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IDENTIFICATION OF THE PLA₂ RESPONSIBLE FOR PROSTANOID SYNTHESIS
IN RESPONSE TO INFLAMMATORY CYTOKINES

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University.

by

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List of Abbreviations

AA	Arachidonic acid
ATCC	American Type Culture Collection
C-1-P	Ceramide-1-phosphate
CaLB	Calcium Dependent Lipid Binding Domain
COX	Cyclooxygenase
cPLA₂	Cytosolic Phospholipase A₂
ELISA	Enzyme Linked Immuno Sorbent Assay
IL-1β	Interleukin-1 beta
iPLA₂	Calcium independent Phospholipase A₂
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
PAF	Platelet Activating Factor
PGE₂	Prostaglandin E₂
RNAi	RNA interference
SIC	Standard Incubator Conditions
siRNA	small interfering RNA
SMase D	Sphingomyelinase D
sPLA₂	secreted Phospholipase A₂
TNFα	Tumor Necrosis Factor-alpha

Abstract

IDENTIFICATION OF THE PLA₂ RESPONSIBLE FOR PROSTANOID SYNTHESIS IN RESPONSE TO INFLAMMATORY CYTOKINES

By Chaminda Fernando

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2005

Major Director: Charles E. Chalfant, Ph.D.
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Preliminary studies from our laboratory showed that cPLA₂α may be responsible for approximately 50-60% of the PGE₂ production in response to inflammatory cytokines. Thus, we hypothesized that a closely-related PLA₂ is responsible for 40-50% of the PGE₂ produced in response to inflammatory cytokines. To this end, we utilized RNAi technology, extensively optimized, to down regulate the expression of closely-related isoforms of phospholipase A₂ in A549 cells and used an enzyme linked immuno sorbent assay (ELISA) to quantitate the PGE₂ produced. These studies found that cytosolic phospholipase A₂α (cPLA₂α) regulated 97.7% of the prostaglandin E₂ (PGE₂) produced in response to inflammatory cytokines (e.g. IL-1β or TNFα), as well as regulating the basal levels of this prostanoid. Furthermore, cPLA₂γ, cPLA₂δ, and iPLA₂ were found to also

regulate the basal levels of PGE₂ production. On the other hand, cPLA₂β was not involved in prostanoid synthesis in A549 cells either in the presence or absence of inflammatory cytokines. Thus, our studies show that cPLA₂α plays the pivotal role in the production of PGE₂ in response to inflammatory cytokines, and suggests that cPLA₂α may be a possible drug target in diseases such as asthma, inflammation, and cancer.

1. Introduction

Inflammation is the first response of the immune system to infection or irritation. The symptoms of inflammation are characterized by rubor (redness), calor (heat), tumor (swelling), and dolor (pain), which have been known since the ancient times [1]. The study of the signal transduction pathway leading to inflammation has recently flourished due to the relationship of inflammation diseases to cancer, arteriosclerosis, and Alzheimer's disease [2-5]. Recent studies have revealed that prostaglandins and leukotrienes operate as key intra- and inter-cellular inflammatory mediators. These bioactive lipids are produced as a result of the catalytic activity on arachidonic acid (AA) by cyclooxygenases (COX) to produce prostaglandins and by lipoxygenases to produce leukotrienes [6]. Initially, it was thought that the AA cascade was a constitutively active pathway generating prostaglandins for homeostatic functions such as maintenance of the renal blood flow and protection of the gastric lining [7]. This concept soon changed with the discovery of an inducible form of COX, COX-2, which led the former form of COX to be called COX-1; both of which are inhibited by aspirin and other forms of non-steroidal anti-inflammatory drugs [8]. Studies have shown that COX-1 is responsible for basal level prostanoid synthesis and COX-2 is important in various inflammatory settings and cancer [8,9].

Prostaglandins were first isolated from semen in the 1930s, and it was thought that these bioactive lipids were synthesized in the prostate gland, hence the name prostaglandin. Since then many studies have shown that prostaglandins are produced in almost all the cells of the body and that they play a pivotal role in mediating disease states such as inflammation, thrombosis, diabetes, and cancer [10]. They are also responsible for the promotion or inhibition of normal bodily functions such as vasodilation, blood clotting, ion transport, cell growth, lipolysis, and immune system response (figure 1). Prostaglandins belong to a subclass of lipids known as eicosanoids because of their structural similarities to the C-20 polyunsaturated fatty acids, the eicosanoic acids. Soon after the synthesis of prostaglandins, they are released from cells through facilitated transport and act in an autocrine or paracrine manner due to their short half lives (seconds to a few minutes) [8]. Therefore, prostaglandins must be synthesized as needed and are not stored free in tissues, and are one of the most potent natural substances known [1,11]. The same prostaglandin can have different functions depending upon the receptor form on the surrounding cells [8]. Several types of prostaglandins are produced in the body based upon subtle differences in their structure and the most abundant COX-2 product is prostaglandin E₂ (PGE₂), and its levels are used as a measure of phospholipase activation.

The binding of cytokines or growth factors to cell receptors or mechanical trauma can initiate a cascade of events leading to the activation of a group of very important

enzymes known as the phospholipases. The hydrolysis of cell membrane lipids by phospholipases facilitates the release of AA, which is the precursor to prostaglandins and leukotrienes, from phospholipids, and this is the initial rate-limiting step in the eicosanoid biosynthetic pathways [12-15]. The enzymes, COX-1 and COX-2, act on the released arachidonic acid to begin the prostaglandin synthetic pathway (Figure 2).

