MOLECULAR REGULATION OF EICOSANOID METABOLISM IN THE INFLAMMATORY RESPONSE

By

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To my parents and grandmothers
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Inflammation is the initial physiological response to infection and tissue injury, involving complex processes including the release of bioactive lipid metabolites of arachidonic acid (AA), the eicosanoids. The first step in eicosanoid metabolism is the release of AA from membrane phospholipids by a phospholipase A2 (PLA2), of which, the best characterized family member is cytosolic phospholipase A2α (cPLA2α). The release of AA by cPLA2α is regulated by a number of mechanisms including, post-translational modifications and increases in intracellular calcium concentrations. Furthermore, changes in the gene expression of cPLA2α by pro-inflammatory cytokines, such as IL-1β, add an additional layer of complexity to its regulation in the inflammatory response.

The data presented in this thesis provide evidence for the induction of cPLA2α gene expression by IL-1β requiring a novel feed forward signaling pathway, where a
downstream metabolite of cPLA$_2$α is required for the activation of cPLA$_2$α gene expression. Evaluation of several signal transduction pathways elucidated a complex mechanism requiring the coordination of p38 MAPK and NF-κB pathways in the induction of cPLA$_2$α gene expression. Furthermore, a regulatory element upstream of the cPLA$_2$α promoter was found to mediate the induction of cPLA$_2$α expression by IL-1β.

Given the importance of eicosanoids to inflammation I also investigated changes in gene expression of many of the enzymes involved in the metabolism of eicosanoids in a mouse model of allergic asthma, including several phospholipase A$_2$s and lipoxygenases. The increase in eicosanoid release in asthma has previously been shown to require cPLA$_2$α, however, the only significant changes observed were in cPLA$_2$γ in the lungs of the asthmatic mice. Furthermore, although 5-Lipxygenase (5-LO) and its metabolites have been implicated in physiological responses causing asthma, drugs inhibiting this pathway have had limited success in its treatment. In our model of asthma there was no change in expression of 5-LO, however, an increase in several other lipoxygenases, 15-LO-1, 15-LO-2 and epidermally derived 12-LO was observed. The data in this thesis identifies novel potential targets, cPLA$_2$γ and the 12- and 15-LOs, in the treatment of asthma, ultimately adding to our understanding of the underlying roles of eicosanoids in this disease.
CHAPTER 1
LITERATURE REVIEW

The initial physiological response to infection and tissue injury is inflammation. Although the processes involved are complex, the release of bioactive lipid metabolites of arachidonic acid (AA), the eicosanoids, is central to inflammation. The eicosanoids include the prostaglandins, prostacyclins, thromboxanes, leukotrienes, hydroperoxy-eicosatetraenoic acids, and lipoxins. These bioactive lipids mediate many aspects of inflammation, including increased vasodilation, vascular permeability, chemotaxis, and the transcription of pro-inflammatory enzymes [1-3]. The eicosanoids are metabolized from AA, released from phospholipid membranes by Phospholipase A$_2$ (PLA$_2$). AA is metabolized into the various bioactive lipids by specific downstream enzymes, and the mis-regulation of eicosanoid metabolism has been implicated in the pathology of several diseases, including: Alzheimer’s disease [4]; rheumatoid arthritis [5,6]; Crohn’s Disease [7]; a plethora of cancers [8-15]; adult respiratory distress syndrome [16]; asthma [17-22]; as well as brain injury and disease [23-27]. In addition to their role as inflammatory mediators, the eicosanoids also arbitrate many general cellular processes, such as cell differentiation [28], apoptosis [29-31], lipid membrane [32] and vascular homeostasis [33,34]. Due to their important role in these processes in general, and in the inflammatory process, in particular, an understanding of the mechanisms controlling the levels of the eicosanoids during inflammation is crucial.
**Eicosanoid Metabolism**

**Physiological Aspects of Arachidonic Acid Metabolites**

PLA$_2$ activity, AA, and the metabolites of AA play many important roles in normal physiology and the pathophysiology of several diseases. In the kidney Prostaglandin E$_2$ (PGE$_2$) can inhibit Cl- reabsorption and renin production [35]. cPLA$_2$α and the cyclooxygenases are expressed in the brain and have been associated with normal neuronal activity, fever induction and the inflammatory response mediated by microglia during infection and after traumatic injury [36-40]. Increased cPLA$_2$α activity, AA and prostanoids have also been found in lesions of Alzheimer’s disease [4] and at lower levels in the brains of schizophrenics [27].

The creation of a cPLA$_2$α knockout mouse (cPLA$_2$α -/-), by Bonventre and colleagues [23], has further demonstrated the important role the enzyme plays in neural activity. In the initial study of the knockout mice, this group analyzed the function of cPLA$_2$α in stroke, via an ischemia-reperfusion model. The knockout mice have a smaller infarct volume and decreased neural deficits when compared to control mice, after 2 hours of ischemia followed by 22 hours of reperfusion; demonstrating the detrimental neurological function of cPLA$_2$α in this model of stroke. This study also investigated the function of cPLA$_2$α in the physiology of pregnancy. cPLA$_2$α -/- mothers are incapable of inducing labor because of the lack of Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) production, known to be involved in the induction of labor, thus demonstrating the necessity of cPLA$_2$α mediated AA metabolism during parturition. cPLA$_2$α -/- mice also have a lower rate of successful mating, where the major difficulty associated with successful mating occurs at the stage of implantation.
In a separate study, Naonori Uozumi and colleagues [41] also disrupted the cPLA$_2$$\alpha$ gene in mice, and demonstrated the role of cPLA$_2$$\alpha$ in lung physiology and fertility. Using a model for allergen induced anaphylaxis, cPLA$_2$$\alpha$ -/- mice exhibited altered physiological responses compared to normal mice. When mice are pre-sensitized to ovalbumin followed by subsequent re-administration of the protein through the lung, ovalbumin acts as an allergen, and mimics allergic asthma. This allergic response normally leads to a hyper-reactivity when methacholine is administered after ovalbumin challenge. Using this model of anaphylaxis, the null mice were shown to have a marked decrease in airway constriction when challenged with ovalbumin. This data clearly illustrates the important role cPLA$_2$$\alpha$ plays in airway responsiveness to allergens and a likely role for its involvement in allergen induced asthma.

There have been several studies connecting eicosanoid metabolism with cancer and tumorogenesis. These include up-regulation of cPLA$_2$$\alpha$ and increased prostanoid levels in non-small cell lung cancer cells [42,43]. Increased prostanoids, cPLA$_2$$\alpha$ and COX-2 enzyme levels were also observed in colorectal cancer [44,45]. AA metabolism is also associated with tumor angiogenesis [46,47], further demonstrating the significant role AA plays in tumorogenesis. Finally, the pathophysiology of several autoimmune diseases, such as rheumatoid arthritis, is also connected with improper cPLA$_2$$\alpha$ activity, with an increase in lipid mediators of inflammation in the rheumatic joints. The prostaglandins are known to affect bone metabolism, regulating both bone formation and re-absorption. cPLA$_2$$\alpha$ expression is higher in rheumatic joints, and this mis-regulation leads to an increase in eicosanoids [5,6,48,49]. Therefore, an unchecked inflammatory response in the joint leads to an increase in local eicosanoid production, in turn disrupting proper
bone metabolism, and ultimately resulting in rheumatic disease. These examples illustrate the critical roles eicosanoids play in normal cellular function as well as a wide variety of disease pathologies.

**Phospholipase A\(_2\)s**

The initial and rate-limiting step in eicosanoid production is the hydrolysis of AA from membrane phospholipids. This reaction is catalyzed by the phospholipase A\(_2\) (PLA\(_2\)) family of enzymes [50,51]. The hydrolysis of membrane phospholipids by PLA\(_2\)s also releases lysophospholipids, giving rise to another bioactive phospholipid, platelet activating factor (PAF). To date, there have been 19 PLA\(_2\)s identified in mammals [52]. These isoforms are divided into three groups based on size, calcium requirements, sequence similarities and fatty acid specificity. In humans there are 10 low molecular weight (14-19 kDa) secretory isozymes of PLA\(_2\) (sPLA\(_2\)s), six isoforms of the calcium independent PLA\(_2\)s (iPLA\(_2\)s), and four isoforms of the cytosolic PLA\(_2\) (cPLA\(_2\)s) identified [52].

**Secreted PLA\(_2\)s**

The sPLA\(_2\) family is comprised of the groups I, II, V and X. These enzymes have conserved calcium binding domains and catalytic domains, and there are six conserved disulfide bonds plus two unique disulfide bonds in each enzyme. The sPLA\(_2\)s require high (millimolar) calcium concentration for enzymatic activity and do not have strict fatty acid selectivity. sPLA\(_2\)-I is found predominantly in the pancreas and acts as a digestive enzyme, processing dietary phospholipids. Groups II and V are closely related forms and are involved in inflammation. Both have been found at increased levels at the sites of inflammation, and their expression is increased in response to pro-inflammatory stimuli. There have been five members of the sPLA\(_2\)-II group cloned (sPLA\(_2\)-IIA, -IIC, -IID, -IIE,
and –IIF) [53,54]. The different Group II forms along with the lone sPLA$_2$-V isoform are expressed in various tissues in an isoform specific manner, and affect cell and tissue specific immune responses [54]. sPLA$_2$-X is expressed in pancreas and immune cells and is involved in both digestive and immune function [54].

**Calcium independent PLA$_2$s**

There are several forms of the iPLA$_2$ family, with a wide range of molecular weights and tissue expression. The iPLA2s show little sn-2 specificity and possess some sn-1 lysophospholipase activity. The iPLA$_2$s are believed to be mainly involved in membrane remodeling and don’t seem to play a role in inflammation[52].

**Cytosolic PLA$_2$s**

In humans there are four cPLA$_2$ isozymes (cPLA$_2$$\alpha$, cPLA$_2$$\beta$, cPLA$_2$$\gamma$ and cPLA$_2$$\delta$); each sharing two highly homologous catalytic domains with intervening isotype specific sequences and high specificity for AA in the sn-2 position of membrane phospholipids [52,55-57]. cPLA$_2$$\alpha$ and cPLA$_2$$\beta$ have a homologous C2 domain (phospholipase C-like membrane binding domain) and require micromolar calcium concentrations for translocation to and docking with the membrane. cPLA$_2$$\gamma$, on the other hand, is isoprenylated, anchoring the enzyme at the membrane surface, in close proximity with the phospholipid substrate [58].

cPLA$_2$$\alpha$ was the first cPLA$_2$ cloned and its function is the best understood. cPLA$_2$$\alpha$ is expressed ubiquitously and has been linked to the increase in lipid mediators of inflammation in response to pro-inflammatory stimuli. Although the functions of cPLA$_2$$\beta$, cPLA$_2$$\gamma$ and cPLA$_2$$\delta$ still need to be fully elucidated, they do not appear to be
redundant enzymes. The expression patterns of cPLA$_2$$\beta$, cPLA$_2$$\gamma$ and cPLA$_2$$\delta$ are more restricted and specific, suggesting these enzymes affect tissue specific responses.

**Cellular Regulation of cPLA$_2$$\alpha$**

cPLA$_2$$\alpha$ enzymatic activity is regulated in response to pro-inflammatory stimuli, by receptor mediated signaling cascades [59]. The regulatory mechanism is multifaceted, requiring an increase in the intracellular Ca$^{2+}$ concentration and protein phosphorylation [60]. Dessen and colleagues [61,62] resolved the crystal structure of cPLA$_2$$\alpha$, enabling a more in depth analysis of the protein and the domains involved in the regulation of cPLA$_2$$\alpha$. The N-terminus contains a phospholipase C like (C2) domain, while the C-terminal half harbors the catalytic domain and the phosphorylation sites. The C2 domain directs the calcium dependant translocation of cPLA$_2$$\alpha$ from the cytosol to the nuclear and endoplasmic reticulum membranes [63-65]. This is an essential step in the activation of the enzyme, by bringing the protein into contact with its phospholipid substrate. The preferential translocation to the perinuclear membrane arises from the phospholipid specificity of the C2 domain for membranes containing high levels of phosphatidylcholine [66]. The docking of the C2 domain with the membrane results in proper interface of the catalytic domain with the lipid surface/substrate. Additionally, the translocation of cPLA$_2$$\alpha$ to the perinuclear membrane is noteworthy in that cyclooxygenases-1 and -2 (COX-1 and -2) microsomal Prostaglandin E$_2$ Synthase (mPGES) Five Lipoxygenase Activating Protein (FLAP) and 5-lipoxygenase (5-LO), the most important downstream enzymes co-localize with cPLA$_2$$\alpha$ at this location [67,68]. Hence, the AA produced from cPLA$_2$$\alpha$ activation is in close proximity with the enzymes involved in its downstream metabolism.
In addition to the Ca\(^{2+}\) mediated translocation, cPLA\(_2\alpha\) is phosphorylated, and this is required for full enzymatic activation. Binding of a stimulus to surface receptors activates the Ras/Raf and MEKK signaling cascades, leading to the phosphorylation of cPLA\(_2\alpha\) at Ser505 and Ser727 by MAPK and MAPK-activated kinases [59,60,69,70]. cPLA\(_2\alpha\) is also phosphorylated at Ser 515 by Ca\(^{2+}\)/calmodulin kinase II, also increasing enzymatic activity [60]. Although there are examples from in vitro studies of calcium free activation by phosphorylation alone [71], both an increase in calcium and phosphorylation are required for full enzymatic activation in vivo [60].

**Molecular Regulation of cPLA\(_2\alpha\)**

The cDNA of cPLA\(_2\alpha\) has been cloned from several animals, including human, mouse, rat, chicken and fish. The human protein contains 749 amino acids and has a predicted molecular weight of 85 kDa [72]. The expression of cPLA\(_2\alpha\) is increased in response to several pro-inflammatory cytokines, including IL-1\(\beta\), TNF-\(\alpha\) and IFN-\(\gamma\). This increased expression is a result of de novo transcription as shown by nuclear run-on assay. Treatment with glucocorticoids effectively down regulates both basal and stimulus dependent gene expression [73].

The human and rat promoters have been identified and further studied by several groups including our own. The transcriptional start site was determined by rapid amplification of 5’-cDNA ends (5’-RACE) [74,75]. Analysis of the upstream sequence demonstrated the promoter is similar to housekeeping genes, as it is void of both a TATA and a CAAT box, however the promoter does not contain any SP1 sites nor is it GC rich (34.5%) [74,76]. Based on consensus sequences, several sites have been suggested as putative regulatory elements, including several IFN-\(\gamma\) response elements, an IFN-\(\gamma\) activated sequence, a glucocorticoid response element, several AP-1 sites and a NF-\(\kappa\)B
However, until recently, there have not been any reported observations of functional inducible regulatory elements in the proximal 5' region. In fact, our laboratory has shown there are no functional inducible regulatory elements in the first 3.4 kb of upstream sequence in several cell types [73]. Another potentially interesting characteristic of the proximal promoter is the existence of a 48 bp CA dinucleotide repeat at position –268 to –221 [74]. CA/GT dinucleotide repeats in other mammalian genes have been shown to confer a negative regulatory effect on the promoter activity and results from our lab confirm this effect on the basal cPLA$_2$α promoter [73].

The human and mouse cPLA$_2$α genes have been completely sequenced during the compilation of their completed genomes. The human gene spans 160,000 bp of genomic sequence and resides on the q arm of chromosome 1 at position 1q31.1. The genomic sequence encodes 18 exons resulting in an mRNA that is 2.9 kb.

**Metabolism of Arachidonic Acid by Cyclooxygenase**

The cyclooxygenases (Cox-1 and -2) mediate the production of Prostaglandin H$_2$ (PGH$_2$) from AA in a two step process; PGH$_2$ is then metabolized by specific synthases into the prostaglandins (PGE$_2$, PGD$_2$, PGF$_{2\alpha}$), prostacyclin (PGI$_2$), or thromboxane (TXA$_2$). These mediators control a wide variety of the functions of inflammation including vascular dilation and constriction, lymphocyte chemotaxis and infiltration.

