THE ROLE OF POLYUNSATURATED FATTY ACID AND EICOSANOID BIOSYNTHESIS IN THE PATHOGENESIS OF CYSTIC FIBROSIS

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ABBREVIATIONS

CF…… Cystic Fibrosis
CFTR…… Cystic Fibrosis Transmembrane Regulator
ENaC…… Epithelial Sodium Channel
PI…… Pancreatic Insufficient
PS…… Pancreatic Sufficient
FEV_1…… Forced Expiratory Volume in 1 Second
PUFA……Polyunsaturated Fatty Acid
POA…….. Palmitoleic Acid
OA……Oleic Acid
MA…….. Mead Acid
LA…… Linoleic Acid
LNA……... Alpha-Linolenic Acid
AA…… Arachidonic Acid
EPA…….. Eicosapentaenoic Acid
DHA…….. Docosahexaenoic Acid
Δ5D……Δ5-Desaturase
Δ6D……Δ6-Desaturase
$cPLA_2$……... Cytosolic Phospholipase A_2
COX-2…….. Cyclooxygenase-2
mPGES-1……Microsomal Prostaglandin E_2 Synthase-1
5-LOX……... 5-Lipoxygenase
LTA_4H…….. Leukotriene A_4 Hydrolase
FLAP……5-Lipoxygenase Activating Protein
LC-MS/MS…….. Liquid Chromatography-Tandem Mass Spectrometry
PGE_2……Prostaglandin E_2
LTB_4…… Leukotriene B_4
LTC4…… Leukotriene C_4
LTD4…… Leukotriene D_4
LTE4…… Leukotriene E_4
LXA_4…… Lipoxin A_4
RvE……Reslovin E
RvD…… Resolvin D
NPD…… Neuroprotectin
MaR……Maresin
GPCR…….. G Protein-Coupled Recptor
EP…… E Prostanoid Receptor
BLT…… LTB_4 Receptor
CysLT…….. Cysteinyl Leukotriene Receptor
IL…… Interleukin
PaF…… *Pseudomonas aeruginosa* Filtrate
A23187…… Calcium Ionophore
NS-398……COX-2 Selective Inhibitor
AA-861…… 5-LOX Selective Inhibitor

MK-886…….. FLAP Selective Inhibitor
SC-57461-A……LTA_4H Selective Inhibitor

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CHAPTER I

INTRODUCTION

Cystic Fibrosis

Disease History and Overview

Cystic fibrosis is the most common genetic disorder in white populations, affecting 1 in 2500 live births. It was first described in 1938 as "cystic fibrosis of the pancreas", after autopsy studies of malnourished infants uncovered mucus plugging in glandular ducts[1]. The term "mucoviscidosis" was used as an alternative description of this disease in 1944, due to the accumulation of thick viscous that plugged the ducts of mucus glands[2]. Soon after, CF was considered to be a genetic disorder caused by a defect in a single gene because of its autosomal recessive pattern of inheritance[3]. The discovery of defective sweat sodium and chloride balance meant that the basic defect of the disease was not a disorder of mucus production[4]. In 1983, defective chloride transport accompanied by increased sodium reabsorption was identified as the main culprit in the development of CF[5]. This was verified by the discovery of the CF gene in 1989, which encoded a chloride channel named the cystic fibrosis transmembrane conductance regulator (CFTR)[6]. When CF was first described in 1938, the life expectancy of a CF patient was

around 6 months[2]. However, with the advancement in therapy and care, the life expectancy has risen to 37 years of age in the United States[7].

CFTR

CFTR is an ion channel and a member of the ATP-binding cassette (ABC) superfamily that is widely expressed at the apical side of epithelial cells[8]. It consists of two membrane-spanning domains (MSD) with six transmembrane helices each, two nucleotide-binding domains (NBD), and a regulatory domain (RD)[9]. *CFTR* is regulated by cAMP-dependent phosphorylation of RD by protein kinase A (PKA), which allows ATP binding to NBD1 and NBD2 and the consequent dimerization of the two domains to form a pore that allows for ion movement across the channel[10].

CFTR Mutation Classes

Since the discovery that CF is caused by mutations in the gene encoding CFTR, there have been close to 2000 identified mutations[11]. Mutations fall into six classes based on the general mechanism of *CFTR* dysfunction[10]:

- Class I mutations, defective protein production: These are nonsense, frame shift and splicing mutations that lead to premature transcription termination causing defective protein synthesis.
- Class II mutations, defective protein processing: the missense mutations in this class disrupt the protein folding and trafficking to the apical membrane.
- Class III mutations, defective channel regulation: Mutated *CFTR* protein is produced, processed and delivered properly to the cell membrane. However, the regulation of the channel is disrupted so that it no longer opens in response to channel agonists.
- Class IV mutations, defective channel conduction: like class III mutations, *CFTR* in this class is delivered correctly to the membrane; however, the mutations produce a misshaped channel pore that restricts ion movement.
- Class V mutations, reduced protein synthesis: *CFTR* protein produced by this class of mutations is produced at extremely reduced levels resulting in less protein at the cell membrane.

- Class VI mutations, reduced protein stability: although *CFTR* reaches the membrane, mutations of this class cause a reduction in protein stability and increase its turnover, which results in low CFTR levels.

A phenylalanine deletion at position 508 (ΔF508) causes the most common *CFTR* mutation[7]. Nearly 90% of CF patients carry at least one copy of this mutation[11]. ΔF508 is considered a class II mutation, where the protein is misfolded and tagged for degradation without reaching the membrane. However, it is a complex mutation that has characteristics of Classes III and VI in that even if the protein reaches the membrane it will have a gating defect and will be unstable[11]. Class I and class III mutations account for 7% and 3% of all CF mutations, respectively. Classes I, II, and III cause a complete loss of *CFTR* function and are considered severe, whereas, classes IV, V, and VI allow residual ion transport and are therefore viewed as mild mutations[7].