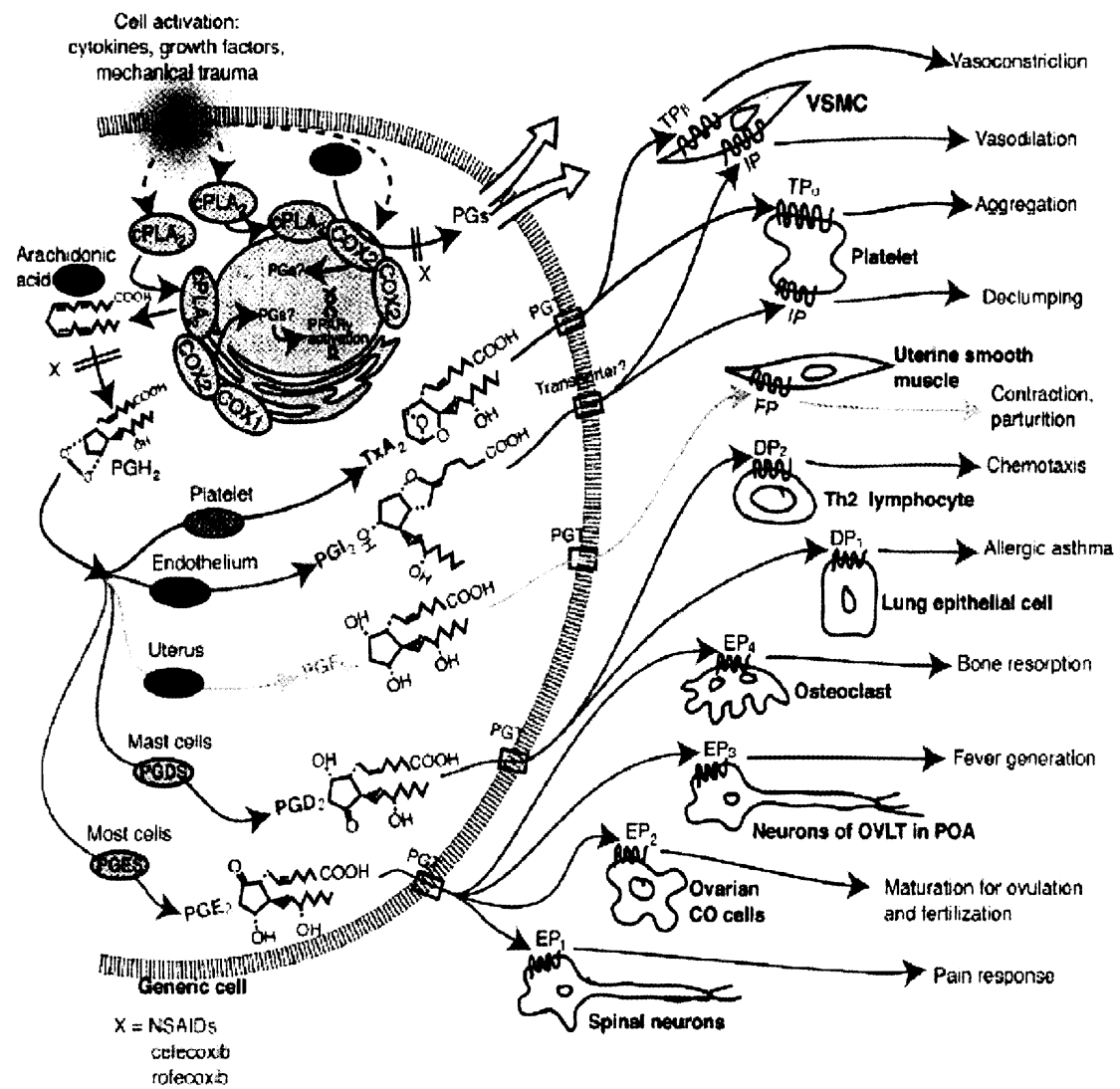


FIGURE 1. Prostaglandin biosynthesis is carried out by most cells of the body (Funk D, Collin. *Lipid Biology* 2001, 294: 1872)

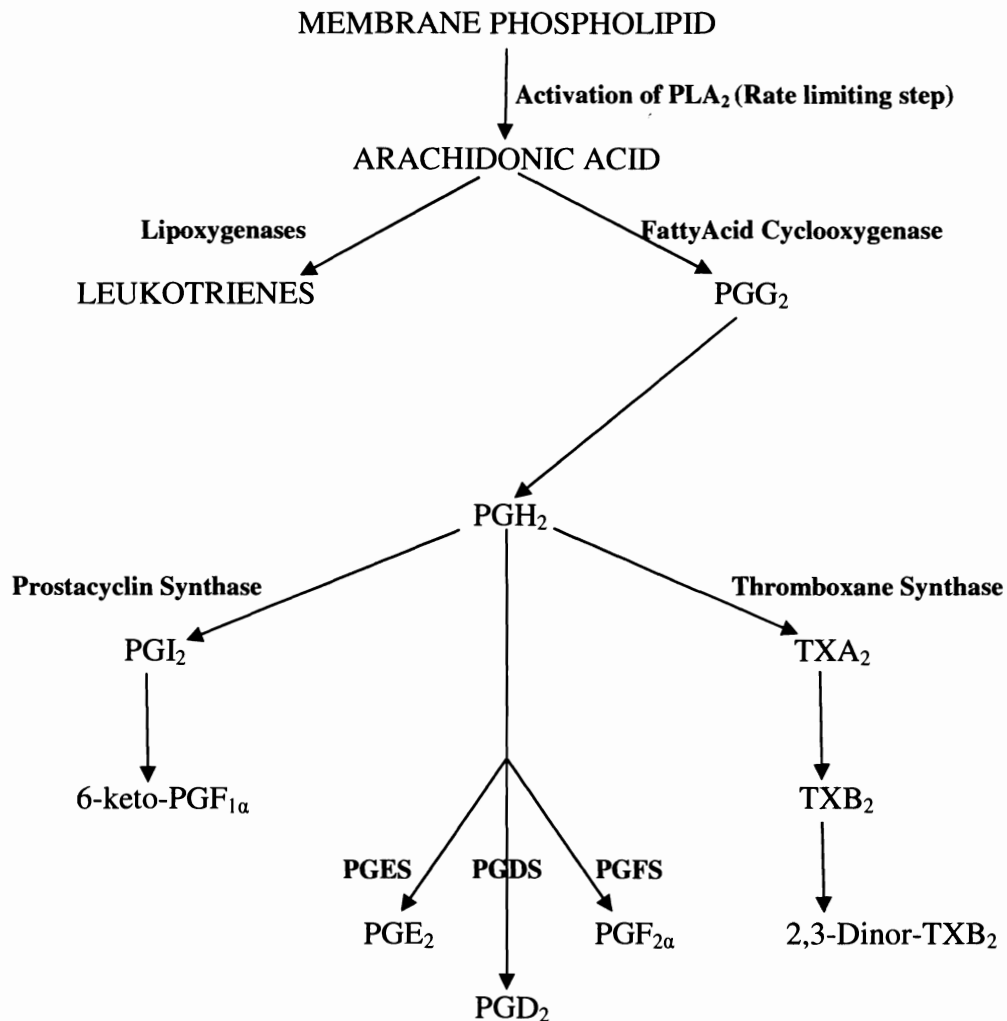


FIGURE 2. Eicosanoid biosynthesis. Activated PLA₂ releases arachidonic acid by hydrolysis of membrane phospholipids. Arachidonic acid can be utilized by either lipoxygenases to generate leukotrienes or by cyclooxygenases (COX-1 and COX-2) to generate the intermediate, PGG₂ followed by PGH₂. The activity of prostacyclin synthase, PGE synthase, PGD synthase, PGF synthase, and thromboxane synthase gives rise to prostaglandins and thromboxanes.

Phospholipase A₂ (PLA₂) enzymes specifically catalyze the hydrolysis of the sn-2 ester bond of phospholipids to release free fatty acids and lysophospholipids (Figure 3) [12,15,16]. Therefore, this group of enzymes plays a critical role in cellular lipid metabolism, thereby regulating energy storage, membrane remodeling, and the production of potent inflammatory mediators (e.g. prostaglandins and leukotrienes) in response to inflammatory cytokines such as IL-1 β and TNF α .

