The Role of Hepoxilins in an Asthma-Like Mouse Model

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science

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Abstract

Asthma is a chronic respiratory illness where the airway occasionally constricts, becomes inflamed, and is lined with excessive amounts of mucus. There are many lipid mediators involved in this inflammatory response. Hepoxilins are biologically active hydroxyepoxy eicosatrienoic acid metabolites of arachidonic acid, formed through the 12-lipoxygenase pathway. Our goal in this study was to identify if hepoxilins had a direct effect on smooth muscle contractions, to identify the source of hepoxilins in asthmatic-like lungs, and to determine if blocking this pathway reduced inflammation associated with asthma. We found that hepoxilins had no direct effect on smooth muscle contraction. We identified the bronchiolar epithelium as a major source of hepoxilins in OVA-challenged mice. We demonstrated that baicalein inhibits total lung resistance in the OVA-treated mice, but also found that the drug in-vivo is not specific for 12-lipoxygenase.
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List of Abbreviations

AA: arachidonic acid

ACD: acid citrate dextrose

BSA: bovine serum albumin

CAMS: cell adhesion molecules

CC10: clara cell secretory protein 10

CCCP: carbonyl cyanide m-chloro phenyl hydrazone

DMSO: Dimethyl sulfoxide

ECL: enhanced chemiluminescence

FLAP: 5-lipoxygenase activating protein

HPLC: higher performance liquid chromatography

HxA3: hepoxilin A3

HxB3: hepoxilin B3

IL: interleukin

LC-MS-MS: liquid chromatography mass spectrometry

LDL: low-density lipoprotein

LFOT: low frequency forced oscillation

LTs: leukotrienes

MAPK: mitogen-activated protein kinase

NSAIDS: Nonsteroidal anti-inflammatory drugs
OVA: ovalbumin

PEEC: pathogen-elicited epithelial chemoattractant

PFA: paraformaldehyde

PGs: prostaglandins

PMN: polymorphnuclear

TCPO: trichloropropene ocide

TX: thromboxane

VCAM: vascular cell adhesion molecule-1

WBP: whole-body barometric plethysmography

5-HETE: 5-hydroxyeicosatetraenoic acid

12-HETE: 12-hydroxyeicosatetraenoic acid

15-HETE: 15-hydroxyeicosatetraenoic acid

5-LO: 5-lipoxygenase

12-LO: 12-lipoxygenase

15-LO: 15-lipoxygenase
Chapter 1

1 General Introduction and Rationale

1.1 The Pathophysiology of Asthma

The etiology of asthma is complex and involves the interaction between genetic factors and environmental stimuli. The symptoms of asthma – dyspnea, wheezing, coughing, and chest tightness – are caused by airflow obstruction. Another clinical feature of asthma is increased airway responsiveness to various stimuli (1). This means that smaller concentrations of an inhaled bronchoconstrictor agonist are needed to induce narrowing of the airway in subjects who have asthma than are needed in normal subjects. Also, the maximal response to the bronchoconstrictor is greater in subjects with asthma.

Airway hyperresponsiveness, variable airflow obstruction, and the symptoms of asthma are consequences of a characteristic form of cellular inflammation and structural changes in the airway wall of patients with asthma. Among the cells involved in asthma are eosinophils, lymphocytes, macrophages, and an increased number of mast cells, which have been identified in both BAL fluid and airway-tissue samples from subjects who have asthma. The inflammatory mediators include cytokines and growth factors (peptide mediators), as well as eicosanoids, which are products of arachidonic acid metabolism and include leukotrienes (LTs), prostaglandins (PGs), thromboxane (TX), and lipoxins (2). The structural changes described in the asthmatic airway, which appear to be characteristic of the disease, are likely caused by persistent inflammation of the airway (3). These structural changes include patchy desquamation...
of the airway’s epithelium, thinning of the reticular collagen layer of the basement membrane, and hypertrophy of the airway’s smooth muscle (4).

The mechanisms that lead to airflow obstruction in asthma are bronchoconstriction caused by the contraction of the airway’s smooth muscle, mucosal edema caused by vascular leakage, increased secretion of mucus, and an inflammatory-cell infiltrate that is rich in eosinophils. Leukotrienes (LTs) have been shown experimentally to play a role in each of these mechanisms.

1.2 Asthma and Genetics

Research has shown that genetic inheritance plays a role in asthma (5-7). For example, methacholine responsiveness has been shown to be bimodally distributed in asthmatic families compared to non-asthmatic families (8). Twin studies estimate the heritability of asthma anywhere from 35%-75% (9-12), with one recent study by Laitinen et al. (13) showing that in families with one asthmatic parent, genetic factors could account for 87% of the variation and susceptibility to asthma. Although genetic loci and possible candidate genes have been associated with asthma, there has been no definitive identification of major susceptibility genes (5, 14), with several studies showing a relationship between asthma and a combination of several possible genes (15-19).

1.3 Environmental factors and Asthma

It’s important to note that while twin studies clearly indicate the important role of genetic factors, these studies also show that a large part of the variability associated with asthma is caused by factors unique to the individual, especially with regards to the surrounding environment. Within
the last twenty years, the prevalence of the disease in Westernized countries has doubled (20). Because the genetic makeup of a stable population is unlikely to significantly change in less than a century, providing evidence that exposures play a significant role in the development of the disease. For example, studies have shown that asthma occurs more frequently in low-income populations and developed areas (7, 21). And while demographic factors, such as race and age, can be factors for the development and progression of the disease (22), most research shows that asthma’s increasing prevalence and severity can be attributed to air pollution, smoking behaviour, and workplace air quality. For example, a cross-sectional study in Switzerland found an association between long-term exposure to ambient air pollution and asthma (23). Several other recent studies have also found an association between air pollution and increased asthma related hospital visits (24-26).

1.4 Asthma and Respiratory Infection

In addition to genetics and the environment, exposure to infectious agents has also been shown to lead to asthma. Measles, hepatitis A, and tuberculosis have been associated with a reduction in the rate of atopy (27). Respiratory syncytial virus (RSV) and viruses that cause lower respiratory tract infections also increases atopy and wheezing (28). Epidemiological studies have linked RSV with asthma, but many of these studies are lacking adequate control groups, lack of knowledge as to whether or not the infants wheezed prior to contracting RSV, and short follow-up times.

It should be noted, however, that while some respiratory viruses increase the risk of developing asthma, there is rapidly growing evidence suggesting that some childhood infections can actually
protect against the development of asthma. An epidemiological study by Ball et al. (29) found that children who wheezed during their preschool years had a lower chance of developing asthma by the age of 13 compared to ‘non-wheezing’ children. Scientists postulate that as children are exposed to infections and environmental antigens, they develop a cell-mediated immunity that helps to reduce the chance of developing diseases such as asthma later in life.

1.5 Inflammation in Asthma

A six-step model of inflammation demonstrating the roles of cytokines, lipid mediators, and inflammatory cells can be used (Figure 1).

1.5.1 Triggering

The initial phase of inflammation almost always involves a triggering event, such as an allergen. Other triggers include occupational antigens, aspirin, and viral infections. Although a variety of cell types, including macrophages and lymphocytes, may initially respond to the triggering event, the strongest evidence points to mast cells (30)

Increased amounts of the specific mast cell mediator tryptase have been measured in the lungs of allergic asthmatics after exposure to allergen, and studies of lavage fluid and biopsy specimens from asthma patients have shown elevated numbers of mast cells (31, 32). This is important as activated mast cells release a variety of products, including LTs. Allergen challenge has shown increased concentrations of LTs in lavage fluid, indicating increased mast cell activation (33). Another eicosanoid mediator, PGD₂, is also increased in the lungs of asthmatic subjects (34).
Figure 1. Asthma inflammation shown in six-step model integrating the role of inflammatory cells, lipid mediators, and cytokines. From Wenzel (5).
1.5.2 Signaling

Following the initial stimulus, a multitude of signals are released from mast cells and other resident cell types in the airways. These signals (i.e. eicosanoids, chemokines, and cytokines) are biochemical mediators that act on and communicate with numerous other cell types (35).

1.5.3 Migration

The migration of inflammatory cells into the lung is likely controlled by at least two processes; release of chemoattractants and up-regulation of adhesion molecules. Chemoattractants are released from the signaling cells and include LTB$_4$ and chemokines. With asthma, the migration of eosinophils into the lung is a key event in cell trafficking. Eosinophils appear to act as key effector cells in asthma because they release a variety of lipid mediators that can produce bronchoconstriction, edema, and inflammation (35).

The second factor in cellular migration is the up-regulation of cell adhesion molecules (CAMs) on the endothelial surfaces of the lung vasculature (30). The cell adhesion molecules serve as tethers that attach to cells circulating in the bloodstream and draw them toward the site of inflammation. Firm attachment of the circulating leukocyte to the endothelium is mediated by other CAMS, such as the vascular cell adhesion molecule-1 (VCAM-1) (36). After they have been firmly attached, circulating cells migrate through the endothelial cell layer and into the extravascular spaces, where they can release their mediator products.

1.5.4 Activation

The presence of a specific cell type in the airways is insufficient to initiate asthma. Only activated inflammatory cells release the inflammatory substances (i.e. proteases, catonic proteins,
superoxide radicals, LTs and other eicosanoids) that lead to the pathophysiological processes underlying the development of asthma symptoms (30). It has been demonstrated that eosinophils are activated once they are present in the airways. Levels of eosinophil cationic protein, a marker of eosinophil activation, were found to be greater in patients with asthma than in healthy subjects, and increased with the severity of disease (37).

1.5.5 Tissue Stimulation and Damage

Cell activation leads to the release of a variety of pro-inflammatory substances that have the capacity to damage the lung. In addition to eosinophils, tissue damage can also result from the release of superoxide and other reactive oxygen species. While the LTs may not directly cause tissue damage, they do promote the secretion of thick, viscous, mucus that can lead to plugging of the airways. They can also promote increased vascular permeability that leads to airway edema (38).