CF Disease Phenotype and Pathogenesis

The loss of *CFTR* function causes a defect in Cl^- reabsorption by epithelial cells in the reabsorptive duct in the sweat glands, which leads to high salt concentrations in the sweat of CF patients[12]. This disease feature has been used reliably as a diagnostic test for CF[13]. *CFTR* interacts both directly and indirectly with many proteins in the cell[14]. The epithelial sodium channel (ENaC) is responsible for sodium movement across membranes in the opposite direction of Cl^-. Importantly, it is one of the proteins that are affected

by *CFTR*[15]. In this setting *CFTR* activation inhibits the activity of ENaC and prohibits the movement of Na^+. Therefore, with the loss of *CFTR* function, ENaC is no longer restricted, and sodium flow is increased into the cell. This influx of sodium into the cell is followed by water, which decreases liquid volume in the lumen[16], leading to the development of thick viscous mucus that can lead to various organ dysfunctions[17]. The main organs affected in CF are the lungs, pancreas, GI tract, and the male reproductive system. In the GI tract the accumulation of thick mucus causes intestinal obstruction and lead to malabsorption. Meconium ileus is present in 15% of neonates, which if left untreated is lethal.

The fat and protein malabsorption is exacerbated by pancreatic dysfunction in CF[10]. In this organ, the presence of thick dehydrated mucus leads to plugging of the pancreatic ducts and has two major consequences[7]. First, it prohibits the delivery of pancreatic enzymes to the duodenum and therefore the digestion of lipids and proteins is halted and will lead to malnutrition. Second, the trapped enzymes will cause inflammation and autolysis of the exocrine pancreas and its eventual replacement by fat. This leads to pancreatic insufficiency (PI) in almost 90% of CF infants, which is characterized by greasy stools, flatulence, abdominal bloating, and most notably, poor weight gain. PI can be present at birth or may develop and progress over the first year of birth. Males with CF are infertile due to congenital bilateral absence of the vas deferens, which is extremely sensitive to CFTR dysfunction. Increased water reabsorption in the lungs of CF patients

causes mucus plugging and a significant decrease in the airway surface liquid (ASL) volume, which leads to impaired mucocilliary action and chronic infection and inflammation[17].

Cell Culture and Animal Models of CF

Several immortalized cell models of CF have been developed from tissues important in CF pathology from both CF and non-CF individuals. While there are pancreatic-derived cell models, like CFPAC-1 cells, most of the developed cell models are derived from lung epithelial cells, such as IB3-1, CFBE45o-, and 16HBE14o-. [18]. To further our understanding of the complex mechanisms of CF disease in humans, mouse models of this disease were developed. Several mouse models of CF have been developed and extensively used over the past two decades, such as the *CFTR*^{tm1UNC} [19]. While CF mouse models have provided valuable insights in CF pathology, they lack some of the important abnormalities that CF patients develop early in life in organs such as the lungs and pancreas. This has led to the development of pig and ferret models of CF, which develop most of the defect seen in CF patients, including predisposition to lung disease[20,21].

Lung Inflammation in Cystic Fibrosis

The main cause of morbidity and mortality in CF is progressive pulmonary failure that is characterized by a vicious cycle of obstruction, infection, and inflammation, which leads to tissue destruction (Fig 1). As stated above, defective *CFTR* function in the lungs compromises mucociliary clearance, causing obstruction of respiratory passages by thick, viscid mucus, which leads to luminal dilation in the submucosal glands[22]. This creates a suitable environment for non-resolving infections, since pathogens clearance is defective[23]. *Pseudomonas aeruginosa* is the major bacterial pathogen in CF airways[24]. By the time CF patients reach middle teenage years, most are infected with *Pseudomonas* and will subsequently never be free of this pathogen. This is due to the manner by which *Pseudomonas* adapts to the CF airway microenvironment whereby it produces copious amounts of extracellular capsular polysaccharides that gives the bacteria a mucoid appearance and indicates the formation of a biofilm[25]. The development of this phenotype signals an accelerated decline in lung function as it protects the bacteria from host defenses and leads to the formation of immune complexes around the biofilm, which in-turn exacerbates the inflammatory response[26].

Enhanced inflammation appears to play a significant role in CF pathophysiology. The airway epithelium contributes significantly to the inflammatory response by producing a variety of molecules that aid in recruiting immune cells[27]. Infected CF airways exhibit marked inflammation, characterized by profuse neutrophil infiltration and production of pro-

inflammatory oxygenated fatty acid metabolites and cytokines, such as leukotriene B_4 (LTB_4) and interleukin (IL)-1β, IL-6, and IL-8[28]. Recruited neutrophils, in turn, elicit more release of pro-inflammatory mediators and lead to a further increase in the perpetuating inflammatory cycle.

While chronic bacterial infections are the leading cause for neutrophilic inflammation in CF lungs, there may be other possible contributing factors[26]. First, the inflammatory response in CF could be disproportionate to levels of infection. A study that investigated the inflammatory response in CF mice after intrabronchial instillation of PA agarose beads showed that CF mice had a higher mortality rate. In addition, they had a greater concentration of several inflammatory mediators in their BAL fluids in response to the same bacterial load as their WT littermates[29]. Second, the airway epithelium may be inherently pro-inflammatory and the inflammatory response may be increased even in the absence of infection. Several studies have evidence to support this theory. Using a human fetal tracheal graft in-vivo model[30], Tirouvanziam *et al*. showed that uninfected CF airway grafts, but not matched WT controls, underwent neutrophil-driven inflammation that led to lung tissue destruction. Moreover, several studies reported the presence of inflammation independent of the presence of pathogens in the lungs of children that are 15 months or younger[31], infants 6-months of age[32], even in the lungs of a CF fetus[33]. These findings imply that *CFTR* mutations may establish a uniquely pro-inflammatory state, although the mechanisms are poorly understood.