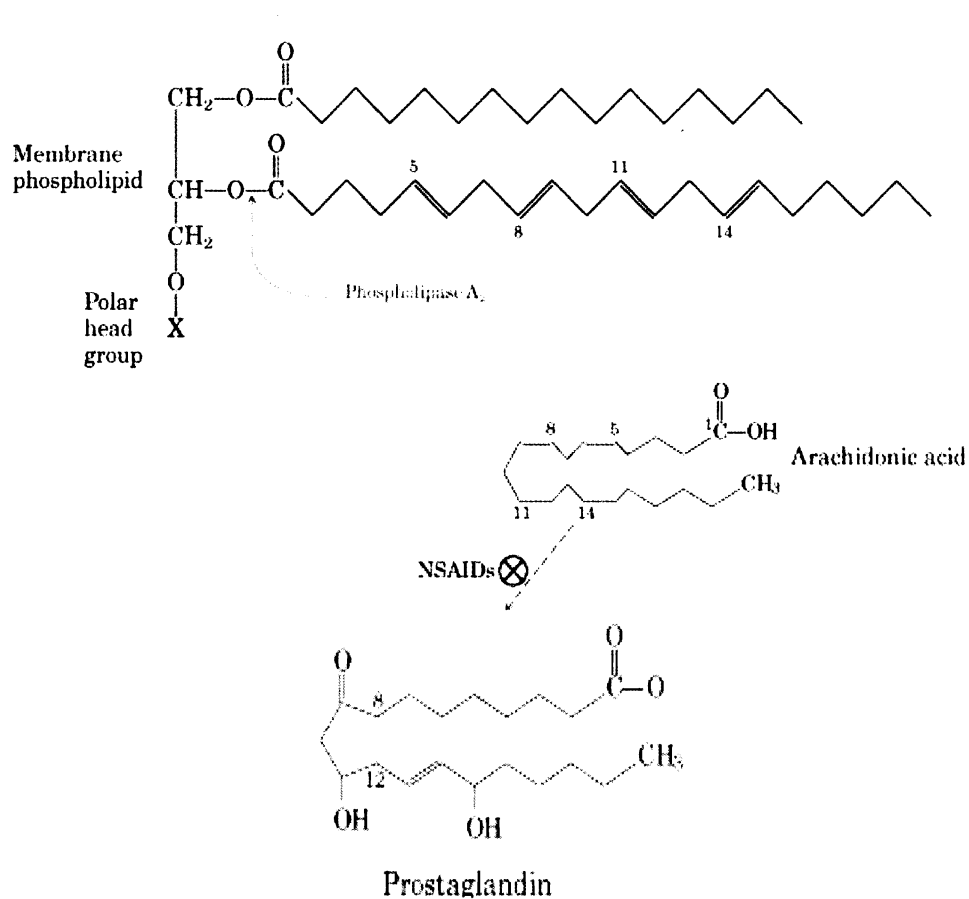


Figure 3. Hydrolysis of membrane phospholipids. Activated PLA₂s cleaves membrane phospholipids at the *sn*-2 position to release arachidonic acid. The catalytic reactions of cyclooxygenases ultimately lead to the production of prostaglandins. The cyclooxygenases are inhibited by non-steroidal anti-inflammatory drugs (NSAIDs).

Mammalian cells contain structurally diverse forms of PLA₂, classified into groups I, II, III, IV, V, VI, VII, VIII, IX, X, XI, and XII (Table 1) [16,17]. Functionally, these groups are recombined into four types of PLA₂s: **secreted PLA₂** (sPLA₂), which are stored in cytosolic granules or synthesized upon stimulation and then secreted extracellularly, consisting of groups I, II, III, V, IX, X, XI, and XII; **cytosolic PLA₂** (cPLA₂), which are located in the cytosol and translocated into the membrane, consisting of groups IVA, IVB, IVC, and IVDelta; **calcium independent PLA₂** (iPLA₂), which are located both in the cytosol and in membrane fractions, consisting of groups VIA, and VIB; and **platelet-activating factor (PAF) acetylhydrolases** consisting of groups VII and VIII [18-20]. The cPLA₂ family of phospholipases is further divided into four isoforms, cPLA₂α (group IVA), cPLA₂β (group IVB), cPLA₂γ (group IVC), and cPLA₂δ (group IVDelta).

Type	Group	Ca ⁺⁺ dependent binding to membranes	Notes
Secreted PLA ₂ (sPLA ₂)	I, II, III, V, IX, X, XI, XII	–	Stored in cytosolic granules or synthesized upon stimulation and then secreted extracellularly
Cytosolic PLA ₂ (cPLA ₂)	IVA, IVB, IVC, IVDelta	cPLA ₂ α-Yes cPLA ₂ β-Yes cPLA ₂ δ-Yes cPLA ₂ γ-No	Located in cytosol and translocated into membrane, except for cPLA ₂ γ, which is membrane bound
Calcium independent (iPLA ₂)	VIA, VIB	No	Located both in cytosol and membrane fractions
Platelet Activating Factor (PAF) Acetylhydrolases	VII, VIII	–	–

Table 1. Classification of phospholipases.

The cDNAs of human PLA₂ isoforms, cPLA₂α, cPLA₂β, cPLA₂γ, cPLA₂δ, and iPLA₂ encode polypeptides of 749, 1012, 541, 818, and 782 amino acids, respectively. Close examination of the primary structures of the group IV isoforms reveal sequence homology among the members, which can be related to the amino acid sequence of iPLA₂ (figure 4). The mRNA for cPLA₂ is widely expressed in the brain, lung, kidney, heart, and spleen [21-24]. All 5 of the phospholipases contain two catalytic domains, A and B, with gene-unique sequences separating the domains. The amino acid residues that

are important for catalytic activity of cPLA₂α, Arg200, Ser228, Asp549, and Arg566, are all conserved in cPLA₂β, cPLA₂γ, and cPLA₂δ [25-27]. Mutagenesis studies have shown that amino acid residues Ser483 (catalytic domain A) and Asp627 (catalytic domain B) in iPLA₂ are involved in catalytic activity and that these residues are homologous to Ser228 and Asp549 in cPLA₂α [18].

Numerous findings on the structure of cPLA₂α, cPLA₂β, and cPLA₂δ have revealed that there is a 120 amino acid sequence near the N-terminus that encodes for the calcium-dependent lipid binding domain (C2/CaLB) [28-30]. In cPLA₂β and cPLA₂δ there are 120 and 135 amino acid inserts, respectively, between the C2 domain and catalytic domain A. However, in cPLA₂α, the C2 domain and the catalytic domain A are adjacent to each other. The C2/CaLB domain functions to facilitate the calcium-dependent binding of cPLA₂α, cPLA₂β, and cPLA₂δ to membranes. Studies have shown that calcium, at physiologically relevant concentrations, is required for the translocation of these cPLA₂ isoforms from the cytosol to the cellular membranes. Numerous studies have revealed that calcium binding to the C2 domain functions as an electrostatic switch. Thus, calcium binding decreases the polarity on the surface of this domain by neutralizing negative charges and promotes penetration of hydrophobic residues into the membrane interior [24]. In contrast, cPLA₂γ is calcium-independent and is farnesylated at the C-terminus, which facilitate this enzyme to bind to membranes.