1.5.6 Resolution

In asthma, inflammation associated with the initial response can persist for an indeterminate duration due to the loss of the reparative mechanisms that return the airways to normal (30). It is still unclear whether this is due to persistent exposure to triggers, like allergens, or to alterations in the inflammatory response. What is known, however, is that this prolonged inflammation leads to worsening of the condition and eventual structural changes in the lung. Consequently, symptomatic treatment, such as bronchodilators, is less than optimal.
1.6 Arachidonic Acid Metabolism

Phospholipids are a major constituent of cell membranes. After stimulation by trauma, infection, or inflammation, translocated phospholipases, especially phospholipase A\textsubscript{2}, act on membrane phospholipids to liberate arachidonic acid (Figure 2). Arachidonic acid is stored mainly esterified to phospholipids but is also found in neutral lipids (mainly triglycerides) and in cholesterol esters. Only in tissues where the mass of neutral lipids matches or exceeds that of phospholipids, such as the adipose tissue or the adrenal cortex, will the AA be found in the free form (39). Generally saturated fatty acids are esterified in the \textit{sn}-1 position while unsaturated fatty acids are commonly found in the \textit{sn}-2 position (40). In the mammalian membrane, AA is almost exclusively located in the \textit{sn}-2 position. AA is released from membranes quite quickly (anywhere from 5-60 seconds) and is typically accompanied by turnover of inositol-controlling phospholipids (41).

As mentioned, most of the arachidonic acid is released via the activation of phospholipase A\textsubscript{2}. The main controlling factor for arachidonic acid liberation in-vitro is intracellular calcium levels, with Chang et al. (42) demonstrating that PLA\textsubscript{2} requires calcium for activity. Once released, arachidonic acid can be metabolized via several different enzymatic pathways, including the cyclooxygenase pathway—which gives rise to PGs—and the lipoxygenase pathways (38). Of importance in asthma are the 5-lipoxygenase (5-LO), the 12-lipoxygenase (12-LO), and 15-lipoxygenase pathways (2, 30, 43, 44). The specific lipoxygenase involved determines which products are formed; LTs, lipoxins, or hydroxyeicosatetraenoic acids (HETEs).
Figure 2. Arachidonic acid cascade. Of particular interest to our study are the 12-LO and 5-LO pathways.
1.7 Cyclooxygenase Pathway

Cyclooxygenase is the first enzyme of arachidonic acid metabolism. The cyclooxygenase pathway produces prostacyclin, thromboxane A$_2$, and the prostaglandins D$_2$, E$_2$, and F$_3$ (45). Eicosanoids synthesized via the cyclooxygenase pathway are referred to as prostanoids. Cyclooxygenase is bound to heme and generates PGH$_2$ from PGG$_2$. Formation of the biologically active eicosanoids from PGH$_2$ occurs via the actions of a set of isomerases and synthases called PGH-PGD isomerase, PGH-PGE isomerase, PGF synthase, PGI$_2$ synthase, and TX synthase (46). Most prostaglandin-forming cells produce only one of these products due to the predominance of a single PGH$_2$ metabolizing enzyme. Human blood platelets, for example, contain predominantly TX synthase, resulting in the formation of TxA$_2$ only (47).

Biologically, thromboxane A$_2$ is the predominant product of the metabolism of arachidonic acid from platelets and is a potent aggregating agent. TxA$_2$ is also a vasoconstrictor and a bronchoconstrictor, and has been implicated as a mediator in diseases such as stroke, myocardial infarction, and asthma (45, 48). Prostaclycin, in contrast, is a potent inhibitor of platelet aggregation, and is thought to antagonize the formation of TxA$_2$ and serve as a vasodilator in some vascular systems (49). Prostaglandin D$_2$ is formed from mast cells upon antigen binding to IgE receptors (45). In addition to acting as a vasodilator and enhancing vascular permeability, PGD$_2$ can modulate the release of other inflammatory mediators, and like histamine, acts as a potent bronchoconstrictor (50). PGD$_2$ is also a potent inhibitor of platelet aggregation, with only small quantities of the prostanoids being produced by platelets (51). Prostaglandin E$_2$ is a vasodilator and bronchodilator, while PGF$_2$ is a vasoconstrictor and bronchoconstrictor. A study by Widdicombe and colleagues (52) showed that human airway epithelial cells produce both PGE$_2$ and PGF$_2$ in response to inflammatory stimuli.
1.8 5-Lipoxygenase Pathway

The 5-lipoxygenase enzyme catalyzes the first step in the formation of leukotrienes. Activation of the 5-LO enzyme involves the enzyme docking to the 5-lipoxygenase activating protein, or FLAP, located on the perinuclear membrane (30). 5-LO possesses two enzymatic activities. The first of these catalyzes the conversion of arachidonic acid into the intermediate 5-hydroperoxyeicosatetraenoic acid (5-HPETE), while the second then converts 5-HPETE intermediate to the epoxide LTA₄. LTA₄ in turn can then be metabolized in one of two ways. In neutrophils LTA₄ epoxide hydrolase catalyzes the conversion of LTA₄ into LTB₄, while in eosinophils and mast cells LTC₄ synthase produces the first of the sulfidopeptide-LTs, LTC₄ (53).

1.8.1 Leukotrienes and Airway Inflammation

The effect of leukotrienes on respiratory functions has attracted special interest because of their mediator role in asthma. The cytokines interleukin (IL)-3, IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor are important in the propagation of allergic inflammation (54, 55) and are known to prime human basophils, eosinophils, and neutrophils for enhanced release of LTC₄ after stimulation by a second agonist (56).

LTC₄, LTD₄, and LTE₄ are potent stimulators of airway smooth muscles from different species and mediate mucus production (57). The EC₁₀₀ values (the concentration required to increase the resting tension by 100%) for LTD₄ and LTE₄ were 0.6 and 50 nM in guinea pig lung strips and trachea, while LTC₄ was four times more potent than histamine (57). Additionally, airway smooth muscle from rabbits and rats do not respond to LTC₄ and LTD₄, while monkey, guinea pig, and human smooth muscle are sensitive to both (58). Structure-activity studies on LTC₄ and LTD₄ analogs on guinea pig airways also showed that the position of the C-6 peptide substituent
Figure 3. Schematic diagram of leukotriene synthesis. Pathway inhibitors act on either the FLAP or 5-LO protein.
is important for maximum activity and that partial saturation of the triene (to the n7-hexahydro compounds) reduced smooth muscle stimulating activity (59).

The role of LTs in increased airway vascular permeability, which leads to edema, has been demonstrated in animal studies, in which LTD\(_4\) was 10-fold less active than platelet-activating factor (PAF) (60). Intradermal application of LTC\(_4\), LTD\(_4\), and LTE\(_4\) in humans has also been shown to stimulate inflammation (61, 62). Additionally, an increased volume of mucus is formed in asthma by sub-mucosal glands and LTs have been shown to be some of the most potent stimulants for mucus secretion (61-64).

### 1.9 Inhibition of Leukotriene Formation or Action

Pharmaceutical companies have developed a number of agents capable of modulating the actions of LTs. This is usually done through inhibiting LT synthesis by reducing the activity of either FLAP or 5-LO. Initial human studies found that these compounds inhibited LT biosynthesis in whole human blood removed from test subjects following administration of these agents (1). Additionally, in cold air or exercise-induced bronchoconstriction, 5-LO inhibitors reduced the maximal bronchoconstrictor response by approximately 50% (1, 65-68). The selective FLAP inhibitor MK-886 was chosen in our study because a previous study by Henderson et al. (69) using an OVA mouse model found the drug effective at blocking several symptoms associated with the disease.
1.10 15-Lipoxygenase Pathway

15-hydroxyeicosatetraenoic acid (15-HETE) is the main arachidonic acid metabolite synthesized by the human trachea, bronchi, and parenchyma (70-72), in addition to being produced by several cells such as macrophages and eosinophils (73-75). 15-HETE arises from the metabolism of AA through the 15-lipoxygenase (15-LO) pathway (Figure 4).

12/15-LO in mice and other animals is the ortholog to human 15-LO. There are many similarities between human 15-LO and animal 12/15-LO. The expression pattern of human 15-LO and murine 12/15-LO is similar (76), and the cytokines IL-4 and IL-3 induce the expression of 12/15-LO in both murine and human macrophages (77).

1.10.1 12/15 Lipoxygenase and Asthma

Unlike the 5-LO pathway, the 12/15-LO pathway has been much less extensively studied in asthma. In humans, Chu et al. (78) found that the amount of 15-LO is significantly increased in the bronchial tissue of asthmatic patients compared with control subjects. Smokers, particularly smokers with chronic bronchitis, also display an increased expression of 15-LO in bronchial biopsies (79). Several studies have also shown that 15-HETE can be rapidly metabolized or reincorporated into cellular lipids either spontaneously or after stimulation (80-83). 15-HETE can also be reincorporated into phospholipids of pulmonary epithelial cells, while 15-LO is modulated by cytokines like IL-4, a cytokine that plays a major role in asthma inflammation. (84). Ozone exposure also increases 15-HETE production and reincorporation in airway epithelial cells, resulting in reduced synthesis of PGE$_2$, a cyclo- oxygenase metabolite of AA associated with anti-inflammatory activities in airways (85). Additionally, increased 15-HETE levels were reported in stable asthmatics, along with an increased percentage of 15-LO
Figure 4. 15-lipoxygenase pathway. 15-LO acts on arachidonic acid to form 15(S)-HPETE
mRNA positive macrophages in induced sputum (86). These findings suggest that 15-HETE plays a major role in the regulation of cell activation and on the release of inflammatory mediators.

In animals, Andersson et al. (43) used 12-LO knockout mice and an OVA allergen model to show that the knockout group developed significantly less airway inflammation than the wild type mice upon local allergen challenge. The researchers also noted that the 12-LO knockout mice were still able to produce allergen-specific anaphylactic antibodies, suggesting 12-LO is critical for the local manifestation of an allergic airway inflammatory response.