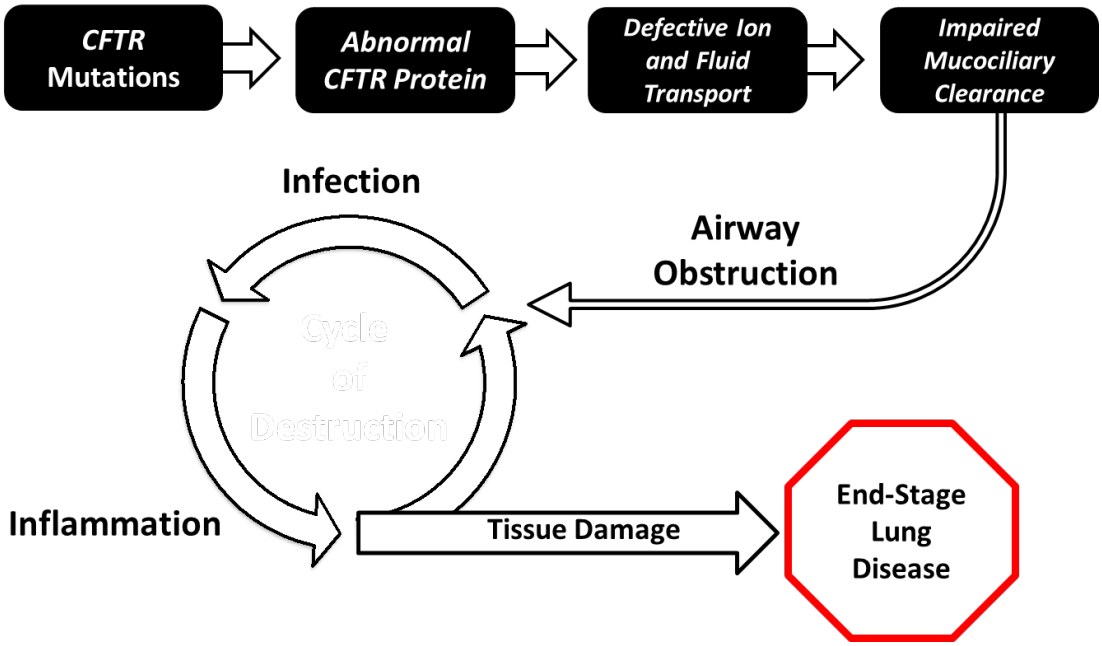

Figure 1:

Lung disease is the main cause of mortality in CF. Lung disease in CF is characterized by a vicious cycle of mucus plugging, chronic infections, and non-relenting neutrophilic inflammation.

Fatty Acids and Their Oxygenated Metabolites in CF

Fatty Acids

A fatty acid is comprised of an aliphatic carbon chain, that can be saturated or unsaturated, connected to a carboxylic group. The term polyunsaturated fatty acid (PUFA) is used to describe any fatty acid with 2 or more double bonds. Unsaturated fatty acids fall into four major groups based on the position of the last double bond from the methyl end. These groups are n-3, n-6, n-7, and n-9, where the last double bond in each group is 3 carbons, 6 carbons, 7 carbons, or 9 carbons away from the methyl end, respectively. While n-7 and n-9 fatty acids can be synthesized de-novo, n-3 and n-6 fatty PUFAs are essential fatty acids (EFA), in that mammals cannot synthesize them and must obtain them from dietary sources[34].

Palmitic acid (16:0) can be the precursor of both the n-7 and the n-9 pathways, where its chain length and desaturation is increased by the actions of elongase and desaturase enzymes (Fig 2). Elongase enzymes add two carbons, while desaturase enzymes remove two hydrogens and introduce a double bond to the fatty acid. Alpha linolenic acid (LNA, 18:3n-6) and linoleic acid (LA, 18:2n-6) are the precursor fatty acids in the n-3 and the n-6 pathways, respectively. Both LNA and LA are converted to their downstream PUFA in the n-3 and n-6 pathways via a series desaturation and elongation followed by a final peroxisomal β-oxidation step (Fig 3). EFA dietary intake levels have great implications in the inflammatory process. In particular, the intake of n-6 PUFA increases the availability of arachidonic acid (AA, 20:4n-6)

in cell membranes and may exacerbate inflammatory diseases by the production of oxygenated metabolites, which play a significant role in inflammation[35]. Since both the n-3 and n-6 pathways compete for the same desaturase and elongase enzymes, the dietary intake ratio of n-6/n-3 PUFA can determine the dominance of each pathway. Historic n-6 to n-3 ratio used to be close to 1:1. However, as the human diet changed this ratio has risen dramatically to be closer to 20:1[36]. Many disorders such as cardiovascular disease, inflammatory and autoimmune disorders have been shown to be enhanced by a high n-6/n-3 ratio, while they were suppressed when that ratio is decreased[37].

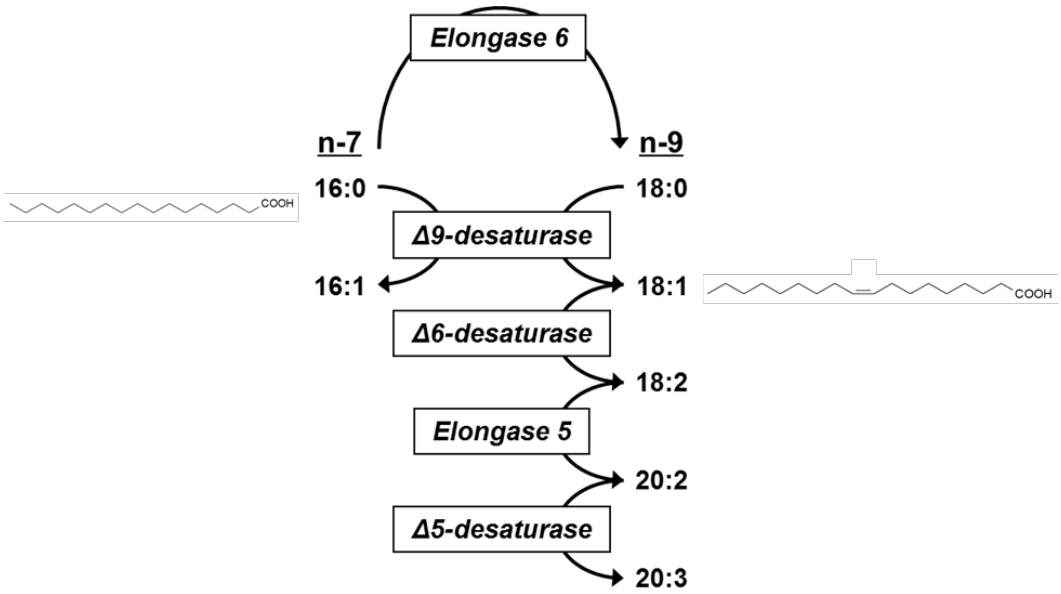

Figure 2:

The n-7 and n-9 biosynthesis pathways.