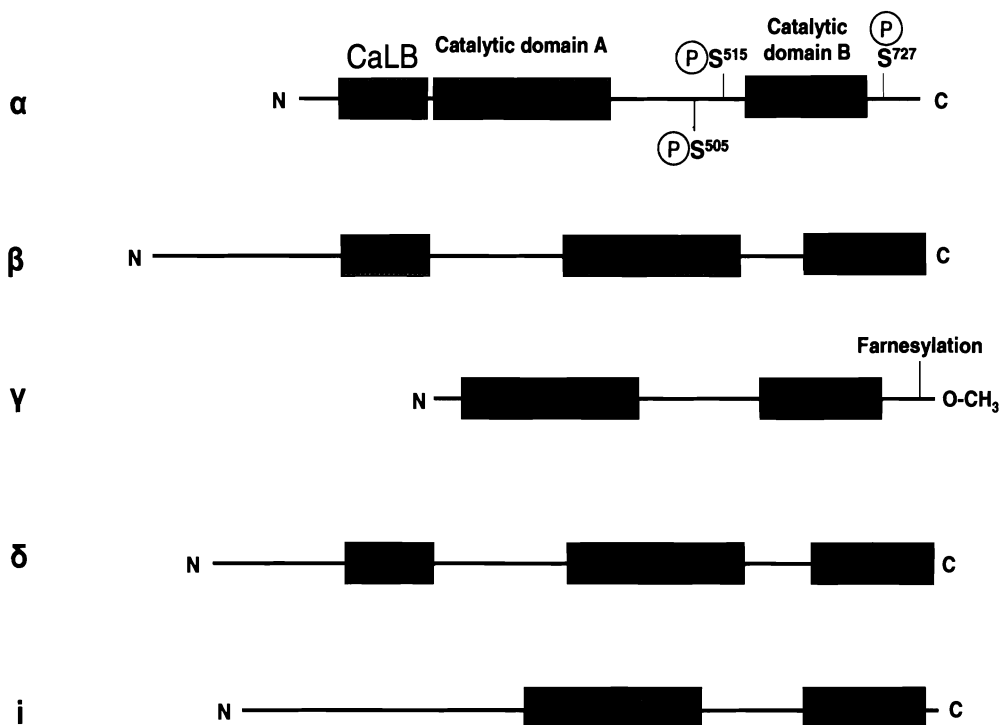


Figure 4. Structure of PLA₂ isoforms. All PLA₂ isoforms carry 2 catalytic domains, A and B. The amino acid residues essential for catalytic activity, Arg200, Ser228, Asp549, and Arg566, are all conserved in cPLA₂α, cPLA₂β, cPLA₂γ, and cPLA₂δ. In iPLA₂, amino acid residues Ser483 in catalytic domain A and Asp627 in catalytic domain B are involved in catalytic activity. These residues are homologous to Ser228 and Asp549 in cPLA₂α. The C2/CaLB domain found in cPLA₂α, cPLA₂β, and cPLA₂δ facilitates the calcium dependent translocation of these enzymes to Golgi/perinuclear regions. cPLA₂γ is membrane bound through farnesylation at the C-terminus.

Research has shown that there is an essential component interacting with the CaLB domain of cPLA₂, which can regulate the membrane association of cPLA₂. Ceramide-1-phosphate (C-1-P), formed by the phosphorylation of ceramide by ceramide kinase is one such lipid regulator of cPLA₂ and interestingly ceramide kinase has similar tissue distribution as cPLA₂ (Figure 5) [11,22]. In other studies, the main component of the venom from the brown recluse spider, *Loxosceles reclusa*, is the enzyme sphingomyelinase D (SMase D) [11,31]. SMase D is responsible for the hydrolysis of sphingomyelin to produce C-1-P and the bite of this spider causes an intense inflammatory response mediated by arachidonic acid and prostaglandins. This led our laboratory to hypothesize that C-1-P may function as a pathophysiologic link in the activation of cPLA₂ and the inflammatory response mediated by arachidonic acid and prostaglandins and that there might be a mammalian counterpart to SMaseD, which led to the discovery of a biology for the enzyme, ceramide kinase.

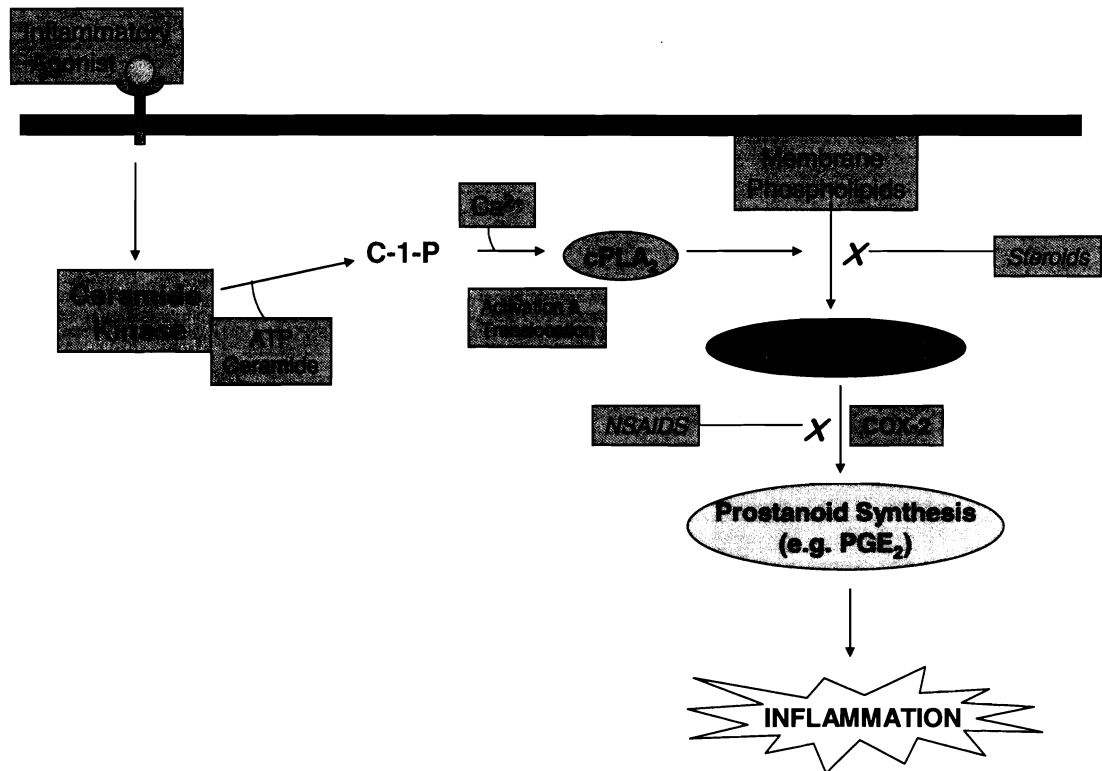


Figure 5. C-1-P, the “missing link” in inflammatory agonist mediated eicosanoid biosynthesis. Binding of inflammatory agonists to cell membrane receptors activates ceramide kinase resulting in the production of C-1-P, which goes on to activate cPLA₂. cPLA₂ hydrolyses membrane phospholipids to release arachidonic acid and this step can be inhibited by steroids. The released arachidonic acid is utilized by COX-2, which is inhibited by NSAIDS, to produce prostanoids ultimately resulting in inflammation.

Published results from our laboratory establish ceramide kinase and subsequent C-1-P production as a “missing link” in eicosanoid biosynthesis in response to inflammatory agonists [11,22,23,]. This data is important because they revealed a specific biology regulated by C-1-P. In the same study, our laboratory showed that down-regulation of ceramide kinase decreased arachidonic acid release implicating that C-1-P is required for arachidonic acid release in response to inflammatory agonists [11]. Furthermore, C-1-P induced translocation of full-length cPLA₂ from the cytosol to the Golgi apparatus/perinuclear regions, and cPLA₂ siRNA completely inhibited the induction of arachidonic acid release by C-1-P [11]. Unpublished findings disclose that cPLA₂ α is responsible for approximately 50-60% of the production of PGE₂. Thus, we hypothesized that cPLA₂ β , cPLA₂ γ , cPLA₂ δ , or iPLA₂ are responsible for the remaining 40-50% of the PGE₂ production, and are possible targets for C-1-P.

2. Materials and Methods

2.1 Maintenance of A549 Human Adenocarcinoma Cells

A549 cells, thawed from cultures stored in liquid nitrogen (freeze media: 10% sterile DMSO, 40% FBS, 50% supplemental media), were obtained from American Type Culture Collection (ATCC). The cells were grown in 250 mL canted, vented tissue culture flasks (Falcon) containing A549 culture media. This media is composed of 50% DMEM (Invitrogen) supplemented with L-glutamine, 50% RPMI 1640 (Invitrogen), 100 U/mL of penicillin G sodium (Invitrogen), 100 µg/mL of streptomycin sulfate (Invitrogen), and 10% (v/v) heat inactivated fetal bovine serum (Invitrogen). Cell cultures were maintained at 37° C in a Heraeus incubator with 5% CO₂ (verified by gas analyzer) and 95% humidity (Standard Incubator Conditions (SIC)). For experiments, cells were counted using trypan blue dye and a hemocytometer (Fisher Scientific) and plated on 24-well plates at 1.25x10⁵ cells/mL per treatment. The 24-well plate was incubated overnight at SIC.

