major role in glomerular upregulation of ICAM-1. Another cytokine with a well-documented effect ***(116)*** on the endothelial expression of adhesion molecules is IL-1. Some cytokines; e.g., IL-4 and IL-13, appear capable of inducing the expression of 15-LO in macrophages (**Fig. 4**), and could theoretically augment LX generation. Diapedesis therefore requires a coordinated system that engages adhesion molecules (possibly E-cadherin) in a manner that allows modulation of the gap junction (by cytokines, kinins, eicosanoids, other autacoids) and interaction of the pseudopodium with the extracellular matrix.

Basic information on the effects of LXs on renal function has been acquired through experimental animal studies. Like LTD_4, LXA_4 reduces glomerular filtration rate (GFR) when administered into the renal artery of anesthetized rats ***(108)***, and is thought to bring about falls in ultrafiltration coefficient (Kf) by interacting with the same binding site as LTD_4 ***(137)***. However, LXA_4 is a partial agonist on the mesangial LTD_4 receptor , and could antagonize the deleterious effect of LTD_4 on glomerular function by competitive inhibition. Unlike LTD_4 which is a renal vasoconstrictor, LXA_4 dilates arterioles in the kidney ***(108)*** and other vascular beds. In contrast to the renal effects of LXA_4, LXB_4 causes reductions of both GFR and RBF ***(108)*** when infused into the renal artery. Several groups working with the rat model of nephrotoxic serum nephritis (NSN) have obtained findings indicating that an elevation of sp-LTs may impair the ability of the glomerulus to form an ultrafiltrate of plasma. This possibility is in line with the observation ***(108)*** that the selective LTD_4 antagonist SKF104353 abolished the increase in protein excretion that typically occurs within 2 h after administration of nephrotoxic serum (NTS).

Possible induction of a 15-LO (or a 12-LO) during NSN has been critically examined by Badr and coworkers ***(53)***. Using a rat model of NSN, the latter group observed marked stimulation of glomerular 15-LO in the first 48 h after injection of the NTS, and presented convincing evidence, based on RT-PCR

amplification of glomerular mRNA, that IL-4 may direct the expression of the 12-LO/15-LO(s) during this phase of the disease. Badr and colleagues ***(53)*** suggested that macrophages might be the source of the 12-LO/15-LO mRNA that abruptly appeared in the glomerulus after 12–24 h, but no histological findings were presented, and these workers could not state unequivocally that the macrophage was responsible. It is certainly possible that changes in 15-LO expression may occur in more than one glomerular cell-type (structural, resident, infiltrating) during NSN.

In summary, our understanding of the medical significance of LTs, HETEs, and LXs has advanced to the point where clinical benefits have materialized. Well-characterized models of inflammation and immune injury have been used gainfully, to indicate new therapeutic approaches. Besides LT antagonists and inhibitors of the 5-LO, other strategies under consideration for drug development are inhibitors of the FLAP and the LTA_4 hydrolase. The initial exploratory work on the possible anti-inflammatory activity of LXs also suggests novel pharmacologic approaches, such as administration of stable synthetic LXs, or augmentation of endogenous LXs by cytokines or synthetic compounds.

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17

Whole Blood Assays for Evaluation of Thromboxane Synthase Inhibition

Eric G. Spokas, Nancy S. Nicholson, Osman D. Suleymanov, Gerald M. Walsh, and David Tuffin

1. Introduction

Researchers in the eicosanoid field have frequently applied whole blood assays ***(1–3)*** for evaluation of enzymic inhibitors in preclinical and clinical studies. In numerous investigations of antiplatelet agents, the capacity of blood to generate thromboxane A_2 (TXA_2) in response to an agonist has been assessed, and similar assays figure importantly in the current clinical trials ***(4)*** of cyclooxygenase-2 (COX-2) inhibitors. Early work by Sun ***(5)***, carried out with human platelet microsomes, established the reaction conditions for studies of thromboxane synthase, and demonstrated that thromboxane synthase is not inhibited by non steroidal anti-inflammatory agents, such as aspirin. It now seems clear that other blood elements, notably monocytes, also contain thromboxane synthase. When the objective is to manipulate, pharmacologically, arachidonic acid (AA) metabolism, or to track routes of transcellular metabolism of AA (discussed in Chapter 16), convenient assays are often set up to measure stable products, such as 6-keto-$PGF_{1\alpha}$ and TXB_2; hydrolysis products of prostacyclin (PGI_2) and TXA_2, respectively. Depending on the specific purpose, different agonists (e.g., thrombin, collagen, endotoxin) may be used to elicit formation of eicosanoids by blood elements.

Measurement of eicosanoid production in response to an activator of phospholipases, added directly to whole blood, can be very useful for defining structure–activity relationships, especially when many synthetic compounds need to be tested. The Biology Department (High Wycombe, UK) and Cardiovascular Diseases group (Skokie, IL) at Searle R & D routinely performed ex vivo assays for evaluation of potential thromboxane synthase inhibitors, seeking

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lead compounds that could actually augment prostacyclin synthesis by diversion of prostaglandin endoperoxide intermediates through the prostacyclin synthase pathway; i.e.; enhancement of leukocyte-derived PGI_2 production ***(6,7)***. Work by McCullagh, Manley, and the Biology group at Searle-High Wycombe in the UK ***(8)*** indicated that ex vivo collagen-induced whole blood TXB_2 formation could be used as an in vitro model of the platelet–leukocyte/vascular endothelial cell interaction. The objective was to "mimic" in an isolated system the shunting of the PGH_2 intermediate that may occur in vivo.

Our group relied on a straightforward collagen stimulation assay which is the primary subject of this chapter. These assays were set up at the Searle labs in the United States and United Kingdom, and led to identification of a novel series of imidazole ester inhibitors of TXA_2 synthase ***(9,10)*** capable of increasing the pool of PG endoperoxides available for other PG pathways. The agent selected as the lead compound was SC-41156 (2,2-dimethyl-6 [2-{1H-imidazol-1-yl}-1-[[{4-methoxyphenyl] methoxy}-methyl}ethoxy} hexanoic acid); this selection was based on work with various platelet preparations, including human lysed platelet or sheep platelet microsomal thromboxane synthase ***(8)***. Kinetic analyses conducted with partially purified human platelet microsomal TXA_2 synthetase demonstrated that the compound was a competitive inhibitor of the human platelet enzyme, with a K_i close to 0.1 μ*M*. Concentrations as high as 100 μ*M* did not suppress the prostacyclin synthetase activity of aortic microsomes (guinea pig).

As part of oral efficacy studies with SC-41156, we set up an assay for measurement of collagen-induced whole blood TXB_2 formation and demonstrated dose-dependent inhibition of TXA_2 synthase after single dose administration (**Fig. 1**). All of our assays at Searle Research and Development, Cardiovascular Research Department (Skokie, IL), were carried out with blood from spontaneously hypertensive rats (SHR), since we were also interested in the possibility that selective TXA_2 synthase blockade might be associated with a hypotensive effect. However, SC-41156 exerted little effect on arterial blood pressure after 14 d of daily dosing by gavage (**Table 1**).

In addition to evaluating SC-41156 in the ex vivo collagen stimulation assay, we determined the effect of SC-41156 on circulating levels of immunoreactive TXB_2 ($_iTXB_2$) (*see* **Subheading 3.2.**). A significant reduction of circulating $_iTXB_2$ levels was observed after 14 d of treatment (i.g.) with either 3 or 10 mg/kg/d SC-41156 (**Table 1**).

Both assays are useful tools for monitoring, ex vivo, the activity of the COX and TXA_2 synthase enzymes ***(3)***. Although numerous laboratories have reported values for "basal" levels of $_iTXB_2$ in serum or plasma from human subjects ***(3)*** and experimental animals ***(2,11)***, investigators sometimes encounter excessive variability in the data. In our experience, the variation does not stem from the

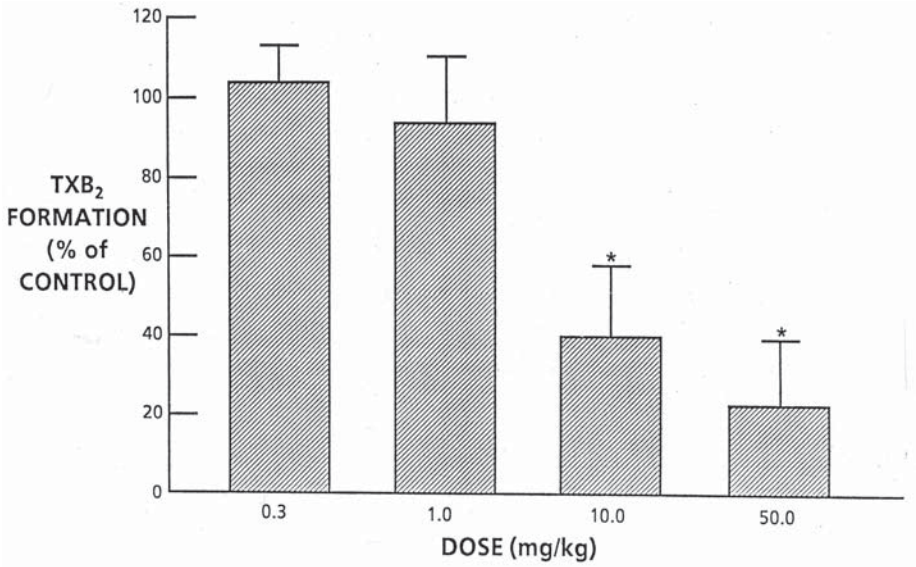

Fig. 1. Effect of orally administered SC-41156 on ex vivo collagen-induced whole-blood immunoreactive thromboxane B_2 formation ($_iTXB_2$) formation in 5-mo-old spontaneously hypertensive rats (SHR). Groups of four rats were treated with carboxymethyl cellulose/ PEG vehicle or SC-41156 (0.3–50 mg/kg) and anesthetized with sodium pentobarbital. Blood was collected from the abdominal aorta into tubes containing trisodium citrate (0.38% final concentration). Blood collections were timed such that 1 h elapsed between oral dosing and ex vivo collagen challenge. Absolute values for collagen-induced $_iTXB_2$ formation in whole blood from the age-matched vehicle control group averaged 33.2 ± 9.6 ng/mL. $*p < 0.05$ as determined by ANOVA with modified *t*-statistics (Bonferroni method).