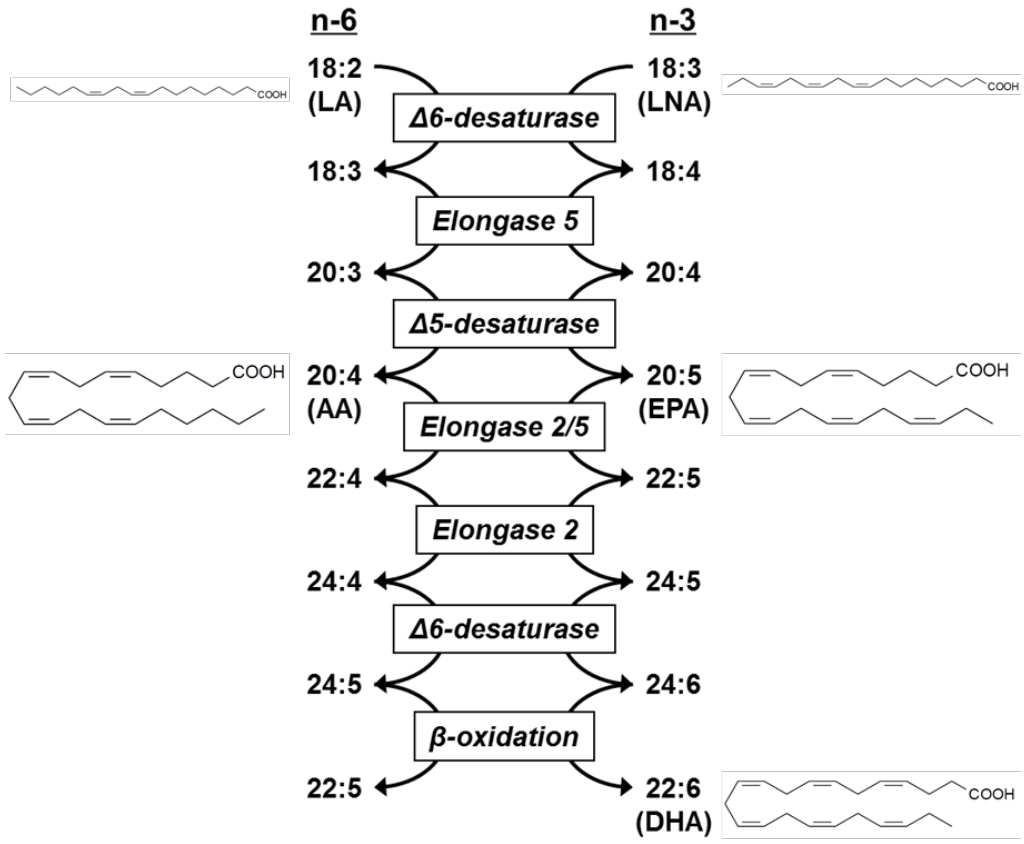

Figure 3:

The n-6 and n-3 biosynthesis pathways.

Oxygenated Metabolites of Fatty Acids

While a small fraction of fatty acids occur as free fatty acids, the majority are esterified to form cholesterol esters, triglycerides, or phospholipids. The latter is usually the most abundant component of cell membranes[38]. Phospholipase A_2 (PLA_2) is the enzyme responsible for hydrolyzing the fatty acid from the *sn*-2 position in membrane phospholipids, which is most frequently occupied by PUFAs, such as AA. PLA_2 enzymes fall into three main categories: cytosolic PLA2 ($cPLA_2$), which has a high molecular weight and depends on micromolar intracellular calcium concentrations, secretory PLA_2 ($sPLA_2$) which is stored in granules and secreted upon activation, has a low molecular weight, and depends on millimolar calcium concentrations for activation, and calcium-independent PLA_2 ($iPLA_2$), which is present in the cytosol and membrane fractions[39]. The enzymatic action of $cPLA_2$ appears to play a pivotal role in the inflammatory process, as it is the only phospholipase that preferentially hydrolyzes *sn*-2 bound AA upon inflammatory stimulation[40].

The importance of $cPLA_2$ relates to the formation of eicosanoids, which are oxygenated metabolites that are produced from 20-carbon PUFAs, with AA as the major substrate. Once AA is released from membrane phospholipids by $cPLA_2$, it is rapidly converted to a variety of metabolites mainly via the enzymatic actions of cyclooxygenase (COX) and lipoxygenase (LOX) pathways (Figs 4,5). AA can be converted into a variety of eicosanoids via the cyclooxygenase pathway that has two main isoforms COX-1 and COX-

2. While COX-1 is constitutive and is ubiquitously expressed, COX-2 is inducible and is expressed in response to stimulation and inflammation. These enzymes convert AA to the unstable intermediate prostaglandin (PG) H_2, which in turn is converted to the biologically active prostanoids PGE_2, PGD_2, $PGF_2\alpha$, PGI_2, and thromboxane (TX) A_2 by specific synthases[41]. There are three distinct isoforms of the PGE_2 synthases (PGES): cytosolic (cPGES), microsomal (mPGES-1) and mPGES-2. Of those enzymes mPGES-1 is an inducible isoform that is associated with COX-2 and inflammation[42].

Lipoxygenase enzymes are classified as 5-, 12, or 15-LOX based on the position of oxygen insertoin in AA. Leukotriene A_4 (LTA_4) is produced from AA via the enzymatic action of 5-LOX, which requires Ca^{2+} for activation, aided by the 5-LOX activating protein (FLAP), which is an accessory protein that binds to the AA released by PLA_2. LTA_4-hydrolase (LTA4H) can convert this unstable intermediate to LTB_4, which is a very potent neutrophil chemoattractant. Alternatively, LTA_4 can be conjugated with glutathione (GSH) by LTC_4 synthase (LTC4S) to form LTC_4, which can be metabolized into LTD_4 and LTE_4. LTC_4, LTD_4, and LTE_4 are called cyteinyl LTs (cysLT) because of their similar structures and biological functions, as they are involved in both bronchoconstriction and vasoconstriction[43].