**Lipoxygenase Metabolism of Arachidonic Acid**

AA can also be metabolized by several different lipoxygenases, all mediating the peroxidation of AA into specific hydroxyeicosatetraionic acids (HETEs). Most significant to inflammation, 5-, 12- and 15-Lipoxygenases (5-, 12-, and 15-LO) produce 5-, 12-, or 15-HETEs, respectively. The 5-LO product, 5-HETEs, is further metabolized into the cysteinyl leukotrienes (cys-LTs) via LTA$_4$ hydrolase and LTC$_4$ synthase. The cys-LTs
are key regulators in the asthmatic response and several asthma drugs target cys-LT signaling. The lipoxins are potent anti-inflammatory mediators formed by the dual metabolism of AA by 5- and 15-LO. The 12- and 15-HETEs have both pro- and anti-inflammatory roles, as well as being ligands for Peroxisome Proliferator Activated Receptors (PPARs).

**Allergic Asthma**

Allergic asthma is a common respiratory disease developing from a Th2-directed immune response towards allergens. It is characterized by airway inflammation with elevated IgE production, eosinophilia, tissue remodeling and mucus hypersecretion culminating in airway hyperreactivity (AHR) [78-80]. From a classic viewpoint, asthmatic reactions develop from the effects of Th2 cytokines including IL-5 (associated with eosinophilia) along with IL-4 and IL-13 (associated with hyper-IgE production). Eosinophils are likely one of the main effector cells in conjunction with IgE secreting B cells and mast cells/basophils which perpetuate the disease through eosinophil recruitment by histamine release.

**Immune Response and Pathophysiology of Allergic Asthma**

Asthma is an inflammatory disease of the lung typified by reversible airway obstruction, increased airway mucous production as a result of goblet cell hyperplasia, chronic airway eosinophilia and bronchial smooth muscle hypertrophy. These symptoms eventually lead to loss of proper epithelial cell repair and ultimately remodeling of the airway. In allergic, or allergic asthma these symptoms result in airway hyper-responsiveness upon re-exposure to allergen [81,82]. These asthmatic symptoms are a result of airway remodeling mediated by immune cell infiltration and the resultant increase in cytokines, chemokines and lipid signaling molecules. The immune response
is an important component of allergic asthma and requires pre-exposure to the antagonizing allergen in order to prime the immune response. Physiological exposure typically occurs through the airway, however, epicutaneous and other routes can also lead to sensitization. Priming of the immune system to an allergen occurs by the uptake, processing and presentation of the antigen(s) to T-cells by antigen presenting cells (APCs).

In asthma, dendritic cells are the main APCs although; macrophages, eosinophils and even the airway epithelia can also act as APCs. Dendritic cells (DCs) are found in the upper and lower airways both above and below the basement membrane. From these loci immature DCs extend projections into the lumen of the airway without disturbing the epithelial barrier. Immature DCs have high allergen uptake and processing capabilities, but cannot stimulate T-cells. Dendritic cell maturation occurs when allergen uptake coincides with additional stimuli, and in the process of maturation, DCs lose the ability to uptake allergen, becoming professional APCs. In this process of maturation, the mature DCs express the chemokines and co-stimulatory molecules required for stimulation of naïve T-cells. The mature DCs migrate to draining lymph nodes where they present immunogenic peptides derived from phagocytosed allergen to naive T cells. When mature DCs come in contact with naïve or specific T cells they form an immunologic synapse, leading to T-cell activation, division and differentiation. Dependent upon the lineage, maturation, environment where the DC resides and the nature of the allergen, DCs can express different patterns of co-stimulatory factors and express different chemokines and cytokines. These are important points as they direct and influence the interaction with T-cells and the subsequent outcome.
Interestingly, DCs, in their function as APCs (in the development of asthma) act as the link between innate and adaptive immunity. In the absence of noxious stimuli with an otherwise benign allergen, it does not appear that DCs can prime the system. Eisenbarth and colleagues [83] have demonstrated the requirement of toll-like receptor (TLR) stimulation as a requirement for sensitization to ovalbumin (OVA) when sensitizations are delivered intra-nasally. TLR signaling is a fundamental element of the innate response and thus the Eisenbarth study suggests the requirement for the innate response in priming the adaptive (Th2) response.

One hallmark of allergic asthma is increased circulating and lung IgE levels, and more specifically allergen specific IgE [84]. Increased IgE production is a direct result of the ligation of T-cells with mature DCs, which present immunogenic targets to B-cells. This stimulates antibody production by B-cells and the Th2 cytokines IL-4 and IL-13 cause a class switch from IgG to IgE. Allergen specific IgE produced and secreted by activated B cells binds to the FcεRI immunoglobulin receptor on the surface of mast cells. Mast cells are effector immune cells and reside between the basement membrane and epithelia, thus situating them in close proximity to the area of allergen exposure. Upon subsequent allergen challenge, there is a crosslinking of multiple IgE/ FcεRI complexes on the surface of mast cells which initiates the signaling cascades with release of preformed mediators, including histamines, cysLTs, PAF, PGD2, TNFα, TGFβ, interleukins -4, -5, -8, -13, as well as tryptase and chymase. These mediators have a plethora of effects culminating in the immediate asthmatic response including: enhanced vascular permeability, initial leukocyte recruitment, bronchospasm, and enhanced
mucous secretion. Left unchecked these factors lead to a more prolonged disease state with chronic airway inflammation, airway remodeling and airway hyperresponsiveness.

Eosinophils are one of the main immune cells infiltrating the lung in response to cytokines and chemokines secreted by T-cells and mast cells after allergen challenge. When recruited to active sites of allergic inflammation, eosinophils, along with resident lung cells, produce lipid mediators leading to chronic asthmatic symptoms. Although the full role of eosinophils in the development of asthma is controversial, their importance to asthma and particularly an involvement in the lung remodeling associated with chronic asthma has been demonstrated [85,86].

In separate studies, two groups created mice genetically deficient in eosinophils, and both demonstrate the importance of eosinophils for the airway remodeling associated with asthma. However, these studies differed in their assessment of the role of eosinophils on airway hyper-responsiveness. Lee and colleagues [86] concluded eosinophils are required for increased mucous secretion and airway remodeling leading to increased airway hyper-responsiveness. While Humbles and colleagues [85] concluded eosinophils are important to airway remodeling but are not necessary for other allergen-induced lung dysfunction. Both of these studies support an important role for eosinophil-targeted therapies in chronic asthma. The differences in the findings are likely due to the differences in mouse strain background and mode of eosinophil ablation. These points will likely be addressed by studies in mice crossed to the complimentary strains and it will be interesting to see the results of such studies in the future.

**Lipoxygenase Metabolites in Asthma**

Leukotrienes are derived from the oxidative metabolism of arachidonic acid (AA). As previously stated, intracellular AA is liberated from membrane phospholipids by the
action of phospholipase A\(_2\). A principal downstream enzyme 5-lipoxygenase (5-LO) oxygenates AA at C-5 in conjunction with the AA-binding protein, 5-LO-activating protein (FLAP), generating the epoxide intermediate LTA\(_4\). LTA\(_4\) can be either hydrolyzed to LTB\(_4\), a potent leukocyte chemoattractant/activator or combined with glutathione to form LTC\(_4\). This metabolite serves as the source for cysteinylic LTs (cysLTs), including LTD\(_4\) and LTE\(_4\), all of which are critical in the pathogenesis of asthma. CysLTs can evoke systemic allergic responses including: increased mucus secretion, decreased mucociliary clearance, bronchoconstriction, vasodilation, and vascular permeability. The importance of these metabolites in allergic inflammatory responses prompted the development of a line of leukotriene antagonists and 5-LO inhibitors, and since the early 1990s have been utilized as anti-asthma therapies.

Although 5-LO/FLAP inhibitors and cys-LT antagonists have some clinical efficacy in asthmatic patients, these compounds are not more effective than inhaled corticosteroids alone. However, they are effective as an add-on therapy to inhaled corticosteroids and have the advantage of oral administration without significant side effects.

Arachidonate is metabolized by 15-lipoxygenase (15-LO) through oxygenation of AA at the C15 position. This generates either the reduced alcohol form, 15S-hydroxyeicosatetraenoic acid (15S-HETE), or the peroxide form, 15S-hydroperoxyeicosatetraenoic acid (15-HPETE). These compounds elicit a variety of pro-inflammatory responses correlating with events in allergic asthma including: increases in mucus secretion, pulmonary artery and capillary pressure, vascular permeability and immune cell recruitment. Analogously, allergen challenge in asthmatic subjects causes an elevation in 15S-HETE levels in bronchoalveolar lavage fluid. In addition, the Th2
cytokines, IL-4 and IL-13, known to be elevated in allergic asthma, are reported to induce 15-LO expression in cell culture models. These pathological responses demonstrate 15-LO metabolites can also potentially propagate some of the early pro-inflammatory events in allergic asthma. However they have been somewhat ignored, given the focus on 5-LO products such as the cys-LTs in the etiology of allergic asthma.

The lipoxins are lipids with anti-inflammatory effects formed by the trans-cellular transformation of 15-LO products, 15-HETE and 15-HPETE or the 5-LO products 5-HETE and 5-HPETE through the action of the converse lipoxygenase. Eosinophils, macrophages, monocytes, dendritic cells and/or airway epithelial cells containing 15-LO, actively produce 15-HETE and 15-HPETE which are then transformed by infiltrating leukocytes harboring 5-LO activity. The 15S-hydro(peroxy)-DiH(p)ETE produced by 5-LO activity is then rapidly converted to a 5(6)-epoxytetraene and, through the action of hydrolases, then converted to either 5S,6R,15S-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid, termed lipoxin A₄ (LXA₄), or 5S,14R,15S-trihydroxy-6,10,12-trans-8-cis-eicosatetraenoic acid, termed lipoxin B₄ (LXB₄) [87]. Conversely, the activity of 15-LO in resident lung cells on the 5-LO products produced by infiltrating immune cells also leads to lipoxin production. These potent anti-inflammatory mediators inhibit airway hyperresponsiveness, immune infiltration and decrease edema, as well as redirect the cytokine/chemokine balance. Furthermore, the biosynthesis of the lipoxins is accompanied by a concomitant decrease in leukotriene synthesis at the 5-LO level [88]. Hence, although both 5-LO and 15-LO, and their AA metabolites are linked to the etiology of asthma, the products of the trans-cellular metabolism by these enzymes potentially have beneficial effects and may act to self restrict the detrimental effects of the mono-
The role lipoxins play in asthma is still not fully understood, however, in models of allergic asthma, administration of a stable lipoxin analog decreased airway inflammation and hyperresponsiveness [89] suggesting an important role for these lipid mediators in the development of asthma. Decreased lipoxin synthesis has likewise been observed in the lungs of cystic fibrosis patients, and is believed to be an important factor in the uncontrolled inflammatory response in these patients [90].

**Transcriptional Regulation of Gene Expression**

Transcriptional regulation of gene expression involves interactions between protein transcription factors with the DNA in the promoter and enhancers of the gene. These interactions permit spatial and temporal differences in gene expression in response to various cellular and physiological stimuli. Different intra- and extra-cellular stimuli activate specific transcription factors and these transcription factors, and their cofactors, are regulated through reversible post translational modifications such as phosphorylation, ubiquitination and acetylation, or inactivated through such mechanisms as proteolysis.

Transcription factors are proteins that bind DNA at a specific promoter or enhancer region or site, where it regulates transcription. Transcription factors can be selectively activated or deactivated by other proteins, often as the final step in signal transduction. Transcription is initiated by an increase in the amount of a transcription factor and the subsequent interaction with its cognate target DNA sequence. DNA sequences farther from the initiation site are known as enhancers and aid in the assembly of the pre-initiation complex with RNA polymerase II at the promoter. The pre-initiation complex consists of the ubiquitous transcription factors TFIIA, TFIIIB, TFIIID, TFIIE, TFIIF, and TFIIH. These factors interact with sequences in the core promoter region surrounding the transcription start site. The activation of transcription can also be regulated by changes in
chromatin structure, which alter the accessibility of the DNA for trans-acting regulatory factors.

**Transcriptional Activation by NF-κB**

Nuclear Factor-kappa B (NF-κB) is a ubiquitous transcription factor often associated with cellular responses to stress and extra-cellular stimuli. NF-κB is particularly important in the inflammatory response. NF-κB exists as a heterodimer, and all the members of this family share a REL homology domain in their N-termini. A subfamily including RelA, RelB and c-Rel also have a trans-activation domain in their C-termini and a nuclear localization signal (NLS). The NF-κB1 and NF-κB2 subunits are synthesized as large precursors, p105 and p100, which undergo processing to generate the second subfamily of NF-κB subunits, p50 and p52, respectively. The processing of p105 and p100 is mediated by the ubiquitin/proteasome pathway and involves selective degradation of their C-terminal region containing ankyrin repeats. While the generation of p52 from p100 is a tightly regulated process, the processing of p105 into p50 is constitutive. The prototypical subunits are NF-κB 1 (aka p50) and RelA (aka p65), although other subunits exist, with cell and stimuli specific functions. In unstimulated cells, the NF-κB dimer is bound by the Inhibitor of kappa B (IkB), masking the nuclear localization sequence (NLS) on NF-κB, thus retaining the NF-κB dimer in the cytosol. In response to extracellular stimuli, IkB is phosphorylated by the IKK complex, leading to the ubiquitination of IkB, and in turn causing its proteosomal degradation. Unmasking of the NLS allows the nuclear translocation of the NF-κB dimer and ultimately sequence specific DNA binding [91,92]. When unphosphorylated at ser276, p65 interacts with Histone Deactelyase 1 (HDAC1), and when phosphorylated this interaction is replaced by the acetyltransferase, CBP/p300.
The Effects of Changes in Chromatin Structure on Gene Expression

The term chromatin was first coined by Walther Flemming in 1882; using basophilic dyes, he observed thread-like structures in the nucleus and called them chromatin. Although at the time the true function of chromatin was not understood, his observations proved to be quite significant, being one of the fundamental findings leading to DNA as the basis of inheritance. In addition to simply packaging the DNA into the nucleus, chromatin has proven to be important to the regulation of gene expression through inherent structural changes. One of the main regulators of chromatin structure is acetylation. Chromatin in the regulatory regions of active genes is hyperacetylated leading to a more open structure, while hypoacetylation in inactive genes causes a tighter compaction of the chromatin. This balance is controlled by the activity of acetyltransferases, most notably CBP/p300, and histone deacetylases (HDACs). In this manner, chromatin is held in a silent conformation by the activity of HDACs, and in active genes, acetyltransferases are recruited by transcription factors in response to specific stimuli, acetylating the chromatin, leading to a transcription-permissive chromatin conformation [93-97].
CHAPTER 2
MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA Ligase, and Klenow fragment of E.coli. DNA Polymerase were purchased from New England Biolabs, (Ipswich, MA). Bovine Serum Albumin (BSA-A7511) and Dimethyl sulfoxide (DMSO-D8779) were purchased from Sigma Chemical Company (St. Louis, MO). Random Primers DNA Labeling System (18187-013) and proteinase K (25530-015) were purchased from Invitrogen technologies (Carlsbad, CA). QIAquick Nucleotide Removal kit (28304), QIAquick Gel Extraction kit (12162), QIAprep Spin Miniprep kit (27106), Qiagen Plasmid Midi kit (12144), Qiagen Plasmid Maxi kit (12162) were purchased from Qiagen. Hyperfilm MP (RNP 1677K, RNP30H), and ECL Western Blotting Analysis System (RPN2108), were purchased from Amersham Biosciences (Piscataway, NJ). [α-32P] dATP, and dTTP (3,000 Ci (111 TBq)/mmol) (BLU013H), [γ-32P] ATP (3,000 Ci (111 TBq)/mmol) (BLU002A) were purchased from Perkin Elmer, Boston, MA. Fugene6 transfection reagent (1-815-091), Interleukin-1β (IL-1β), Tumor Necrosis Factor (TNFα) and complete protease inhibitor cocktail (1-697-498) were purchased from Roche Technologies. Zeta-Probe Blotting membrane positively charged nylon transfer membrane (162-0159) was purchased from Cuno, Meriden, CT. QuickChange Site-Directed Mutagenesis Kit and XL-10 competent cells were purchased from Stratagene (200518). Bay 11-78902 (196870), NDGA (479975), Luteolin (440025), TSA (647926), ALLN (208750), U0126 (662005),
SP600125 (420119), PD98059 (513000), SB203580 (559389), SB202190 (559388), L-α-lyso-lecithin (440154) were purchased from Calbiochem, San Diego, CA.