2.2 siRNA Transfection

The plate was observed under an inverted microscope the following day in order ensure that the cells were in the confluency range of 40-70% prior to siRNA transfection. The media was removed and each well was washed with 1 mL of Optimem (Invitrogen). Then, 200 μ L of optimem were added to each well, and the plate was returned to SIC. The siRNA reagent, Dharmafect (Dharmacon), was prepared by diluting 2 μ L of Dharmafect per treatment with 5.5 μ L of optimem per treatment. This solution was incubated at room temperature for 10 minutes. siRNA (Dharmacon) 20 μ M stock solutions were made by adding 250 μ L of 1X Dharmacon siRNA buffer to 5 nmol of lyophilized siRNA. Then, 2.5 μ L per treatment of a respective siRNA stock solution was diluted in 40 μ L per treatment of Optimem and the diluted Dharmafect was added to each siRNA solution at 7.5 μ L per treatment. The solutions were mixed by pipetting up and down gently three times, and the siRNA/Dharmafect complex was incubated at room temperature for 15-20 minutes. After the incubation period, the siRNA/Dharmafect mix was added drop wise to the appropriate wells at 50 μ L per well. The plate was swirled gently to mix well and then placed at SIC for 4 hours. Following the incubation period, 125 μ L of 3X growth media (1.8% Penicillin/Streptomycin, 30% Fetal Bovine Serum, 68.2% Optimem) was added to each well and returned to standard incubation conditions for 24 hours. After the incubation period, A549 media was added to each well and the plate was gently swirled to mix, which was followed by removal of the media. 1 mL of

A549 media was then added to each well, and the cells were returned to standard incubation conditions for 24 hours.

2.3 Treatment with Cytokines

On the day of the treatment, the cells were rinsed twice with resting media (2% Fetal Bovine Serum, 96.2% DMEM, 1.8% Penicillin/Streptomycin) and the cells were observed for any changes in confluency and/or morphology. The cytokines, IL-1 β and TNF α were diluted in resting media at 5 ng/mL and 10 ng/mL, respectively. The media was removed and resting media with the appropriate cytokine was added to each well at 1 mL per well. The control wells received equal amounts of PBS diluted in resting media (sham control). The plate was incubated at SIC.

2.4 WST-1 Assay

Following cytokine treatment, 750 μ L of media from each well was collected. To the remaining 250 μ L of media in the wells, 25 μ L of WST-1 reagent (Roche Diagnostics) was added and the plate was placed back in the incubator for 30 minutes. After the incubation period, the optical density in each well of the plate was measured (at 450 nM vs. a reference of 630 nM) using microplate spectrophotometer (BIO-TEK KC Junior). These data were used to normalize the proceeding PGE₂ results.

2.5 Enzyme Linked Immuno Sorbent Assay (ELISA)

The standard was prepared by obtaining eight 1.5 mL microfuge tubes and numbering them #1 through #8. To tube #1, 180 μ L of 1 in 40 dilution resting media in 1X EIA buffer (Cayman Chemical) was added. To tubes #2 through #8, 100 μ L of the same solution was added. Next, 20 μ L of the standard PGE₂ (Cayman Chemical) was added to tube #1 and mixed thoroughly. In order to serially dilute the samples, 100 μ L was removed from tube #1 and placed in tube #2 and mixed thoroughly, from tube #2 100 μ L was removed and placed in tube #3 and mixed thoroughly. This process was repeated until standard #8. The ELISA plate (Cayman Chemical), coated with goat anti-mouse IgG was loaded at 50 μ L per well of standard/sample, where the sample was diluted at 1 in 40 (sample : 1X EIA), 50 μ L of PGE₂ EIA AChE tracer (Cayman Chemical), and 50 μ L of PGE₂ monoclonal antibody (Cayman Chemical). The control wells received 50 μ L of 1X EIA buffer along with 50 μ L of PGE₂ EIA AChE tracer and 50 μ L of PGE₂ monoclonal antibody. The plate was covered and placed in 4° C for 16 hours.

After the incubation period, all the liquid from the wells were removed and the plate was washed with wash buffer (Cayman Chemical) five times. Next, 200 μ L of Ellman's reagent (Cayman Chemical) was added to each well and the plate was covered and allowed to develop in the dark with low shaking at room temperature for 90 minutes.

Following the developing step, absorbance in each well at 405 nM was read using a microplate spectrophotometer (BMG Labtech FLUOStar Optima). Wells containing Ellman's reagent alone served as the blank for absorbance background.

2.6 Western Immunoblotting Against cPLA₂ α

A549 cells were counted using trypan blue exclusion counting, and were plated into 6-well plates at 2.5×10^5 cells/35 mm well (4 mL total media) per treatment. The 6-well plate was incubated overnight at SIC. The plate was observed under an inverted microscope the following day in order ensure that the cells were in the confluency range of 40-70% prior to siRNA transfection. The media was removed and each well was washed with 1 mL of Optimem. Then, 600 μ L of Optimem were added to each well and the plate was placed back in the incubator. Next, 7.5 μ L per treatment of its respective siRNA 20 μ M stock solution was diluted in 120 μ L per treatment of Optimem. The siRNA reagent, Dharmafect (Dharmacon), was prepared by diluting 6 μ L of Dharmafect per treatment with 16.5 μ L of optimem per treatment. This solution was incubated at room temperature for 10 minutes. Then, the diluted Dharmafect was added to each siRNA solution at 22.5 μ L per treatment. The solutions were mixed by pipetting up and down gently three times. These siRNA/Dharmafect complexes were incubated at room temperature for 15-20 minutes. After the incubation period, the siRNA/Dharmafect mix was added drop wise to the appropriate wells at 150 μ L per well. The plate was swirled

gently to mix well and then placed at SIC for 4 hours. Following the incubation period, 375 μL of 3X growth media was added to each well, and the plate was returned to SIC for 24 hours. On the following day, the cells were observed for any changes in confluency and/or morphology. Then, 1 mL of A549 media was added to each well and the plate was gently swirled to mix. Next, the media was removed, and 1 mL of A549 media was added to each well. The plate was returned to SIC for an additional 24 hours. After 24 hours, the cells were lifted using a cell lifter, and the media with the cells were transferred into 15 mL conical tubes. The tubes were centrifuged at 4^o C under low speed in order to pellet the cells, and the supernatant was discarded. The cell pellet was washed with 1X cold PBS and the tube was centrifuged again. The supernatant was discarded, and the cell pellet was re-suspended in 100 μL of 1X cold PBS and transferred to 1.5 mL microfuge tubes with screw caps. Next, 100 μL of 2X lysis buffer (200 μL 1X Binding buffer, 10 μL Leupeptin, 1 μL Aproprotinin, 5 μL Pepstatin, 10 μL PMSF, 100 μL Glycerol) was added to the tubes, and the samples were boiled for 10 minutes.

30 μL of the total protein lysate was subjected to 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) and blocked in 5% milk, 1 \times PBS-T (M-PBS-T) for 2 h. The membrane was incubated with anti-cPLA₂ α polyclonal goat IgG (Santa Cruz Biotechnology) or anti- α -tubulin mouse mono-clonal IgM (Santa Cruz Biotechnology) for 2 h in M-PBS-T followed by 3 washes with PBS-T. The membrane was then incubated with a secondary antibody of horseradish peroxidase-

conjugated affinipure donkey anti-goat IgG (anti-cPLA₂α) (Jackson Immuno Research Labs Inc.) or horseradish peroxidase-conjugated anti-mouse IgM (Cal-Biochem) (anti-α-tubulin) for 45 min followed by 3 washes with PBS-T. Immunoblots were developed using Pierce ECL reagents and Bio-Max film.