Another important class of AA-derived eicosanoids is lipoxins (LX), which have anti-inflammatory properties such as inhibiting neutrophil trafficking and promoting macrophage phagocytic actions of apoptotic neutrophils, are produced by two different LOX enzymes that usually come

from two different cell types during cell-cell interactions. For example, AA can be converted to 15-hydroxyeicosatetraenoic acid (HETE) by 15-LOX in epithelial cells, which can then be converted by 5-LOX from infiltrating neutrophils to form LXA_4[44].

Eicosanoids usually signal, in both autocrine and paracrine fashion, via G protein—coupled receptors (GPCR). PGE_2, PGD_2, $PGF_2\alpha$, PGI_2, and TXA_2 signal through E prostanoid (P), DP, FP, IP, and TP, respectively. There are four subtypes of the PGE_2 receptor that differ based on their GPCR signaling. EP_1 signals through G_q and increases free Ca^{2+}, EP_3 signals through G_i and inhibits adenylate cyclase, and finally, EP_2 and EP_4 signal through G_s and increase cAMP concentrations. Similarly, leukotriens signal through GPCRs. LTB_4 signals through BLT_1 or BLT_2, while LTC_4, LTE_4, and LTD_4 signal through $cysLT_1$ or $cysLT_2$[45].

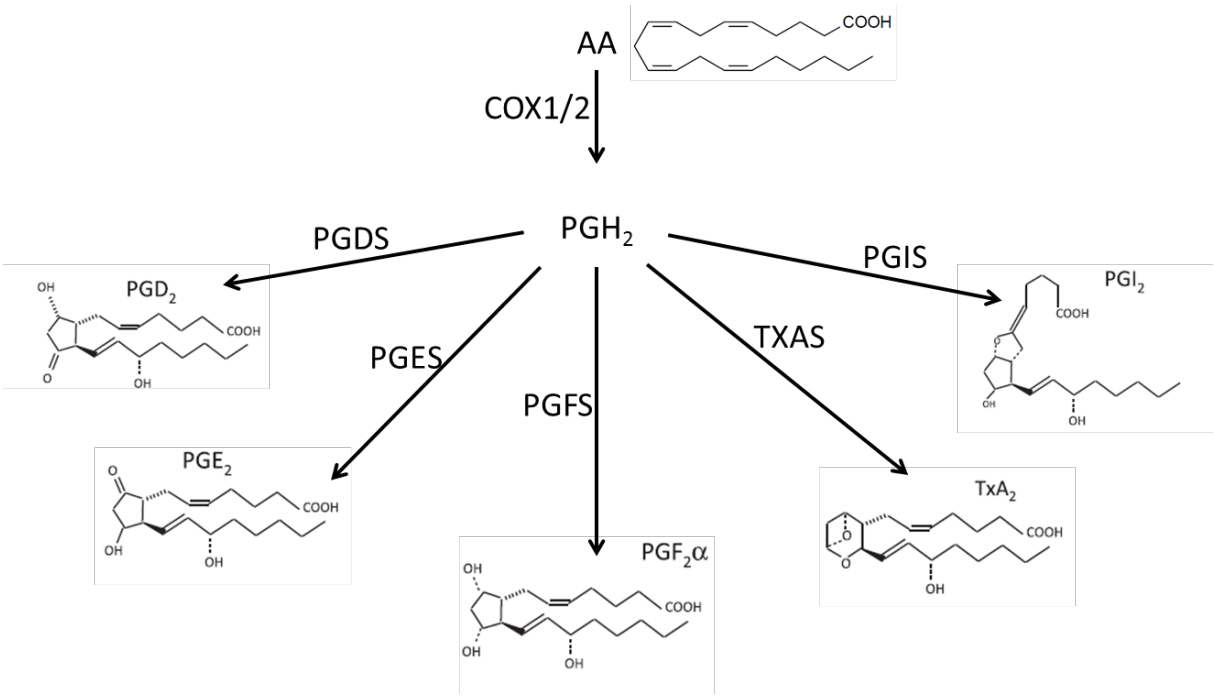

Figure 4:

AA-derived eicosanoid production through the COX pathway.

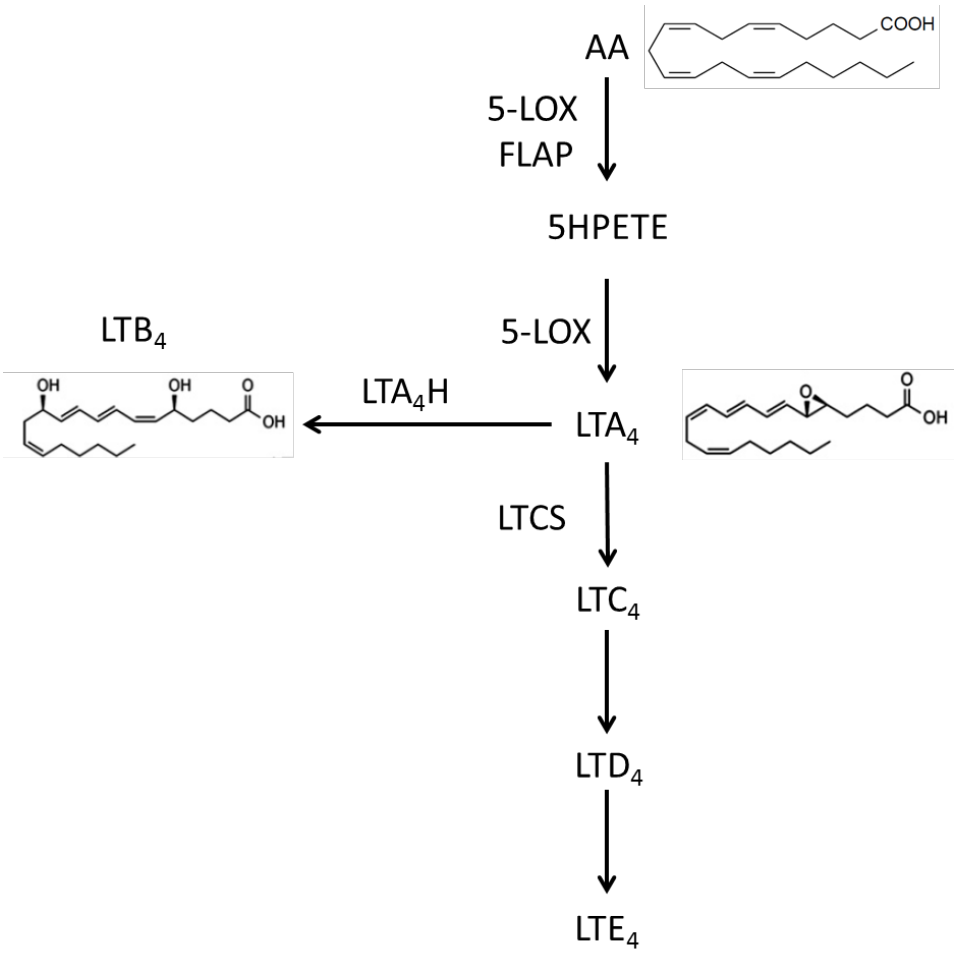

Figure 5:

AA-derived eicosanoid production through the 5-LOX pathways.