The cPLA$_2$$\alpha$ antibody (4-4B-3C; sc454) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody to phosphor ser505 cPLA$_2$$\alpha$ (2831) was purchased from Cell Signaling Technologies, Danvers, MA. Criterion 4-20% Tris-HCl PAGE gels (345-0033) and Trans-Blot Transfer Medium (162-00115) were purchased from BioRad, Hercules, CA. BCA protein assay (23223/1859078) was purchased from Pierce, Rockland, IL.

**Methods**

**Tissue Culture**

Human Fetal Lung Fibroblast (HFL-1) cells from ATCC were grown in Hams F12K medium (Life Technologies-N-3520) supplemented with 10% fetal bovine serum, 10 µg/ml penicillin G, 0.1 mg/ml streptomycin, and 0.25 µg/ml amphotericin B at 37°C in humidified air with 5% CO$_2$. Experimentally, cells were grown to 70-75% confluency before treatment with pharmacologic agents, and/or cytokines. In reporter gene assays cells were transfected in a batch format. Five µg of DNA and 15 µl of Fugene were combined in serum free media to a final volume of 500 µL before addition to the cells in 100 mm tissue culture plates. Three hours after addition of the DNA/Fugene mixture, cells were washed with PBS two times and fresh media added. Cells were grown overnight and split one to two, and again allowed to recover overnight. On the following day cells one plate was treated with indicated cytokine for eight or 24 hours as indicated; the control plate was left untreated. After incubation with cytokine RNA was isolated as described below.
RNA Isolation

Total cellular RNA was isolated by the acid guanidinium thiocyanate extraction method described by Chomczynski and Sacchi (1987) with the following modifications. To collect RNA, cells were washed once with room temperature PBS, and then with 0.5 ml 3 guanidinium thiocyanate solution (GTC) (4M guanidinium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol) per 100mm plate. The homogenate was transferred to a 1.5 ml microcentrifuge tube and 0.1 volume of 2M sodium acetate pH 4.0, an equal volume of water saturated phenol and 0.2 volumes of chloroform:isoamyl alcohol (IAA) (49:1) was added, mixed vigorously and incubated on ice for 15-30 min. The organic extraction mixture was centrifuged for 10 min. at 4°C. Following centrifugation, the upper aqueous phase was transferred to a fresh tube and mixed with an equal volume of isopropanol and placed at -20°C for at least one hour to precipitate the RNA. Following incubation at -20°C, tubes were centrifuged for 10 min at 4°C at maximum velocity in an Eppendorf microcentrifuge. The supernatant was discarded; the RNA pellet was dried briefly and resuspended 75 µl of DEPC treated water and incubation at 50°C for 15 min. 25 µl of 8 M LiCl was added and the tubes were placed at -20°C for at least one hour. Tubes were centrifuged for 10 min at 4°C and maximum velocity in an Eppendorf microcentrifuge. 200 µl of 100% EtOH was added to each tube to reduce the viscosity of the LiCl solution. The pellets were washed with 300 µl of 70% EtOH, then resuspended in 75 µl of DEPC treated H₂O. RNA concentration was determined by measuring the absorbance at 260nm in a Beckman DU-64 Spectrophotometer (Beckman Instruments, Inc.).

To isolate total RNA from the lungs of mice, dissected lungs were flash frozen in liquid nitrogen and stored at -80°C. The frozen lungs were pulverized with a frozen
mortar and pestle. The powdered tissue was transferred to a glass mortar with 3 mL of GTC lysis solution and homogenized with a Teflon pestle. The homogenate was transferred to a 15 mL conical tube and 1 volume of acidic phenol was added with 0.1 volumes of 2 M sodium acetate, pH 4.0. The samples were kept on ice for 30 minutes, and 0.2 volumes of chloroform:isoamyl alcohol (IAA) (49:1) was added and mixed vigorously, then centrifuged for 30 min at 4°C. The aqueous layer was transferred to a 50 mL polycarbonate tube and an equal volume of isopropanol was added. To precipitate the RNA the samples were stored at 4°C for at least 1 hour. The pelleted RNA was resuspended in 500µL of GTC and further processed as in the isolation from cultured cells.

Northern Analysis

20 µg of total RNA was lyophilized and resuspended in 28 µl of loading buffer containing 50% formamide, 6.6% formaldehyde, 6 mM sodium acetate pH 7.4, 0.5 mM EDTA pH 8.0, 20 mM 3-(N-morpholino) propane sulfonic acid (MOPS) pH 7.0. RNA was dissolved through incubation at 50°C for 5 min followed by incubation at 65°C for 5 min. 5 µl of loading dye containing 0.4% xylene cyanol FF, 0.4% bromophenol blue, 1mM EDTA pH 8.0, 50% glycerol, and 0.3 µg/µl of ethidium bromide was added prior to gel loading. The ethidium bromide allowed for visualization of ribosomal RNA under UV light, giving an estimate of the equivalence of loading.

Total RNA was size-fractionated on a 1% agarose-formaldehyde gel overnight. The gel was washed in distilled water, and then washed two times for 30 min with 50mM TBE (10 mM Tris-HCl pH8.0, 1 mM EDTA pH8.0). The RNA was electrotransferred to a charged nylon membrane (ZetaProbe, BioRad) in 40mM TBE for 1h and covalently cross-linked for 3 min by UV irradiation. The membrane was hybridized with randomly
primed double stranded (Random primers DNA labeling system) $^{32}$P-labeled probes over night in hybridization solution (450 mM Na$_2$HPO$_4$, 6 % SDS, 1 mM EDTA and 1% BSA). Hybridized blots were washed three times in ash buffer (40 mM Na$_2$HPO$_4$, 2 mM EDTA and 1% SDS) at a temperature 5 degrees higher than hybridization temperature (Church & Gilbert, 1984). Blots were exposed to autoradiograph film. All autoradiographs shown are the result of a minimum of 3 independent experiments. Densitometry was done using NIH Scion Image analysis. Statistical analysis was done using a T test: Paired two sample for Means, two-tailed p values were used.

**Protein Isolation and Western Analysis**

Control and IL-1β cells were lysed in RIPA buffer (50 mM Hepes pH 7.3, 150 mM NaCl, 1.5 mM MgCl$_2$, 0.1 mM EGTA, 1% Triton X-100, 10 % Glycerol, 1 mM DTT, and protease inhibitor cocktail from Boehringer) and homogenized with a hand held homogenizer (Fisher, Fairlawn, NJ) in a 1.5 ml microcentrifuge tube. Lysed cells were centrifuged at 14,000 X g for 15 min and 4°C to remove cellular debris. The supernatant was transferred to pre-chilled 1.5 ml microcentrifuge tubes. Ten µl aliquots were used to determine protein concentration with the Pierce BCA kit (23223/1859078); the standards were done in triplicate.

For SDS-PAGE analysis, 30 µg to 50 µg of each protein sample were mixed with equal volumes of 2X Laemmlie buffer. Samples were boiled for 5 min, centrifuged briefly, and the supernatant was loaded onto 4-20% Tris-HCl Criterion gels, run for approximately 2hr at 100V using Tris buffer. SDS PAGE gels were transferred to nitrocellulose membranes (BioRad) overnight at 30V at 4°C. Membranes were subsequently blocked with 7.5% non-fat dry milk or 7% BSA (Santa Cruz) in TBS with 0.1% Tween (TBST) overnight at 4°C. Blots were washed for 15 min three times in
TBST at RT. Primary antibodies were prepared in 7% BSA/TBST and incubated either overnight at 4°C. Following primary antibody incubation, blots were washed as previously stated and incubated with secondary antibodies made up in 7 BSA/TBST for 1h at RT, followed by washes with TBST.

**DNase I Hypersensitivity Analysis and Southern Blotting**

HFL-1 cells were grown to 75% confluency, then either left untreated or treated for six hours with 2 ng/ml of IL-1β. Ten plates of HFL-1 cells were used for each group. To permeabilize the cells they were initially trypsinized for 10 min at 37°C in a 1X trypsin solution in PBS without EDTA. The trypsinized cells from each group were pooled in 30 milliliters of media containing serum, and trituated to dissociate the monolayers into single cells. The pooled cells were pelleted by gentle centrifugation at 1500 rpm for five minutes at 4°C in a Beckman GS-6KR. The pelleted cells were resuspended in 10 ml ice cold DNase I Buffer (150 mM Sucrose, 80 mM KCl, 35 mM HEPES, pH 7.4, 5 mM K₂HPO₄, 5 mM MgCl₂, 0.5 mM CaCl₂), then pelleted as before and resuspended in four ml of ice cold DNase I Buffer. Four milliliters of room temperature 0.1% L-α-lysolecithin in DNase I Buffer is added to the resuspended cells and incubated on ice for two minutes. To stop the permeabilization process, the cell solution was brought to a final volume of 40 ml with DNase I Buffer. Cells are again pelleted by centrifugation at 1800 rpm for 5 min at 4°C. Each group of permeabilized cells were resusupended in 1.8 ml of DNase I Buffer and kept on ice prior to immediate DNase I digestion.

During the final centrifugation step, duplicate sets of 2.0 ml microcentrifuge tubes are prepared with increasing concentrations [0-36 Units] of DNase I (DPFF; Worthington Biochemical corp). 300 µL of permeabilized cell suspension was added to the prepared tubes. The aliquoted cells are gently mixed and incubated for four minutes at 37°C. The
DNase I digestion was stopped and the cells were lysed by addition of 300 µL of a 2X DNA lysis buffer (4% SDS, 0.2 M EDTA, and 800 µg/ml Proteinase K. Cell lysates were incubated overnight at 50°C. The DNase treated DNA was isolated by organic extraction and isopropanol precipitation, resuspended and washed by ethanol precipitation.

After ethanol precipitation the DNA was resuspended in 75 µL of TE and allowed to rest at 4°C for several days to fully resuspend the DNA pellet. The resuspended DNA was restriction digested with BamH I, overnight in a final volume of 100 µL and 100 Units of enzyme. The concentration of digested DNA was determined by spectrophotometric analysis of the samples and 30 µg of each sample was loaded on a 0.8% agarose gel in TBE overnight and electrotransfered to nylon membrane as described. The transferred DNA was probed with a randomly labeled DNA probe to a sequence in exon 1 as depicted in the genomic map of the cPLA2α gene.

**Generation of Growth Hormone Reporter Gene Constructs**

Different regions of the cPLA2α and cPLA2γ promoters were cloned into a human growth hormone reporter plasmid [98]. BAC and PAC clones, containing the sequences of interest were obtained from the Genetics Institute (cPLA2α) or the Sanger Center (cPLA2γ), containing the entire human MnSOD gene was used to clone regions of interest into the growth hormone vector by PCR. The -14 kb cPLA2α promoter was amplified using the Long Range PCR kit (Roche, Switzerland), with the forward primer 5’-CAGGGTACCAGAGTTGGGATGGAGAAGGTTG-3’ and the reverse primer 5’-CGAGTCGGATCCGCTTACAGTTCCCAGAGTTACC-3’ containing a Kpn I and BamHI restriction sites on the ends respectively. Initially the PCR products were cloned into the TOPO TA cloning vector pCR-XL. Subsequently, the -14 kb fragment was
digested from pCR-XL with BamHI and cloned into a BamHI digested pØGH plasmid.

An endogenous BamH I site at the 3’ end of the fragment and a BamHI I site conveniently located in the multi cloning site of the pCR–XL vector were utilized for the cloning. The -4.8 kb and -6.8 kb promoter constructs were created by digestion and religation of the -14 kb construct with HincII or HindIII respectively. Construction of the cPLA_2γ promoters was conducted in a similar manner. The -3.8 and -1.2 kb promoters were amplified separately with the 5’-TGGCTTCTTCCTCCTCGTCC-3’ and 5’-TGTTGATACTCCTGCCTTG-3’ forward primers respectively and the 5’-GCTTCTGTGGTCCCTCTGC-3’ reverse primer. Forward primers contained a HindIII restriction site and the reverse primer contained a BamHI site. The TOPO cloned PCR fragments were digested with BamHI and HindIII and cloned into a BamHI-HindIII digested plasmid. The 588 bp plasmid was created by digestion and religation with KpnI.

**Mouse Strains**

The primary Cftr knock-out strain used for these studies was the Cftr S489X -/- neo insertion in C57BL/6 mice developed initially at the University of North Carolina and then modified with the transgenic overexpression of gut-specific expression of human CFTR from the fatty acid binding protein (FABP)- promoter in order to prevent intestinal obstruction and improve viability. These mice demonstrate the same lung phenotype as the original mice. Age and sex matched C57BL/6 mice were used as control mice for all experiments. Since some FVB/NJ genetic background remains in the Cftr S489X -/-; FABP-hCFTR (+/+) mice thus, FVB/NJ mice were also used as control mice.
Aspergillus Sensitization and Challenge

Five to six week old Cftr S489X -/-; FABP-hCFTR (+/+), C57BL/6 and FVB/NJ mice were housed in the SPF mouse colony of the University of Florida according to NIH guidelines and allowed food and water ad libitum. All experimental procedures were approved by the IACUC of the University of Florida. Animals were sensitized to Aspergillus fumigatus crude protein extract (Af) (Greer Laboratories). Briefly, animals were administered with intraperitoneal (i.p.) injections of 200 ug of Af total crude protein extract dissolved in 100 ul of PBS on days 0 and 14. Aerosol challenge was performed with 0.25 % Af for 20 min in a 30x30x20 cm acrylic chamber using a jet nebulizer Pari model LC-D with an air flow of 6 liters/min on days 28, 29 and 30. Non-sensitized control mice received i.p. injections with PBS alone and were challenged with AF along with sensitized mice.

Real Time PCR

Isolated total RNA was reverse transcribed to cDNA using Superscript II First Strand Synthesis Kit from Invitrogen. Briefly, 1µg of total RNA was mixed with oligo(dT) primers and dNTP mix and incubated at 65°C for 5 min. This was followed by addition of 5 mM MgCl2, 10mM DTT, reverse transcriptase reaction buffer [1X] and 1µl RNaseOUT Rnase Inhibitor. Mixture was incubated at 42°C for 2 min, followed by addition of 1 µl (50 units) of Superscript II reverse transcriptase, and incubation at 42°C for 50 min. The reaction was terminated by 15 min incubation at 70°C, followed by a brief 4°C incubation, and addition of 1 µl RNase H to degrade any remaining RNA for 20 min at 37°C. For PCR reaction, 79 µl of water was added to each tube. For real-time PCR analysis two µl of this cDNA was used in the PCR reaction with a final concentration of 300 nM of each primer and 1X of SYBR Green in 96 well optical plate
with an optically clear coverslip. Real-time analysis was conducted on an ABI SDS 7000. Each RT sample was run in triplicate; the cycle threshold was determined for each sample and then averaged. Multiple replicates of each animal group or cellular condition was done on at least two separate days, and the average of the $C_T$ of each condition was taken. Cyclophillin A was used as a reference gene to determine the fold changes in gene expression by the $\Delta\Delta C_T$ method. The $\Delta C_T$ for a sample is derived by subtracting the average $C_T$ of the gene of interest from the average $C_T$ of the internal reference gene for a given sample. To calculate the relative fold changes ($2^{\Delta\Delta C_T}$), the $\Delta\Delta C_T$ was calculated by subtracting the $\Delta C_T$ of a given condition from the $\Delta C_T$ of the control condition. Standard deviations were determined by propagating the error for each real-time reaction and differences in each group. The primers used in all of the real-time reactions had an annealing temperature between 58°C and 60°C, with a GC content between 50% and 60%. The sequences of the primers are provided in Appendix A.