1.10.2 Pathophysiological Role for 12/15-LO

Inflammatory vascular disease encompasses a spectrum of disorders that includes atherosclerosis, diabetes and hypertension. A central involvement of the immune system in the pathophysiology of these diseases is recognized (77). The role of 12/15-LO in mediating the pathogenic lipid peroxidation associated with inflammatory vascular disease has been studied for many years. Sparrow et al. (87) demonstrated that soybean 15-LO incubated with low-density lipoprotein (LDL) and phospholipase A₂ caused fatty acids to oxidize. Additional studies also showed that intracellular 12/15-LO could oxidize extracellular LDL forming specific 12/15-LOX-derived free and esterified products (88), demonstrating that 12/15-LO possessed vascular damaging properties.
1.11 12-Lipoxygenase Pathway

The 12-lipoxygenase pathway allows for hepxilin formation, with the 12-LO enzyme acting on arachidonic acid to form the intermediate 12(S)-HPETE. Hepxilins are hydroxy epoxide metabolites formed through the rearrangement of 12(S)-HPETE, the initial product resulting from the action of 12-lipoxygenase on arachidonic acid (89). Two hepxilins are formed from this reaction: 8 (S/R)-hydroxy-11S, 12S-trans-epoxyeicosa-5Z, 9E,14-trienoic acid (hepxilin A₃, HxA₃) and 10(S/R)-hydroxy-11S, 12S-trans-epoxyeicosa-5Z, 8Z, 14Z-trienoic acid (hepxilin B₃, HxB₃) (Figure 5). The ‘A’ and ‘B’ designation refers to the carbon position of the hydroxyl group, where ‘A’ is the hydroxyl group on C8 and ‘B’ is the hydroxyl group on C10 (89). Hydrolysis of the epoxide generates the trihydroxy metabolites 8(S/R), 11R, 12S-trihydroxy-eicosa-5Z, 9E, 14Z-trienoic acid (trioxilin A₃, TrXA₃) and 10(S/R), 11S, 12R-trihydroxy-eicosa-5Z, 8Z, 14Z-trienoic acid (trioxilin B₃, TrXB₃). HxA₃ is normally unstable biologically and consequently metabolized into trioxilin A₃ through the reaction of an epoxide hydrolase (90). HxA₃ exhibits increased stability and potency in its methyl ester form, and methods for the rapid extractive methylation have been described (91). The activity of the free acid form of HxA₃ is dependent upon the type of vehicle used (i.e. ethanol allowing for calcium release but not DMSO), while the methyl ester is equally active in either vehicle (92). When the activity of the hepxilin epoxide hydrolase is inhibited by trichloropropene oxide (TCPO), HxA₃ is further metabolized through a glutathione transferase pathway to form a glutathione conjugate of hepxilin termed HxA₃-C. The metabolite is formed via the addition of glutathione to the epoxide moiety to form the 11-glutathionyl-12-hydroxy metabolite (93, 94). Hepxilin B₃ in contrast is resistant to both enzymatic and non-enzymatic hydrolysis (89).
Figure 5. The 12-Lipoxygenase pathway. 12-LO acts on arachidonic acid to form 12(S)-HPETE. Two hepoxilins are formed from this reaction: 8 (S/R)-hydroxy-11S, 12S-trans-epoxyeicosa-5Z, 9E,14-trienoic acid (hepoxilin A3, HxA3) and 10(S/R)-hydroxy-11S, 12S-trans-epoxyeicosa-5Z, 9E, 14Z-trienoic acid (hepoxilin B3, HxB3). The ‘A’ and ‘B’ designation refers to the carbon position of the hydroxyl group.
The formation of hepoxilins was first shown in human platelets and lung, pancreas, liver, brain, aorta, and pineal gland of the rat (93, 95, 96). The hepoxilin pathway is also seen in tropical marine algae (97) and Aplysia brain (98). Most of the biological activities appear to be exerted by HxA₃, making it the most bioactive hepoxilin (89, 95, 99).

1.12 Biological Actions of Hepoxilins

1.12.1 Biological Effects

Hepoxilins have been shown to exhibit a variety of biological actions, including stimulating glucose-evoked secretion of insulin in rat pancreatic islets (100), regulation of cell volume through the activation of K⁺ channels in human platelets (101), potentiating bradykinin-evoked vascular permeability in rat skin (102), potentiating norepinephrine-induced vascular contraction on rat aorta (103), and potentiating neurokinin A-induced guinea pig trachea (104). HxA₃ has also been shown to inhibit the aggregation of normal human platelets (105).

1.12.2 Effects on Calcium Mobilization and Transport

The primary biological action of hepoxilins is to release calcium from intracellular stores through a receptor-mediated mechanism. Previous studies indicate that receptor appears to be an intracellular hepoxilin-specific binding protein (106), and may be G-protein coupled, as its actions are inhibited by pertussis toxin (107). Hepoxilin A₃ has been shown to increase free Ca²⁺ transport across a visceral yolk sac membrane from a pregnant guinea pig (108). Studies by Dho and colleagues (107) using dye-loaded human neutrophils found the neutrophils increased their free intracellular calcium levels in the presence of hepoxilin A₃. This increase was brought on via an initial rapid rise in intracellular calcium followed by a slower influx from the extracellular
medium. Studies suggest that the initial rapid phase is caused by a release of calcium from intracellular stores in the endoplasmic reticulum while the slower rate of decline is thought to be caused by calcium influx as it is abolished in zero intracellular calcium medium (89, 109). A study by Reynaud et al. (92) also determined that the addition of 1µM of lanthanum chloride, an inorganic calcium channel blocker, prior to the addition of hepoxilins, caused the calcium peak to return to normal baseline levels as in a zero intracellular calcium condition.

Calcium response to hepoxilins varies intracellularly. Mills et al. (109) eliminated hepoxilin-evoked calcium sequestration via exposure to CCCP, a mitochondrial uncoupler. CCCP also eliminated the plateau phase of the calcium response in cell suspension, suggesting that the phase was associated with mitochondrial function rather than calcium influx alone. These results demonstrate that hepoxilins help release calcium from the calcium stores in the endoplasmic reticulum, which is then taken up and sequestered by organelles.

1.12.3 Inflammatory Mediators

A recent study by Mrsny et al. (110) identified HxA₃ as a pathogen-elicited epithelial chemoattractant (PEEC) in intestinal inflammation. PEECs are naturally occurring mediators that stimulate G-protein–coupled Ca²⁺ mobilization. The PEECs were distinguished from other known polymorphnuclear (PMN) attractants via high performance liquid chromatography (HPLC), which identified the specific chemical composition of PEEC as HxA₃. The Mrsny (110) study found that the HxA₃ was secreted by apical epithelial cells and secreted from the surface in response to inflammatory events. Importantly, it was found that blocking the 12-LO pathway with baicalein dramatically reduced inflammation.
1.13 12-Lipoxygenase Inhibition

While there are numerous 5-LO specific inhibitors, there are significantly fewer for 12-LO. One cited 12-LO specific inhibitor is baicalein, a plant flavanoid derived from *Scutellaria baicalensis*, which has been shown to induce apoptosis in breast, colon, prostate, and pancreatic cell lines (111-114). The potency of baicalein is thought to be due to the selective inhibition of 12-LO, thereby interrupting only part of the arachidonic acid metabolic pathway (115-121).

1.14 Mouse Models and Limitations

1.14.1 History of Animal Models

As with many human diseases, animal studies have produced much of what we think we know regarding the mechanisms of asthma. The relevance and validity of these studies relate to how accurately researchers can re-produce accurate animal equivalents of human asthma. Optimizing these animal models is still very much a work in progress; while many of the features of asthma are now recapitulated in many animal models, almost every model misses some aspect of the human syndrome (122). Additionally, very few animals naturally develop a condition with any similarity to asthma, with the most similar being an allergic syndrome in cats (123) and heaves in horses (124).

Early animal asthma models were developed in a number of species (125) and focused on airway hyperresponsiveness. The challenging agent was often a smooth muscle agonist, such as methacholine or histamine. The agonists were intended to mimic the actions of mediators released as part of immune response that was associated with allergic asthma. The motivation
behind these models was the idea that asthma was primarily a matter of excessive shortening of the airway smooth muscle (126). By the 1980s, however, a greater awareness of asthmatic inflammation developed due to an increased understanding of allergic immunology and the observation that human asthmatic patients frequently exhibited increased symptoms after undergoing allergen sensitization (126). Eosinophils were firmly linked to asthma pathogenesis (127), followed by the identification of the role T-cells play in the disease (128).

Despite the wealth of knowledge we know about asthma, there are still unanswered questions, resulting in the continued reliance of animal models. As with much biomedical research, mice are the predominant animal model used because of the many molecular and immunological tools available to study them (129, 130).

### 1.14.2 Mouse Models of Allergic Asthma

Mouse asthma models of asthma are generated by sensitizing an animal to a foreign protein, the most common being ovalbumin (OVA). OVA is a 45 kDa egg-white glycoprotein and is a member of the serpin (serine protease inhibitor) family of protease inhibitors (131). OVA is a well-known food allergen that comprises approximately 54% of the total egg-white. Additional antigens include house dust mice, cockroach antigens, and ragweed extracts. Sensitization is typically done by injecting the protein intraperitoneally along with an adjuvant, such as aluminum hydroxide. In a process not entirely understood, the adjuvant acts to enhance the protein’s immunogenicity (132). After the immune system has had a chance to mount a reaction against the antigenic protein, which can take several days, the animal undergoes further sensitization either via aerosolization or postnasal drip. This treatment results in a Th-2 response in the lungs, with varying levels of eosinophilia and airway responsiveness to bronchoconstricting agents (133). The differences in response between strains for a given antigen
sensitization is seen as an advantage of mouse asthma models because it allows for the identification of both genetic and cellular mechanisms of airway responsiveness and inflammation (133-136). The recent expansion of transgenic technology also makes the mouse an ideal candidate for asthma research as a single molecular pathway can be switched off, up-regulated, or suppressed help understand the importance the pathway plays in the development of the asthmatic phenotype (137). Such studies in asthma mouse models have developed our understanding of the important role that cytokines IL-4, IL-5, and IL-13 play in allergic reactions of human asthmatic (138-140). By identifying the important role these cytokines and mediators play in the allergic process, most of which have been studied in mice, many new therapeutic targets have been identified (141).

1.14.3 Limitations of Mouse Models

While there are a number of benefits of using a mouse model to study asthma, there are also a number of issues relating to the appropriateness of mice as models for human asthma (142-145). One of the main concerns is that the inflammatory response in sensitized mice, depending on the dose of antigen used, results in a massive influx of eosinophils-dominated inflammatory cells into the airways (146). Eosinophils, in this case, can represent up to 60% of the cells obtained from the bronchiolar lavage (BAL), and the pattern of inflammation is more similar to allergic alveolitis instead of the asthmatic phenotype that is being modeled (143, 147). There is also controversy regarding the importance of eosinophils in mouse asthma models, as eosinophils rarely degranulate in mice, while human eosinophils readily granulate (148, 149). Another potential problem mice asthma models is that the pattern of mediators released by mast cells. Specifically, rodent mast cells release serotonin, which is not thought to play a role in human asthma (150).
Another limitation associated with mouse asthma models is the airway hyperresponsiveness to bronchoconstricting agents. In human asthmatic airways, the lung often develops an early-phase response following allergen inhalation that is characterized by acute bronchoconstriction and usually subsides within an hour (150). In approximately 50% of cases, a late-phase response develops that also demonstrates marked bronchoconstriction. In mice, the airway hyperreactivity is the primary physiological outcome; however, the acute responses to allergen inhalation are poorly defined, and it is still unclear whether mice models are capable of exhibiting a physiological late-phase constriction in the lung (151).