RIA method, but is likely the result of differences in the extent of platelet activation during blood collection (*see* **Note 1**). Besides describing the ex vivo collagen assay in this chapter, we describe our experimental protocol for measurement of serum $_iTXB_2$. The collagen challenge assay is adapted from the procedure of Defreyn et al. ***(12)***.

2. Materials

1. Indomethacin stock and working solutions. A concentrated solution (10 m*M*) (3.57 mg/mL, pH 8.5) is prepared on the day of the experiment according to the following procedure: 26.7 mg sodium carbonate monohydrate is dissolved in 4 mL 0.9% saline. This solution is added to a graduate cylinder containing a slurry of 71.4 mg indomethacin and 3 mL of 0.9% saline. Indomethacin is then solubilized by vigorous agitation of the graduate cylinder for approx 10 min in a bath of hot tap water. The volume of the resultant solution is brought up to 20 mL

Table 1
Effects of Daily Gavage with Vehicle or SC-41156 on Mean Arterial Blood Pressure (MABP) and Basal Levels of Serum Immunoreactive Thromboxane B_2 ($_iTXB_2$) in Conscious SHR[a]

	SC-41156 Dose (mg/kg/day, i.g.)		
	Vehicle	3	10
MABP (mmHg)	187 ± 3	172 ± 2	183 ± 5
	(n = 16)	(n = 4)	(n = 6)
Serum $_iTXB_2$ (ng/mL)	5.77 ± 1.72	1.07 ± 0.34[b]	2.53 ± 0.50[b]
	(n = 8)	(n = 4)	(n = 5)

[a]Animals (males, 16 wk of age) received SC-41156 at the indicated dose or the vehicle (2% aqueous solution of carboxymethyl cellulose in polyethylene glycol 400, 2.5 mL/kg/d) for a period of 14 d. Arterial blood pressure was recorded (4 h after the final dose) via an implanted arterial cannula; blood was collected from the same cannula after recording blood pressure.

[b]$p < 0.05$ compared to vehicle group.

with 0.9% sterile saline. A working solution of 1 m*M* is prepared by diluting the concentrated stock 10-fold with sterile 0.9% saline. These solutions can be stored at room temperature for 1 d.

2. Heparinized saline to maintain catheter patency. A solution of sodium heparin (30 U/mL) is freshly prepared in sterile saline.
3. Prosil® 28 (PCR Incorporated, Gainesville, FL) siliconizing solution. All of the glass tubes used for blood collection, as well as Pasteur pipets (used to withdraw aliquots of serum), are siliconized with Prosil® according to the manufacturer's instructions. Siliconized cuvets and siliconized magnetic stir bars designed to fit in the well of the aggregometer are obtained from Payton Scientific, Inc. (Buffalo, NY).
4. Collagen solution. This reagent is obtained from Hormon-Chemie (Munich, Germany) and used at a final concentration of 10 µg/mL. This final concentration is considerably higher than that usually employed for studies of platelet aggregation using plasma or platelet suspensions. Dilutions are freshly made for each assay, using the buffer provided with the collagen reagent. These dilutions are stored on ice until used.
5. Citrate solution added to whole blood as anticoagulant. A solution of 3.8% trisodium citrate (w/v) is prepared in distilled water. For blood collection, 1 vol of 3.8% citrate solution is added to 9 vol of blood. We recommend filling 5 mL plastic syringes with 0.5 mL of this citrate solution in preparation for the collagen challenge assay (*see* **Note 2**).
6. Commercial radioimmunoassay (RIA) kits. Levels of $_iTXB_2$ in plasma samples from the collagen challenge assay, and in serum samples, are determined by RIA using New England Nuclear [^{3}H]-TXB_2 Kits (DuPont NEN, NEN Life Sciences, Kit# NEK-007).

   Caution: Tritium emits β radiation. Consult with the Radiation Safety Officer at your organization for safe handling and disposal of this isotope.

7. Sterile saline. Used for preparing the above solutions of indomethacin and heparin.
8. Radioimmunoassay buffer. Serum samples assayed by our method (without extraction) typically require substantial dilution. The phosphate buffer provided with the RIA kit should be used for diluting the plasma samples.

3. Methods

3.1. Ex Vivo Studies of the Effects of Test Agents on Collagen-Induced TXB_2 Formation in Rats

1. After dosing *(see* **Note 3**) with test agent or vehicle, the animal is anesthetized with sodium pentobarbital (50 mg/kg, ip).
2. It is important to have all assay materials and equipment ready and available prior to blood collection.
3. The peritoneal cavity is exposed by a midline incision, and a butterfly cannula (23 gage, Miniset®, Travenol Laboratories, Inc., Deerfield, IL) is placed in the abdominal aorta.
4. Approximately 4.5 mL of whole blood is collected into the plastic syringe prefilled with citrate anticoagulant (*see* **Note 4** concerning timing of blood collections).
5. An aliquot (750 μL) of the citrated blood is pipeted into a small cuvette and stirred (900 rpm) at 37°C in the well of an aggregometer. After 1 min, collagen is added (10 μg/mL final concentration), and stirring is continued for an additional 5 min (*see* **Note 5**). The incubation is terminated by the addition of indomethacin (final concentration = 10 μ*M*) (*see* **Note 6**) and cooling (5 min) in an ice bath.
6. Plasma is separated by centrifugation (1200*g*, 1 min) and stored frozen (–20°C or lower) until assay (*see* **Note 7**). Levels of $_iTXB_2$ in diluted plasma samples are measured by RIA using methods identical to those used for the serum samples (*see* **Subheading 2.**). Plasma $_iTXB_2$ determinations are obtained using duplicate samples. Blank tubes consisting of collagen buffer alone are included as controls in each assay run.

3.2. Measurement of Serum $_iTXB_2$ in Rats Subjected to Chronic TXA_2 Inhibition

It has not been established whether measurement of plasma TXA_2 offers any real advantage over the analysis of serum thromboxane levels. We elected to measure serum TXB_2 since the problem of nonspecific interference may be reduced to some extent. For pharmacodynamic purposes, measurement of serum TXB_2 is usually adequate (*see* **Note 8**).

Blood samples for determination of circulating serum TXA_2 levels are drawn from an implanted carotid arterial cannula ***(13)*** with the animal in the conscious, ambulatory state. An interval of at least 24 h should be allowed postsurgery before blood collection. These blood samples for measurement of serum TXB_2 levels are collected in the presence of indomethacin, which inhib-

its platelet COX-1, effectively preventing in vitro formation of TXB_2. Accordingly, the measurements are considered to reflect concentrations of TXA_2 "circulating," with minimal activation of blood elements.

1. As indicated, it is desirable to collect the samples from the subject by rapid blood withdrawal while they are not experiencing stress or physical activity.
2. Approximately 2 mL of blood is allowed to flow from an arterial catheter directly into silicone-treated glass tubes containing indomethacin (final concentration = 10 μ*M*).
3. With minimal delay, the test tube is gently inverted, and the blood is allowed to clot at room temperature for 45 min.
4. The serum is separated with silicone-coated pipets and stored frozen (–20°C or lower). Basal serum TXB_2 concentrations are determined by RIA as above.

4. Notes

1. The blood sampling process *per se* can lead to artifactually elevated levels of TXB_2. Clearly, the blood sampling artifact is quantitatively more important when one is performing assays of basal "circulating" plasma thromboxane. To obtain good reproducibility, it is important to collect the sample rapidly, with a wide bore arterial catheter (for sampling from rat carotid artery, PE50 or Tygon® equivalent) or butterfly needle (for sampling from rat abdominal aorta, 23 gage).
2. Heparin is believed to have a direct "antiplatelet" effect and should be avoided. Many investigators feel that EDTA is a suitable alternative ***(3)***. If EDTA is selected, a solution of 77 m*M* sodium EDTA (7.5% v/v) can be used or the tube can be coated with a solution of 4.5 m*M* EDTA.
3. The timing of blood sampling in relation to oral dosing is a crucial consideration. Knowledge of the oral bioavailability and C_{max} after oral dosing is obviously desirable. In the case of SC-41156, pharmacokinetic studies conducted with various species indicated persistent inhibition of TXA_2 synthase. The duration of inhibition in rhesus monkeys was estimated as >24 h ***(8)***, as judged by similar assays of collagen-induced whole-blood TXB_2 formation.
4. In order to carry out acute experiments with an orally administered compound, the interval between dosing and blood collection must not vary by more than a few minutes. In order to carry out the study with SHR shown in **Fig. 1**, blood collections were timed such that 1 h elapsed between oral dosing and ex vivo collagen challenge. Consequently, the animals were anesthetized and surgically prepared for blood collection, shortly after dosing.
5. This collagen challenge assay can be readily adapted to concurrent assessment of functional responses; particularly for studies of platelet aggregation. In order to monitor platelet aggregatory responses, samples of whole blood (50 μL) can be removed from each incubation tube immediately prior to and at 4 min following collagen addition. For this purpose, the samples are immediately diluted with Isoton II diluent (1:20), mixed, and a diluted platelet supernate prepared for counting by rapid centrifugation followed by a second dilution step (1:150) in Isoton II. Blood platelets are counted with a Coulter Counter® (Coulter Electronics, Miami, FL), and the whole-blood platelet count derived by correction for the

total dilution factor (1:3000). Note that these steps are carried out with aliquots of the test sample (whole blood) obtained prior to addition of indomethacin; i.e., indomethacin is added immediately after removal of the final sample for platelet counting.