**Immunohistochemistry Analysis of 15-LO-1 Expression in the Mouse Lung**

Immediately after collection of lavage fluid one of the lungs was tied off and, ice-cold freshly prepared 4% paraformaldehyde in 1 X PBS (pH 7.4) was instilled through a tracheal cannula into the other lung to inflation-fix the lung. Following dissection specimens were immersed in 4% paraformaldehyde at 4°C overnight and dehydrated in a graded series of ethanol solutions. Tissue was embedded in paraffin. Sections were cut at 5 µm thicknesses and mounted. For determination of 15-LO-1 localization, slides were immunostained with rabbit anti-15-LO primary antibody, and a secondary HRP-conjugated anti-rabbit antibody, by the molecular pathology core at the University of Florida.
CHAPTER 3
MOLECULAR REGULATION OF CYTOSOLIC PHOSPHOLIPASE A2 ALPHA

Introduction

Cytosolic phospholipase A2α (cPLA2α) is an important enzyme in the production of arachidonic acid (AA) in response to pro-inflammatory stimuli and the activation of cPLA2α activity by IL-1β, TNFα, IFNγ, LPS and zymosan has been shown [99]. Additionally, the involvement of cPLA2α in inflammatory diseases such as rheumatoid arthritis, asthma, and multiple sclerorisis has been demonstrated. The activation of cPLA2α enzymatic activity requires an increase in intracellular calcium concentration and concomitant phosphorylation by MAP kinases. In many cells, particularly immortalized cancer cell lines and immune cells, the expression of cPLA2α is elevated. However, in untransformed cells the expression of cPLA2α is inducible by pro-inflammatory stimuli. The full induction of AA release in these cell types likely requires an increase in the expression of cPLA2α. Previously our lab has demonstrated the activation of cPLA2α gene expression by the pro-inflammatory cytokines IL-1β, TNFα and IFNγ, culminating in an increase in AA release by cPLA2α [73]. This increase in gene expression requires de novo transcription; however, the cis acting regulatory regions required for induction of cPLA2α gene expression by pro-inflammatory cytokines have not been identified. The proximal promoter is sufficient for basal expression, but is not inducible by pro-inflammatory cytokines when used in a reporter gene assay. Also, the signaling mechanisms required for the induction of cPLA2α expression by cytokines has not been determined. Therefore, determining the signaling cascades and identifying the cis acting
regulatory elements involved in the expression of cPLA$_2$α gene expression will provide important information on the regulation of this gene in inflammatory disease and cancer.

**Results**

**Feed Forward Regulation of cPLA$_2$α Gene Expression:**

The induction of cPLA$_2$α mRNA and protein expression in human lung fibroblasts and epithelial cells by pro-inflammatory cytokines has previously been shown [73]. Similarly, other enzymes involved in the downstream metabolism of AA derived eicosanoids have also been shown to be induced by extra-cellular stimuli [100]. As stated above, the activation of several of these genes involve a feed forward signaling mechanism [101]. A one hour pretreatment of human fetal lung fibroblasts (HFL-1) cells with increasing doses of the cPLA$_2$α inhibitor AACOCF$_3$ blocks the induction of cPLA$_2$α mRNA by an 8 hour exposure to IL-1β (Figure 3-1). This demonstrates cPLA$_2$α is activated by feed forward signaling where the induction of cPLA$_2$α gene expression by IL-1β requires cPLA$_2$α activity.

![Figure 3-1](image)

Figure 3-1. Feed forward induction of cPLA$_2$α gene expression in HFL-1 cells by IL-1β. A representative northern blot of cPLA$_2$α mRNA in HFL-1 cells, pretreated for 1 hour with increasing concentrations of the cPLA$_2$α inhibitor AACOCF$_3$, with or without 8 hours of IL-1β treatment, as indicated. Control cells were either not treated with inhibitor [C] or treated with the vehicle, ethanol [E]. Northern analysis of the expression of L7a on the same blot is included as a loading control.
To further delineate the downstream eicosanoids mediating this feed forward signaling, the general inhibitors of cycloxygenases (indomethacin) and lipoxygenases (NDGA) were utilized. The induction of cPLA$_2$α mRNA by IL-1β is not affected by pre-treating HFL-1 cells with indomethacin (Figure 3-2); however, pre-treating cells with increasing concentrations of NDGA inhibited the induction (Figure 3-3).

![Figure 3-2. Feed forward signaling of cPLA$_2$α induction by IL-1β is not regulated by COX-1 or -2. A representative northern blot of cPLA$_2$α mRNA in HFL-1 pretreated one hour with increasing concentrations of the COX inhibitor indomethacin (INDO) with or without 8 hour IL-1β treatment as indicated. Control cells were either not treated with inhibitor [C] or treated with the vehicle, DMSO [D]. Northern analysis of the expression of L7a on the same blot is included as a loading control.](image)

This demonstrates the requirement of lipoxygenase but not cycloxygenase activity in the induction of cPLA$_2$α expression. NDGA is a general inhibitor of lipoxygenases, blocking 5-, 12-, and 15-LOs. However, the effective concentration of NDGA for the different lipoxygenases differs; the IC$_{50}$ of 5-LO is 0.2 µM and, while the IC$_{50}$ for 12- and 15-LO is 30 µM [102]. The reduction of cPLA$_2$α expression reaches 50 percent between 20 µM and 50 µM of NDGA (Figure 3-3). Therefore, the effective concentration on IL-1β dependent cPLA$_2$α expression is similar to a dose where NDGA inhibits 12- or 15-LO, suggesting that one of these lipoxygenases, and not 5-LO, mediates the induction of cPLA$_2$α mRNA by IL-1β. Additionally, MK886 is an inhibitor of 5-LO.
Figure 3-3. Feed forward induction of cPLA$_2$$\alpha$ by IL-1$\beta$ requires lipoxygenase activity. (A) Representative northern blot of cPLA$_2$$\alpha$ mRNA expression in HFL-1 cells pretreated one hour with increasing concentrations of the lipoxygenase inhibitor NDGA with or without 8 hour IL-1$\beta$ treatment as indicated. Control cells were either not treated with inhibitor [C] or treated with the vehicle, DMSO [D]. Northern analysis of the expression of L7a on the same blot is included as a loading control. (B) Densitometry acquired from 3 identical blots as in (A). Percent inhibition is determined by comparison to the DMSO control. The SEM of the average of the sets of data are shown. * depicts a p value $\leq 0.05$ determined by a two tailed t-test.
Figure 3-4. Feed forward signaling of cPLA₂α induction by IL-1β is not regulated by 5-LO. A representative northern blot of cPLA₂α mRNA in HFL-1 pretreated one hour with increasing concentrations of the 5-LO inhibitor MK 886 with or without 8 hour IL-1β treatment as indicated. Control cells were either not treated with inhibitor [C] or treated with the vehicle, DMSO [D]. Northern analysis of the expression of L7a on the same blot is included as a loading control.

The pretreatment of HFL-1 cells with MK886 does not inhibit the induction of cPLA₂α by IL-1β (Figure 3-4). This further implicates a 12- or 15-LO, and not 5-LO, as the lipoxygenase(s) mediating the feed forward activation of cPLA₂α expression.

Luteolin is a plant-derived flavenoid with potent anti-inflammatory effects and is a known inhibitor of 15-LO in vitro [103]. A one hour pretreatment of HFL-1 cells with Luteolin blocks the IL-1β induction of cPLA₂α mRNA expression (Figure 3-5). The chemical nature of Luteolin makes it an anti-oxidant and it can have non-specific effects. Therefore, HFL-1 cells were also treated with the specific inhibitor of 15-LO, PD146176. Similar to the inhibition by Luteolin, a one hour pretreatment of HFL-1 cells with increasing concentrations of PD146176 caused a dose dependent inhibition of the induction of cPLA₂α gene expression by IL-1β (Figure 3-6). Taken together, these results demonstrate that 15-LO activity is required downstream of cPLA₂α in the feed forward induction of cPLA₂α gene expression. Unfortunately, extra-cellular treatment of cells with 15-HETE alone does not induce cPLA₂α gene expression (data not shown).
Figure 3-5. Feed forward induction of cPLA$_2$α by IL-1β requires 15-LO activity. (A) Northern analysis of cPLA$_2$α mRNA expression in HFL-1 cells pretreated one hour with increasing concentrations of the 15-LO inhibitor Luteolin with or without 8 hour IL-1β treatment as indicated. Control cells were either not treated with inhibitor [C] or treated with DMSO [D]. Northern analysis of the expression of L7a on the same blot is included as a loading control. (B) Densitometry acquired from 3 identical blots as in (A). Percent inhibition is determined by comparison to the DMSO control. The SEM of the average of the sets of data are shown. * depicts a p value $\leq 0.05$ determined by a two tailed t-test.

There are several possible reasons for the lack of response to extra-cellular treatment with 15-HETE in the induction of cPLA$_2$α gene expression: 1) a downstream metabolite of 15-HETE may be required and the enzyme(s) involved in its conversion require activation, 2) signals in addition to the 15-LO metabolite (i.e. chromatin remodeling) may be
required or 3) the 15-HETE effect might occur via intra-cellular mechanisms and may require transport into the cells to cause a response.

**Figure 3-6.** Feed forward induction of cPLA$_2$α by IL-1β requires 15-LO activity. (A) Northern analysis of cPLA$_2$α mRNA expression in HFL-1 cells pretreated one hour with increasing concentrations of the 15-LO inhibitor PD146176 with or without 8 hour IL-1β treatment as indicated. Control cells were either not treated with inhibitor [C] or treated with DMSO [D]. Northern analysis of the expression of L7a on the same blot is included as a loading control. (B) Densitometry acquired from 3 identical blots as in (A). Percent inhibition is determined by comparison to the DMSO control. The SEM of the average of the sets of data are shown. * depicts a p value ≤ 0.05 determined by a two tailed t-test.
MAP Kinase Signaling is Required for cPLA$_2\alpha$ Transcription:

MAP kinases are a vast and varied family of Serine/Threonine protein kinases and regulate many aspects of cellular function including cell survival, proliferation, and division. There are three major families of MAP kinases, Extracellular Regulated Kinases (ERK), Jun N-terminal Kinases (JNK), and p38 Mitogen Activated Protein Kinases (p38 MAPK). This family of kinases recognize a similar consensus site, a serine or threonine preceded by a proline with a single spacer amino acid (PXS/T) [104]. The serine at position 505 on cPLA$_2\alpha$ is such a site, and is phosphorylated by ERK and p38 MAP Kinases in response to variety extra-cellular stimuli in vivo [105-108].

Figure 3-7. MAP kinases are required for the induction of cPLA$_2\alpha$ by IL-1$\beta$.
Representative northern blot of cPLA$_2\alpha$ mRNA expression in HFL-1 cells pretreated one hour with 10 µM of the p38 inhibitors SB203580 (SB203) and SB202190 (SB202), the ERK inhibitor PD 98059 (PD) or the JNK inhibitor SP600125 (SP) with or without 8 hour of IL-1$\beta$ treatment as indicated. Control cells were either not treated with inhibitor [Un] or treated with the vehicle, DMSO [D]. Northern analysis of the expression of L7a on the same blot is included as a loading control.

Although the role of phosphorylation in the regulation of cPLA$_2\alpha$ activity is debatable, the general consensus is that phosphorylation at ser505 is an important component required for the full enzymatic activation. Both ERK and p38 MAP kinases phosphorylate cPLA$_2\alpha$ in response to bacteria, LPS, Zymosan, TNF$\alpha$ and amyloid beta peptide, [59,69,109,110]. Thus, I hypothesized that MAP kinases are involved in the
regulation of cPLA2α mRNA expression through phosphorylation of cPLA2α at ser505. As depicted in Figure 3-7, using inhibitors of ERK, JNK and p38 MAP Kinases, the expression of cPLA2α was decreased by SB203580 and SB202190, two analogous inhibitors of p38 MAPK; however, neither PD98059 nor SP600125, inhibitors of ERK and JNK respectively, block the induction of cPLA2α expression induced by IL-1β.

![Figure 3-8](image)

**Figure 3-8.** The induction of cPLA2α by IL-1β requires p38 MAPK signaling. A representative northern blot of cPLA2α mRNA expression in HFL-1 cells pretreated one hour with increasing concentrations of the p38 inhibitor SB203580 (SB203) with or without 8 hour of IL-1β treatment as indicated. Control cells were either not treated with inhibitor [C] or treated with DMSO [D]. Northern analysis of the expression of L7a on the same blot is included as a loading control.

![Figure 3-9](image)

**Figure 3-9.** IL-1β leads to an increase in cPLA2α protein expression. Western analysis of cPLA2α protein expression in HFL-1 cells pretreated for one hour with 10 µM of the p38 inhibitor (SB) or the JNK inhibitor SP600125 (SP) with or without 48 hour IL-1β treatment as indicated. Control cells were either not treated with inhibitor [U] or treated with DMSO [D].

cPLA2α expression was dose dependently inhibited by SB203580, with reduction of cPLA2α expression first observed between 0.5 and 1.0 µM, and a return of cPLA2α basal levels with 10 µM SB203580 (Figure 3-8). Additionally, treatment of HFL-1 cells with IL-1β for 48 hours increases cPLA2α protein, and pretreatment with SB203580 blocks the
induction of cPLA₂α protein, while the JNK inhibitor SP600125 has no effect (Figure 3-9).

![Figure 3-10. IL-1β increase the phosphorylation of cPLA₂α at ser505. Western analysis of phosphorylated cPLA₂α protein levels in HFL-1 cells pretreated for one hour with 10 µM of the p38 inhibitor SB203580 (SB203) or left untreated, followed by increasing time of IL-1β treatment as indicated.](image)

This agrees with the hypothesis that MAP Kinases would regulate cPLA₂α expression; however, the increase in the phosphorylation of cPLA₂α induced within one hour by IL-1 is not blocked by a one hour pretreatment with SB203580 (Figure 3-10). Therefore, although p38 is necessary for the induction of cPLA₂α gene expression by IL-1β, the regulation by p38 must occur via an alternative pathway from the activation of cPLA₂α.

**NF-κB is Required for the Induction of cPLA₂α Gene Expression.**

The transcription factor NF-κB is a heterodimer, prototypically consisting of p50-NF-κB and p65-NF-κB subunits, although other subunits exist. p50-NF-κB and p65-NF-κB both contain Nuclear Localization Signals (NLS) and DNA binding domains, while the p65-NF-κB subunit also contains a transactivation domain. When unphosphorylated at Ser276, p65 interacts with Histone Deactelyase 1 (HDAC1), but this interaction is replaced by the acteltransferase CBP/p300 upon phosphorylation. In unstimulated cells the NF-κB dimer is bound by the Inhibitor of kappa B (IκB), masking the NLS on NF-κB, thus retaining NF-κB dimer in the cytosol. In response to extracellular stimuli IκB is phosphorylated by IKK complex, leading to the ubiquitination of IκB, and in turn, causing its proteosomal degradation. The unmasked NLS then allows
the nuclear translocations of the NF-κB dimer. The proteosomal inhibitor ALLN prevents the degradation of IκB and thus inhibits activation of NF-κB [111].

Figure 3-11. Inhibition of the 26S proteosome inhibits the expression of cPLA$_2$α induced by IL-1β. A representative northern blot of cPLA$_2$α mRNA expression in HFL-1 cells pretreated one hour with increasing concentrations of the proteosome inhibitor ALLN with or without 8 hour of IL-1β treatment as indicated. Control cells were either not treated with inhibitor [C] or treated with DMSO [D]. Northern analysis of the expression of L7a on the same blot is included as a loading control.

Pre-treating cells with ALLN blocks the induction of cPLA$_2$α by IL-1β (Figure 3-11) demonstrating the requirement of an active proteosome in the induction of cPLA$_2$α expression by IL-1β, and suggesting a role for NF-κB in this transcriptional activation. Likewise, Bay11-7802, an inhibitor of IKK complex, blocks the phosphorylation of IκB by IKKs, also inhibiting NF-κB activation [112]. Co-treating cells with Bay11-7802 at the time of stimulation blocks the IL-1β induced gene expression in a dose dependent manner (Figure 3-12). The ALLN and Bay 11-7802 data taken together strongly implicate a role for NF-κB signaling in the induction of cPLA$_2$α gene expression.
Figure 3-12. Induction of cPLA$_2$α expression by IL-1β requires the transcription factor NF-κB. (A) A representative northern blot of cPLA$_2$α mRNA expression in HFL-1 cells pretreated one hour with increasing concentrations of the IKK inhibitor Bay 11-7082 with or without 8 hour IL-1β treatment as indicated. Control cells were either not treated with inhibitor [C] or treated with the vehicle, ethanol [E]. Northern analysis of the expression of L7a on the same blot is included as a loading control. (B) Densitometry acquired from 3 identical blots as in (A). Percent inhibition is determined by comparison to the DMSO control. The SEMs of the average of the sets of data are shown. * depicts a p value ≤ 0.05 determined by a two tailed t-test.
Figure 3-15. Induction of cPLA$_2$α expression by IL-1β is inhibited by curcumin. (A) Northern analysis of cPLA$_2$α mRNA expression in HFL-1 cells pretreated one hour with increasing concentrations of curcumin (CURC) with or without 8 hour IL-1β treatment as indicated. Control cells were either not treated with inhibitor [C] or treated with the vehicle DMSO [D]. Northern analysis of the expression of L7a on the same blot is included as a loading control. (B) Densitometry acquired from 3 identical blots as in (A). Percent inhibition is determined by comparison to the DMSO control. The SEM of the average of the sets of data are shown. * depicts a p value $\leq 0.05$ determined by a two tailed t-test.