3. Results

3.1 Optimization of the media used in the transfection of A549 cells with siRNA

Initially, we used DMEM as the media to wash the cells prior to transfection and to incubate the cells that have been transfected with siRNA. We observed that most of the cells in the center of the wells died just 24 hours post-transfection. Evidently, DMEM proved to be toxic to RNAi transfected cells. Then, we replaced the DMEM with optimem for the washing and incubation steps and the cell survival rate increased dramatically. Thus, optimem was used as the media of choice in the transfection of A549 cells with siRNA.

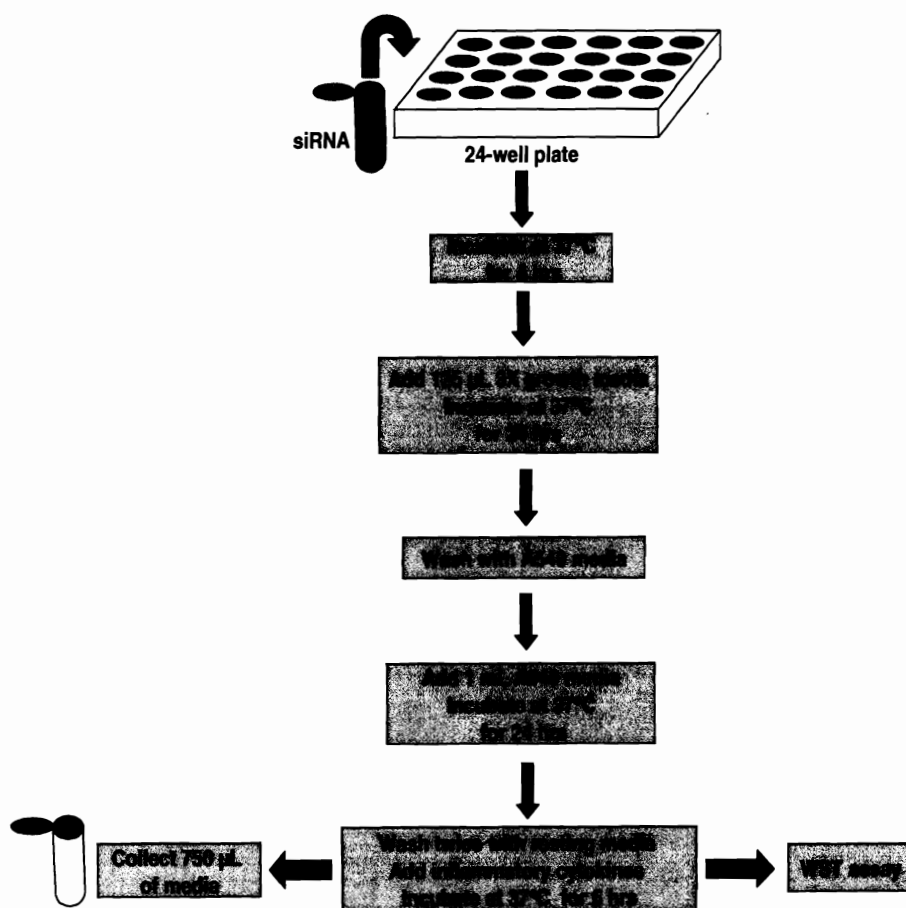


Figure 6. Optimized protocol used to transfect A549 cells with siRNA

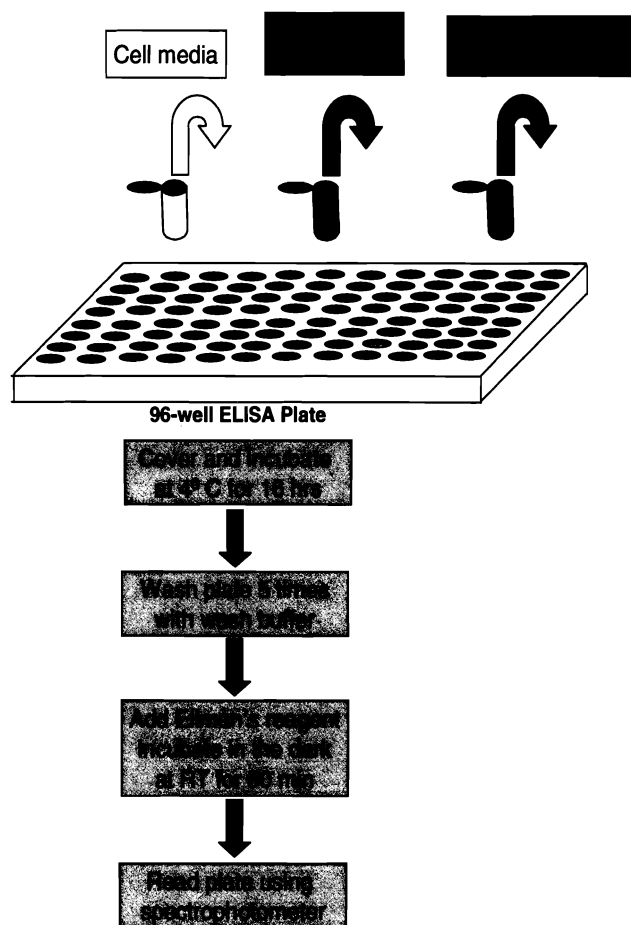


Figure 7. Transferring the media collected from the cytokine treated cells on to an ELISA plate to quantitate PGE₂.

3.2 Optimization of the transfection protocol for siRNA

During the initial stages of the project, we were using Oligofectamine transfection reagent (Invitrogen) to introduce siRNA to A549 cells. This protocol generated very good results until the manufacturer changed the reagent, causing non-reproducible results. We examined Dharmafect, the RNAi transfection reagent manufactured by the same company that produces siRNA, as a vehicle to introduce siRNA into A549 cells. This transfection reagent worked very effectively to generate reproducible results, and Dharmacon siRNA transfection protocol is the standard protocol now used by the Chalfant laboratory.

3.3 Optimization of the stable maintenance of PGE₂ production in A549 cells

One of the obstacles that we had to overcome was the elevated basal levels of PGE₂ production from the non-cytokine treated A549 cells. We attributed this to over stressing the cells. We incorporated a washout step with A549 complete media 24 hours post-transfection to overcome the elevated basal PGE₂ production. This resulted in relatively low basal PGE₂ levels. Thus, this step was incorporated into the siRNA transfection protocol.

3.4 cPLA₂α siRNA down-regulates target protein

To determine if cPLA₂α was downregulated by RNAi transfection, a Western immunoblot utilizing an anti-cPLA₂α antibody was used. The normalized densitometry data indicated that cPLA₂α was down-regulated by approximately 83% (figure 8). The data demonstrated that siRNA was specific to cPLA₂α and that down-regulation of cPLA₂α was not due to any off-target effects of siRNA. Furthermore, siRNA to the closely related cPLA₂δ and iPLA₂ had no effect on cPLA₂α levels, also demonstrating specificity. Therefore, the transfection was successful and cPLA₂α siRNA actually down-regulated the target protein.