Another criticism of most mouse models is the lack of chronicity response to allergen exposure following sensitization (152). When mice are sensitized systematically with an antigen and then challenged repeatedly, studies show that these animals can develop a tolerance to the allergen, where the immunological response is then suppressed (153). There are, however, mouse models that use house dust mite extract, an antigen that is clinically significant in humans which induces a pathology that is similar to human asthma with airway eosinophilia and long-term sensitivity to bronchoconstricting agents (154).

Despite these limitations, mice are currently the most popular model because of the technology available to manipulate their biology. These models offer an effective means of generating hypotheses that can be tested in human asthmatics.
1.15 Research Question

Do hepoxilins, as inflammatory mediators, play a role in asthma inflammation, and if so, by what mechanism?

1.15.1 Rationale

While there is significant evidence that the 12-LO pathway and hepoxilins play a role in inflammation, very little is known about what, if any, role they play in asthma. If 12-LO is shown to play a contributing factor to airway inflammation, then by identifying where this pathway is found and blocking 12-LO could help reduce some of the symptoms associated with asthma.

1.15.2 Hypothesis

12-LO, and by extension hepoxilins, is present in large airway epithelium of asthma-like lungs, and blocking this pathway with 12-LO inhibitors will reduce airway inflammation associated with OVA sensitization.

1.15.3 Aims

1) To determine if hepoxilins have a direct effect on airway smooth muscle (ASM) contractility in a naive murine mouse.

2) To determine the source of hepoxilins in OVA-treated mouse.

3) To determine if blocking the 12-LO pathway will reduce total lung resistance in mice.
1.15.4 Clinical Relevance

The prevalence of asthma in Westernized countries has doubled within the last two decades. An estimated 15 million Americans suffer from the disease, and mortality associated with asthma is increasing in industrialized nations (27). Additionally, the annual cost of caring for asthmatics is more than six billion dollars per year in the United States alone, with the worldwide market for asthma medication estimated to be more than 5.5 billion dollars annually (155). Because of the increasing prevalence and the costs associated with the disease, any new means of treating some of the symptoms associated with asthma is very important. Since very little is known about 12-LO and asthma, demonstrating that this novel pathway is indeed involved with airway inflammation could provide clinicians with another means of treating the disease. Research could focus on finding 12-LO inhibitors, which could then be combined with pre-existing asthma medications.
2 Materials and Methods

2.1 Materials

Adult (41-48 days) female Balb/c mice from Charles River (Wilmington, MA, USA).
Precipitated ovalbumin from Sigma Chemical (St. Louis, MO, USA). Alum from J.T. Baker Chemical (Phillipsburg, NJ, USA). Methacholine from Sigma-Aldich (St. Louis, MO, USA).
Ethyl ether from Caledon Laboratories (Georgetown, ON, Canada). MK-886 and Baicalein drugs from Biomol (Plymouth Meeting, PA, USA). WBP chambers from Buxco Electronics (Troy, NY, USA). Flexivent mouse ventilator from SCIREQ (Montreal, QB, Canada). OCT from.
Cryomold Vinyl Specimen Molds from Sakura Finetek (Torrance, CA, USA). A Pixcell laser capture microscope from Arcturus Engineering (Mountain View, CA, USA). A myograph machine from DMT (Sarasota, FL, USA). Paraformaldehyde from BDH Inc. (Toronto, ON, Canada). Xylene from Sigma-Aldrich (St. Louis, MO, USA). 100% ethanol from Commercial Alcohols (Brampton, ON, Canada). Acids, hydrogen peroxide, Superfrost/Plus microscope slides, and glass coverslips from Fisher Scientific (Markham, ON, Canada). Trisodium citrate dehydrate from BioShop (Burlington, ON, Canada). Bovine serum albumin from Sigma-Aldrich (St. Louis, MO, USA). Normal donkey serum from Sigma-Aldrich (St. Louis, MO, USA). Avidin-biotin-peroxidase complex immunohistochemistry kits and DAPI mounting media from Vector Laboratories (Burlingame, CA, USA). RNA PicoPure isolation kit from Arcturus (Mountain View, CA, USA). Histogene Staining Kit from Arcturus (Mountain View, CA, USA). UltraPure distilled water from Invitrogen (Carlsbad, CA, USA). 5-lipoxygenase rabbit antibody AbCam (Cambridge, Great Britain). 12-lipoxygenase goat antibody from Santa Cruz (Santa Cruz, CA, USA). 12-lipoxygenase rabbit antibody kindly provided as a gift from Dr. Jerry
Nadler at the Eastern Virginia Medical School (Virginia, MA, USA). Donkey anti-goat and goat anti-rabbit secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sorvall centrifuge, model GLC-2B from Thermo Fisher labs (Waltham, MA, USA). Siliconized 100 µl inserts with polymer springs from Varian (Palo Alto, CA, USA). Crimp top vial from Chromatographic Specialties (Brockville, ON, Canada). Aggregometer from Bio/Data Corp (Horsham, PA, USA).

2.2 Acute Asthma-Like Model

All animal experiments were conducted in accordance with the Canadian Council for Animal Care guidelines. Approval for all experiments was obtained from the Hospital for Sick Children’s Animal Care Review committee. Female Balb/c mice aged 41-48 days were sensitized and challenged with OVA. To sensitize the mice, the animals were given an ip injection of 5 µg chicken OVA and 0.5 mg alum in 0.1 ml of saline on days 0 and 7. A control group was sensitized using the same protocol except that only alum and saline were used. All OVA mice were challenged with 5% OVA dissolved in 10 ml saline for 40 minutes every other day starting on day 14 until day 21 (Figure 6). Unrestrained mice were placed in a plastic holding chamber and the OVA solution was nebulized into the chamber. Control mice were challenged by saline only.
Figure 6. Acute asthma-like model. OVA/saline injections on days 0 and 7 followed by 4 days of nebulization of either OVA or saline beginning on day 14.
Figure 7. LO inhibitors Baicalein (6mg/kg) and MK-886 (3 mg/kg) are injected beginning day 10 and then every other day until day 21.
2.3 Lipoxygenase Inhibitor Selection and Preparation

We chose to use the five lipoxygenase-activating protein inhibitor MK-886 based on a previous study by Henderson et al. (69) that found that 3mg/kg of MK-886 successfully reduced airway inflammation in OVA-treated mice. Because there were no similar animals studies to help us determine what dosage of baicalein should be used, we used the same concentration as the MK-886. Initial total lung resistance measurements, however, showed that baicalein was having no significant effect at this concentration. When we doubled the dose to 6 mg/kg, a significant reduction in resistance was seen.

Both the 5-LO (MK-886) and 12-LO (baicalein) inhibiting drugs were dissolved in 50% DMSO, and then diluted 5x by the addition of saline. The drugs were then shaken for 20 minutes before being given through ip injection starting on day 10 of the protocol (Figure 7).

2.4 Measuring Total Lung Resistance

Total lung resistance was measured using a Scireq Flexivent mouse murine ventilator (Figure 8). The Flexivent is an integrated platform for pulmonary research that combines a mechanical ventilator with accurate measurements of respiratory mechanics. It quantitatively assesses ‘airway constriction’, and also characterizes the complex relationship between pressure, volume, and flow.

Both OVA and saline-treated control mice were anaesthetized with xylazine (10 mg/kg) and ketamine (150 mg/kg) via ip injection. The mice were paralyzed with 1 mg/kg pancronium bromide with additionally doses given with any evidence of muscular activity. The trachea was
then cannulated and connected to the Flexivent, where forced oscillation measurements were taken. Mice were ventilated with an average breathing frequency of 150 breaths/min. Using Total Lung Capacity (TLC), the following parameters were determined: mean displaced volume (Vend), Vend relative to weight, and mean delivered volume. A ‘snapshot perturbation’ maneuver was used to measure resistance (R) of the whole respiratory system (airways, lung, and chest wall).

After performing all perturbations at baseline level airway hyperreactivity was measured. Mice were challenged with increasing methacholine aerosols, generated with an in-line nebulizer and administered directly with increasing concentrations of 0, 2.5, 5, 10, and 20 mg/ml. A snapshot maneuver recorded resistance against methacholine concentrations.

2.5 Tissue Preparation for Immunostaining

The mice were sacrificed 22 days after the start of the OVA/saline treatment by inhalation of ether. The thoracic cavity was opened and an incision made to the diaphragm to collapse the lung. The trachea was then cannulated and the pulmonary vascular system flushed with PBS to clean the lungs. PBS-buffered 4% (wt/vol) paraformaldehyde (PFA) under a constant airway pressure of 20 cm H₂O with air was used to fix the lungs. The pressure was maintained during fixation to prevent recoiling of the lung. The lungs were then extracted and placed in 4% PFA solution overnight at 4°C. The tissue was then dehydrated by immersion in increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin at random orientations. Lung sections of 7µm thickness were cut and mounted on Superfrost/Plus slides and allowed to dry overnight before proceeding to the immunostaining procedures.
Figure 8. The Scireq Flexivent rodent module combines a mechanical ventilator with accurate respiratory measurements in mice.
2.6 Tissue Preparation for Frozen Lung Tissue

The mice were sacrificed 22 days after the start of the OVA/saline treatment by inhalation of ether. The mice were then placed on ice to preserve the tissue, the thoracic cavity opened and an incision made to the diaphragm to collapse the lung. For samples that were to be used for immunostaining, the trachea was cannulated and the pulmonary system flushed with 1xPBS to clean out the lungs. This step was skipped on tissue to be used for RNA extraction to save time and minimize RNA degradation. The lungs were then perfusion-fixed using a 1:1 solution of OCT and 1xPBS under a constant airway pressure of 25 cm H$_2$O with air, which allowed the lungs to reach total lung capacity. The lungs were then embedded using a Cryomold vinyl specimen mold and OCT at random orientations and placed on a bed of dry ice and 100% (vol/vol) ethanol. Once the samples had set, they were then placed in the -80°C freezer. A cryostat maintained at a constant temperature of -22°C was used to make sections 7µm in thickness, which were then mounted on Superfrost/Plus slides and kept in the cryostat at -22°C until they were moved directly to the -80°C freezer.