6. Some workers have recommended the use of a higher concentration of indomethacin (100 μ*M*) but in our experience, 10 μ*M* is adequate to terminate the collagen challenge assay, and has been widely used for collection of blood samples in plasma TXB_2 assays. Although we recommend using the cyclo-oxygenase inhibitor indomethacin, for similar assays other laboratories prefer lysine acetylsalicylic acid ***(14)*** in a final concentration of 1 mg/mL to prevent formation of TXB_2 during blood sampling. Note that Simpson et al. ***(7)*** included aspirin (500 μ*M* final concentration) in their blood collection tubes; these tubes served as blanks in their studies of ex vivo TXB_2 generation in canine blood activated with thrombin.
7. Freezing and thawing of plasma samples should be avoided since artifactually high values might result. This elevation has been attributed ***(3)*** to release of TXB_2 from monocytes and platelets in frozen plasma. The group at Searle, UK, routinely saved plasmas at –30°C until RIA for TXB_2.
8. Indomethacin is included in our blood collection tubes to prevent the in vitro formation of TXB_2. It should be emphasized that measurements of "true" circulating plasma levels of TXB_2 may be extremely difficult or impossible to obtain, because of the inherent invasiveness of the sampling procedure. Plasma TXB_2 measurements are sometimes used, however, as an index of "circulating" levels in a nonactivated state. Extraction of plasma (e.g., to remove interfering substances) may improve assay reliability but if this approach is taken, recoveries must be monitored and one must be alert to other problems, such as effects of organic solvents on the RIA procedure.

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18

Novel Eicosanoids

Isoprostanes and Related Compounds

L. Jackson Roberts, II, Cynthia J. Brame, Yan Chen, and Jason D. Morrow

1. Introduction

This chapter will summarize the biochemistry and biological actions of isoprostanes (IsoPs) and related compounds. Several comprehensive reviews of the IsoPs have been published recently ***(1–3)***. Therefore, many aspects of this subject will only be summarized briefly for sake of completeness. Rather, in this chapter, we have provided an update on previously published reviews by focusing in more detail on new information and new emerging areas related to the IsoPs.

2. Isoprostane Biosynthesis

Over two decades ago, it had been shown that prostaglandin-like compounds could be formed by nonenzymatic autoxidation of linolenic acid ***(4,5)***. However, this remained a curiosity and was not taken further. In the course of analyzing for isomeric F-ring metabolites of PGD_2 in plasma by mass spectrometry, we noted a series of prostaglandin F-ring compounds, the levels of which increased ~100-fold during storage of plasma for several months at –20°C. Further studies undertaken in an attempt to understand this curious finding led to the discovery that these were PGF_2-like compounds that were being generated by autoxidation of plasma arachidonic acid during storage ***(6)***. The mechanism envisioned by which these compounds were formed is shown in **Fig. 1**. As noted, three arachidonoyl radicals are initially formed that are then converted to four peroxyl radicals following addition of oxygen. These undergo endocyclization to yield endoperoxide intermediates, which are then reduced to

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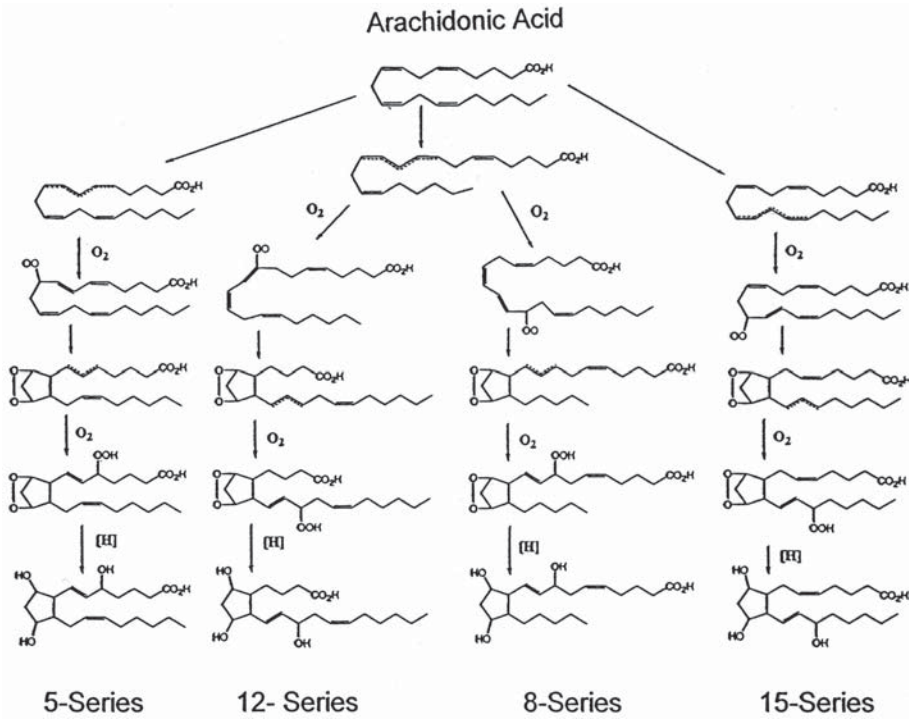

Fig. 1. Mechanism of formation of F_2-IsoPs by oxidation of arachidonic acid. Four regioisomers are formed, each of which is theoretically comprised of eight racemic diastereomers. In accordance with the approved nomenclature system for the IsoPs *(7)*, the four regioisomers are designated by the carbon number on which the side-chain hydroxyl group is located.

yield four F-ring regioisomers. Each of these regioisomers is comprised of eight racemic diastereomers for a total of 64 compounds. Because these compounds are isomeric to cyclooxygenase-derived $PGF_{2\alpha}$, they have been termed F_2-isoprostanes (F_2-IsoPs). A nomenclature system for the IsoPs has been approved by the Eicosanoid Nomenclature Committee for the designation of the individual compounds *(7)*. In accordance with this nomenclature, the four regioisomers are designated by the carbon number at which the side-chain hydroxyl group is located, as indicated in **Fig 1**. Recently, Waugh and Murphy carried out studies to confirm the presence and compare the relative abundance of each of the four regioisomers formed in vitro *(8)*. This revealed that the 5-series and 15-series regioisomers are formed in somewhat greater abundance than the 12-series and 8-series compounds. This is understandable from the proposed mechanism of formation in that a single arachidonoyl radical gives rise to the 12-series and

8-series compounds, whereas the 5-series and 15-series compounds each derive from a single arachidonlyl radical. Thus, in the absence of any influencing factors that may favor the formation of one of the arachidonoyl radicals over others, one would predict a ratio of 1.0:0.5:0.5:1.0 for the relative abundance of the 5-, 12-, 8-, and 15-series compounds, respectively.

Although this was a curious finding that had potentially important ramifications for the analysis of eicosanoids, we explored the possibility that this same process might also occur in vivo. This led to the intriguing discovery that these compounds are also generated in prodigous quantities in vivo ***(9)***. As was found in vitro, we also found that the 5-series and 1 5-series regioisomers are formed in greater abundance than the 12-series and 8-series compounds in vivo ***(10)***. The method of assay developed for quantification of F_2-IsoPs is a stable isotope-dilution-negative ion-chemical-ionization gas chromatography mass spectrometric assay, which has a precision of ±6% and an accuracy of 96% ***(11)***. Interestingly, levels of F_2-IsoPs in human biological fluids, e.g., urine and plasma, exceed levels of prostaglandins derived from the cyclooxygenase enzyme by more than an order of magnitude.

A unique aspect of the formation of IsoPs was the discovery that these compounds are initially formed *in situ* esterified in phospholipids and then released in free form ***(12)***. This finding is consistent with the fact that almost all arachidonic acid in cells exists esterified in phospholipids. F_2-IsoPs esterified in tissue lipids can be measured as free compounds following hydrolysis of a Folch lipid extract of tissue ***(11)***. In addition to the presence of measurable quantities of F_2-IsoPs in free form in all biological fluids, F_2-IsoPs can also be detected esterified in virtually all tissues in the body thus far examined. The formation and release of esterified IsoPs can be a very dynamic process ***(12,13)***. The phospholipase(s) that hydrolyze IsoPs from phospholipids has not yet been established.

In the formation of F_2-IsoPs, the IsoP endoperoxide intermediates are reduced to the F-ring. Recently we found that a key effector of the reduction of the IsoP endoperoxides is glutathione *(14)*. However, if the endoperoxides are not rapidly and efficiently reduced, they can undergo rearrangement. In this regard, we have reported that PGE_2-like compounds (E_2-IsoPs), PGD_2-like compounds (D_2-IsoPs), and thromboxane-like compounds (isothromboxanes) are also produced in abundant quantities in vivo as rearrangement products of the IsoP endoperoxides ***(15,16)*** (**Fig. 2**).

3. Newly Discovered Reactive Products of the IsoP Pathway

3.1. Isolevuglandins

In 1985, Salomon and colleagues reported the discovery of γ-ketoaldehydes that are formed as rearrangement products of the cyclooxygenase-derived PGH_2 endoperoxide ***(17,18)***. These compounds have been termed levuglandin

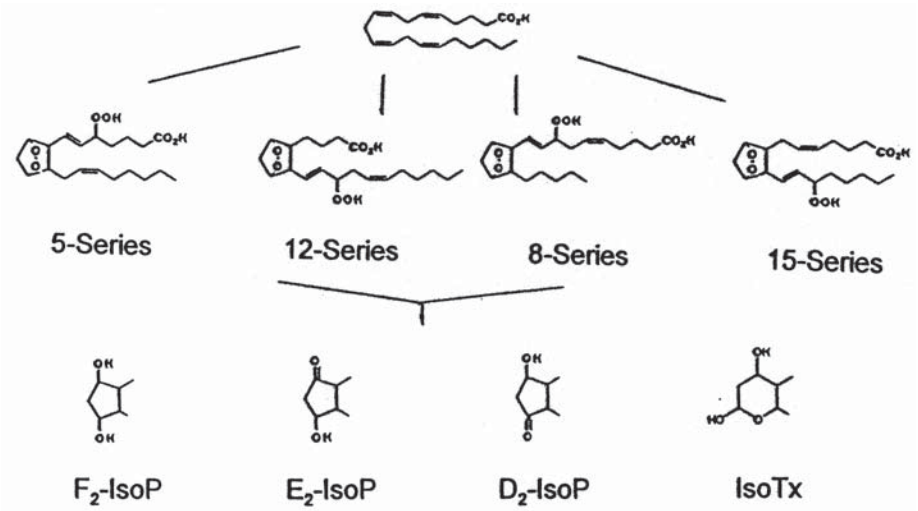

Fig. 2. Additional compounds that have been shown to be formed as products of the IsoP pathway *in vivo*. The four IsoP endoperoxide intermediates are reduced to form F_2-IsoPs as depicted in **Fig. 1** but also undergo rearrangement to form E_2-IsoPs, D_2-IsoPs, and isothromboxanes (IsoTx).