PUFAs from the n-3 family can also be converted to oxygenated metabolites (Fig 6). EPA is a 20-carbon n-3 PUFA that can compete with AA for both phospholipid presence and enzymatic action of COX and LOX pathways, by which it can be converted to 3-series prostanoids and 5-series leukotrienes. These oxygenated products are similar to the ones derived from AA; however, they are far less potent. Additionally, both EPA and DHA can be converted to anti-inflammatory oxygenated metabolites by the action of various LOX enzymes or acetylated COX-2. EPA can be converted to "resolution-phase interaction products" or resolvins of the E-series (RvE), whereas, DHA can be converted to D-series resolvins (RvD), neuroprotectins (NPD), and "macrophage mediators in resolving inflammation" or maresins (MaR)[46].

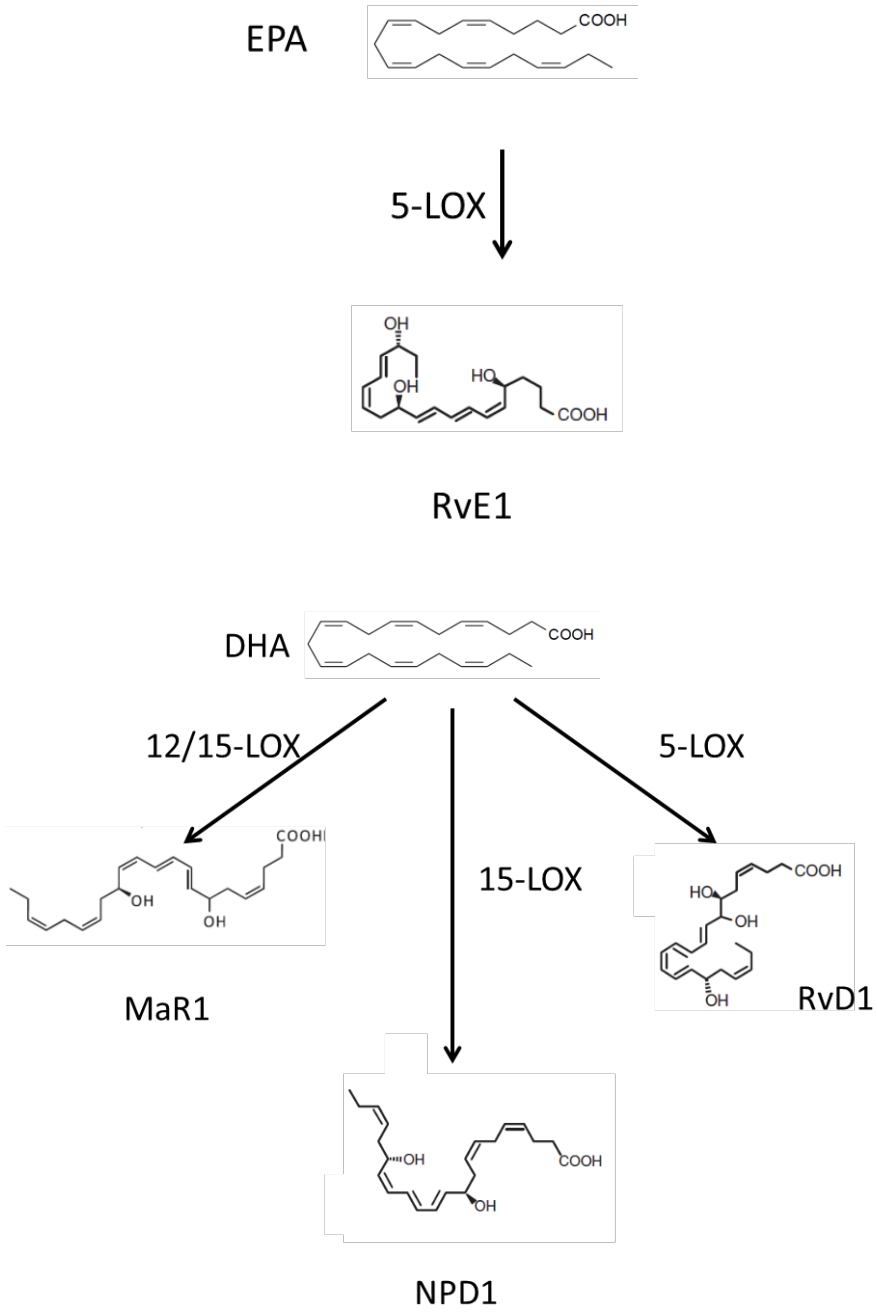

Figure 6:

The common production pathways of n-3 PUFA-derived oxygenated metabolites.

Role of Fatty Acid Oxygenated Metabolites in Inflammation

Temporal production of PUFA-derived lipid mediators plays an important role in the initiation and resolution of inflammatory events. The introduction of an insult to tissue, such as bacterial infection, elicits the production and release of AA-derived eicosanoids as a part of the inflammatory response. Release of LTB_4 at the initial stages of inflammation enhances chemoattractant signals and leads to augmented neutrophil recruitment, while the production of PGE_2 increases blood flow and vessel permeability to allow for leukocyte migration to the site of insult. As inflammation progresses, several events initiate the signal for inflammatory resolution[47]. Oxygenated metabolites contribute significantly to this process via their "class switching" ability, by which the production of proinflammatory metabolites is decreased and the production of pro-resolving metabolites is initiated[48]. In particular, PGE_2 has been shown to have the ability to trigger this "class switching" event by inhibiting the expression of 5-LOX and inducing the expression of 15-LOX, which decreases LTB_4 while increasing LXA_4, and leads to inhibition of neutrophil recruitment and the eventual resolution of inflammation. This resolution phase is further enhanced by the production of n-3 PUFA-derived anti-inflammatory mediators, such as RvE, RvD, NPD, and MaR[44].