2.7 Immunofluorescence

Frozen lung sections were fixed in a 1:1 mixture of methanol and acetone at -20°C for 7 minutes. The sections were then washed in PBST (3 x 10 min) and blocked with 10% (vol/vol) NGS in 1% (wt/vol) BSA in PBS (blocking solution) for a minimum of 1 hour at room temperature. The primary antibodies 12-LO and 5-LO at dilutions of 1:100 were added to blocking solution and sections incubated overnight at 4°C. The sections were then washed in 1xPBST (3 x 5 min) and the secondary antibody (Alexa-Fluor 488 conjugated goat-anti-rabbit IgG) was added at a 1:200...
dilution in blocking solution at room temperature for 1 hour. After a final washing in PBST (3 x 5 min) the slides were mounted using a 4’,6-diamidino-2-phenylindole (DAPI) medium and viewed under a Leica DM6000B fluorescence microscope.

### 2.8 Western Blots

Frozen tissue was thawed and homogenized in cold 200µL RIPA buffer (50mM Tris HCL, pH7.4, 150mM NaCl, 1mM EDTA, 1% Triton -X-100, and 1% (vol/vol) complete protease inhibitor cocktail), and incubated on ice for 10min. The samples were stirred using an orbital shaker for 2 hours at 4ºC, and then centrifuged at 14,000 x g at 4ºC for 5 min to collect the supernatant which was stored at -80ºC. The total protein concentration was determined using the Bradford Protein Assay. 50 µg of protein diluted with sample buffer was loaded in each well on an 8% (wt/vol) stacking and 10% (wt/vol) resolving SDS-PAGE gel and transferred for 1h at 100V in a tris-glycine 20% methanol transfer buffer. Transfer efficiency was determined using Ponceau S staining. Nonspecific binding was blocked with 5% (vol/vol) powdered skim milk in PBS plus 0.05% (vol/vol) Triton-X-100 (blocking solution) at room temperature for 1h. The membranes were then incubated with either goat 12-LO or rabbit 5-LO polyclonal antibodies at dilutions of 1:250 and 1:500 respectively, in blocking solution at 4ºC overnight. The slides were washed in TBST (3 x 10 min), and then incubated with either HRP- conjugated goat-anti-rabbit and donkey-anti-goat IgG (1:20 000) in blocking solution at room temperature for 1 hour. The membranes were washed with TBST (3 x 10 min) and the signal visualized by enhanced chemiluminescence (ECL) on BioMax films. β-actin was used to determine equal protein loading and transfer.
2.9 Platelet Aggregation

2.9.1 Isolation of Human Platelets

With the help of Denis Reynaud, blood was drawn from healthy human subjects who had not taken NSAIDS (nonsteroidal anti-inflammatory drugs) for at least two weeks. Blood was drawn into plastic syringes containing ACD (acid citrate dextrose). The samples were immediately centrifuged at 23°C at 200g for 15 minutes. The platelet rich plasma was transferred into fresh plastic tubes and centrifuged at 400g for 5 minutes. The supernatant was discarded and the platelet sediment was re-suspended in fresh medium containing NaCl (137mM), KCl (1mM), NaH$_2$PO$_4$ (0.42mM), glucose (5.5 mM), HEPES (20 mM), and CaCl$_2$ (1 mM) and allowed to stand at room temperature for 30 minutes. A platelet count was made, which determined the volume of platelet suspension needed to make up 350 x 10$^6$ cells for each measurement.

2.9.2 Measurements of Platelet Aggregation

Half an ml of platelet suspension was added to siliconized glass tubes (four samples at a time) and heated with magnetic stirring to 37°C for 1 minute in a platelet aggregometer (PAP-4C). CaCl$_2$ was added initially to give a final concentration of 1 mM in the tube. Either vehicle alone (ethanol, 1 µl) or hepoxilins at concentrations of 5 µg were added, followed by the agonist (collagen, 2 µg) 2 minutes later. The response was recorded for the next 6 minutes. Since an aggregometer is a photo-optical tool, platelet aggregation measurements are based on the amount of light that is able to filter through the mixture. This means that more light will be able to pass through a suspension of aggregated platelets.
2.10 Measuring Contraction Force

Naïve Balb/c mice were sacrificed via cervical dislocation, the secondary bronchi (average diameter 100 µm and a length of approximately 3 mm) were removed with the help of Ms. Jingyi Pan of Dr. Jaques Belik’s lab and mounted on a wire myograph. Briefly, tissue was bathed in 4 ml of Krebs-Henseleit buffer (115 mM NaCl, 2.51 mM KCl, 1.38 mM NaHPO₄, 25 mM NaHCO₃, 2.46 mM MgSO₄, 1.91 CaCl₂, 5.56 mM dextrose), maintained at 37ºC and continuously gassed with 95% O₂ and 5% CO₂. After 1 hour of equilibrium, the optimal resting tension of the tissue was determined by repeated stimulation with 128 mM KCl until maximumal active tension was reached. All subsequent force measurements were obtained at optimal resting tension. Bronchial muscle force generation was evaluated by stimulation with methacholine (10⁻⁹ to 10⁻⁴ M). Isometric force changes were digitized and recorded online. Contractile responses were normalized to the tissue cross-sectional area as follows: (width x diameter) x 2 and expressed as mN/mm². The methyl ester hepxoxilins were stored at -80ºC in benzene. Once removed from -80ºC, the hepxoxilins were dried to a powder using nitrogen gas and then dissolved in ethanol. To test whether hepxoxilins had a direct effect on bronchi contraction, various doses (10⁻⁹ to 10⁻⁴ M) were added to the Krebs bath and allowed to sit for 45 minutes before being stimulated by methacholine.
2.11 Laser Capture Microscopy and RNA Isolation

2.11.1 Slide Preparation

Slides were removed from -80°C and thawed at room temperature for 30-60 seconds without drying. The slides were immediately immersed in 70% (vol/vol) ice-cold ethanol for 30 seconds that was kept at -20°C. The tissue was re-hydrated in LCM DEPC-grade water for 1 minute to remove the OCT, and then Arcturus Histogene staining solution was applied for 30 seconds. The tissue was de-hydrated by placement in 75%, 95%, and 100% (vol/vol) ethanol for 30 seconds each and then xylene for 5 minutes. The slides were allowed to dry in the fume hood for 1 minute before being vacuum-sealed in a slide desiccator and carried to the LCM machine. This procedure was carried out as quickly as possible in order to reduce possible RNA degradation.

2.11.2 Laser Capture Microdissection

One of the goals of this project was to identify whether large airway epithelium is the source of hepoxilin production. One of the difficulties of studying airway epithelial cells is the inability to isolate pure samples for DNA analysis. Laser Capture Microscopy (LCM) was created more than twenty years ago by the National Institutes of Health as a means of isolating cells from specific microscopic regions of tissue (156). As cells of interest are transferred to a polymer film in one step, any possible contamination is avoided, while the precision of transfer itself is within 1µm in diameter. The remaining tissue on the slide is still fully accessible for further capture, allowing for a comparative molecular analysis of adjacent cells. Without this technique, it would be nearly impossible for us to isolate airway epithelium without possible cross-contamination.
Briefly, after Histogene staining, an Arcturus PixCell LCM machine with an infrared diode laser was used to isolate the epithelial layer from the large airways from both OVA- and saline-treated mice. The dehydrated tissue section was overlaid with a thermoplastic membrane mounted on optically transparent caps, and cells captured by the melting of the membrane via a laser pulse. The cap with the attached cells was then carefully removed and immersed in extraction buffer (Figure 9). Only bronchiolar epithelial tissue was isolated (Figure 10).

2.11.3 RNA Extraction

RNA extraction on LCM samples was carried out using the Arcturus PicoPure RNA Isolation kit. Briefly, once the desired tissue was isolated, the cells were incubated in 50 µl of extraction buffer at 42°C for 30 min using sterile Biorad tubes. The samples were centrifuged for 2 minutes at 800 x g and transferred onto a new column membrane. 50 µl of 70% (vol/vol)
Figure 9. The laser capture microscopy process. Tissue is placed on slides and a transfer film cap placed on top of the tissue. A laser pulse isolates any cells of interest. The film is then lifted off the tissue with selected cells attached. The cap is then placed in an epindorph tube with extraction buffer and placed at 42C for 30 min.
Figure 10. LCM-captured lung epithelium. A) the lung tissue prior to being pulsed with the laser. B) removal of the bronchiolar epithelium after LCM laser pulse.
ethanol was added and the entire column centrifuged at 100 x g for 2 minutes, followed by 16,000 x g for 30 seconds to remove flow through. 100 µl wash buffer I was added to the column and then centrifuged at 8000 x g for 1 minute. 100 µl of wash buffer II was then added and centrifuged at 8000 x g for 1 minute. The same amount of wash buffer II was added a second time and then centrifuged at 8000 x g for 2 minutes. The column was transferred to a new 500 µl PicoPure tube and 15 µl of elution buffer added. Finally, the tube plus column was centrifuged at 1000 x g for 1 minute to distribute the elution buffer, followed by 1 minute at 8000 x g to elute RNA. RNA was then stored at -80ºC until needed.

2.12 RNA Isolation of Whole Lung Tissue

Lungs were taken from both OVA-treated and saline control mice and flash frozen in liquid nitrogen. Total RNA was isolated using TRIzol reagent according to the manufacturer’s instructions. Briefly, after samples were homogenized in 1mL TRIzol, 200µL of chloroform was added followed by vigorous mixing by inversion for 15 sec and then resting at room temperature for 10 min. The samples were centrifuged at 14,000 X g for 15 min at 4ºC. The upper of the three resulting phases was transferred to fresh Eppendorf tubes and 50µL of isopropanol added. The tubes were inverted to mix their contents and left at room temperature for 5min. The samples were centrifuged at 14,000 X g for 10 min at 4ºC and the Isopropanol removed by aspiration. The RNA pellets were washed 3 times in 100 µL ice cold 75% (vol/vol) ethanol followed by centrifugation at 14,000 g for 5 min at 4ºC, air dried, and then dissolved in 35µL RNase-free DEPC-treated water at 60ºC for 15 min. Optical density measurements and gel electrophoresis
analysis of 5 µg samples from each pool on a 1% (wt/vol) MOPS agarose gel was used to ensure quality RNA was obtained.

2.13 Reverse Transcriptase

2 µg of total RNA from each sample was reversed transcribed with random heximers using the Superscript II RT enzyme. In the first step the RNA sample, DEPC water, 1.25 µL of random heximers, and 1.25 µL of dNTP (total volume of 12.5 µL) were incubated at 65ºC for 5min to denature the RNA. The samples were then placed directly on ice. In step two, 5 µl of 5x Standard Buffer, 2.5 µl of 0.1M dithiothreitol (DTT), 1.25 µl RNase Inhibitor, and 1.25 µl of Superscript II was added to each sample to bring the total reaction volume to 25µL. The reaction was incubated at 42ºC for 1h. In step three, incubating the samples at 70ºC for 15min stopped the reaction. The samples were then stored at -20 ºC until needed.