(LG) D_2 and E_2 because of their structural similarity to levulinaldehyde. Recently we have found that similar compounds (IsoLGs) are also generated as rearrangement products of the IsoP endoperoxide intermediates during oxidation of arachidonic acid in vitro (manuscript submitted) (**Fig. 3**). The amounts of these compounds formed during oxidation of arachidonic acid was somewhat unexpectedly quite high in comparison to the amounts of IsoPs formed. The amount of IsoLGs formed was 1241 ± 388 ng/mg arachidonic acid, which was only slightly less than the amount of E_2/D_2-IsoPs formed (1826 ± 413 ng/mg arachidonic acid) and greater than the amount of F_2-IsoPs formed (785 ± 289 ng/g arachidonic acid).

Our interest in the possibility that IsoLGs may be formed as lipid peroxidation products of the IsoP pathway stems from the fact that LGs exhibit a remarkable proclivity to form covalent adducts with proteins and DNA and form crosslinks, effects that could be highly relevant to the pathobiology of oxidant injury ***(19,20)***. Despite the fact that IsoLGs are formed in abundance during oxidation of arachidonic acid in vitro, exhaustive attempts to detect their formation in biological systems have not been successful. We hypothesized that this may be because of rapid adduction of these compounds to proteins or other amines present in biological systems. This notion was supported by experiments in which we examined the time-course for adduction of LGE_2 to protein, using albumin as a model. Adduction was assessed by determining the decline in the

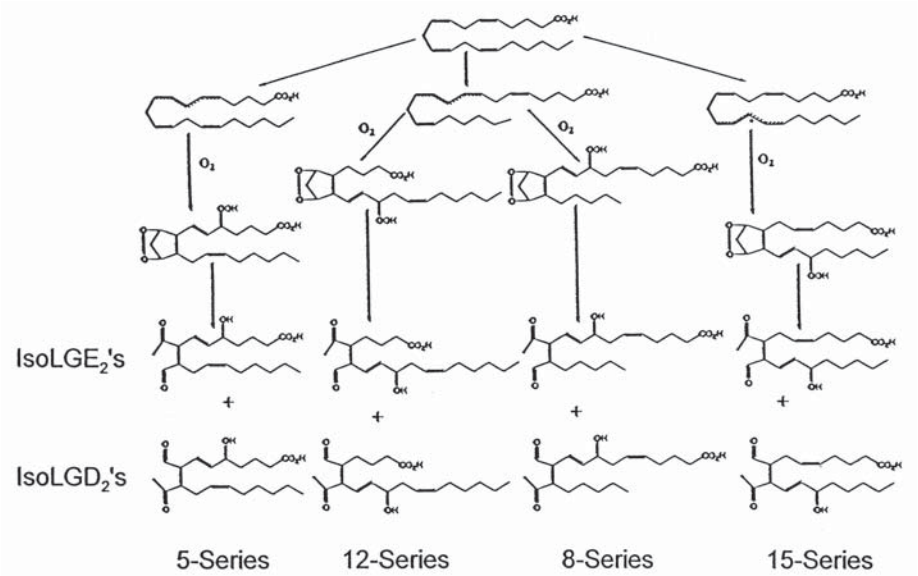

Fig. 3. Formation of Isolevuglandin (IsoLGE$_2$ and IsoLGD$_2$) compounds by rearrangement of the IsoP endoperoxide intermediates.

amount of free LGE$_2$ present in aliquots removed over time from an incubation of LGE$_2$ with a 3-molar excess of albumin. Levels of free LGE$_2$ dropped precipitously; more than half of the LGE$_2$ had adducted within 20 s and the adduction was essentially complete by 5 min. Interestingly, we compared the rate of adduction of LGE$_2$ to albumin with that of 4-hydroxynonenal, which is considered to be one of the most reactive products of lipid peroxidation thus far identified, and found that the rate of adduction of LGE$_2$ exceeded that of 4-hydroxynonenal by several orders of magnitude; after 80 min, approx 45% of 4-hydroxynonenal had still not adducted to albumin. This rate of adduction with albumin found for 4-hydroxynonenal is consistent with that previously reported ***(21)***.

Therefore, we undertook studies to elucidate the nature of the adduct formed with the ε-amino group of lysine. Products formed following incubation of equimolar amounts of radiolabeled lysine with LGE$_2$ were purified by reversed-phase HPLC and analyzed by electrospray tandem mass spectrometry. Salomon and colleagues had provided evidence that LGs form pyrrole adducts ***(22)***. We were able to detect the predicted MH$^+$ ion consistent with the formation of a LGE$_2$-pyrrole lysine adduct, but this was an inconsistent finding. Rather, we consistently detected the presence of compounds 16 and 32 Daltons higher than the predicted MH$^+$ for the pyrrole adduct. These were consistent with lactam and hydroxylactam adducts, which have been shown to be formed by

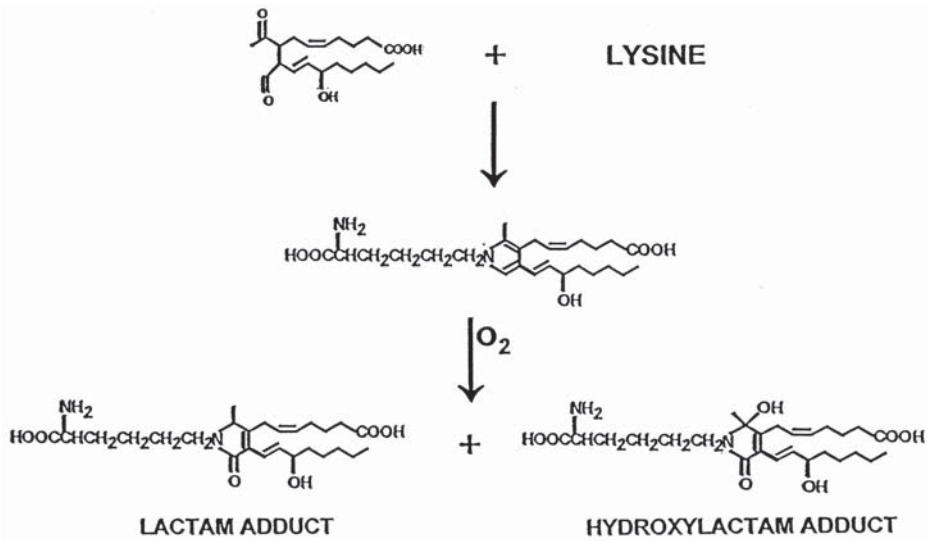

Fig. 4. Formation of LGE_2 adducts with the ε-amino group of lysine. The initial adduct formed is a pyrrole that readily undergoes autoxidation to form lactam and hydroxylactam adducts.

autoxidation of pyrroles *(23)* (**Fig. 4**). To confirm that IsoLGs also formed these adducts, arachidonic acid was oxidized in the presence of lysine. Analysis of products formed revealed multiple IsoLG lactam and hydroxylactam lysine adducts, as would be predicted resulting from the formation of multiple $IsoLGE_2$ and $IsoLGD_2$ species. These studies have now provided the necessary background information required to initiate the development of an analytical approach to assess the formation of IsoLGs in vivo as protein adducts.

3.2. Cyclopentenenone IsoPs

PGE_2 and PGD_2 are unstable molecules under certain conditions that undergo dehydration to form cyclopentenone prostaglandins, PGA_2 and PGJ_2, respectively. Relevant to this process in biological systems is that albumin, and perhaps other proteins, can catalyze the dehydration *(24)*. Cyclopentenone prostaglandins are characterized by an α,β-unsaturated carbonyl which confers chemical reactivity to these molecules. These compounds have been shown to exert interesting biological properties; they undergo Michael addition with thiols, form Schiff base adducts with proteins, induce heat shock protein transcription, and modulate tumor and viral proliferation *(25)*. More recently, it has been reported that they can activate peroxisome proliferator-activated receptor-γ, but this requires very high concentrations (μ*M*) that are very unlikely to be achieved in vivo *(26)*.

Evidence that cyclopentenone prostaglandins derived from the cylooxygenase enzyme are formed in vivo is limited to a single report in which small quantities of Δ^{12}-PGJ_2 were identified in human urine ***(27)***. However, the possibility that the Δ^{12}-PGJ_2 detected was not formed in vivo in the absolute sense, but occurred as a result of dehydration of PGD_2 in bladder urine prior to voiding, remains a possibility. The reactive nature of these compounds may make it very difficult to detect the formation of these compounds in free form in vivo because of their potential to form covalent adducts. In this regard, we had previously shown that the vast majority, if not all, of the radioactivity that is excreted into the bile and urine of the rat following infusion of radiolabeled Δ^{12}-PGJ_2 was in the form of a polar conjugate ***(28)***.