PUFA Alterations in CF

Fatty acid abnormalities in the blood and tissue of CF patients were first reported in 1962[49]. Since then, consistent PUFA alterations, mainly decreased LA and DHA levels and changes in AA metabolism, have been associated with CF. Many studies have examined fatty acid levels in serum, plasma, erythrocytes, and whole blood samples from CF patients[50-67]. Decreased LA levels were reported in all of those studies, while decreased DHA and increased palmitoleic acid (POA, 16:1n-7) and mead acid (MA, 20:3n-9) in CF patients were observed in the majority of those studies. On the other hand, AA was either unchanged or found to be decreased in the blood of CF patients in a minority of studies. Similar findings have been shown in other tissues from CF patients. LA and DHA levels were decreased in the nasal and rectal biopsies of CF patients[63]. AA levels were significantly higher in the nasal biopsies of CF patients in this study, and was similarly increased in the BAL fluid of CF patients in a different study[68]. These alterations were also found in animal and cell culture models of the disease. In both CFTR KO and ΔF508 CF mouse models, investigators found decreased LA and increased AA levels in multiple tissues, including the small intestines, pancreas, and lungs.

These alterations were observed in CF airway epithelial cells as well[69,70]. Importantly, studies suggest that these alterations were due to an increased expression and activity of specific metabolic enzymes involved in fatty acid metabolism. Radiolabeled studies showed that the decreased LA levels are attributed to an increased flux in the n-6 pathway, driven by an

increased expression of the rate-limiting enzyme Δ6-desaturase in addition to Δ5-desaturase[71]. Similar radiolabeled studies demonstrated that the increased POA and MA levels in the n-7 and n-9 pathways is due to increased flux in these pathways. This increased metabolism was dependent on higher expression of Δ9-desaturase and elongase-6 in addition to Δ5D and Δ6D[72]. The mechanism behind low DHA levels in CF is less clear. Radiolabeling studies have shown that the flux of eicosapentaenoic acid (EPA, 20:5n-3) to DHA is decreased in CF cells, while the flux of docosapentaenoic acid (DPA, 22:5n-3) to DHA appeared to be the same in both CF and WT cells[71]. This suggests that there could be a defect in the elongation step between EPA and DPA in the n-3 pathway. Alternatively, EPA might be shunted into another pathway, such as eicosanoid production, rendering it unavailable for metabolism to DHA. Another possible mechanism involves the retroconversion of DHA back to EPA via modified peroxisomal β-oxidation[73-75]. While assessing this pathway is technically challenging, its activity may be estimated by comparing EPA and DHA levels after DHA supplementation. Using this method in an airway epithelial cell model, CF cells showed a 20 times higher rate of retroconversion compared to WT cells, which may account for the lower DHA levels[71]. Additional studies are required to elucidate the retroconversion pathway and its contribution to the fatty acid abnormalities in CF.

Significance of Fatty Acid Alterations in CF

PUFA alterations in CF were thought to be secondary to pancreatic insufficiency and malabsorption. However, there are several reports that proved otherwise. A study by Rogiers *et al*. showed that LA levels were decreased in both free fatty acid and cholesterol ester fractions, independent of pancreatic status in CF patients[58]. Moreover, persistent PUFA alterations were not corrected in CF patients with malabsorption adequately treated by nutritional supplementation or pancreatic enzyme replacement[62,65]. In fact, these alterations were recapitulated in airway epithelial cell culture models of CF, which provides further evidence that these alterations are part of an intrinsic defect in CF not related to lipid digestion or absorption[69,71]. Moreover, the consistent nature of these alterations may suggest they are not merely phenomenological, but rather may play a significant role in the pathophysiology of this devastating disease. This is supported by studies that examined correlations between PUFA alterations and disease status of CF patients. A report by Strandvik *et al*. found that LA levels were significantly lower in patients with severe mutations, while patients with mild mutations had LA levels comparable to levels observed from normal controls[52]. Van Biervliet *et al*. reported similar findings regarding lower LA levels in CF patients with severe mutations, in addition to higher 20:3n-6, 22:5n-6, and MA, when compared with patients that have moderate mutations[76]. Additionally, these PUFA alterations were found in both patients homozygous for CFTR mutations and their heterozygous parents, albeit they were more pronounced in the

homozygous group[51]. Furthermore, PUFA alterations correlated with the clinical status[54], and pulmonary function of CF patients[60,77]. Interestingly, inhibiting Cl^- conductance by chloride channel blocker anthracene 9-carboxylate in primary airway epithelial cell significantly lowered LA levels[78].

PUFA are important constituents of membranes, where they may play an important role in both membrane structure and direct protein interaction. Therefore, PUFA alterations may influence CF pathophysiology by changing membrane composition or regulating membrane-associated proteins. In support of this hypothesis, there have been studies that looked at the effect of AA on Cl^- conductance by CFTR. Linsdell *et al*. demonstrated by using patch clamp recordings from CFTR-transfected baby hamster kidney cells that, when applied to the cytoplasmic side of the excised membrane patches, AA is able to inhibit CFTR Cl^- conductance[79]. On the other hand, Li *et al*. showed that AA has a dual regulatory effect on CFTR Cl^- conductance in airway cells, where it inhibits the conductance in excised membrane patches, but stimulates conductance in intact cell monolayers[80]. This implicates AA as a regulator of CFTR, which may offer a partial explanation for the observed PUFA alterations.

PUFA are precursors of many biologically active oxygenated metabolites that play a significant role in inflammation. As mentioned above, there is plenty of evidence that implicates inflammatory dysregulation in the pathogenesis of CF. PUFA alterations in CF may affect pathophysiology via the dysregulated production of oxygenated metabolites. Of those metabolites,

AA-derived eicosanoids have a profound part in driving the inflammatory response. This may implicate specific PUFA alterations in the inflammatory response in CF. In particular, the decreased LA and variably increased AA levels that have been attributed to increased metabolism from LA to AA in CF cells. This increased metabolism leads to increased availability of AA for the production of proinflammatory eicosanoids.