3.5 cPLA₂α regulates PGE₂ generation at basal levels and in response to inflammatory cytokines

Preliminary data from our laboratory inferred that cPLA₂α may be responsible for approximately 50-60% of PGE₂ production in response to inflammatory cytokines, but the RNAi technology was not optimized. To further verify this finding, and determine the percentage of PGE₂ produced in response to inflammatory cytokines regulated by cPLA₂α, RNAi transfection was again employed to down regulate the expression of cPLA₂α. An optimized ELISA, to quantitate the PGE₂ produced, specifically demonstrated that siRNA targeted to cPLA₂α inhibited approximately 80% of the PGE₂ produced in response to either IL-1β or TNFα. Normalizing this percentage of inhibition

to the percentage of down regulation of cPLA₂α indicated that 96.4% of the PGE₂ produced in response to inflammatory cytokines was due to cPLA₂α. Furthermore, siRNA targeted to cPLA₂α inhibited 95.6% of the basal levels of PGE₂ (figure 9).

3.6 cPLA₂γ, cPLA₂δ, and iPLA₂ regulate the basal levels of PGE₂ production and cPLA₂β is not involved in prostanoid synthesis in A549 cells

According to preliminary data, 50-60% of PGE₂ produced in A549 cells in response to inflammatory cytokines was regulated by cPLA₂α, thus we hypothesized that the remaining 40-50% is produced by cPLA₂β, cPLA₂γ, cPLA₂δ, or iPLA₂. As stated above, optimization of the siRNA transfection demonstrated that approximately 100% of the PGE₂ produced in response to inflammatory cytokines was due to cPLA₂α. To further verify this finding, RNAi technology was utilized to down regulate the aforementioned PLA₂ isoforms individually. cPLA₂γ, cPLA₂δ, and iPLA₂ inhibited the basal level of PGE₂ production, but as expected these phospholipases had no effect on cytokine mediated production of PGE₂ (figure 8). In addition to the above findings, the data indicated that cPLA₂β did not have a role in the cytokine induced or the basal level of PGE₂ production (figure 10). Therefore, these findings verify that cPLA₂α is the PLA₂ responsible for PGE₂ production in response to inflammatory cytokines as down regulation of closely related PLA₂s had no effect. Furthermore, possible roles for iPLA₂, cPLA₂γ cPLA₂δ in basal PGE₂ synthesis have been implicated.

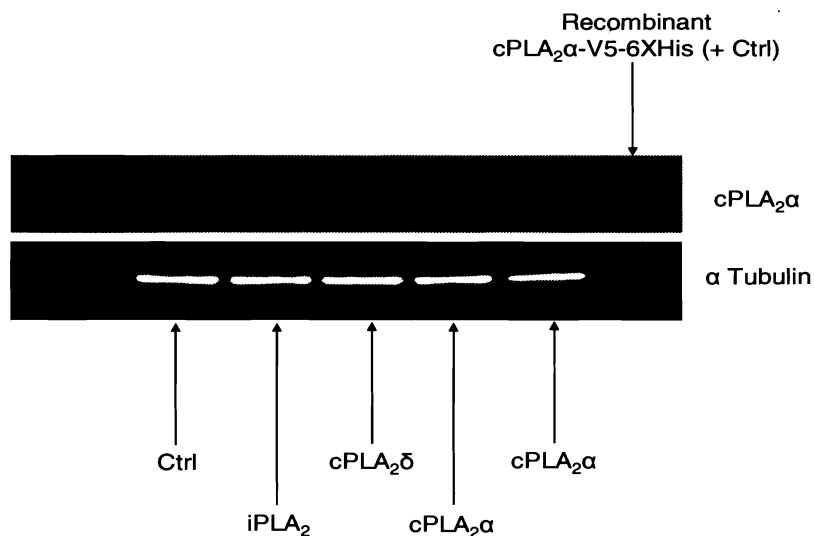


Figure 8. Down regulation of cPLA₂α by RNAi transfection. Cells were transfected with respective siRNA and cell lysates were subjected to 10% SDS-PAGE analysis, transferred to a polyvinylidene difluoride membrane, and immunoblotted as described in “material and methods” section. The top panel shows the down regulation of cPLA₂α in cells transfected with siRNA as compared to cells transfected with control, iPLA₂, and cPLA₂δ siRNA. The bottom panel shows the blot normalized with α tubulin. Recombinant cPLA₂α-V5-6XHis was used as positive control. This figure is representative of two independent experiments.

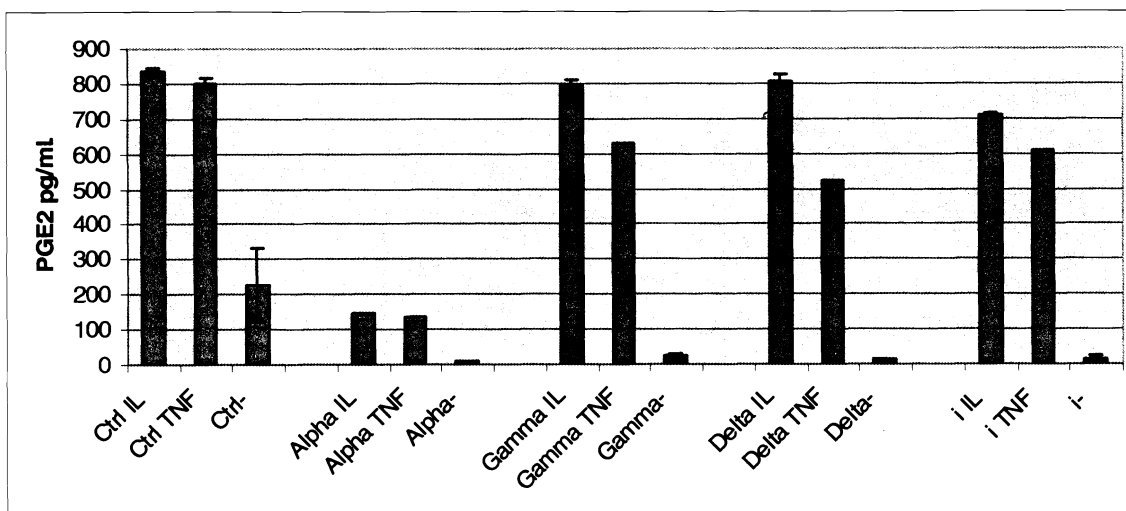


Figure 9. Down regulation of PLA₂ isoforms using RNAi transfection resulted in decreased PGE₂ production. Cells were transfected with siRNA and treated with IL-1 β (IL), TNF α (TNF), or PBS (-) as described in the “material and methods” section; **Ctrl IL** = transfected with control siRNA and treated with IL-1 β ; **Ctrl TNF** = transfected with control siRNA and treated with TNF α ; **Ctrl-** = transfected with control siRNA and treated with PBS; **Alpha IL** = transfected with cPLA₂ α siRNA and treated with IL-1 β ; **Alpha TNF** = transfected with cPLA₂ α siRNA and treated with TNF α ; **Alpha-** = transfected with cPLA₂ α siRNA and treated with PBS; **Gamma IL** = transfected with cPLA₂ γ siRNA and treated with IL-1 β ; **Gamma TNF** = transfected with cPLA₂ γ siRNA and treated with TNF α ; **Gamma-** = transfected with cPLA₂ γ siRNA and treated with PBS; **Delta IL** = transfected with cPLA₂ δ siRNA and treated with IL-1 β ; **Delta TNF** = transfected with cPLA₂ δ siRNA and treated with TNF α ; **Delta-** = transfected with

cPLA₂δ siRNA and treated with PBS; **i IL** = transfected with iPLA₂ siRNA and treated with IL-1β; **i TNF** = transfected with iPLA₂ siRNA and treated with TNFα; **i-** = transfected with iPLA₂ siRNA and treated with PBS. This figure is representative of two independent experiments.