However, we considered the possibility that cyclopentenone IsoPs may be formed by dehydration of D_2/E_2-IsoPs while esterified in tissue phospholipids and that the formation of conjugates/adducts may not occur to an appreciable extent until they are released in free form from membrane phospholipids (**Fig. 5**). Studies undertaken to explore this possibility convincingly demonstrated the formation of cyclopentenone IsoPs in vivo (manuscript submitted). These compounds were detected esterified in liver phospholipids from normal rats at a level of 5.1 ± 2.3 ng/g liver. Following administration of CCl_4 to induce an oxidant injury in the liver, levels increased strikingly by a mean of 24-fold. Following administration of CCl_4 to rats, large quantities of F_2-IsoPs appear free in the circulation ***(9,13)***. However, even in this setting of massive oxidant injury, in which levels of cyclopentenone IsoPs esterified in liver lipids increased dramatically, free compounds in the circulation could not be detected. This would be consistent with the concept discussed above and outlined in **Fig. 5**, in which these compounds may rapidly form adducts following their release in free form from phospholipids. We recently demonstrated that one of the E_2-IsoPs that is formed in vivo is 1 5-E_{2t}-IsoP (8-iso-PGE_2) ***(29)***. Therefore, we generated 15-A_{2t}-IsoP by acid-catalyzed dehydration of 15_{2t}-E-IsoP and found that it rapidly undergoes Michael addition with GSH in the presence of GSH-transferase; approx 80% had conjugated within 2 min and the conjugation was essentially complete by 10 min. It was also found to form covalent adducts with protein, using albumin as a model, albeit at a slower rate; adduction was essentially complete by 2 h. Evidence for the rapid formation of adducts in vivo was also obtained by the finding that virtually none of the radioactivity excreted into the urine following iv infusion of radiolabeled 15-A_{2t}-IsoP into a normal human volunteer could be extracted into methylene chloride, suggesting the presence of a polar conjugate(s). The discovery that reactive cyclopentenone IsoPs are produced in vivo elucidates a new series of reactive molecules that are produced as products of the IsoP pathway, which opens up new interesting avenues for investigation regarding the

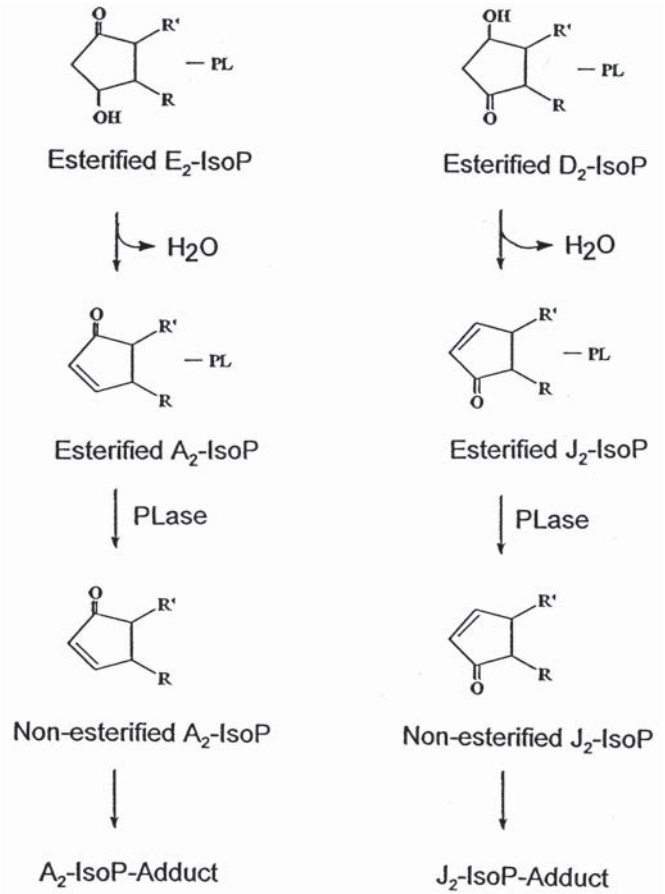

Fig. 5. Scheme depicting the hypothetical fate of cyclopentenenone IsoPs *in vivo*. E_2-IsoPs and D_2-IsoPs are initially formed *in situ* esterified to tissue phospholipids (PL). These then undergo dehydration to form esterified A_2-IsoPs and J_2-IsoPs. While esterified in membrane lipids, A_2-IsoPs and J_2-IsoPs may largely be prevented from forming covalent adducts. However, once released in free form by action of phospholipases (PLase), they rapidly form adducts, e.g., with glutathione and proteins.

biological actions of these compounds as may be relevant to the pathobiology of oxidant injury.

4. IsoP-Like Compounds Formed by Oxidation of Other Fatty Acids

4.1. F_3-IsoPs Formed from Eicosapentaenoic Acid

In addition to F_2-IsoPs derived from oxidation of arachidonic acid, Nourooz-Zadeh and colleagues have recently reported the formation of F_3-IsoPs in vitro

from eicosapentaenoic acid (C20:5ω3) ***(30)***. These compounds differ from IsoPs derived from arachidonic acid by having an additional double bond present, and because of the presence of an additional double bond in the precursor fatty acid, six regioisomers are formed. However, only relatively small amounts of F_3-IsoPs would be expected to be formed in vivo in humans under normal circumstances because eicosapentaenoic acid is not an abundant fatty acid in humans. However, there has been interest in the health benefits of supplementation with fish oil, which contains relatively high levels of eicosapentenoic acid. Thus, measurement of F_3-IsoPs could have applications as a marker of lipid peroxidation in humans who consume large amounts of fish in their diet or whose diet is supplemented with high amounts offish oil. However, even under these unique circumstances, it remains to be established whether measurement of F_3-IsoPs would offer any advantage over measurements of F_2-IsoPs as an index of oxidative stress status in vivo.

4.2. IsoP-Like Compounds (Neuroprostanes) Formed from Docosahexaenoic Acid

More recently, we have described the formation of significant quantities of IsoP-like compounds in vivo from oxidation of docosahexaenoic acid (DHA) (C22n6) ***(31)***. Whereas DHA is also not an abundant fatty acid is most tissues, it is highly enriched in the brain, where levels vary between 20 and 60% of total fatty acids present in various regions of the brain ***(32,33)***. Therefore, we hypothesized that measurement of DHA-derived IsoP-like compounds might provide a unique marker of oxidative neuronal injury. Because DHA is highly enriched in brain neurons and because it would not be appropriate to refer to these compounds as IsoPs, we have termed them *Neuroprostanes* (NPs). The mechanism of formation of F-ring NPs is depicted in **Fig. 6**. As noted, eight regioisomers are formed, each of which are theoretically comprised of eight racemic diastereomers, for a total of 128 compounds. In accordance with the approved nomenclature system for IsoPs, the different regioisomers are designated according to the carbon number at which the side-chain hydroxyl is located. NP's have four double bonds; thus the designation F_4-NPs.

Initially, we explored whether F_4-NPs are formed during oxidation of DHA in vitro. The method used for the analysis of these compounds utilized modifications of the TLC purification procedure previously employed for detection of F_2-IsoPs using $[^2H_4]$ $PGF_{2\alpha}$ as an internal standard ***(11)***. Peak integration was used for quantification of the total amount of NPs formed. Oxidation of DHA with iron/ADP/ascorbate resulted in the formation of a series of compounds that were confirmed using a number of mass spectrometric approaches to be F_4-NPs. The time-course of formation was rapid, reaching a maximum level of approx 5 μg/mg DHA at 50 min. Interestingly, the amount of F_4-IsoPs

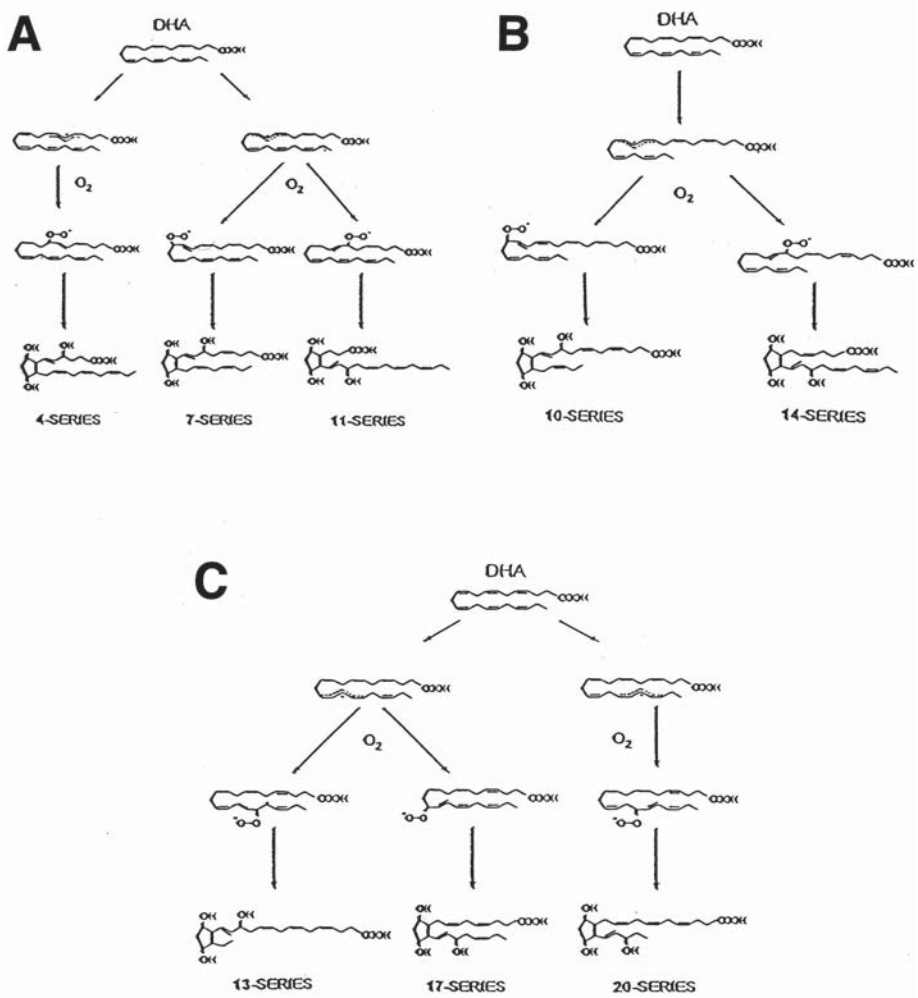

Fig. 6. Mechanism of formation of F_4-NPs by oxidation of docosahexaenoic acid (DHA). Eight regioisomers are formed, each theoretically comprised of eight racemic diastereomers. The regioisomers are designated by the carbon number at which the side-chain hydroxyls are located

formed exceeded the amount of F_2-IsoPs formed during co-oxidation of equimolar amounts of DHA and arachidonic acid by a mean of 3.4-fold. This finding was consistent with the fact that, among all natural occurring fatty acids, DHA is the most easily oxidized ***(34)***. This data also supports the notion that measurement of NPs may provide a uniquely sensitive marker of oxidative neuronal injury. We then analyzed for the presence of these compounds in vivo

esterified in brain lipids of the normal rat and newborn pig and compared levels present with levels of F_2-IsoPs. In whole rat brain, the levels of esterified F_2-IsoPs and F_4-NPs measured were not significantly different; 10.3 ± 3.1 and 7.0 ± 1.4 ng/g, respectively ($p > 0.05$). In contrast, the levels of F_4-NPs measured in newborn pig brain cortex (13.1 ± 0.8 ng/g) were significantly higher than the levels of F_2-IsoPs (2.9 ± 0.4 ng/g) ($p < 0.0001$). These findings are consistent with the fact that the relative amounts of DHA can differ significantly in different areas of the brain ***(32,33)***. This also suggests that there may be region-related differences in the sensitivity of measuring F_4-NPs and F_2-IsoPs as markers of oxidative injury in the brain.