2.14 PCR Reaction

2 µg of cDNA was added to a master mix of 10 µl of 5x Buffer, 4 µl of MgCl, 4 µl of dNTP, 2 µl of commercial Taq polymerase, and 1 µl of the 5-LO (forward primer: ATGGTGCTGAAGCGGTCTAC; reversed primer: AAAGGATGACATIGGCCTTG) and 12-LO (forward primer: CACGTCCATCAAACGATCACC; reversed primer: CCTCACATGGGCTACCAGCAGC) primers. Reaction parameters are as follows: 95ºC for 5 min, 95ºC for 30 sec, 62ºC for 30 sec, 72ºC for 30 sec, 72ºC for 7 min run for 35 cycles. We used
Lipopolysaccharide (LPS) injected rat tissue as our positive control, as LPS is one of the most powerful bacterial virulence factors in terms of pro-inflammatory properties. Many studies have used LPS to induce inflammatory mediator expression \((157, 158)\)

### 2.15 Mass Spectrometry

All LC-MS-MS work, including sample preparation, was kindly done by Denis Reynaud and Michael Leadley at the Analytical Facility for Bioactive Molecules at the Hospital for Sick Children using methods established by Dr. C Pace-Asciak.

#### 2.15.1 Sample Preparation

Lung tissue was kept at -80°C until extraction. 25 mg of frozen tissues were placed into pre-weighed (pierced) siliconized Eppendorf tubes and lyophilized overnight. The weight of lyophilized tissue was then measured, and samples transferred into 15 ml flat bottom siliconized tubes containing 3 ml of 100% ethanol. A mixture of internal standards (deuterated eicosanoids, 1 ng each) was added to each sample. Tissues were homogenized on ice using a Polytron and then centrifuged at 1000 g for 5 minutes. Supernatant was carefully removed using siliconized Pasteur pipets and transferred to a new set of siliconized 15 ml round bottom glass tubes. Pellets were re-suspended in a same volume of ethanol, mixed with a vortex, and after centrifugation, the supernatants were removed and pooled. Ethanol was taken to dryness under nitrogen gas into a fume hood. Residues were dissolved into a small amount of ethanol, 1 ml of double distilled water was added, and after mild acidification, eicosanoids were extracted 3 times with ethyl acetate. For each extraction, after a short centrifugation, the organic top layers were collected.
using siliconized Pasteur pipets, washed twice with water to neutrality and collected into 15 ml conical siliconized glass tubes. Ethyl acetate was evaporated to dryness under nitrogen gas. Residues from each sample were reconstituted into a mixture of acetonitrile/water (1/1), transferred into siliconized 100 µl inserts with polymer springs with siliconized micro-pipets. Inserts were inserted into 1.5 ml crimp top vial, sealed with aluminum tops with Teflon/rubber septa and kept at -20°C until liquid chromatography mass spectrometry (LC-MS-MS) analysis.

2.15.2 LC-MS-MS

Quantitation was carried out by comparing the deuterium-to-protium ratio of the prostanoids in the sample with standard lines generated from authentic mixtures of eicosanoids. An Agilent HPLC 1100 was at the front end, equipped with a short Zorbax SB-phenyl column (3.0 X 50mm, 3.5-m spherical size; Chromatographic Specialties, Brockville, ON, Canada). The mass spectrometry (MS) source temperature was maintained at 500°C and the ion source voltage at 4,500 V. Compounds were separated on HPLC with a direct inlet into the MS source. HPLC followed the following program: 80:20 (vol/vol) water-acetonitrile at sample injection and maintained for 2 min, 75:25 (vol/vol) for 0.5min, 50:50 (vol/vol) by 5 min, 45:55 (vol/vol) by 6.2 min, and 0:100 (vol/vol) by 11 min. The latter solvent was maintained for another 1.5 min before being replaced by 80:20 (vol/vol) water-acetonitrile for the next run. The flow rate was at 400 l/min. MSMS parameters were established through infusion (20 µl /min) of each authentic standard (Table 1) separately. The Q1 spectrum was first obtained, followed by selection of the M-1 fragment ion, and recording of a Q3 spectrum after collision-induced decomposition (CID). Optimization of the parameters was carried out either manually or by running the quantitative optimization program to establish conditions for use in the analysis by the metabolic rate monitor. The CID gas was nitrogen. Authentic standards in appropriate dilutions (1 ng deuterated
prostanoids of interest mixed with 50 pg-1 ng of undeuterated prostanoids) were prepared, and standard concentrations of eicosanoid were analyzed at the same time as the samples containing unknown amounts of the compound. Typically, 1 ng of deuterated standard was added to each unknown sample, and 20% (vol/vol) of the sample was injected for analysis. Samples were then analyzed and data presented as ng/25mg tissue.

2.16 Data Presentation

All values are presented as mean ± standard error of the mean (SEM) with at least four animals per test group. One-way analysis of variance (ANOVA) was used to determine statistical significance (p<0.05), followed by post-hoc analysis using Tukey test when significant differences were found between groups.
<table>
<thead>
<tr>
<th>Analyte Peak Name (By Elution Order)</th>
<th>Analyte Mass Transitions (amu)</th>
<th>Internal Standard Peak Name</th>
<th>Internal Standards Mass transitions (amu)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>TXB2-D4</td>
<td>373.2/169.0 amu</td>
</tr>
<tr>
<td>PGF2</td>
<td>353.2/309.2 amu</td>
<td>PGF2-D4</td>
<td>357.3/313.2 amu</td>
</tr>
<tr>
<td>PGE2</td>
<td>351.3/271.3 amu</td>
<td>PGE2-D4</td>
<td>355.3/275.4 amu</td>
</tr>
<tr>
<td>PGD2</td>
<td>351.3/189.1 amu</td>
<td>PGD2-D4</td>
<td>355.3/193.2 amu</td>
</tr>
<tr>
<td>LTB4</td>
<td>335.3/317.3 amu</td>
<td>LTB4-D4</td>
<td>339.2/321.4 amu</td>
</tr>
<tr>
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<td>5HETE-D8</td>
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</tr>
<tr>
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<td>12HETE-D8</td>
<td>327.3/184.1 amu</td>
</tr>
<tr>
<td>15HETE</td>
<td>319.3/175.1 amu</td>
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<td>303.2/259.3 amu</td>
<td>AA-D8</td>
<td>311.3/267.3 amu</td>
</tr>
</tbody>
</table>

amu = atomic mass unit

Table 1. Internal standards for the desired arachidonic acid pathway metabolites.
3 Results

3.1 Methyl Ester HxA<sub>3</sub> Significantly Decreased Collagen Induced Platelet Aggregation Compared to the free acid form of HxA<sub>3</sub> and HxB<sub>3</sub>

We initially tested whether hepoxilins have a direct effect on ASM contractility using a commercially made free acid form of HxA<sub>3</sub>. When no significant effect was seen (data not shown), we wanted to determine whether the free acid was still biologically active. To test biological activity, both HxA<sub>3</sub> and HxB<sub>3</sub> (free acid form) were added to a collagen platelet suspension and platelet aggregation was measured for 6 minutes. We also tested the methylated form of HxA<sub>3</sub> kindly provided by Dr. Pace-Asciak at the Hospital for Sick Children to compare biological activity between the two forms.

Results showed that the methyl ester form of HxA<sub>3</sub> significantly reduced the collagen-induced aggregation of human platelets. In contrast, neither of the free acid forms of hepoxilins appeared to significantly inhibit platelet aggregation (Figure 11). Because the methyl ester was shown to be more biologically active, the methyl ester form of HxA<sub>3</sub> was used in all further experiments.
Figure 11. The biological activity of hepxilins. To test the biological activity of both the commercially bought FA HxA3 and B3, and the methylated HxA3 provided by Dr. Pace-Asciak, each was mixed with collagen to determine their effect on collagen induced platelet aggregation. After being stirred for 2 minutes, collagen was added. The collagen only control saw 88% platelet aggregation occur. The methylated HxA3 was the most biologically active, reducing aggregation to 11%. In contrast, the FA forms had very little biological activity, reducing platelet aggregation to around 60% (HxA3) and 70% (HxB3).
3.2 Hepoxilins Had no Direct Effect on Smooth Muscle Contractions in Naïve Mice

After isolating the fourth generation pulmonary bronchi, force generation measurements were taken using a Myograph. After allowing the bronchi to bathe in Krebs solution for 1h, varying doses ($10^{-8}$ to $10^{-6}$ M) of methylated HxA$_3$ were added to the solution and contraction measurements were taken. The contraction force measurements confirmed that HxA$_3$ did not have a direct effect on airway smooth muscle of naïve mice, as there was no significant difference between the bronchi bathed in HxA$_3$ and the untreated controls (Figure 12).
Figure 12. HxA3 contraction graph. Isolated bronchi were bathed in hepoxilin concentrations ranging from 10^-8 to 10^-6 M. N=4.
3.3 Higher 12-LO Protein Concentrations Found in OVA-treated Lungs

Western blot analysis was used to assess the concentration of 12- and 5-LO in both OVA-treated and saline control lungs. Approximately 50 µg of protein from each sample was loaded into each well. Results indicate that both 12- and 5-LO protein expression was higher in OVA treated samples vs. controls (Figure 13). To ensure equal loading and transfer, the membrane was also blotted with β-actin (Figure 13).
Figure 13. 12-LO and 5-LO western blot. Lanes 1 and 2 represent saline treated control tissue. Lanes 3 and 4 have asthma tissue. Each lane was loaded with 50 mg of protein. β-actin was used to determine equal loading and transfer.
3.4 Bronchiolar Airway Epithelium is one of the cellular Sources of Hepoxilin Production in OVA-treated Lungs

In order to determine where in the lung 12-LO and 5-LO are present, frozen tissue sections were stained with either 12- or 5-LO antibodies. The sections were also stained with the epithelial marker CC10. Results show that in the saline controls little to no 12-LO and 5-LO reactivity was noted in larger airways, whereas both 12- and 5-LO could be seen in the large airways from OVA-treated lungs (Figures 14 and 15). Both the 5 and 12-LO co-localized with CC10, a bronchial epithelial marker, in the large airways (Figures 16 and 17). These findings appear to support our initial hypothesis that the bronchiolar airway epithelium is the source of hepoxilin production in OVA-treated lungs.