Curcumin is a naturally occurring anti-inflammatory derived from the spice turmeric, and is a reported inhibitor of NF-κB activity [113,114]. Pretreatment of HFL-1 cells with curcumin for one hour also inhibits the IL-1β mediated increase in cPLA$_2$α mRNA expression further implicating NF-κB in the induction of cPLA$_2$α (Figure 3-15).
However, curcumin also inhibits the activity of the transcription factors AP-1 and Egr-1 [114,115], and more recently has been implicated as an inhibitor of acetyltransferase activity [116-118]. The effects of curcumin on acetyltransferases are interesting as this could explain the pleitropic effects of a single compound on several dissimilar transcription factors.

**Histone Acetylation Regulates cPLA$_2$$\alpha$ Expression**

The regulation of gene expression is known to be controlled by the state of chromatin condensation, where the chromatin in the regulatory regions of active genes are hyperacetylated leading to a more open chromatin state, and those in inactive genes are hypoacetylated causing a condensation of the chromatin. This balance is controlled by the activity of acetyltransferases, most notable CBP/p300, and histone deacetylases (HDACs). In this manner, chromatin is held in a silent conformation by the activity of HDACs, and in active genes, acetyltransferases are recruited by transcription factors in response to specific stimuli, acetylating the chromatin, leading to a permissive chromatin conformation [93-97].

In light of the duplicate role curcumin can play as an inhibitor of NF-κB and acetyltransferases, the role of histone acetylation in cPLA$_2$$\alpha$ gene expression was analyzed. Trichostatin A (TSA) is an inhibitor of class 1 and 2 HDACs and treatment of cells with TSA leads to a global increase in histone acetylation [119]. Interestingly, treatment of HFL-1 cells with TSA quickly activates cPLA$_2$$\alpha$ gene expression. Induction of cPLA$_2$$\alpha$ expression began after 4 hours of treatment, and continued to increase, with a maximal induction achieved between 8 and 24 hours, and lasting for at least 48 hours (Figure 3-14). The time course of the induction by TSA is similar to the induction by IL-$\beta$ [73].
Figure 3-14. Increased histone acetylation leads to an increase in cPLA₂α expression.  
(A) Representative northern blot of cPLA₂α mRNA expression in HFL-1 cells treated for increasing amounts of time with 1 µM of the HDAC inhibitor Trichostatin A (TSA) as indicated. Control cells were either not treated with inhibitor [C], treated with the vehicle, DMSO [D] or with IL-1β. Northern analysis of the expression of L7a on the same blot is included as a loading control.  (C) Average from densitometry on three identical blots as in (A and B), the SEM are shown and * depicts a p value ≤ 0.05 as determined by a two tailed t-test.
However, unlike the time course of cPLA\(_2\alpha\) expression by IL-1β, where the cPLA\(_2\alpha\) mRNA levels maximize between 8 and 12 hours, and return to basal levels by 24 hours, the induction by TSA was sustained through 48 hours of treatment with only a qualitatively minor decrease in mRNA levels (Figure 3-14). This suggests the cPLA\(_2\alpha\) gene is kept silent by HDAC activity, and subsequently activated by the recruitment of actelytransferase in response to IL-1β. NF-κB could be the factor recruiting CBP/p300 to the promoter, as the phosphorylation of p65 at ser276 has been previously implicated in recruiting CBP/p300.

**Chromatin Structure and Promoter Analysis of the cPLA\(_2\alpha\) Promoter**

The trichostatin A studies demonstrated a role for chromatin acetylation and remodeling in the regulation of cPLA2α gene expression. Therefore, in an attempt to further investigate the chromatin structure of the upstream cPLA2α promoter, DNase I hypersensitivity analysis was performed on the first 18 kb upstream of the cPLA2α promoter in HFL-1 cells (Figure 3-15). A single constitutive hypersensitive site is present between approximately 8-9 kb upstream of the transcriptional start site. Interestingly, the hypersensitive site is present whether or not the cells were treated for six hours with IL-1β. As stated above, histone acetylation creates a more open chromatin structure. Therefore, one would expect there would be a change in the hypersensitive site. In other genes constitutive hypersensitive sites have been shown to regulate gene expression, and undergo structural changes when studied at higher resolution.

Previous studies in our lab analyzing the cPLA\(_2\alpha\) promoter have demonstrated that the proximal promoters starting at -3.6 kb and smaller are not inducible in response to IL-1β. To further study the cPLA\(_2\alpha\) promoter containing this constitutive hypersensitive site, a -14 kb cPLA\(_2\alpha\) promoter was cloned into a human growth hormone reporter
plasmid. Additionally, -4.8 kb and -6.8 kb constructs were created by restriction digest and recloning of the -14 kb promoter with Hinc II and Hind III respectively (Figure 3-15).

Figure 3-15. DNase I hypersensitivity analysis of the cPLA$_2$$\alpha$ promoter. (A) A schematic of the cPLA$_2$$\alpha$ promoter region with the restriction sites used in DNase I HS analysis and promoter cloning (Figure 3-16), as well as the site of the probe for Southern blotting are. An approximation of the location of the hypersensitive site is also depicted (*). (B) Southern blot of DNase treated HFL-1 cells demonstrating the DNase I hypersensitivity site.
Transfection of HFL-1 cells with the -14 kb construct lead to a 2.5 fold induction of reporter gene expression after 8 hours of IL-1β treatment (Figure 3-16). However, neither the -4.8 kb nor the -6.8 kb constructs were capable of inducing reporter gene expression (data not shown). Taken together, the presence of a hypersensitive site and the inducible nature of the -14 kb promoter construct demonstrate the presence of an IL-1β responsive cis-acting element in the distal regions 5’ to the promoter of cPLA2α.

Figure 3-16. Induction of the -14 kb cPLA2α promoter, by IL-1β, in a reporter gene assay. Densitometric analysis from northern blots of the expression of hGH mRNA expressed from a hGH reporter plasmid containing the -14 kb cPLA2α promoter transfected into HFL-1 cells. Identical plates were either treated for 8 hrs with 2 ng/ml of IL-1β (IL-1), or left untreated (Ctrl). The fold increase was determined by comparing the expression of hGH in the IL-1β treated plates to the untreated plates. The densitometric data is the average of 4 individual experiments, and the SEM is shown. A two tailed t-test was performed and * indicates p value ≤ 0.05.

Discussion

Eicosanoids are important intra-cellular and extra-cellular signaling molecules, and the regulation of their metabolism is important to inflammation, as well as, normal cellular function and cancer. The regulation of eicosanoid metabolism is tightly
regulated by intracellular calcium concentrations, post-translational protein modification, and differential gene expression. These processes control the eicosanoid production by mediating which enzymes are present and active in specific cell types under various physiological conditions. Additionally, in response to pro-inflammatory mediators the gene expression profile can change, thus altering the eicosanoids produced. Interestingly, several of the enzymes involved in eicosanoid metabolism are regulated by their own products in a feed forward, or positive feedback, manner, as well as, negative feedback inhibition of other eicosanoid metabolizing enzymes. As shown in the data presented here, cPLA$_2$$\alpha$ is likewise regulated by eicosanoids in a feed forward manner. This feed forward signaling requires downstream metabolism by a 15-LO and additional signals as depicted in the model presented in Figure 3-17.

AA released by cPLA$_2$$\alpha$ in response to IL-1$\beta$ is metabolized by a 15-lipoxygenase, and acting through an as-of-yet unidentified process, the 15-HETE produced is necessary for the activation of cPLA$_2$$\alpha$ gene expression. In addition, signaling from p38 MAPK is required. Although the downstream target of p38 MAPK in this system is unknown, its effects are not mediated through the phosphorylation of cPLA$_2$$\alpha$ and must occur through a parallel pathway. NF-$\kappa$B activity and histone acetylation are also required for the activation of gene expression. As there is a putative NF-$\kappa$B binding site in the cPLA$_2$$\alpha$ promoter, and the phosphorylation of p65-NF-$\kappa$B has been linked to acetyltransferase recruitment, it is possible to speculate that NF-$\kappa$B binding to the promoter recruits histone acetyltransferase (i.e. CBP/p300), causing an increase in histone acetylation, and opening of the chromatin to a permissive status.
As previously stated, the phosphorylation of p65-NF-κB at Ser276 is known to recruit CBP/p300. Additionally, p38 MAPK signaling can phosphorylate Ser276 on p65 in response to pro-inflammatory stimuli, and this phosphorylation of p65-NF-κB is thought to occur via the downstream kinase MSK-1 [120]. Therefore, it is tempting to speculate that the role of p38 MAPK in the regulation of cPLA$_2$α gene expression is mediated by the activation of MSK-1, which in turn phosphorylates NF-κB. However, H89, an inhibitor of MSK-1, did not block the induction of cPLA$_2$α expression by IL-1β (data not shown). Therefore, this study has identified several aspects of the signaling cascades involved in the induction of cPLA$_2$α by IL-1β have been identified. However,
the connections between these pathways need to be further elucidated in order to fully understand the regulation of cPLA$_2$α gene expression.

The proximal promoter of the cPLA$_2$α gene is not responsive to extra-cellular stimuli. Some studies with over-expression of transcription factors or oncogenic proteins have shown modest increases in expression from the proximal promoter, however, none have demonstrated stimulus dependent activation. The DNase I hypersensitivity analysis identified a constitutive hypersensitive site in the distal regulatory element approximately located between -8 to -9 kb, in relation to the transcriptional start site. This hypersensitive site likely represents a putative element in the upstream of the cPLA$_2$α promoter. In agreement, the reporter gene data further demonstrate the presence of an IL-1β responsive regulatory region upstream of the cPLA$_2$α promoter, located between -14 and -6.8 kb. Therefore, the IL-1β responsive element is likely located in the region of the hypersensitive site, between -8 to -9 kb. Further delineation of this element and its coordinated regulation, via the defined signaling cascades, will be important to understanding the mechanisms of cPLA$_2$α gene expression.
CHAPTER 4
GENE PROFILE ANALYSIS OF EICOSANOID METABOLISM IN ALLERGIC ASTHMA

Introduction

Allergic asthma is an inflammatory disease of the lung leading to airway hyperreactivity and obstruction. Allergic asthma is caused by the prior sensitization of inhaled allergens. *Aspergillus fumigatus* is a common and ubiquitous fungus, and sensitization to this organism has been reported in 16-38% of asthmatics. An allergic response to this fungus manifests in the lung as *Aspergillus*-induced asthma (AIA) or can develop into more severe lung disease, such as Allergic Bronchopulmonary Aspergillosis (ABPA). There is a positive correlation with *Aspergillus* sensitive asthmatics and severity of airway obstruction [121], and sensitization to *Aspergillus* is a particular compounding factor with cystic fibrosis (CF) patients.

We recently developed a model of allergic aspergillosis in normal and CF mice [122]. In this model, mice are sensitized to *Aspergillus* with two intraperitoneal injections of 200 µg of crude *Aspergillus fumigatus* (*Af*) extract on days 1 and 14. On days 28-30, the *Af* and mock sensitized mice are challenged with vaporized *Af* extract for 30 min each day, and the lungs from the challenged mice are harvested 48 hours after the final challenge. The *Af* sensitized mice develop a Th2 mediated allergic inflammatory response typical of asthma, including elevated levels of the Th2 cytokines, IL-4, IL-5 and IL-13, increases in total serum IgE, goblet cell hyperplasia and airway eosinophilia. More specific to the development of *Aspergillus*-induced asthma and ABPA, an increase
in *Aspergillus* specific IgE and IgG are found in the serum of sensitized mice, demonstrating an *Aspergillus* specific immune response [122].

Although the processes involved in the development of asthma are complex, the release of bioactive lipid metabolites of arachidonic acid (AA), the eicosanoids, is central to the inflammatory response associated with asthma. These bioactive lipids mediate many physiological aspects of the inflammation associated with asthma including, increased vasodilation, vascular permeability, immune cell chemotaxis, mucous production and bronchoconstriction. The eicosanoids are metabolized into these various bioactive lipids by specific downstream enzymes from AA released by Phospholipase A$_2$ (PLA$_2$). The metabolism of AA into the eicosanoids occurs via two distinct pathways, the activity of cyclooxygenases on AA into prostaglandin H$_2$ and then the downstream prostaglandins, prostacyclin or thromboxane by specific synthases, or the peroxidation by a variety of lipoxygenases into different hydroperoxyeicosatetraenoic acids and subsequently their downstream metabolites.

Gene ablation studies, biochemical analysis of the lung, and clinical use of pharmaceuticals targeting leukotriene metabolism and signaling have previously implicated eicosanoids in the etiology of asthma. Naonori Uozumi and colleagues [41] demonstrated the importance of the cPLA$_2\alpha$ gene in the development of allergic asthma utilizing mice genetically deficient of the cPLA$_2\alpha$ gene in an ovalbumin model of allergic asthma. The cPLA$_2\alpha$ deficient mice did not have the bronchoconstriction or the airway hyper-responsiveness central to the development of asthma. Likewise, studies with mice genetically deficient in 5-LO, FLAP, and the cysLT receptors illustrated the necessity of these genes in the development and progression of asthma [123].
The mis-regulation of eicosanoid metabolism has been also implicated in the etiology of CF, most notably, by the recent demonstration of reduction of lipoxin production in connection with CF [90]. Cystic fibrosis is a pulmonary disorder caused by loss of chloride conductance due to mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) chloride channel, leading to increased and more viscous mucous secretions in the lung. The full understanding of the role CFTR plays in the progression of the disease is yet to be fully appreciated; however, loss of chloride conductance in the pulmonary epithelia is a major component. The increased mucous viscosity decreases the clearance of common, normally benign pathogens such as *Pseudomonas aeruginosa*, and *Aspergillus fumigatus*, resulting in infection and chronic inflammation.

In the study presented here, the gene expression levels of many of the enzymes involved in eicosanoid metabolism are analyzed by quantitative real-time PCR, after *Af* extract sensitization and challenge in normal (C57BL/6) and CF (Cftr -/-) mice, to investigate the changes in gene expression of these enzymes in this model of *Aspergillus*-induced asthma.

**Results**

**Development of Th2 Inflammatory Response in a Mouse Model of Allergic Asthma**

Asthma is a chronic inflammatory disease of the lung. The pathophysiological characterization of the disease is reversible airway obstruction, increased mucous secretion as a result of goblet cell metaplasia, chronic pulmonary eosinophilia and bronchial smooth muscle cell hypertrophy. These symptoms eventually lead to loss of proper epithelial cell repair and ultimately remodeling of the airway culminating in airway hyper-responsiveness. Asthma is controlled by the secretion of IL-4, IL-5, IL-9,
IL-10, IL-13 and GM-CSF by Th2 cells [124,125], while mast cells and eosinophils exert effector cell functions [126-128]. In allergic asthma the released Th2 cytokines increase immune cell recruitment through increased cellular adhesion and chemokine release, as well as increased IgE levels and bronchial hyper-reactivity. In collaboration with Christian Muller and Dr. Terrence Flotte, we developed a model of allergic aspergillosis in normal and CF mice. In this model, mice are sensitized to *Aspergillus* with two intraperitoneal injection of 200 µg of crude *Aspergillus fumigatus* (*Af*) extract on days 1 and 14, on days 28-30 the *Af* and mock sensitized mice are challenged with vaporized *Af* extract for 30 min each day, and the lungs from the challenged mice are harvested 48 hours after the final challenge. The *Af* sensitized mice develop a Th2 mediated allergic inflammatory response typical of asthma, increases in total serum IgE, goblet cell hyperplasia and airway eosinophilia are observed in *Af* sensitized mice when compared to mock sensitized mice. More specific to the development of *Aspergillus*-induced asthma and ABPA, an increase in *Aspergillus* specific IgE and IgG are found in the serum of sensitized mice, demonstrating an *Aspergillus* specific immune response [122]. Additionally, the expression levels of several cytokines and chemokines of interest to asthma were studied. An increase in the mRNA expression levels of the cytokines IL-2, IL-6, IL-4, IL-10 and IL-13, as well as the chemokines Eotaxin and KC (aka IL-8), is observed in the mice sensitized to *Aspergillus* compared to the unsensitized control animals (Figure 4-1). Interestingly, there were several differences in the magnitude of induction of several of these genes in the Cftr -/- mice compared to C57BL/6 mice. The induction of IL-2, IL-10 and the chemokine IL-8/KC were greater, while the induction of eotaxin and IL-4 were lower, in the Cftr -/- mice. Additionally, the basal level of
expression of IL-8/KC was four fold higher in the Cftr -/- mice. The increase in cytokines IL-4 and IL-13 represent a typical Th2 mediated asthmatic response and the differences in expression of IL-4 and IL-8/KC is analogous to the exaggerated IL-8-like chemokine response seen in human CF patients infected with *Pseudomonas aeruginosa*, where IL-8/KC negatively affects the expression of IL-4.