Although these initial studies focused on the identification of the formation of F_4-NPs, analogous to the formation of IsoPs it is reasonable to suspect that the NP endoperoxides also undergo rearrangement to form E-ring compounds, D-ring compounds, and thromboxane-ring compounds. The possibility that these compounds may exert receptor-mediated biological activity remains to be explored. Nonetheless, it is provocative that the synthetic prostaglandin $F_{4\alpha}$ compound that would be formed if DHA were a substrate for the cyclooxygenase, although this has been shown not to be the case, was approximately equipotent as $PGF_{2\alpha}$ in contracting gerbil colon smooth muscle strips ***(32)***. However, the endoperoxides likely may also undergo rearrangement to form highly reactive IsoLG-like compounds, and the E-ring and D-ring NPs, if formed, may also undergo dehydration to form reactive cyclopentenone NPs. Therefore, the discovery of the formation of NPs has opened up numerous new avenues for further study that may yield new information relevant to the pathobiology of oxidative injury in the brain.

5. ISOPs AND NPs as Markers of Oxidant Injury

5.1. Measurement of F_2-IsoPs to Assess Oxidative Stress Status

One of greatest needs in the field of free radical research has been the availability of a reliable test of oxidative stress status *in* vivo. Most methods that had been developed for this purpose in the past were recognized to have serious shortcomings regarding either specificity or sensitivity or were too invasive for use in human investigation ***(35)***. However, we have accumulated considerable evidence suggesting strongly that measurement of F_2-IsoPs represents a major advance in this area. First, these are stable compounds that are not subject to degradation during sample processing. F_2-IsoPs can also be detected in free form in all biological fluids and esterified in all tissues, thus allowing the definition of a normal range. Levels of F_2-IsoPs have also been shown to increase dramatically in animal models of oxidant injury ***(9,13)***. Furthermore, levels increase in animals rendered deficient in antioxidant defenses, even in the absence of administration of an agent to induce oxidant injury, and levels can be suppressed by administration of antioxidants ***(9,36–41)***.

Some general concepts should be mentioned regarding the use of measurements of F_2-IsoPs to assess oxidative stress status in vivo. The most commonly used approach is to measure levels of F_2-IsoPs in readily accessible biological fluids, i.e., plasma and urine. There are distinct advantages and disadvantages associated with both approaches. With plasma measurements, precautions must be taken to avoid artifactual generation of IsoPs by autoxidation of plasma arachidonic acid. Although we have found that this can occur readily during storage of plasma at –20°C ***(6)***, it does not occur in plasma stored at –70°C, at least for up to 6 mo (unpublished observation). Generation of IsoPs by autoxidation in urine is not a concern because lipids are present at very low concentrations. In fact, we have found that incubation of urine at 37°C for 1 wk did not result in an increase in levels of F_2-IsoPs ***(9)***. One of the advantages of measuring IsoPs in plasma is that it provides a sensitive index of total systemic production of IsoPs. This is not the case with urine. Although it is presumed that IsoPs in the circulation are filtered and excreted into the urine by the kidney, we have obtained data that suggests that unmetabolized F_2-IsoPs in urine in part derive from local production of IsoPs in the kidney ***(2)***. This enhances the sensitivity and specificity of measuring urinary IsoPs to assess oxidant injury in the kidney but dampens the sensitivity and specificity of measuring urinary IsoPs as an index of systemic oxidative stress. One of the potential disadvantages of plasma measurements is that it only provides a determination of IsoP production at a single point in time. This can be a drawback in situations in which the production of IsoPs fluctuates significantly over time.

A very attractive approach to assess oxidative stress status in vivo is to measure a metabolite(s) of an F_2-IsoP that can be detected preferably both in the circulation and in urine. The formation of a metabolite is an enzymatic process that cannot occur by autoxidation, thus eliminating that potential problem. Furthermore, measurement of a metabolite provides an index of total systemic production of IsoPs, which circumvents the problem of measuring unmetabolized F_2-IsoPs in urine that, as discussed, in part appear to derive from renal production of IsoPs. Another advantage of measuring a urinary metabolite of IsoPs is that measurements can be performed in urine that has been collected over a period of several hours, which provides an integrated index of IsoP production over time. Toward this goal, we recently carried out a study in which we identified the major urinary metabolite of the F_2-IsoP, 15-F_{2t}-IsoP, in humans as 2,3-dinor-5,6-dihydro-1 5-F_{2t}-IsoP ***(42)***. With this information in hand, it is anticipated that methods of assay for this metabolite or other metabolites with be forthcoming in the near future.

Another general concept involved with the use of measurements of F_2-IsoPs to assess oxidant injury relates quantification of esterified F_2-IsoPs. Although measurements of free IsoPs or their metabolites in plasma or urine can provide

a valuable general index of oxidative stress status, measurement of esterified IsoPs in tissue lipids can be utilized to assess oxidant injury in a specific organ/tissue of interest. Although this approach is most applicable in experimental animal models because it is invasive, the sensitivity of the mass spectrometric assay does allow measurement of levels of esterified IsoPs in small human biopsy specimens (unpublished observations). Another useful but relatively noninvasive application where this approach can be utilized is to assess the extent of oxidation of plasma lipoproteins. Such information can be of value in studies exploring the oxidation hypothesis of atherosclerosis ***(43)***. Almost all lipids present are contained in lipoproteins. Therefore, for such studies, plasma lipids are extracted and free F_2-IsoPs are then measured following alkaline hydrolysis ***(11)***. Utilizing this approach, we obtained evidence that plasma lipoproteins in individuals who smoke exhibit enhanced oxidation compared to controls, providing a possible link between smoking and accelerated atherosclerosis ***(44)***. We have also shown that it is possible to fractionate the lipoproteins and determine levels of F_2-IsoPs in individual lipoprotein subclasses while avoiding the potential problem of autoxidation during separation of lipoprotein subclasses using a rapid isolation technique (45). Thus, it is also possible to obtain information regarding the extent of oxidation from F_2-IsoP measurements in lipoprotein subclasses of interest, e.g., LDL.

Two of the most common tests used to detect lipid peroxidation are measurement of malondialdehyde (MDA) by the thiobarbituric acid reacting substances (TBARS) assay and measurement of lipid hydroperoxides. Two of the major problems with measuring MDA and the TBARS assay is that MDA is not a specific product of lipid peroxidation and the TBARS assay is not specific for MDA ***(21)***. Nonetheless, because of its widespread use, we carried out studies directly comparing measurements of F_2-IsoPs with TBARS and lipid hydroperoxides both in vitro and in vivo ***(46)***. In peroxidizing microsomes, formation of TBARS and F_2-IsoPs closely paralleled each other. We then compared these measures of lipid peroxidation in vivo in rats following administration of CCl_4 to induce lipid peroxidation. Remarkably, levels of F_2-IsoPs esterified in the liver 2 h following administration of CCl_4 increased >80-fold, whereas levels of MDA measured by the TBARS assay only increased less than threefold. Similarly, the formation of lipid hydroperoxides and F_2-IsoPs during oxidation of LDL in vitro were found to closely parallel each other ***(47)***. We then compared the formation of these two markers of lipid peroxidation in CCl_4-treated rats ***(38)***. In these experiments, we measured levels of both F_2-IsoPs and lipid hydroperoxides esterified in the liver and free levels in the circulation. In these studies, lipid hydroperoxides were measured by a highly sensitive and specific mass spectrometric assay following reduction of the hydroperoxides to alcohols. The fold-increase in the levels of F_2-IsoPs esterified in the

liver following administration of CCl_4 (mean 76-fold) greatly exceeded that of lipid hydroperoxides (mean sevenfold). Of further importance also was that there was a dramatic increase (mean 25-fold) in the levels of F_2-IsoPs in the circulation, whereas lipid hydroperoxides were undetectable, even in this setting of severe lipid peroxidation induced by CCl_4.

The results of the above studies greatly highlight the value of measuring F_2-IsoPs as a marker of oxidative stress in vivo. Measurements of F_2-IsoPs to assess oxidant stress has been applied in numerous studies both in experimental animals and humans that have yielded important new findings and insights into the role of free radicals in the pathogenesis of disease processes. These include smoking ***(44)***, atherosclerosis ***(45,48,49)***, hypercholesterolemia ***(45,50)***, ischemia/reperfusion injury to the heart, kidney, and liver in experimental animals ***(45,51–53)***, coronary angioplasty in humans ***(54)***, diabetes mellitus ***(45,55)***, hepatorenal syndrome ***(56)***, acetaminophen overdose ***(56)***, scleroderma ***(57)***, diquat poisoning ***(9,37,58)***, bile duct obstruction (manuscript submitted), rhabdomyolysis (manuscript submitted), ozone inhalation ***(59)***, acute Cr(IV) poisoning ***(60)***, organophosphate poisoning ***(61)***, halothane-induced liver injury ***(62)***, age-related decline in renal function ***(41)***, renal cellular injury induced by cisplatin ***(40)***, alcoholic liver disease ***(63)***, iron overload ***(64)***, high homocysteine levels ***(45)***, antiphospholipid antibody syndromes ***(65)***, experimental brain injury ***(66)*** and O_2-induced lung injury in premature infants ***(67)***. Readers are referred to recent reviews and original articles for details of these studies.