To further strengthen our immunofluorescence findings, we used LCM to carefully isolate the large airway epithelial layer from both OVA-treated and saline-treated frozen tissue slices. Following RNA extractions, RT-PCR showed that 12- and 5-LO mRNA was mainly expressed in the larger airway epithelium of OVA-treated tissue, with no expression seen in saline-treated tissue (Figure 18). GAPDH was also used to demonstrate that mRNA was present in all LCM-extracted samples (Figure 18).
Figure 14. 12-LO immunofluorescence staining in frozen lung tissue. Saline controls are column 1. OVA-treated mice are in column 2. Strong 12-LO signal can be seen in the bronchiolar epithelium of the OVA-treated mice, with little to no signal seen in the controls. 1:100 dilution factor was used.
Figure 15. 5-LO immunofluorescence staining in frozen lung tissue. Saline controls are shown in column 1 and OVA-treated mice are in column 2. Strong 5-LO signal can be seen in the bronchiolar epithelium of the OVA-treated mice, with little to no signal seen in the controls. 1:200 dilution factor was used.
Figure 16. CC10 and 12-LO immunofluorescence staining. CC10 was used because it is a bronchiolar epithelial marker. 12-LO signal can be seen in the same region as CC10, supporting our hypothesis that the large airway epithelium is a possible source for 12-LO production in asthmatic airways.
Figure 17. CC10 and 5-LO immunofluorescence staining. CC10 was used because it is a bronchiolar epithelial marker. 5-LO signal can be seen in the same region as CC10, supporting the theory that the large airway epithelium is a possible source for 5-LO production in asthmatic airways.
Figure 18. 12-LO and 5-LO RT-PCR using LCM-captured bronchiolar epithelial tissue. The first 3 lanes contain saline-treated samples. The next 3 lanes have mRNA from OVA-treated samples. The positive control (PC) is LCM captured mRNA from an LPS-injected rat. The negative control is water only. No 12-LO or 5-LO expression can be seen in samples from saline-treated mice. 12-LO and 5-LO expression is present in the OVA-treated samples. N=3 per saline and OVA group.
3.5 Inhibition of the 12-LO Pathway Significantly Reduces Total Lung Resistance in Asthmatic Mice

For the purpose of data presentation, the saline only controls and the original OVA mouse models are included to show the regular response to OVA. The groups that follow are ‘Vehicle Saline’, which are mice injected with the drug vehicle and exposed to saline, and then ‘Vehicle OVA’, which are our sensitized and challenged mice injected with the drug vehicle, and finally ‘Baicalein OVA’ and ‘MK-886 OVA’, which are our OVA sensitized and challenged mice injected with the respective drugs. Resistance measurements were also taken for ‘Baicalein Saline’ and ‘MK-886 Saline’, which are mice injected with drug and exposed to saline. No significant difference was seen between these groups and saline only.

As previously mentioned, we initially injected 3 mg/kg baicalein per mouse, but found that this dose had no significant impact on reducing total lung resistance in OVA-treated mice. After doubling the dose to 6 mg/kg baicalein per mouse, resistance measurements showed that OVA mice treated with the 12-LO inhibitor baicalein had reduced total lung resistance compared to OVA mice injected with vehicle only (Figure 19). Similar results were seen in OVA-treated mice with the 5-LO inhibitor MK-886 compared to OVA-treated mice injected with vehicle only (Figure 20).

Resistance measurement for the OVA-treated vehicle group was also significantly higher than the OVA only group (Figures 19 and 20). This data indicates that the vehicle for both baicalein and MK-886 increased methacholine sensitivity. A possible explanation is DMSO causes an increase in inflammatory mediators, such as 5-HETE and prostaglandins. This theory is
supported by the mass spectrometry data showing a higher level of AA metabolites in vehicle-
treated OVA samples compared to non-treated OVA only samples.
Figure 19. Methacholine vs. resistance for the 12-LO inhibitor baicalein. *p<0.05 for Baicalein OVA compared to Vehicle OVA. N=6.
Figure 20. Methacholine vs. resistance for the 5-LO inhibitor MK-886. *p<0.05 for MK-886 OVA vs. Vehicle OVA. N=6.
3.6 Baicalein Significantly Reduces 12-HETE and Several other Arachidonic Acid Metabolites in Whole Lung Tissue

For the purpose of data presentation, the saline only controls and the original OVA mouse models are included as the two first bars in the graphs to show the regular response to OVA. The groups that follow are ‘Vehicle Saline’, which are mice injected with the drug vehicle and exposed to saline, and then ‘Vehicle OVA’, which are our sensitized and challenged mice injected with the drug vehicle, and finally ‘Baicalein OVA’ and ‘MK-886 OVA’, which are our OVA sensitized and challenged mice injected with the respective drugs. Measurements were also taken for ‘Baicalein Saline’ and ‘MK-886 Saline’, which are mice injected with drug and exposed to saline. No significant difference was seen between these groups and saline only.

Interestingly, mass spectrometry analysis revealed that vehicle alone (saline vs. vehicle saline and OVA vs. vehicle OVA) increased in inflammatory metabolites. Mass spectrometry analysis also showed that the 12-LO inhibitor baicalein is not specific to 12-LO, but instead inhibits several of the arachidonic acid inflammatory pathways. Both 12-HETE and HxA3 were significantly reduced (Figures 21 and 22). Figures 23 and 24 also show that baicalein significantly reduced both the 5- and 15-LO pathways based on HETE measurements. Further analysis of prostaglandins and thromboxane showed that baicalein also significantly reduced thromboxane B2 and prostaglandins D2 and E2 (Figures 25, 26, 27). In contrast, the 5-LO inhibitor MK-886 only inhibited 5-HETE (Figure 22), confirming MK-886 as a 5-lipoxygenase specific inhibitor. While baicalein did not significantly reduce arachidonic acid (data not shown) the concentration of all samples, including saline controls, were so high that we believe saturation of the mass spectrometer detector may have occurred.
Figure 21. Effects of baicalein and MK-886 on 12-HETE formation in whole lung tissue. *p<0.05 for Baicalein OVA compared to Vehicle OVA. N=4.
Figure 22. Effects of baicalein and MK-886 on HxA3 formation in whole lung tissue. *p<0.05 for Baicalein OVA compared to Baicalein Vehicle. N=4
Figure 23. Effects of baicalein and MK-886 on 5-HETE expression in whole lung tissue. *p<0.05 for MK-886 OVA compared to Vehicle OVA and **p<0.05 for Baicalein OVA compared to Vehicle OVA. N=4.
Figure 24. Effect of baicalein and MK-886 on 15-HETE formation in whole lung tissue. *p<0.05 for Baicalein OVA compared to Vehicle OVA. N=4.
Figure 25. Effects of baicalein and MK-886 on TXB2 formation in whole lung tissue. *p<0.05 for Baicalein OVA compared to Vehicle OVA. N=4.
Figure 26. Effects of baicalein and MK-886 on PGD2 formation in whole lung tissue. *p<0.05 for Baicalein OVA compared to Vehicle OVA. N=4.
Figure 27. Effect of baicalein and MK-886 on PGE2 formation in whole lung tissue. *p<0.05 for Baicalein OVA compared to Vehicle OVA. N=4.
3.7 Baicalein Significantly Reduces 12-HETE and 5-HETE Expression in BAL

Mass spectrometry analysis was also carried out in BAL samples to determine if baicalein specifically inhibited 12-LO activity in airway epithelium. As in our whole lung tissue analysis, baicalein reduced both 12- and 5-HETE expression compared to vehicle-injected OVA controls (Figure 28), giving further support that baicalein is not specific to the 12-LO pathway. Once again, MK-886 appears specific to the 5-LO pathway (Figure 29).
Figure 28. Effects of baicalein and MK-886 on 12-HETE formation in BAL. Baicalein appears to reduce 12-HETE expression in Baicalein OVA compared to the Vehicle OVA group. N=2.
Figure 29. Effects of baicalein and MK-886 on 5-HETE expression in BAL. Both baicalein and MK-886 appear to reduce 5-HETE expression in BAL. N=2
4 Discussion

4.1 Assessment of Hepoxilins on Smooth Muscle Contractions

We had initially hypothesized that the addition of hepoxilins to the Krebs solution would potentiate methacholine-induced contractions of the fourth generation bronchi (Figure 30) as earlier experiments (104) had found that HxA₃ potentiated neurokinin A-induced contractions in guinea pig trachea. Laneuville et al. (103) also showed that hepoxilins potentiated the contractile effects of noradrenaline on the rat thoracic aorta. According to the authors, these findings support the theory that the 12-LO pathway is important for modulating control of inflammation and contraction. Our research, however, found that hepoxilins have no direct effect on fourth generation bronchi (Figure 30) in naïve mice.

There are several reasons why there was no change to bronchial contractions with the addition of hepoxilins. The first is that while hepoxilins might act as cellular messengers, they do not act on any receptors specific to the bronchi. Another possibility, while highly unlikely, is the difference in constricting agents used. Neurokinin-A is the predominant neuropeptide released from sensory nerve endings by mechanical inflammatory stimuli (159, 160). When inhaled, neurokinin has been shown to induce rapid bronchoconstriction in both asthmatic and non-asthmatic subjects (161). Similarly, methacholine is also listed as a powerful bronchoconstrictor that acts mainly on the central airways by stimulating muscarinic receptors, resulting in smooth muscle contraction (162). Methacholine is the primary bronchoconstricting agent used for asthma tests because methacholine acts directly on bronchial smooth muscle cells, while substances such as neurokinin act on other cell types such as mast cells and neurons (163). While a difference in
target cells between the two bronchoconstrictors could explain the different results, this explanation seems unlikely, as our research has shown the bronchial epithelium to be a source of 12-LO in OVA-treated lungs. If anything, therefore, the methacholine should have had more of an effect than the neurokinin because it acts directly on bronchial smooth muscle.