The more common model of asthma in mice uses ovalbumin as an allergen rather than the *Aspergillus* extract utilized in this study. However, in developing our model of *Aspergillus* sensitive asthma we compared the *Aspergillus* extract with the more commonly used ovalbumin antigen. In this system mice were either mock sensitized or sensitized to ovalbumin (OVA) or *Af* on days 0 and 14 and then challenged with the relevant antigen. Adjuvant was included with the OVA sensitization, as an immune stimulant is necessary to activate an immune response toward ovalbumin. No adjuvant is required for the *Af* extract as there are natural immune activating epitopes due to the complex nature of the extract. The expression of the cytokines was significantly more pronounced in the *Aspergillus* sensitized and challenged animals compared to the ovalbumin, and this is particularly true of the induction of the immune relevant Th2 cytokines IL-4 and IL-13 (Figure 4-2). Therefore, the *Aspergillus* extract appears to be a stronger allergen and given the ubiquitous nature of *Aspergillus* fungi potentially a better and more natural stimuli for a model of allergic asthma.
Figure 4-1. Graphs depicting changes in the expression of cytokines and chemokines in Aspergillus-induced asthma. The results from real-time PCR analysis of the mRNA levels of the indicated cytokines and chemokines in the lungs of C57BL/6 and Cftr -/- mice. The mice were challenged in the airway with Aspergillus fumigatus extract and either mock sensitized (PBS) or sensitized to Aspergillus fumigatus extract (AF). Relative expression levels are determined by comparing each condition to the C57BL/6 mock sensitized mice. The standard deviations are included.
Figure 4-2. Graphs depicting changes in the expression of cytokines and chemokines in two models of allergic asthma. The results from real-time PCR analysis of mRNA levels of the indicated cytokines and chemokines in the lungs of C57BL/6 mice challenged in the airway with *Aspergillus fumigatus* or ovalbumin and either mock sensitized (PBS), sensitized to ovalbumin (OVA), or sensitized to *Aspergillus fumigatus* extract (Af). Relative expression levels are determined by comparing the expression in the OVA and Af sensitized mice to the mock sensitized mice. The number of animals in each sample group and the standard deviations are indicated.
Changes in the Gene Expression of Cytosolic Phospholipase A2s in a Mouse Model of Aspergillus-Induced Asthma

Given the important role eicosanoids play in the etiology of lung homeostasis, asthma and CF, I hypothesized that the development of allergic asthma in a mouse model would result in changes in gene expression of the enzymes involved in eicosanoid metabolism and, due to the increased prevalence and severity in CF patients, there may be important differences between the Cfr -/- and normal (C57BL/6) mice.

Figure 4-3. Graphs depicting changes in the expression of the cytosolic phospholipase A2s in two models of allergic asthma. The results from real-time PCR analysis of mRNA levels of the indicated phospholipases in the lungs of C57BL/6 mice challenged in the airway with Aspergillus fumigatus or ovalbumin and either mock sensitized (PBS), sensitized to ovalbumin (OVA), or sensitized to Aspergillus fumigatus extract (AF). Relative expression levels are determined by comparing the sensitized mice to the control mice. The number of animals in each sample group and the standard deviations are indicated.

Combining the clear role of cPLA2α in the development of asthma presented by Naonori Uzuomi et al., [41] and the knowledge of the transcriptional activation of cPLA2α mRNA expression in response to pro-inflammatory stimuli [73], I hypothesized...
that an increase in the expression of cPLA$_2$$\alpha$ would coincide with the development of asthma. However, contrary to this hypothesis, the expression of cPLA$_2$$\alpha$ does not increase (Figure 4-3 and 4-4). In addition to cPLA$_2$$\alpha$, the relative level of expression of the cPLA$_2$$\alpha$ homologues, cPLA$_2$$\beta$ and cPLA$_2$$\gamma$, were also analyzed. The expression of cPLA$_2$$\beta$ does not change in the sensitized animals; surprisingly however, the relative expression of cPLA$_2$$\gamma$ significantly increases in Af sensitized and challenged C57BL/6 mice (Figures 4-3 and 4-4).

![Graph](image)

**Figure 4-4.** Graphs depicting changes in the expression of cytosolic phospholipase A$_2$s in Aspergillus-induced asthma. The results of real-time PCR analysis of the mRNA levels, of the indicated cytosolic phospholipase A$_2$s, in the lungs of C57BL/6 and Cftr -/- mice. The mice were challenged in the airway with *Aspergillus fumigatus* extract and either mock sensitized (PBS) or sensitized to *Aspergillus fumigatus* extract (AF). Relative expression levels are determined by comparing each condition to the C57BL/6 mock sensitized mice. The number of animals in each sample group and the standard deviations are indicated.

Similar to the trend observed in the induction of the cytokines tested (Figure 4-1), the induction of cPLA$_2$$\gamma$ was greater in the Af sensitized mice compared to the ovalbumin sensitized C57BL/6 mice (Figure 4-3). In the C57BL/6 mice, cPLA$_2$$\gamma$ levels increased 26
fold compared to the unsensitized control mice. There was no significant difference in the basal expression level of cPLA\(_{2\gamma}\) the unsensitized Cftr -/- mice compared to the C57BL/6 mice. As in the C57BL/6 mice, cPLA\(_{2\gamma}\) mRNA levels increased in the Cftr -/- sensitized mice after allergen challenge. However, the expression was to a lesser extent (5 fold) than in the C57BL/6 mice (Figure 4-4).

**Determining the Differential Induction of cPLA\(_{2\gamma}\) in C57BL/6 and Cftr -/- Mice:**

Eicosanoid metabolism affects the etiology of CF as well as asthma, and *Aspergillus* sensitization is a compounding factor in CF patients. As stated above, CF is a pulmonary disorder caused by loss of chloride conductance due to mutations in the CFTR. Ultimately, this leads to a decrease in the clearance of common, normally benign pathogens, such as *Pseudomonas aeruginosa* and *Aspergillus fumigatus*, resulting in infection and chronic inflammation. As there was a significant difference in the expression of cPLA\(_{2\gamma}\) in the C57BL/6 and Cftr -/- mice, one potential reason may be a result of the loss of a functional CFTR on gene expression. However due to the complex genetics of these mice, there remains a degree of FVB in the genetic background of the Cftr -/- mice. In accordance with the difference being a result of strain variance, the induction of cPLA\(_{2\gamma}\) in the *Af* sensitized and challenged FVB mice is only 2 fold. Therefore, the induction in FVB mice is lower than in the Cftr -/- mice (Figure 4-5), and the lower expression in the Cftr -/- is likely a result of the remaining FVB background. Likewise, treatment of the Cftr -/- mice with AAV-CFTR, thus re-expressing a functional CFTR, had no significant effect on the expression of cPLA\(_{2\gamma}\) compared to untransduced or AAV-GFP transduced mice (Figure 4-5).
Figure 4-5. Graph depicting differences in the expression of cPLA₂γ in *Aspergillus* induced asthma. The results from real-time PCR analysis of cPLA₂γ mRNA levels in the lungs of C57BL/6, Cftr -/-, or FVB mice challenged with *Aspergillus fumigatus* extract and either mock sensitized (PBS) or sensitized to *Aspergillus fumigatus* extract (AF). Cftr -/- mice were also treated with adeno-associated virus containing either a functional Cftr or GFP. Relative expression levels are determined by comparing each condition to the mock sensitized C57BL/6 mice. The number of animals in each sample group and the standard deviations are indicated.

Eosinophils are one of the main immune effector cells migrating to the lung in human asthmatics and in animal models of asthma [85,86]. Eosinophils can act as antigen presenting cells [129,130], are known to uptake soluble antigen, and to phagocytose live *Aspergillus* conidia [131]. Additionally, several components of *Aspergillus* are Toll like receptor (TLR) ligands, and eosinophils are a known source of eicosanoids [132]. One of the significant differences observed in our previous publication was the difference in the eosinophilic infiltrate in the bronchoalveolar
lavages (BALs) of the sensitized mice. Where in the C57BL/6 mice eosinophils comprised 71.2% of the cells in the BAL, while eosinophils were only 52.5% of the BAL in the Cfr -/- mice [122]. To further illustrate the expression of cPLA2γ in its relation to asthma and address the difference in the induction of cPLA2γ in the mouse strains, the expression of cPLA2γ in the human eosinophilic cell line, EOL-1, was tested. Treatment of EOL-1 cells with increasing doses of crude Af extract for eight hours induces cPLA2γ mRNA expression up to 14 fold in a dose-dependent manner (Figure 4-6). The increase in cPLA2γ expression likely occurs via TLR signaling as LPS caused a similar induction in EOL-1 (data not shown). Further demonstrating the role of TLR signaling in the expression of cPLA2γ, treatment of bone marrow derived macrophages (BMDM) with LPS causes up to a 20 fold increase in cPLA2γ expression in a time dependent manner (Figure 4-6). This finding suggests eosinophils are likely one of the cells expressing cPLA2γ in the lung in Aspergillus-induced asthma. Although TLR signaling may be required in the induction of cPLA2γ, there is a requirement for pre-exposure to allergen as challenge with Aspergillus extract in unsensitized animals does not induce cPLA2γ expression. It is most likely that allergen sensitization primes the immune system to Aspergillus and upon allergen challenge, eosinophils are recruited to the lungs of the sensitized animals where they mediate their effector cell function, including the release of eicosanoids. As stated above, CF is a disease caused by mutations in CFTR expressed in the lung epithelia. IB3.1 cells are bronchial epithelial cells derived from a CF patient and contain the ΔF508 and W1282X mutations common to CF. S9 cells are control cells created by reintroducing a full length functional CFTR into the IB3.1 cells, and thus correcting the chloride conductance [133,134]. As shown in our previous publication and
Figure 4-6. Graphs showing increases in the expression of cPLA$_2$$\gamma$ mRNA in cultured immune cells. The results are from the real-time PCR analysis of cPLA$_2$$\gamma$ mRNA levels in (A) the human eosinophil cell line (EOL-1) with increasing concentrations of *Aspergillus* extract, or (B) the mouse bone marrow derived macrophages treated with LPS for increasing time. Relative expression levels are determined by comparing to the untreated control cells. The standard deviations in (A) are derived from three individual replicates; (B) is a representative experiment.
Figure 4-1, increased levels of IL-4, IL-5 and IL-13 are found in the lungs of the sensitized and challenged mice. While none of these stimuli induce cPLA$_2$$\gamma$ expression (data not shown), treatment of the IB3.1 and S9 bronchial epithelial cells with TNF$\alpha$ for 8 or 24 hours significantly induces cPLA$_2$$\gamma$ mRNA levels. Although initially TNF$\alpha$ levels were not addressed in our study, the release of pre-made TNF$\alpha$ by mast cells in asthma is documented and increased TNF$\alpha$ levels have been observed in asthmatic lungs [135-142]. In agreement with this observation, TNF$\alpha$ mRNA was detectable in the lungs and there was a minor increase in expression (2 fold) in the C57BL/6 and Cftr -/- sensitized animals respectively. This is indicative of prepackaged TNF$\alpha$ in the lungs of these animals and a ramping of production in the sensitized animals after antigen challenge to replenish the stores. Additionally, Lindbom et al. [143] previously demonstrated an increase in cPLA$_2$$\gamma$ mRNA by TNF$\alpha$ in lung epithelial cells.

The expression of cPLA$_2$$\gamma$ in S9 cells increased 18 fold, while in the IB3.1 cells the expression increased 32 fold after 24 hours of TNF$\alpha$ treatment. As the induction of cPLA$_2$$\gamma$ by TNF$\alpha$ in IB3.1 cells exceeded the level of induced expression in the S9 cells after prolonged exposure, this further indicated that the difference in induction of cPLA$_2$$\gamma$ in the mouse models does not appear to be an effect of loss of functional CFTR, but more likely a result of the mixed genetic background of the Cftr -/- mice and/or differences in the eosinophilic infiltration.

**Transcriptional Activation of the cPLA$_2$$\gamma$ Promoter**

To further test the underlying mechanism controlling transcriptional activation of cPLA$_2$$\gamma$ expression, the human promoter was cloned into a human growth hormone reporter plasmid [98]. Three constructs were created: the first starting at -3.8 kb, the second at -1.2 kb, and the third at position -588 bp relative to the transcriptional initiation
site. Each fragment also contains an additional 150 bp 3' of the transcriptional start site (Figure 4-8). To evaluate the functionality of these promoters, each construct was transiently transfected into the IB3.1 and S9 cells. The following day the cells were split one to two, re-plated and incubated overnight. On the third day, one plate was treated for 24 hours with TNFα, and the other was left untreated.

Figure 4-7. A graph depicting the results from real-time PCR analysis of mRNA levels of cPLA$_2γ$ in the human bronchial epithelial cell lines S9 and IB3.1. The bronchial epithelial cells were treated with or without TNFα for 24 hours. IB3.1 cells were cultured from a CF patient with ΔF508 and W1282X CFTR mutations. The S9 cells are control cells derived from IB3.1 cells but expressing full length functional CFTR. Relative expression levels are determined by comparing each condition to the untreated S9 control cells. The data is the average from three individual replicates and the standard deviations of the experiment are shown.
Following incubation with TNFα, total cellular RNA was extracted and reporter gene expression was evaluated by northern analysis for expression of human growth hormone. Treatment with TNFα induces expression through the cPLA2γ promoter (Figure 4-8), illustrating the functionality of the cPLA2γ promoter in response to TNFα. Current studies in the laboratory are targeting the identification of the TNFα specific regulatory elements.
Analysis of Relative Gene Expression Levels of Secretory Phospholipase A2s in a Mouse Model of Allergic Asthma

We also evaluated the expression levels of several of the sPLA2 enzymes in our mouse model of asthma. One of the most interesting observations of the sPLA2s is the differences between the C57BL/6 and Cftr -/- mice. The basal level of expression in Cftr -/- mice of sPLA2-IIC is 59% as much as the basal level in C57BL/6, while the basal expression of sPLA2-IIE is only 2% as much as the expression observed in C57BL/6. Although sPLA2-IIE expression levels didn’t change in response to sensitization, the difference in basal expression between the two mouse strains was one of the most striking in the enzymes studied herein, and thus could be of interest in further studies of

![Graph](image_url)

Figure 4-9. Graphs depicting changes in the expression of the secretory phospholipase A2s in Aspergillus-induced asthma. The results of real-time PCR analysis of the mRNA levels, of the indicated secretory phospholipase A2s, in the lungs of C57BL/6 and Cftr -/- mice. The mice were challenged in the airway with Aspergillus fumigatus extract and either mock sensitized (PBS) or sensitized to Aspergillus fumigatus extract (AF). Relative expression levels are determined by comparing each condition to the C57BL/6 mock sensitized mice. The number of animals in each sample group and the standard deviations are indicated.
eicosanoid metabolism in CF. Additionally, there is an increase in sPLA$_2$-V and -IID in the Af sensitized Cfr -/- mice after allergen challenge (Figure 4-9). The differences in the increased expression of sPLA$_2$-V is of importance to future studies of Aspergillosis and Aspergillus complicating diseases in CF, as it is one of the few genes observed with differential expression between mouse strains, and solely induced in the Cfr -/- mice.