5.2. Measurement of NPs to Assess Oxidant Injury in the Brain

In the studies described previously, we were able to document the formation of F_4-NPs in vivo by demonstrating their presence esterified in brain lipids of experimental animals. Although measurements of NPs esterified in brain tissue may have application in assessing oxidative brain injury in experimental animal models, the application of this approach in humans would primarily be limited to measurements in postmortem samples obtained at autopsy, which may be problematic because of the possibility of artifactual generation of these compounds by autoxidation during the time interval between death and sample procurement. Therefore, we explored whether F_4-NPs are present in detectable quantities in human cerebrospinal fluid. Further, because of the evidence that has accumulated implicating a role for free radicals in the pathogenesis of Alzheimer's disease ***(68)***, we explored whether levels of F_4-NPs are higher in cerebrospinal fluid obtained from patients with well-documented Alzheimer's disease compared to age-matched controls. We found that levels of F_4-neuroprostanes in cerebrospinal fluid of patients with pathologically documented Alzheimer's disease (110 ± 12 pg/mL) were significantly higher than in age-matched con-

trol subjects (64 ± 8 pg/mL) ($p < 0.05$). Essentially an identical fold increase above normal was also found in levels of F_2-IsoPs ***(69)***. These initial data were obtained in ventricular cerebrospinal fluid obtained within 4.5 h following death, both in the patients with Alzheimer's disease and controls. Although autoxidation would not be expected to be a problem in spinal fluid because it is not a lipid-rich fluid, we have since obtained virtually identical data in lumbar fluid obtained from living patients (unpublished data). Much of the evidence for oxidant injury in Alzheimer's disease has been obtained from analysis of brain tissue for products of free radical damage. To our knowledge, this is the only marker of free radical injury that has been shown to be increased in cerebrospinal fluid obtained *intra vitum* in patients with Alzheimer's disease. Thus, the potential impact of being able to utilize these measurements in the diagnosis Alzheimer's disease in living patients is considerable. Although we may also find elevated levels of F_4-NPs in cerebrospinal fluid in other neurological conditions as our studies in this area expand, most neurological diseases can be distinguished from Alzheimer's disease on clinical grounds alone. The major exception to this is the dementia caused by cerebrovascular disease. Therefore, it will be critical as it relates to the specificity for the diagnosis of Alzheimer's disease to establish whether patients with cerebrovascular disease-associated dementia have a normal or increased levels of F_4-NPs in cerebrospinal fluid; such studies are currently underway.

5.3. IsoP and NP Measurements to Define the Clinical Pharmacology of Antioxidants

Another great need in the free radical field is information regarding the clinical pharmacology of antioxidants. In situations in which evidence for oxidative stress is obtained, proof that free radical generation is involved in the pathogenesis of the disorder requires demonstrating that suppression of free radical generation ameliorates the disease process. This is in essence a fulfillment of Koch's postulates. The key questions that need to be answered are:

1. What are the most effective antioxidants?
2. What are the most effective doses of individual antioxidants?
3. What are the most effective combinations of antioxidants? and
4. How long should therapy be given before an assessment of effect is determined?

Unfortunately, little reliable information is available to inform us about these important aspects of antioxidant therapy. This can be attributed to the fact that there has not been a reliable means to assess these effects in humans. However, measurement of F_2-IsoPs should allow us to do that. Although data in this regard is still limited, one study demonstrated that treatment with vitamin C (2 g/d) for 5 d significantly reduced the increased urinary excretion of F_2-IsoPs that occurred in individuals who smoke, but vitamin E (800 U/d) had no effect ***(70)***.

Although this was a very small study involving only 4–7 subjects, the effect of vitamin C and lack of effect of vitamin E may be attributed to the fact that smokers have been shown to be selectively deplete of vitamin C ***(44)*** and the short duration of treatment (5 d) may not have been sufficient to observe an effect of vitamin E, which is highly lipophilic and thus accumulates very slowly in tissues. On the other hand, we recently found that treatment of 48 Finnish males with 200 mg/d vitamin E reduced plasma concentrations of F_2-IsoPs by 27.4% ($p = 0.012$), whereas treatment with 500 mg/d vitamin C had no effect (manuscript submitted). We have also shown that administration of high doses of vitamin E, vitamin C, and β-carotene for a short period of time (2 wk) reduced both circulating concentrations of free F_2-IsoPs and levels esterified in plasma lipids by up to 60% ***(45)***. In another study, administration of 100 and 800 mg/d vitamin E was found to suppress the increased production of F_2-isoprostanes in patients with hypercholesterolemia by 34 and 47%, respectively ***(50)***. These data provide "proof of concept" that measurement of F_2-IsoPs should allow us to define the clinical pharmacology of antioxidants. This information will be of great value in the interpretation of the results of clinical trials that have been completed or are underway assessing the effect of antioxidant therapy in the prevention of disease, e.g., cardiovascular disease, and will greatly inform the rational design of future clinical trials. In addition, measurements of F_4-NPs (and/or F_2-IsoP) levels in cerebrospinal fluid in patients with Alzheimer's disease should for the first time permit a critical assessment of the efficacy of antioxidant therapy to effectively suppress the enhanced lipid peroxidation in the brain that appears to occur with this disease.

6. ISOPs, ISOLGs, and NPs as Effector Molecules

6.1. Receptor-Mediated Biological Actions

In addition to the value of measurement of F_2-IsoPs to assess oxidative stress status, these compounds can also exert biological activity and thus participate as pathophysiologic mediators of oxidant injury. Information regarding the spectrum of the biological actions of the IsoPs at this time is somewhat limited because of the limited availability of compounds in synthetic form for biological testing. However, several groups are actively pursuing the synthesis of IsoPs, which should eventually allow us to greatly expand our understanding of the biological actions of the IsoPs.

Unlike cyclooxygenase-derived prostaglandins, the side-chains of the IsoPs are predominantly oriented *cis* in relation to the cyclopentane ring ***(71)***. Two of the IsoPs that we have documented to be formed in vivo are 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) and, more recently, 15-E_{2t}-IsoP (8-iso-PGE_2) ***(29,72)***. These compounds differ from cyclooxygenase-derived $PGF_{2\alpha}$ only by inversion of the stereochemistry of the upper side-chain. Both of these compounds have been

available in synthetic form for biological testing and studies exploring their biological activity have yielded findings of considerable interest. Both compounds have been shown to be very potent vasoconstrictors of the renal vascular bed ***(9,15,52)***. It is of interest that 15-E_{2t}-IsoP is a vasoconstrictor since PGE_2 derived from the cyclooxygenase is a vasodilator. This suggests that the stereochemistry of the side-chains is an important determinant of biological activity of the IsoPs.

Initially, we found that the renal vasoconstricting effects of 15-F_{2t}-IsoP could be abrogated by SQ29548, a thromboxane receptor antagonist ***(52)***. This initially suggested that the vascular effects of 15-F_{2t}-isoP were mediated by an interaction with thromboxane receptors. However, 15-F_{2t}-IsoP was subsequently shown to be only a very weak agonist of platelet aggregation and acted more potently as an antagonist of thromboxane agonist-induced platelet aggregation ***(73,74)***. Similar data was obtained with 15-E_{2t}-IsoP ***(75)***. Further studies also demonstrated that 15-F_{2t}-IsoP was very ineffective in displacing ligands from the thromboxane receptor ***(74,76)***. Collectively, these findings suggested that 15-F_{2t}-IsoP may interact with a unique receptor. More recently, this notion was greatly supported by radioligand binding studies and functional studies, both of which clearly distinguished the localization of thromboxane and putative 15-F_{2t}-isoP receptors on different cell types ***(77,78)***. Although these studies suggest that 15-F_{2t}-IsoP can exert biological effects at low n*M* concentrations by interaction with a receptor that is distinct from the thromboxane receptor, this does not eliminate the possibility that it can also exert effects by interacting with thromboxane receptors at very high concentrations, as has been shown and concluded in some studies ***(79,80)***. However, it is highly questionable whether the high concentrations required to activate thromboxane receptors are ever achieved in vivo.

In addition to acting as a vasoconstrictor of renal vasculature, 1 5-F_{2t}-IsoP has also been found to potently contract coronary, pulmonary, retinal, and cerebral arterial vasculature, the portal vein, and lymphatics ***(80–88)***. The vascular actions of 15-F_{2t}-IsoP and 15-E_{2t}-IsoP may be relevant to the vasoconstriction that can accompany oxidant injury, for example, during ischemia/reperfusion. In this regard, it is of interest that thromboxane receptor antagonists have been shown to have a protective effect in canine models of myocardial infarction, whereas aspirin is ineffective ***(89)***. This finding is difficult to ascribe to an effect of the thromboxane receptor antagonist to antagonize the effects of thromboxane, but could be explained by an effect to prevent coronary vasoconstriction induced by IsoPs generated in this setting. The finding that 15-F_{2t}-IsoP is a very potent vasoconstrictor of retinal vessels is also of potential importance in that it may contribute to the pathogenesis of ischemia/reperfusion retinopathies, such as the retinopathy of prematurity and of diabe-

tes. In this regard, we recently demonstrated enhanced formation of IsoPs during retinal ischemia/reperfusion injury to the retina ***(78)***. As mentioned previously, overproduction of IsoPs has also been demonstrated in the renal failure of rhabdomyolysis and hepatorenal syndrome, both of which are characterized by intense renal vasoconstriction, the cause of which is not well understood. Thus, it is attractive to consider the possibility that the renal vasoconstriction in these disorders may be mediated in part by IsoPs. The ability of 15-F_{2t}-IsoP to contract the portal vein is also of interest in regard to rupture of esophageal varices in alcoholics. Alcohol administration has been shown to be accompanied by enhanced production of IsoPs ***(63)***, and rupture of esophageal varices in patients with alcoholic cirrhosis frequently occurs after a bout of heavy drinking, which may be linked to an increase in portal pressure caused by enhanced generation of IsoPs.

Other interesting biological actions of 15-F_{2t}-IsoP and 15-F_{2t}-IsoP have also been observed. 15-F_{2t}-IsoP has been shown to induce the release of endothelin ***(90)*** and induce mitogenesis in rat aortic vascular smooth muscle cells ***(76)***. It also caused plasma exudation in guinea pig lungs and induced alterations in monolayer barrier function in cultured pulmonary artery endothelial cells ***(85,91)***. These latter observations may be relevant to the occurrence of altered vascular permeability and fluid transudation in settings of oxidant injury. An additional interesting finding was that 1 5-E_{2t}-IsoP but not 15-F_{2t}-IsoP induces alkaline phosphatase activity, calcification, and differentiation of calcifying vascular cells but inhibits differentiation of bone-derived preosteoblasts ***(92)***. These effects were also seen with minimally oxidized LDL. Based on these observations, it was speculated that 15-E_{2t}-IsoP, perhaps esterified in oxidized LDL, may be a factor underlying the pathogenesis of the coexistence of atherosclerotic calcification and osteoporosis in some patients.