Another possibility for the different results is inter-species variation. Research has shown that airway responsiveness to methacholine is variable among species, due in part to the differences in airway smooth muscle (ASM) (164). Guinea pigs have more ASM than several other species of animal (165), which leads us to believe that they would be more sensitive to any potential effects that the hepoxilins might have on ASM. Additionally, the size of the tissue used could have also been a contributing factor to the different results, as the effects of agonists are usually greater in the larger airways (164). For the purpose of our research we chose to focus on the bronchi because of the central role it plays in asthma. While repeating this experiment using the trachea could have given us different results, it would have little bearing to our research, as the trachea plays a limited role in asthma. Finally, although we found hepoxilins had no direct effect on smooth muscle contractility in un-sensitized mice, hepoxilins might effect OVA-sensitized animals. Hepoxilins could work synergistically with the OVA-induced airway inflammation to increase methacholine sensitivity. Further investigations are required.
4.2 Increased Concentrations of 12-Lipoxygenase in Asthmatic Lung Tissue

Western blot analysis showed a higher concentration of 12-LO in OVA-treated lung tissue compared to saline treated tissue. These results are important as they show that increased 12-LO is associated with OVA-induced airway inflammation. These findings support human studies that have examined the expression and activation of 15-lipoxygenase pathway in severe asthma. Chu et al. (78) Profita et al. (86) have found that sputum and BAL from severe asthmatics contained significantly higher levels of 15-HETE than sputum and BAL from non-asthmatic controls, indicating that 15-HETE in humans is associated with airway fibrosis.

We used 5-LO for comparison because 5-LO is recognized as an important inflammatory pathway in asthma, and studies show that 5-LO is increased in asthmatic airways (2, 108). As previously discussed, 5-LO acts to convert arachidonic acid to the intermediate 5-HPETE, which is then converted into LTA<sub>4</sub>. LTA<sub>4</sub> can then form several classes of leukotrienes, including LTB<sub>4</sub>, a potent chemoattractant compound that promotes the recruitment of eosinophils into the lung and stimulates mucus secretion, contributing to the inflammatory processes observed in asthma (2, 30, 33, 38, 44, 69, 166-168). Western blot analysis of our samples showed that the 5-LO protein content was also increased in OVA-sensitized tissue compared to controls.
4.3 Bronchiolar Airway Epithelium is a Source of Hepoxilins in Airways of OVA-Treated Mice

RT-PCR analysis of LCM-captured tissue from upper airway bronchi (estimated 1\textsuperscript{st} and 2\textsuperscript{nd} generation) (Figure 30) identified large airway epithelium as a source of 12-LO mRNA, and consequently hepoxilin production in OVA-sensitized mice. These findings support our earlier hypothesis that the bronchiolar epithelium is the source of hepoxilins in OVA lungs. As previously discussed, LCM is a reliable method to procure pure populations of targeted cells from very specific regions of tissue (169). It is with some level of confidence, therefore, that we were able to accurately isolate only epithelium tissue from the airway. Furthermore, RT-PCR analysis of captured tissue from control lungs was negative.

In addition to RT-PCR analysis, immunofluorescence staining was used to identify 12-LO in large airway epithelium. The 12-LO immunostaining was done in conjunction with CC10. In mice, clara cells make up more than 90% of the cells in bronchial epithelium. The double stain showed that 12-LO was present in the bronchiolar epithelial Clara Cells, but not in peripheral small airway epithelium, giving further support to our initial hypothesis that the bronchiolar epithelium is the source of 12-LO production.

Finding 12-LO mRNA and protein in bronchiolar airway epithelium suggests that large airway epithelium is the source of hepoxilin production in OVA-treated lungs, supporting the earlier Mrsny et al (110) study which identified the intestinal epithelium as the source of hepoxilin production. In addition to identifying HxA\textsubscript{3} in intestinal epithelium, Mrsny (110) also found that once released from the apical surface of an infected mucosal surface, HxA\textsubscript{3} established a chemical gradient across the epithelial tight junction, stimulating PMN transmigration without
Figure 30. A schematic diagram illustrating upper airway bronchi. First generation Balb/c mouse bronchi are approximately 700-800 μm in diameter, with each successive generation being reduced in size.
premature degranulation. It is this degranulation of PMNs that is suspected of imparting much of the inflammation associated with mucosal infection (170). Mrsny (110) postulated that the orchestration of PMN movement by the intestinal epithelium likely plays a key role in both innate immune responses to food-borne pathogens and in events mediating active flares associated with inflammatory bowel disease. Our research indicates that like intestinal inflammation, hepxilins are secreted from the surface of asthmatic lungs as a response to infection. Whether HxA₃ in the lung acts in a similar way to stimulate PMN transmigration remains to be determined.

4.4 Inhibiting the 12-Lipoxygenase Pathway Reduces Total Lung Resistance in an Asthma-Like Murine Model

As previously mentioned, we initially chose to inject 3 mg/kg per mouse of baicalein, but initial total lung resistance measurements showed that the drug was having no significant effect at this concentration. Upon doubling the concentration, however, resistance measurements of mice treated with the 12-LO inhibitor baicalein showed a significant decrease in resistance measurements compared to vehicle treated OVA groups. (Figure 18). These findings suggested to us that 12-LO plays a role in asthma inflammation, and agreed with earlier work conducted by Mrsny et al. (110) which found the 12-LO product, HXA₃, acted as a pathogen-elicited epithelial chemoattractant (PEEC) in mucousal inflammation.

Subsequent mass spectrometry analysis on whole lung tissue, however, showed that in addition to inhibiting 12-HETE, baicalein also appeared to inhibit the formation of 5- and 15-HETE, several prostaglandins, and thromboxane (Figures 20-26). Analysis of BAL samples also found a
similar trend, with baicalein reducing both 12- and 5-HETE formation. This data indicates that baicalein is not 12-LO specific in either whole lung tissue or airway epithelium. In contrast, 5-LO inhibitor MK-886 only affected 5-HETE formation, indicating 5-LO specificity. It should also be noted that neither baicalein nor MK-886 significantly inhibited LTB₄ (data not shown). However, the LTB₄ graph follows a similar trend to the 5-HETE graph (Figure 22) where both MK-886-treated mice and baicalein-treated mice have much lower concentrations of LTB₄ than vehicle-treated mice.

Our findings were unexpected, as baicalein has been cited as a potent and specific 12-LO inhibitor. None of the earlier studies, however, that used baicalein as a 12-LO inhibitor (110, 171) examined any possible inhibitory effects the drug might have on other inflammatory pathways as we have done.

The question that arises from this study is why did baicalein inhibit almost all of the prostanoids tested? The most obvious answer is that because all prostanoids are formed from arachidonic acid, baicalein acts high enough upstream that all inflammatory pathways arising from arachidonic acid are also affected. As previously mentioned, baicalein is a component of *Scutellariae baicaleinensis* root (or Ogon in many East Asian cultures). Ogon has been listed as a remedy for suppurative dermatitis, diarrhea, and inflammatory diseases in many ancient Chinese texts (172).

A very early study by Sekiya and Okuda (172) found that the addition of baicalein to rat platelets reduced 12-HETE expression compared to non-baicalein treated platelets. IC₅₀ values, however, indicated that baicalein was not selective for 12-LO. Sekiya and Okuda (172) postulated that baicalein might also act as a 5-lipoxygenase inhibitor. A later study by Deschamps and colleagues (173) conducted several tests to determine how effective baicalein was as an inhibitor.
Results of IC$_{50}$ analysis of human platelets also showed that baicalein was not selective against 12-hLO in-vitro.

Kyo and colleagues (174) found that baicalein inhibited the release of arachidonic acid. Nakahata et al. (175) examined the inhibitory effects of baicalein using rat C6 glioma cells to show that the addition of baicalein significantly reduced the release of PGE$_2$. This data in addition to our own mass spectrometry analysis indicates that in order to inhibit all of these pathways the baicalein must either affect arachidonic acid metabolism, or perhaps further upstream at the release step.

A possible mechanism to explain this inhibition is through the MAPK-cPLA$_2$ pathway (Figure 31). Mitogen-activated protein kinase (MAPK) is a family of serine/threonine kinase that plays a role in the arachidonic acid cascade. The Nakahata study used a dose response model to examine the effect baicalein had on the inflammatory cascade. Western blot analysis showed that 30 and 100 µM concentrations of baicalein reduced MAPK phosphorylation in a concentration dependent manner. We feel that further studies are required in our model, however, as the uniformly high arachidonic acid values indicates that the high amounts detected in our assay may have masked any small, localized changes which would parallel the reductions observed for 12-HETE and the other eicosanoids. Additionally, while our own study did not include a dose response analysis of baicalein, we can look to the earlier Nakahata study to support the idea that changing the concentration of baicalein would alter the drugs’ inhibiting effects. Consequently, our results appear to support the idea that baicalein is a potent inhibitor of arachidonic acid release, and consequently several of the metabolite pathways that arise from it.
Figure 31. Baicalein acts to inhibit MAPK kinase kinase (MEKK), thereby reducing MAPK phosphorylation, reducing the cPLA2-MAPK cascade. Cytosolic phospholipase A2 (cPLA2) is involved in cell signalling, leading to the eventual release of arachidonic acid (AA).
4.5 Conclusion

Our study shows that the concentration of 12-LO is increased in ovalbumin-sensitized airways compared to controls and that bronchiolar airway epithelium is the source of hepoxilin production in OVA-treated lungs. Unfortunately, we were unable to fully ascertain how blocking the 12-LO pathway would affect asthma inflammation since the only known 12-LO inhibitor has been shown to in fact block several inflammatory pathways in addition to 12-LO in whole lung tissue. Andersson et al. (43) showed that 12-LO knockout mice exhibit significantly reduced airway inflammation and remodeling compared to wild type controls. Combined, these studies demonstrate that 12-LO plays a role in asthma inflammation (Figure 32) likely mediated through its biologically potent hepoxilin A₃. Additionally, our research showed that baicalein is a potent inhibitor of the arachidonic acid inflammatory cascade.

4.6 Future Experiments

Although our research has answered several questions relating to hepoxilins and asthma, there are still questions to be answered. For example, now that we have shown 12-LO is present in asthmatic airways, future experiments should investigate the importance of hepoxilin A₃ in asthma, including whether reducing the actions of hepoxilins would also reduce resistance and inflammation associated with asthma. Analogs of hepoxilins (PBTs), where the cyclopropyl group has replaced the epoxide moiety at C11 and C12, have been shown to antagonize the actions of natural hepoxilins. Pace-Asciak and colleagues (176) showed that PBTs inhibit the mobilization of intracellular calcium evoked by HxA₃ in human neutrophil suspensions.
Figure 32. A schematic diagram illustrating that baicalein helps to reduce 12-lipoxygenase expression in OVA-sensitized bronchiolar epithelium.
Administering these PBTs prior to the onset of acute asthma to determine if they can control the onset of inflammation and hyperresponsiveness would allow for the identification of another potential asthma therapy.

Additionally, while our research has disproved the claim that baicalein is a 12-LO specific inhibitor, we have shown that the flavonoid is potentially a powerful inhibitor of arachidonic acid release and consequently several of its metabolite pathways. To further verify the usefulness of the drug for treating asthma, more research examining its dose response is required.
References