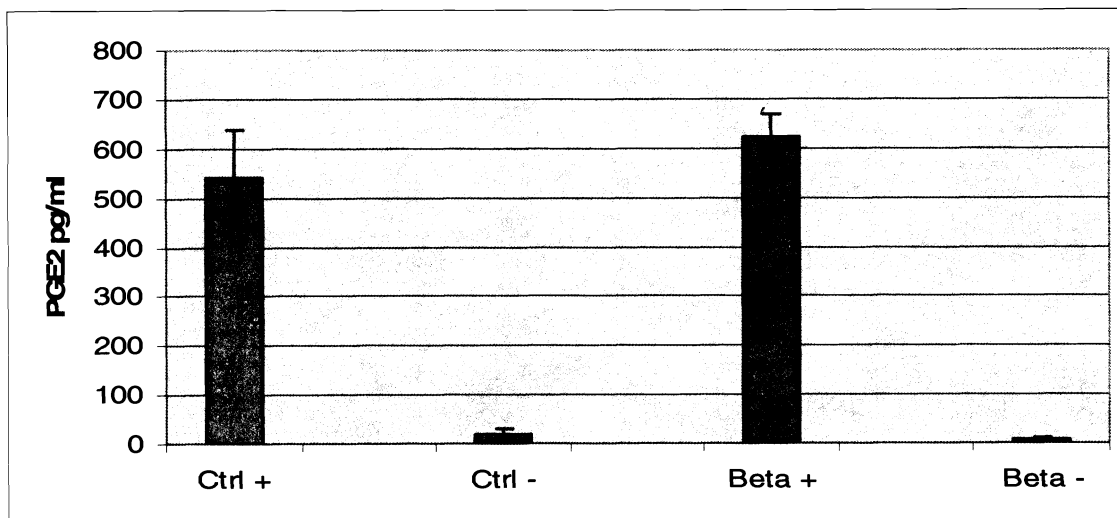


Figure 10. Down regulation of cPLA₂β has no effect on PGE₂ production. Cells were transfected with siRNA and treated with IL-1β (+) or PBS (-) as described in the “material and methods” section; **Ctrl +** = transfected with control siRNA and treated with IL-1β; **Ctrl-** = transfected with control siRNA and treated with PBS; **Beta +** = transfected with cPLA₂β siRNA and treated with IL-1β; **Beta -** = transfected with cPLA₂β siRNA and treated with PBS. This figure is representative of two independent experiments.

4. Discussion

Unpublished findings from our laboratory disclosed that cPLA₂α may be responsible for 50-60% of the PGE₂ produced in response to inflammatory cytokines. In this study, we optimized an RNAi protocol and identified cPLA₂α to be involved in approximately 100% of the PGE₂ production in A549 cells stimulated with inflammatory cytokines. We also showed that cPLA₂α, cPLA₂γ, cPLA₂δ, and iPLA₂ may play roles in basal prostanoid synthesis, and that cPLA₂β is not involved in the prostanoid synthetic pathway in A549 cells. The importance of these findings is as follows: First, cPLA₂α has been confirmed as the major regulator of PGE₂ synthesis in response to inflammatory cytokines in line with previously reported findings involving lipopolysaccharides (LPS) and calcium ionophore, A23187. Second, siRNA targeting cPLA₂α can be developed as a potential therapeutic agent against asthma. Finally, phospholipases may have implications as new drug targets against asthma, inflammation and cancer therapies.

Initially, we spent approximately 8 months on optimizing the protocols that were used in this study. First, when we used DMEM to wash the cells prior to the transfection with siRNA, we observed that most of the cells in the center of the wells died just 24 hours post-transfection. We were able to overcome this obstacle by replacing DMEM, which proved to be toxic to RNAi transfected cells, with Optimem and the cell survival rate increased dramatically. Next, we were using Oligofectamine transfection reagent

(Invitrogen) to introduce siRNA to A549 cells. This protocol, at first, produced a reproducible result, until the manufacturer changed the reagent and protocol for siRNA delivery, causing non-reproducible results. Thus, we examined Dharmafect, the RNAi transfection reagent manufactured by the same company that produces siRNA, and reproducible results were obtained from this reagent. Another obstacle that we overcame was the elevated basal levels of PGE₂ production from the non-cytokine treated A549 cells, which may have been due to over stressing the cells by transfection. We incorporated a washout step with A549 complete media 24 hours post-transfection to overcome the elevated basal PGE₂ production. Finally, allowing for significant observations in cytokine response, an assay for RNAi use in examining PGE₂ production was generated giving very reproducible results, which is now the “Gold standard” used in the Chalfant laboratory.

As aforementioned, unpublished studies from our laboratory demonstrated that cPLA₂α may play a role in the production of 50-60% of PGE₂ in A549 cells treated with inflammatory cytokines. This led to our initial hypothesis that the closely-related PLA₂s, cPLA₂β, cPLA₂γ, cPLA₂δ, or iPLA₂, may be responsible for the remaining 40-50% of the PGE₂ production. By utilizing RNAi technology and down-regulating cPLA₂α, we have demonstrated that cPLA₂α actually is responsible for approximately 100% of the PGE₂ synthesized in response to treatment with cytokines. Furthermore, studies using osteoblastic bone marrow stromal cells from cPLA₂α knockout mice indicated that

cPLA₂α played a key role in PGE₂ production, in response to lipopolysaccharide (LPS) [24,31]. Our studies have similar effects on PGE₂ production by cPLA₂α in response to extracellular mediators. In addition, other PLA₂ isoforms examined in this study had no involvement in cytokine mediated PGE₂ synthesis.

These individual PLA₂ isoforms, cPLA₂α, cPLA₂γ, cPLA₂δ, and iPLA₂, may be involved in basal prostanoid synthesis. In addition, this study revealed that down-regulation of cPLA₂β did not have any significant change in the production of PGE₂ in the presence or absence of cytokines. Thus, a function for cPLA₂β is still elusive.

The findings from this study are also important for several other reasons. First, understanding the individual enzymes involved in the cascade of steps leading to prostanoid synthesis following the envenomation of a brown recluse spider can result in a new treatment regiment to the spider bite. Furthermore, these findings may shed light to the mysterious recurrence of necrotic and hemolytic effects proximal to the brown recluse envenomation site several months after the bite. Second, these findings can lead to the development of siRNA as a potential therapeutic agent, in the form of an inhaler, against asthma. Finally, identification of the most important phospholipases in the prostanoid synthetic pathway can lead to new drug targets in treating asthma, inflammation and cancer. Thus, better understanding of the role of cPLA₂α in the inflammatory pathway can lead to a new approach to bone disease by inhibiting cPLA₂α, especially with the

newly discovered cardiac risks associated with selective COX-2 inhibitors such as Celebrex and Vioxx.

In conclusion, these results demonstrate cPLA₂α as the phospholipase A₂ responsible for the PGE₂ production in response to inflammatory cytokines. Furthermore, these results also demonstrate that cPLA₂α, along with cPLA₂γ, cPLA₂δ, and iPLA₂ are all involved in basal prostanoid synthesis and that cPLA₂β plays no part in the prostanoid synthetic pathway in the presence or absence of cytokines. These findings may have implications in better understanding the pathophysiology of a brown recluse spider bite, incorporating RNAi technology as a potential pharmacological tool, and giving rise to new targets for anti-asthma, anti-inflammatory and anti-cancer therapies.

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Vita

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