Some insights into the mechanism of the vasoconstrictor action of 15-F_{2t}-IsoP have emerged. It had been shown that 1 5-F_{2t}-IsoP causes increased inositol 1,4,5-trisphosphate production in rat aortic smooth muscle cells ***(76)***. As mentioned previously, it has also been shown to induce endothelin release from rat aorta ***(90)***, suggesting that endothelin in part may mediate the vasoconstrictor action of 15-F_{2t}-IsoP. A recent study has also suggested a lack of dependency on intact endothelium and a partial dependency on induction of cyclooxygenase products, mobilization of extracellular Ca^{2+}, and PKC activation in rat aortic rings ***(93)***. Signaling mechanisms involved in the vasoconstrictor action of 15-F_{2t}-IsoP has also been recently studied in some detail in the retinal vasculature from piglets ***(78)***. In this vascular bed, the vasoconstrictor action of 15-F_{2t}-IsoP was found to be completely dependent on cyclooxygenase-generated formation of thromboxane and mobilization of extracellular Ca^{2+} via what appears to be a non voltage-dependent receptor-operated Ca^{2+} channels and to a

lesser extent by endothelin release. The dependency of the vascular action of this IsoP in piglet retinal vessels is consistent with findings in guinea pig lung ***(85)*** and rat aortic rings ***(93)***, but contrasts with its action in the rat kidney and in rat aortic smooth muscle cells where cyclooxygenase inhibitors did not suppress the renal vasoconstrictor effects of 15-F_{2t}-IsoP, nor was there any evidence that it induced thromboxane formation in rat kidney glomeruli or rat aortic smooth muscle cells ***(76)***. Further studies will be required to determine whether these contrasting findings are caused by species differences or vascular bed-specific mechanisms of action.

Interesting findings have also emerged regarding the receptor-mediated effects of 15-F_{2t}-IsoP and 15_{2t}-E-IsoP on canine proximal colon epithelium and muscularis mucosae ***(94)***. The epithelium responded to 15-E_{2t}-IsoP but not to 15-F_{2t}-IsoP, and evidence was obtained that this was mediated by interaction with a prostanoid EP receptor. Both compounds induced contraction of muscularis mucosa strips that was inhibited by thromboxane receptor antagonists. The abrogation in these responses in the muscularis mucosa by thromboxane antagonists was interpreted as indicative of an interaction with thromboxane receptors. However, as discussed previously, this effect could also result from cross blockade of "isoprostane" receptors by these agents.

Only limited information from a single study has been obtained regarding receptor-mediated biological actions of other IsoPs ***(95)***. In this study, 5-F_{2t}-IsoP, 15-F_{2c}-IsoP (12-iso-$PGF_{2\alpha}$), 15-F_{2t}-IsoP, and 8-F_{2t}-IsoP were tested for their interaction with the ciliary $PGF_{2\alpha}$ (FP) receptor. For purposes of clarity, it should be mentioned that the latter two compounds have been previously referred to in the literature as $IPF_{2\alpha}$-I and $IPF_{2\alpha}$-III, respectively, using unconventional nomenclature. Of these compounds, only 15-F_{2c}-IsoP activated the FP receptor, and this was observed only at very high concentrations ($EC_{50} = 5\ \mu M$), which are unlikely to be achieved in vivo.

6.2. Biological Actions Attributed to the Chemical Reactivity of IsoLGs and Cyclopentenone IsoPs

As discussed previously, we have recently identified highly reactive compounds that are produced as products of the IsoP pathway, namely cyclopentenone IsoPs (A_2/J_2-IsoPs) and isoLGs. These compounds can potentially be important effector molecules in the pathobiology of oxidant injury related to their ability to form adducts with critical biomolecules, including proteins, glutathione, and DNA. Although some of the biological actions attributable to the chemical reactivity of cyclooxygenase-derived PGA_2 and PGJ_2 have been studied in some detail ***(96)***, the scope of our understanding of the biological effects exerted by these compounds is very limited. For example, nothing is known about the consequences of adduction of these compounds to

DNA. Furthermore, the reactivity of PGA_2 and PGJ_2 are not identical, suggesting that the biological effects they exert may also differ. For example, we have found that PGA_2 rapidly undergoes Michael addition with glutathione, but this requires catalysis by glutathione-S-transferase (manuscript submitted). In contrast, we previously reported that Δ^{12}-PGJ_2 rapidly conjugates with glutathione in the absence of enzymatic catalysis ***(28)***. Therefore, the spectrum of biological effects that may result from the numerous cyclopentenone IsoPs generated via the IsoP pathway may be quite varied. In regard to the biological consequences of the formation of IsoLGs, there is little that we might predict based on what we know about the specific biological effects of LGs formed from the cyclooxygenase pathway, aside from the fact that they are reactive molecules. In essence, almost all the investigative efforts to date have focused on understanding the chemistry of LGs and the adducts they form rather than specific effects of their formation on biological systems. Therefore, the biological consequences of the formation of IsoLGs and cyclopentenone IsoPs provides new and largely uncharted areas for potentially important scientific investigation.

6.3. Biophysical Effects of Isoprostane and Neuroprostane-Containing Phospholipids

As discussed, both IsoPs and NPs are initially formed *in situ* esterified in phospholipids. Molecular modelling of phosphatidylcholine with palmitate esterified at the *sn*-1 position and 15-F_{2t}-IsoP esterified at the *sn*-2 position revealed it to be a very abnormally distorted molecule that, if present in sufficient concentrations, would be expected to significantly alter the biophysical properties of cellular membranes ***(12)***. In particular, these molecules would likely effect membrane fluidity and integrity, which are well known sequela of oxidant injury ***(97)***. Even more remarkable and distorted are NP-containing phospholipids. Shown in **Fig. 7** is a ball and stick model of phosphatidylserine with palmitate esterified at the *sn*-1 position and a 13-series F_4-NP esterified at the *sn*-2 position. At the top is the polar head group and trailing downward on the right is palmitic acid. Trailing downward on the left and then turning sharply upward is the F_4-NP. The structural feature that effectuates the sharp curve upward in the F_4-NP molecule is the *cis* double bond located at the nadir of the curve. At the top on the left is seen the cyclopentane ring. In sharp contrast, aminophospholipids with unoxidized DHA esterified at the *sn*-2 position are very tightly packed molecules ***(98)***. Thus, a potentially very important aspect of the formation of NPs esterified in lipids in neuronal membranes is the biophysical alterations they induce that could greatly effect neuronal function, e.g., ion channel and amino acid transport function.

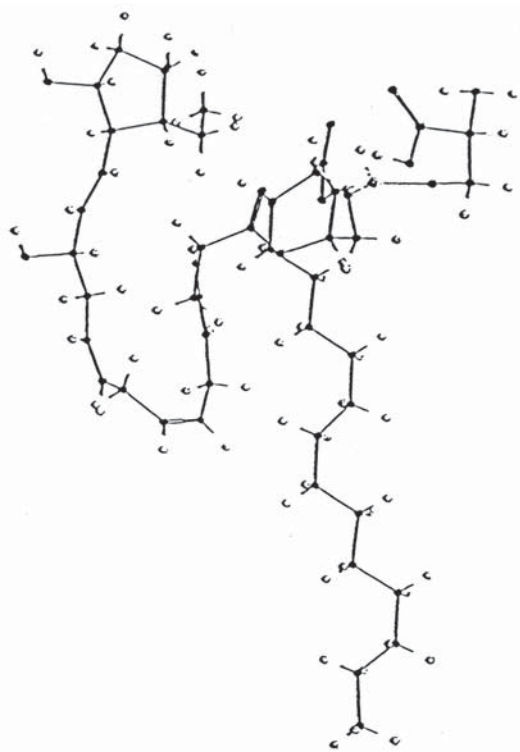

Fig. 7. Ball and wire model of phosphatidylserine with palmitate esterified at the *sn*-1 position and a 13-series F_4-NP esterified at the *sn*-2 position. At the top on the right is the polar serine head group. Descending on the right is palmitic acid. Descending on the left and then turning sharply upward is the 13-series F_4-NP. Effectuating the sharp turn upward is a *cis* double-bond at the nadir of the curve. At the top on the left is seen the cylcopentane ring.

7. Summary

The discovery of IsoPs has been an interesting development for a number of reasons, apart from the fact that it involves novel biochemistry. The simple fact that prostanoids are produced nonenzymatically in prodigous quantities in vivo and in much greater quantities than prostaglandins generated by the cyclooxygenase enzyme was a remarkable finding. The observation that detectable quantities of F_2-IsoPs are present in all tissues and human biological fluids carries interesting implications. Previously, there had been little convincing evidence for the occurrence of lipid peroxidation in vivo except under unusual conditions of severe oxidative stress. However, the finding that F_2-IsoPs can be easily detected in normal humans suggests a continuous level of ongo-

ing oxidative injury that is not completely suppressed by the elaborate system of antioxidant defenses that have evolved. Another very important aspect of the discovery of IsoPs is that it has brought to the field a long sought after reliable approach to assess oxidative stress status in vivo. The continuing and expanded use of measurements of IsoPs for this purpose will contribute in a very valuable way to advancing our understanding of the role of free radicals in human disease processes. Further, the finding that these compounds are not simply markers of oxidant injury but can also exert potent biological actions both by interaction with specific receptors and, in the case of IsoLGs and cyclopentenenone IsoPs, by virtue of their chemical reactivity, has identified several new classes of molecules that are produced by free radical-induced lipid peroxidation that may mediate some of the adverse sequela of oxidant injury. The elucidation of the variety of compounds that are produced as products of the IsoP pathway and more recently the NP pathway provides vast new areas for scientific inquiry that should yield new and interesting information as this area continues to advance.

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