**Gene Expression Analysis of Enzymes Involved in Prostanoid Synthesis**

Arachidonic acid released by phospholipases is metabolized by cyclooxygenases and specific downstream synthases into the prostaglandins, prostacyclin and thromboxane. Similar to the data presented on the phospholipases in our model of asthma, the relative level of expression of several of the enzymes required for the metabolism of the

![Graph](image_url)

**Figure 4-10.** Graphs depicting changes in the expression of the prostanoid synthesizing enzymes in *Aspergillus*-induced asthma. The results of real-time PCR analysis of the mRNA levels, of the indicated prostanoid synthesizing enzymes, in the lungs of C57BL/6 and Cfr -/- mice. The mice were challenged in the airway with *Aspergillus fumigatus* extract and either mock sensitized (PBS) or sensitized to *Aspergillus fumigatus* extract (AF). Relative expression levels are determined by comparing each condition to the C57BL/6 mock sensitized mice. The standard deviations are indicated.
prostanoids were analyzed (Figure 4-10). Interestingly, the expression of 
Cycloxygenase-2 (COX-2) was modestly induced (2 fold) in the sensitized Cftr -/- mice, 
but not in the C57BL/6 mice. There also is a modest increase in microsomal PGE₂ 
synthase expression in the C57BL/6 mice, and it appears to increase in the Cftr -/- mice 
as well, however, due to the large variability of expression in the unsensitized Cftr -/- 
mice it is not possible to say with certainty. Additionally, the expression level of the 
Lipocalin-PGD synthase in the sensitized mice was reduced to 36% and 43% in the 
C57BL/6 and Cftr -/- mice respectively, compared to the expression level in the 
unsensitized C57BL/6 mice.

Changes in the Gene Expression of Lipoxygenase Enzymes in Aspergillus Sensitized 
and Challenged Animals

The peroxidation of AA by the family of enzymes referred to as the lipoxygenases, 
leads to the production of various hydroperoxyeicosatetronic acids. Depending on the 
lipoxygenases present in the cell at the time of AA release, different biologically active 
hydroperoxyeicosatetronic acids (HPETEs) are produced. The HPETEs naturally 
breakdown to hydroeicosatetronic (HETEs) and these products also have biological 
activity. 5-LO requires the cofactor 5-LO Activating Protein (FLAP), which is thought to 
aid in the delivery of AA to 5-LO. The 5-Lipoxygenase (5-LO) metabolite 5-HPETE can 
be further metabolized into the cysteinyl leukotrienes (cysLTs) and LTB₄, both of which 
are important regulators in the lung and in asthma.

The preponderance of data on eicosanoids, with respect to asthma, links these 5-
LO/FLAP metabolites with the disease. Therefore, I hypothesized that there would be an 
increase in the expression of these genes in the sensitized and challenged animals.
Figure 4-11. Graphs depicting changes in the expression of the lipoxygenases in 
*Aspergillus*-induced asthma. The results of real-time PCR analysis of the 
mRNA levels, of the indicated lipoxygenases, in the lungs of C57BL/6 and 
Cftr -/- mice. The mice were challenged in the airway with *Aspergillus 
fumigatus* extract and either mock sensitized (PBS) or sensitized to 
*Aspergillus fumigatus* extract (AF). Relative expression levels are determined 
by comparing each condition to the C57BL/6 mock sensitized mice. The 
standard deviations are indicated.

However, as shown in Figure 4-11 there is no increase in the expression of 5-LO nor 
FLAP. Interestingly, there is an increase in the expression of several other lipoxygenases 
in both sensitized animal groups.

There is an increase in mRNA levels of 15-LO-1 and 15-LO-2. These enzymes 
similarly produce 15-HETEs. In the mouse, 15-LO-1 is also referred to as 12/15-LO and 
leukocyte derived 12-LO, and also produces 12–HETE. 15-LO-1 is the murine 
homologue of human 15-LO-1. However, mice also express a second highly homologous 
enzyme, epidermally derived 12-LO. The expression of epidermally derived 12-LO 
increases in sensitized animals after allergen challenge (Figure 4-11). Also of note, the 
basal level of expression in the Cftr -/- mice is significantly higher compared to the 
C57BL/6 mice, and although this expression is to the same extent as the induced
expression in the C57BL6/J mice, the expression still increased in the sensitized Cfrt -/- after allergen challenge, demonstrating that the induction of epidermally derived 12-LO is a specific response to allergen challenge. In retrospect however, an increase in 15-LO expression is not unlikely. Elevated 15-HETE levels have been shown in human asthmatics and in models of asthma [144-146]. Also, increased expression of 15-LO in human lung epithelia and alveolar macrophages of asthmatics has been shown in response to IL-4 and IL-13 [147-150]. The increase in 15-LO-2 is noteworthy as well, in that it catalyzes the peroxidation of fatty acids producing 15-H(p)ETEs similarly to 15-LO-1. Increased expression of 15-LO-2 has been observed previously in lung macrophages following hypoxia and in malignant lung epithelia [151].

![Graph](image)

Figure 4-12. Graphs depicting changes in the expression of the 15-LO-1 and epidermally derived 12-LO in *Aspergillus*-induced asthma. The results of real-time PCR analysis of the mRNA levels, of the indicated lipoxygenases, in the lungs of C57BL/6 and Cfrt -/- mice. The mice were challenged in the airway with *Aspergillus fumigatus* extract in either mock sensitized (PBS) or *Aspergillus fumigatus* extract (AF) sensitized. Relative expression levels are determined by comparing each condition to the C57BL/6 mock sensitized mice. The standard deviations are indicated.
Immunohistochemical Analysis of 15-LO Protein in Lungs of C57BL/6 and Cftr -/- Mice

Immunohistochemical analysis of the Af-challenged mice demonstrated an increase in 15-LO staining (Figure 4-13). The alveolar macrophages of the control C57BL/6 and Cftr -/- mice positively stain for 15-LO, and there is an increase in staining in the sensitized mice following antigen challenge. This increase in staining in alveolar macrophages suggests that the increase in 15-LO mRNA is at least in part a consequence of increased transcription, as has been previously reported in mouse alveolar macrophages and human lung epithelial cells in response to IL-4 [152-154]. There is diffuse staining in the lung of both sensitized and unsensitized animals. However, there was no increase in the staining for 15-LO-1 after allergen challenge. This is noteworthy as, although there is an increase in IL-4 in the lungs of the challenged mice, increased expression has been reported in the human lung epithelial cell lines in the response to IL-4. One explanation of this observation and apparent disparity with what is known in human cells is a difference in species and the expression of 12- and 15-LOs. Mice express both 15-LO-1 and -2 and the highly conserved epidermally derived 12-LO, while humans do not possess a functional epidermally derived 12-LO homologue. Therefore, it is possible in the mouse, the expression of the individual 12-LO and 15-LOs is cell specific, while in the human the role of both enzymes is fulfilled by 15-LO-1. There is a large eosinophil predominated immune infiltration in the lungs of Af-sensitized animals after allergen challenge. As demonstrated in Figure 4-13 a portion of the infiltration stains positive for 15-LO-1. Thus, a second component of the increase in 15-LO-1 mRNA levels is from the infiltrating 15-LO-1 expressing immune cells.
Figure 4-13. Immunolocalization of 15-LO-1 in the lungs of C57BL/6 and Cfr -/- mice. Immunohistochemistry of 15-LO-1 protein expression in the mouse lung of mock sensitized (PBS) and Aspergillus sensitized (Af) C57BL/6 and Cfr -/- mice stained with anti-15-LO antibody. Red arrows point to 15-LO positive alveolar macrophages in sensitized and unsensitized mice. Black arrows point to the 15-LO-1 positive immune infiltrate present in the Af sensitized mice. The inset in the Cfr -/- Af sensitized mice is a higher magnification of 15-LO-1 positive alveolar macrophages.

Discussion

Eicosanoids are important regulators of the inflammatory response associated with allergic asthma. As demonstrated in the data presented above, we have developed a mouse model of allergic asthma and investigated changes in the relative expression of several of the genes involved in the production of eicosanoids. In this model, mice were sensitized to the ubiquitous fungus Aspergillus fumigatus, which causes allergic disease in humans, particularly in CF patients. Therefore Cfr -/- mice were also utilized to
investigate whether there were differences in the expression of these genes in Af sensitized CF mice.

A decrease in the expression of lipocalin type prostaglandin D synthase (L-PGDS) was observed. The product of L-PGDS, prostaglandin D\(_2\) (PGD\(_2\)), is known to increase vascular permeability, leading to increased airway inflammation and to cause broncho-constriction [155]. Therefore, the decrease in expression of this enzyme may lead toward resolution of inflammation as decreasing the expression of L-PGDS should lead to a decrease in PGD\(_2\) levels.

There were differences observed between the mouse strains in this study, particularly, in the expression of the different sPLA\(_2s\). Comparatively, there was little expression of sPLA\(_2\)-IIE expression in the Cfrt -/- mouse in relation to the C57BL/6 mice. The expressions of sPLA\(_2\)-IID and sPLA\(_2\)-V were increased in the Af sensitized Cfrt -/- mice, but not in the C57BL/6 mice after allergen challenge. Although not addressed here, further studies are required to determine if these differences are disease specific or result from strain differences. There was also a marked difference in the basal expression and the level of induction of the epidermally derived 12-LO. However, as there is no expressed human homolog of this gene determining how this enzyme, or its homologs, affect human disease will not be possible using mouse studies.

The enzymes cPLA\(_2\)\(_{\alpha}\), 5-LO and FLAP are required for the production the cysLTs, which are potent mediators of asthma. Therefore, I hypothesized an increase in the expression of these genes would occur with the development of asthma. Surprisingly, contrary to my hypothesis, the expression of these genes did not change. However, there was an increase in the expression of cPLA\(_2\)\(_{\gamma}\), a homolog of cPLA\(_2\)\(_{\alpha}\). This is the first
observation of an increase in the expression of cPLA$_2$$\gamma$ in a disease, or a model of disease. In contrast to the hypothesized 5-LO, there were increases in the expression of several other lipoxygenases: 15-LO-1, 15-LO-2 and epidermally derived 12-LO.

Interestingly, the induction of cPLA$_2$$\gamma$ expression was muted in the Cftr -/- mice. This observation is provocative as the production of lipoxin in CF is reduced, and this decrease is thought to be a causative factor in the enhanced inflammation observed in the lungs of CF patients [21]. The decrease in cPLA$_2$$\gamma$ levels could explain the abatement in lipoxin generation. However, replacement of a functional CFTR by AAV did not rescue the difference in expression, and the expression of cPLA$_2$$\gamma$ in bronchial epithelial cells from a CF patient was not lower than control cells. As described, the genetic background of the Cftr -/- mice is complex, and a component of FVB mice, as a result of the crosses used to create the transgenic mice, is still present in Cftr -/- mouse background. The induction of cPLA$_2$$\gamma$ in the FVB mice was lower than in the Cftr -/- mice. Therefore, the differences in expression of cPLA$_2$$\gamma$ are likely due to differences in the mouse strains, and are not likely important to the etiology of CF. However, the study of the role of cPLA$_2$$\gamma$ in asthma remains important.

An important component missing from the current study is the knowledge of the cells expressing cPLA$_2$$\gamma$ in the lung during the development of asthma. Unfortunately, attempts to localize the expression of cPLA$_2$$\gamma$ in the lung by immunohistochemistry and in situ hybridization were unsuccessful. However, eosinophils are a key immune effector cells infiltrating the lung in asthma, and the expression of cPLA$_2$$\gamma$ is inducible in human eosinophil cells after treatment with the *Aspergillus* extract. Also the expression of cPLA$_2$$\gamma$ is inducible in human bronchial epithelial cells by TNF$\alpha$, which is released in the
lung by mast cells in an asthmatic response. Therefore, the increase in cPLA$_2$$\gamma$
expression occurring in these two cell types in the development of asthma is plausible.

The roles of 15-LO-1 and its metabolites in asthma are not fully understood. In fact, previous studies have led to conflicting results, and both pro-inflammatory and anti-inflammatory effects by 15-HETE have been demonstrated. The pro-inflammatory effects important to asthma caused by 15-HETE are broncho-constriction, vascular permeability, and immune cell chemotaxis. However, 15-HETE can also act to decrease inflammation, and a decrease in 15-HETE production by human alveolar macrophages has been observed in severe asthmatics [144]. Additionally, aerosolization of 15-HETE into the lung of asthmatics reduces airway hyper-responsiveness [156]. The related 15-LO metabolites, lipoxin and stable lipoxin analogs have anti-inflammatory effects and both severe asthmatics and CF patients have defects in the synthesis of these lipids. At this time, it is not known whether the effects of aerosolization of 15-HETE into the lung is a direct result of 15-HETE or, if the aerosolized 15-HETE is converted to lipoxin, and the anti-inflammatory effects observed are a result of increased concentrations of lipoxin in the lung.

Theoretically the increase in the expression of cPLA$_2$$\gamma$ and 15-LO-1 should lead to an increase in the production of 15-HETE and lipoxin. However, the relationship between cPLA$_2$$\gamma$, 15-LO-1, and an increase in production of the 15-LO metabolites with the development of asthma remains to be elucidated. By combining what is known from the literature and the results presented here, it is possible to speculate interactions of these two enzymes in asthma. Figure 4-14 is a schematic of a model I developed demonstrating the role of 15-LO-1 in the acute and late phases of asthma. Initially AA is
released by cPLA2α and other phosphlipase A2s present in the lung. The increased expression of cPLA2γ, as a result of the allergen challenge in the asthmatic lung, acts to supplement the activity of cPLA2α. This increase in cPLA2γ expression causes an increased and prolonged release of AA, ultimately leading to an increase in 15-HETE or cysLT production depending on cell type and lipoxygenase expression. Initially, the increase in 15-HETE and cysLTs production promotes an increased inflammatory response, which leads to the development of asthma.

As the disease progresses there is an increase in immune infiltration, eicosanoid production, and expression of 15-LO-1. As the increase in 15-LO-1 expression equilibrates with the expression of 5-LO, and the infiltrating immune cells become juxtaposed with the resident lung cells there is an increase in lipoxin biosynthesis by transcellular processes. Simultaneous to the increase in lipoxin biosynthesis is the
reduction in 15-HETE and cysLT production, as 15-HETE and 5-HETE are used to
synthesize lipoxin. Therefore, the increased expression of cPLA$_{2\gamma}$ and 15-LO-1 initially
have pro-inflammatory effects; however, at the later stages of asthma, the increased
expression of these enzymes likely aid in the resolution of asthma. Whether cPLA$_{2\gamma}$ and
15-LO-1 have a pro-inflammatory, anti-inflammatory, or a combinatory effect on
inflammation in the asthmatic lung remains to be determined and will be important for
future studies.
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

Regulation of cPLA$_2$$\alpha$ Gene Expression by the Pro-inflammatory Cytokine IL-1$\beta$

Inflammation is a key component of the body’s natural response to infection and injury. Left unchecked the inflammatory response can lead to inflammatory diseases, such as, inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis and asthma. The eicosanoids mediate many aspects of inflammation, and understanding the regulation of the enzymes involved in their production is important to gaining a full understanding of the role of these enzymes in the inflammatory response. In Chapter 3, the regulation of cPLA$_2$$\alpha$ expression in response to the pro-inflammatory cytokine IL-1$\beta$ is demonstrated. The cPLA$_2$$\alpha$ inhibitor AACOCF$_3$, as well as the 15-LO inhibitors NDGA, Luteolin and PD146178 blocked the activation of cPLA$_2$$\alpha$ expression in response to IL-1$\beta$. This indicates the activation of cPLA$_2$$\alpha$ gene expression occurs by a feed forward mechanism where the induction of cPLA$_2$$\alpha$ expression requires the activity of cPLA$_2$$\alpha$ itself. Also required is the downstream metabolism of AA by a 15-LO. Surprisingly, the addition of exogenous 15-HETE did not induce the expression of cPLA$_2$$\alpha$, nor did it rescue the induction of cPLA$_2$$\alpha$ expression in the presence of lipoxygenase inhibitors (data not shown).

The p38 MAP kinase inhibitor, SB203580, but not inhibitors of either ERK or JNK kinases, inhibited induction of cPLA$_2$$\alpha$ expression by IL-1$\beta$, demonstrating p38 MAPK signaling, but not that of ERK or JNK kinases, was required for induction of cPLA$_2$$\alpha$ expression. As previously stated, cPLA$_2$$\alpha$ is activated in part through phosphorylation by
MAP kinases at ser505. Initially, it was believed the inhibition of cPLA$_2$$\alpha$ expression by SB203580 was caused by inhibiting the phosphorylation, and thus activation of cPLA$_2$$\alpha$. However, SB203580 did not inhibit the phosphorylation of cPLA$_2$$\alpha$. Therefore, the regulation of cPLA$_2$$\alpha$ expression is independent of effects on cPLA$_2$$\alpha$ activity. The downstream targets of p38 MAPK have yet to be determined and identifying these intermediates may provide important information on the regulation of cPLA$_2$$\alpha$ expression.

Utilizing inhibitors of the signaling upstream of the transcription factor NF-κB established a role for this important transcription factor in the regulation of cPLA$_2$$\alpha$ expression. Additionally curcumin, which has been shown to inhibit NF-κB activation \textit{in vivo}, also inhibited the induction of cPLA$_2$$\alpha$ by IL-1β. However, in addition to inhibiting NF-κB, curcumin also inhibits acetyltransferases. Therefore, the role of histone acetylation was further investigated. Trichostatin A is an inhibitor of histone acetyltransferases and leads to global histone acetylation. This inhibitor was sufficient to activate cPLA$_2$$\alpha$ gene expression. Thus, increases in histone acetylation, leading to changes in chromatin structure likely are involved in cPLA$_2$$\alpha$ transcription. Along these lines, a hypersensitive site is present between -8 and -9 kb in the upstream region of the cPLA$_2$$\alpha$ promoter, and transfection of the -14 kb promoter in an hGH reporter system into HFL-1 cells was activated in response to IL-1β. Therefore, the IL-1β mediated increase in cPLA$_2$$\alpha$ expression requires feed forward signaling by cPLA$_2$$\alpha$ itself and downstream metabolism by 15-LO. The induction of cPLA$_2$$\alpha$ expression also requires NF-κB and histone acetylation and there is an element in the distal upstream region of the cPLA$_2$$\alpha$ promoter responsive to IL-1β.
**Future Directions in cPLA$_2$$\alpha$ Gene Regulation**

With the recent identification of a cis-acting element in the distal regions of the cPLA$_2$$\alpha$ promoter, presented in Chapter 3, a finer delineation of the region containing this element is one of the first steps to be taken in future investigations. To do so, deletion analysis of the -14 kb promoter in the reporter plasmid construct will allow initial indications of the location of the regulatory regions. As stated in Chapter 3, deleting the distal 7 kb has already been tested, and this deletion appears to alleviate the expression of reporter gene expression. Further deletion studies of the region from -14 kb to -6.8 kb will help to further establish the region(s) containing the cis element(s). Deletions can be created in multiple ways, including restriction digest, PCR amplification and re-cloning of smaller fragments, as well as, PCR based mutagenesis.

In addition to deletion analysis of the promoter, a higher resolution DNase I hypersensitivity analysis can provide more data on the location of the cis-acting element in the distal regions 5’ to the cPLA$_2$$\alpha$ promoter. Due to the length of the restriction fragment used in the DNase I analysis in Chapter 3, it is hard to estimate the location of the hypersensitive site with accuracy using a standard agarose gel. Therefore, higher resolution DNase I hypersensitivity analysis of the distal regions will allow more accurate assessment of the location of the hypersensitivity site, and depending on the degree of resolution, potentially the location of binding sites within the hypersensitivity site.

Garnering information from the deletion analysis of the promoter it will be possible to choose appropriate restriction sites and probes to allow this higher resolution analysis. Also, once the region containing the cis-element is determined to <1 kb, genomic footprinting can be utilized to determine binding sites at single nucleotide resolution.
The signal transduction results presented in Chapter 3 provide preliminary data on the transcription factors mediating the regulation of cPLA₂α gene expression. NF-κB and acetylated histones have been linked to the regulation of cPLA₂α. However, these results are not able to determine if this affect is by direct binding to the promoter or by indirect means. To investigate whether NF-κB and acetylated histones are in the regulatory regions of cPLA₂α, chromatin immunoprecipitation (ChIP) analysis can be utilized. ChIP analysis can also help further determine the role of these post-translation modifications in the cPLA₂α regulatory regions. To further correlate the inhibitor studies provided in Chapter 3, use of those pharmacological agents in combination with ChIP can demonstrate the effects of these inhibitors on the binding of transcription factors in the regulatory regions of the cPLA₂α gene.

**Alterations in the Expression of Genes Involved in Eicosanoid Metabolism in a Mouse Model of Allergic Asthma**

The data presented in Chapter 4 demonstrates that changes in the expression of several of the genes are involved in eicosanoid metabolism in our model of allergic asthma. Most significantly an increase in the expression of cPLA₂γ and 15-LO-1 were observed in this model. Initially there appeared to be a significant difference in the expression of cPLA₂γ in the C57BL/6 and Cfrt -/- mice. However, several lines of evidence demonstrate this difference is due to the convoluted genetic background of the Cfrt -/- strain and not a disease specific phenomenon. Additionally, the induction of cPLA₂γ expression was observed in human eosinophils and bronchial epithelial cells in response to *Aspergillus* extract and TNFα respectively. Furthermore, the induction of cPLA₂γ is mediated through the proximal promoter, and this region has been isolated to the first 588 bp using a human growth hormone reporter plasmid.
Similar to the increase in cPLA$_{2\gamma}$ expression in our mouse model there was an increase in cPLA$_{2\gamma}$ expression extract in human eosinophils in response to *Aspergillus* and in bronchial epithelial cells by TNF$\alpha$. The expression of 15-LO-1 was observed in alveolar macrophages and a portion of the immune infiltrate by immunohistochemistry in the lungs of the C57BL/6 and Cftr$-/-$ mice.

**Future Directions Investigating cPLA$_{2\gamma}$ and 15-LO-1 in Allergic Asthma**

We identified several genes whose expression changes with the development of allergic asthma. Several of the genes identified were not studied further in the study presented here, and follow up investigations of these genes is obviously important to fully understanding the role of these enzymes in allergic asthma. However, the changes in expression of cPLA$_{2\gamma}$ and 15-LO-1 remain the most intriguing. Although in the data provided above the increase in expression of cPLA$_{2\gamma}$ and 15-LO-1 were investigated more deeply, the data is observatory and does not provide insight into what roles these enzymes play in the etiology of the disease. To investigate these aspects manipulations of cPLA$_{2\gamma}$ and 15-LO-1 *in vivo* will provide the most meaningful data on their effects in asthma. There are several means that can be employed, including traditional gene ablation and RNA interference techniques.

A 15-LO-1$-/-$ mouse is currently available from the Jackson Laboratory that can be used to further investigate the pathophysiological implications of this gene in asthma. There are several layers of complexity to the role of these genes in allergic asthma, including how they are expressed in the resident lung cells and the immune cells. Utilizing the gene ablated 15-LO-1$-/-$ mice in several different assays the roles of infiltrating immune cells and resident lung cells can be determined. Studies comparing the 15-LO-1$-/-$ mice with control mice will provide initial information on the role of 15-
LO-1 in asthma, and how pharmaceutical agents targeting this enzyme may effect asthma. However, more interesting is separately determining the role of 15-LO-1 expressed in the lung and immune cells plays in the development and resolution of inflammation central to asthma. By using adoptive transfer techniques, transfer of wild type bone marrow into irradiated 15-LO-1 -/- mice will result in a mouse with ablated 15-LO-1 in the resident lung cells, while retaining wild type immune cells. Conversely, adoptive transfer of bone marrow from 15-LO-1 -/- mice into irradiated wild type mice will create mice with wild type lungs and 15-LO-1 -/- immune cells. Comparing the pathological and physiological aspects of asthma in these mice with wild type and the 15-LO-1 -/- mice will allow the evaluation of 15-LO-1 in asthma in general, as well in the resident lung cells and the immune cells specifically. An understanding of the roles of 15-LO-1 in these different cell types is important as the changes in expression of 15-LO-1 may have very different functions in these cell types and loss of 15-LO-1 in these different cells may have varying results due to the effects of different metabolites of 15-LO-1 can have.

As discussed in Chapter 1, 15-HETE is a pro-inflammatory mediator, while lipoxin, created by both 5- and 15-LO together, have anti-inflammatory effects and help with the resolution of inflammation. This is significant as loss of 15-LO-1 in these different scenarios could have varying effects, including: a decrease in inflammation due to the loss of 15-HETE production, or an increase in inflammation and worsening of disease due to loss of lipoxin biosynthesis. One possible model is, 15-LO-1 expressed in the resident lung cells produces 15-HETE, which acts as a chemoattractant recruiting immune cells, leading to the immune infiltration in the lungs of asthmatics. As the infiltrating immune cells expressing 5-LO are recruited to the airway, the 15-HETEs
produced by in the lung is converted to lipoxin by transcellular processes. Conversely, the 5-HETE produced by the infiltrating immune cells is also converted to lipoxin by the 15-LO-1 expressed in the lung. In this manner these cells involved work comparatively to self limit the immune response. Taking into account the model presented in Chapter 4, the initial experiments comparing the 15-LO-1 -/- mice with wild type mice, a reduction in the inflammation and disease progression is anticipated. Likewise, adoptive transfer of wild type immune cells into irradiated 15-LO-1 -/- mice will also have a muted inflammatory response, as there will be less recruitment of immune cells due to lack of 15-LO-1 in the lung at the time of challenge. The converse experiment, with transfer of 15-LO-1 -/- immune cells into the wild type animals is harder to speculate on. In one instance there may be no effect if 15-LO-1 is not necessary for immune infiltration and function. However, as shown in Chapter 4 there was strong expression of 15-LO-1 in the immune infiltrate, therefore, it is likely that there will be an effect of loss of 15-LO-1 in the immune cells, but this effect is more difficult to predict without more knowledge on the timing of the expression of 15-LO-1 in these cells, as well as whether or not they express 5-LO. If 15-LO-1 is constitutively expressed in these cells and they do not express 5-LO, then it can be surmised that there would be a beneficial effect by the loss of 15-LO. However, if the expression of 15-LO-1 in these cells is induced upon entering the lung parenchyma, and they do not express 5-LO, then their loss of 15-LO-1 expression will reduce lipoxin synthesis.

The experiments outlines above will also help further the understanding of 5-LO and the effectiveness of anti-leukotriene pharmaceuticals in asthma. As previously stated, although drugs targeted at the 5-LO pathway have some effect on asthma, they are
not as effective as originally predicted. One reason for the partial effectiveness of these
drugs could be the disruption of the normal physiological resolution mechanisms.
Although, the products of 5-LO, the cysLTs, are potent pro-inflammatory mediators and
upon direct exposure in the lung cause symptomatic effects, as with 15-LO-1, lipoxin
biosynthesis requires 5-LO. By inhibiting only one of the lipoxygenase pathways, the
natural resolution mechanisms are disrupted, but the production of the other lipoxygenase
retains its pro-inflammatory effects. Therefore, the beneficial effects of inhibiting cysLT
production may be partially offset by the loss of lipoxin synthesis. In this manner, the
studies described above could help unravel this conundrum. Additionally, similar studies
with double knockouts (5-LO -/- and 15-LO-1 -/-) mice and/or combinations of these
mice in adoptive transfer experiments will help determine how these enzymes interact
with each other to control the inflammatory response and the resolution of inflammation
in asthma.

Another manner of reducing expression of specific genes is by RNA interference
(RNAi). Due to the nature of the lung there are several methods that can be utilized for
RNAi. The simplest manner is by direct administration of short interfering RNAs
(siRNAs) by intra-tracheal instillation. This is simple because gene specific siRNAs are
commercially available from several sources, and by injecting solutions containing these
siRNAs into the trachea is sufficient to get significant knockdown of mRNA and proteins
in the lung for several days [157,158]. One limitation of this system is the longevity of
knockdown of expression, due to the life span of siRNAs. If extended knockdown is
required to see an effect, viral delivery of short hairpin RNAs (shRNA) can also be
utilized.
Currently there is no antibody raised against the murine cPLA\(_2\gamma\) available, and the one available antibody targeting the human protein did not stain above background in the mouse lung. Additionally, attempts to localize the message by \textit{in situ} hybridization also were not able to provide staining above background. Therefore, raising an antibody against the murine cPLA\(_2\gamma\) would allow the immunolocalization of cPLA\(_2\gamma\) in the lung.

Preliminary analysis of the cPLA\(_2\gamma\) promoter has been shown in Chapter 4 and additional analysis of the proximal promoter is currently underway in our lab by another graduate student Justin Bickford. Similar to the analysis of the cPLA\(_2\alpha\) promoter, ChIP analysis, yeast one hybrid screening and genomic footprinting analysis can be employed to determine the transcription factors binding sites mediating the induction of cPLA\(_2\gamma\) expression.

As there is no cPLA\(_2\gamma\) gene ablation mouse available using these RNAi techniques the expression of cPLA\(_2\gamma\) can be reduced. However, the intra-tracheal instillation of the siRNA or shRNA will only reduce expression in the resident lung cells. Reducing expression of genes in immune cells \textit{in vivo} by RNAi is more difficult, and the production of a cPLA\(_2\gamma\) \textendash/- mouse would be more efficient then the complex combinations of genetic, molecular, and cellular manipulation experiments necessary to achieve mice without expression of cPLA\(_2\gamma\) by RNAi. Comparing the effects of knockdown of 15-LO-1 to the pathophysiological characteristics observed in the 15-LO-1 \textendash/- mice, it would be expected that knockdown of 15-LO-1 in the lung by RNAi will mimic the results of the mice where wild type immune cells have been reintroduced into the 15-LO-1 \textendash/- mice. However, one caveat is, if there is difference in the effects on the infiltration or lung function in the RNAi in the lung versus the 15-LO-1 \textendash/- mice with
wild type immune cells this could be a result of knockdown in the immune cells already present in the lung by RNAi.

The irradiation of the mice in adoptive transfer studies ablates the bone marrow and dividing cells. However, it is unclear if the immune cells already present in tissues, such as the lung, are replaced with the new cells. The direct administration of RNAi to the lung will knockdown expression in these immune cells along with the other resident lung cells. Therefore, if there is a difference in the effects of RNAi compared to the knockout mice with wild type adoptive transfer then it is likely this is due to experimental differences which should be taken into account when interpreting results of RNAi targeted at cPLA2γ.
### APPENDIX

**LIST OF PRIMERS USED IN REAL-TIME PCR ANALYSIS**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>cPLA$_{2\alpha}$ Forward</td>
<td>CAG CCA CAA CCC TCT CTT ACT TC</td>
</tr>
<tr>
<td>cPLA$_{2\alpha}$ Reverse</td>
<td>CGG CAT TGA CCT TTT CCT TC</td>
</tr>
<tr>
<td>COX-2 Forward</td>
<td>GGC TTC GGG AGC ACA ACA</td>
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<td>CAA TGC GGT TCT GAT ACT GGA</td>
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<td>Cyclophillin A Forward</td>
<td>GCG GCA GGT CCA TCT ACG</td>
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<td>IL-17E Forward</td>
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<tr>
<td>TGF-$\beta$ Forward</td>
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<td>IFN-G</td>
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<td>IFN-Gamma</td>
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BIOGRAPHICAL SKETCH

John-David Herlihy was born and raised in Rockport, MA. He went to high school at St. John’s Preparatory School in nearby Danvers, MA, graduating in the spring of 1993. John-David then received a BS in Biology from the University of Vermont in the fall of 1998. He followed this with an MS in Biology in 1999. As a student at UVM, John-David became interested in cellular and molecular biology and conducted undergraduate research in the Department of Pharmacology investigating the potential of natural products as anti-cancer drugs. Also during his undergraduate years, John-David participated in Sea Education Associations SEA Semester, where he studied oceanography and sailed on the programs research vessels conducting research on the distribution of the cyanobacteria *Synechococcus* in the northwestern Atlantic. John-David’s graduate research studied a GPI-anchored folate receptor on the surface of Paramecium conducted under the supervision of Dr. Judith Van Houten. In August of 1999 John-David entered graduate studies in the College of Medicine at the University of Florida. Initially studying Chemosensory Neurobiology with Dr. Barry Ache at UF’s Whitney Laboratory in St. Augustine, FL, John-David eventually switched fields and began conducting research on the gene regulation of cytosolic Phospholipase A₂ with Dr. Harry S. Nick of the Department of Neuroscience.