Since AA release from membrane phospholipids by PLA_2 is the rate-limiting step in eicosanoid production, studies have investigated the effect of CFTR dysfunction on this crucial step. Calstedt-Duke *et al*.[81] and Miele *et al*.[82] reported increased AA release in lymphocytes from CF patients and in a CF epithelial cell line carrying a ΔF508 mutation, respectively. The increase in AA production and release is accompanied by significant evidence of higher production of proinflammatory eicosanoids in CF.

Several studies reported increases in both prostaglandins, particularly PGE_2 $PGF_2\alpha$ and their metabolites[71,83-88], and leukotrienes[71,89,90] in CF patients and models of disease. Expression of both COX and LOX enzymes was also augmented in patient tissues and cell models[71,88,91,92]. The levels of these eicosanoids correlated with disease severity[83,87,93], and were shown to be associated with acute pulmonary exacerbations[94]. In contrast, anti-inflammatory LXA_4 levels have been shown to be significantly suppressed in airway fluid of CF patients[95]. Moreover, it was shown that administration of a stable LXA_4 analog was able to decrease bacterial burden, suppress neutrophilic inflammation, and attenuate lung disease severity, in a mouse

model of chronic lung infection and inflammation associated with CF[95]. This may be suggestive of defective inflammation resolution in CF. Importantly, it may also implicate low DHA levels in the pathophysiology of CF, since it is a precursor of a variety of oxygenated metabolites heavily involved in inflammation resolution. In support of this, Freedman *et al*. demonstrated, using a *CFTR*$^{-/-}$ knockout mouse model, that DHA administration reversed the characteristic PUFA alterations, alleviated disease pathology in the GI tract and pancreas, and significantly reduced neutrophilic inflammation in the lungs of CF mice[96].

Therapeutic Potential of PUFA

DHA has been shown to have the ability to reverse the observed PUFA alterations in both airway epithelial cell culture[70,97] and lungs of animal[96,98] models of CF. These studies, in addition to our findings in chapter IV, suggest that n-3 PUFA, particularly DHA, might hold great therapeutic potential for CF patients. Many clinical trials, summarized in (Table 1)[99-114], have investigated this potential by using n-3 PUFA administration in CF patients. While n-3 PUFA administration in all of these studies showed significant reversal of characteristic PUFA alterations in tissues of CF patients, the clinical benefits of this administration were not as clear. This could be due to several issues in study design of these trials. First, most of these studies used a combination of EPA and DHA with varying concentrations, for short periods that most ranged from 2 weeks to 6-8 months, with the longest studies lasting 1 year. Second,

these trials were small, most having 20 patients or less. Finally, the use pulmonary function tests, such as FEV_1, to gauge clinical improvement may not be the most reliable measure, since these tests change due to many factors, such as age and pulmonary exacerbations. Thus, future clinical trials must standardize the dose n-3 PUFA and duration of the study. Moreover, these future studies must identify a better clinical marker of the disease.

Table 1: Summary of n-3 supplementation clinical trials in CF

Ref	N and Age	Dose	Duration	Results
195	9 CF patients (7-20 y)	0.911 g/day n-3 PUFA	1 month	Increased levels of DHA and EPA
196	19 CF patients (12-20 y)	2.7 g/day EPA	12 weeks	Improved neutrophil chemotaxis in response to LTB_4
197	12 CF patients (12.2±5.4 y)	3.2 g/day EPA 2.2 g/day DHA	6 weeks	Increased DHA and EPA incorporation in RBCs
198	14 CF patients (6-16 y)	0.1 g/kg/day n-3 PUFA	2 weeks	Increased levels of DHA and EPA Decreased LTB_4 levels in plasma
199	9 CF patients (14-22 y)	2.7 g/day EPA	6 weeks	Corrected number and ligand affinity of LTB_4 receptor
200	23 CF patients (4-42 y)	0.035 g/kg PUFA	4 weeks	Increased levels of EPA and DHA
201	9 CF patients (12-29 y)	0.150 g/kg IV n-3 PUFA	4 weeks	Increased serum EPA and DHA No improvement in lung function
202	5 CF patients (6-16 y)	1.2-2.7 g/day EPA 1.2-1.8 g/day	1 year	No significant change in number of hospital days
203	30 CF patients (0.8-24 y)	0.4 g/day EPA 0.2 g/day DHA	8 months	Increased levels of DHA in and EPA Decreased AA Improved FEV_1
204	5 CF patients (18-43 y)	0.070 g/kg DHA	6 weeks	Increased levels of DHA Decreased levels of AA only in plasma PC and PE No improvement in lung function
205	20 CF patients (8-20 y)	0.050 g/kg DHA	6 months	Increased DHA levels Decreased AA levels No improvement in lung function

180	17 CF patients (18±9 y)	0.039-1.170 g/day n-3 PUFA	6 months	Increased levels of EPA but not DHA No improvement in lung function
206	9 CF patients 12.1 y (IQR 9.3)	3 g/day (40% DHA)	1 year	Increased levels of EPA and DHA Decreased levels of AA No improvement in lung function
207	17 CF patients (26.4±10.6 y)	0.324 g/day EPA 0.216 g/day DHA	1 year	Increased levels of DHA but not EPA Decreased levels of AA Decreased levels of TNFα Improved FEV_1 Decreased number of exacerbations and number of antibiotic days
208	41 CF patients (6-12 y)	0.1 g/kg DHA, 1 month 1 g/day DHA, 11 months	1 year	Decreased ratio of AA/DHA No improvement in lung function
209	10 CF patients (6-18 y)	0.1 g/kg/day DHA 1 month 0.25 g/kg/day DHA, 5 months	6 months	Decreased levels of IL-8, TNFα, and fecal calprotectin Improved absolute FEV_1 only

Summary and Study Goal

The leading cause of mortality in CF is lung disease that is characterized by a self-perpetuating cycle of infection and inflammation. While the cause of the excessive inflammatory response in CF remains unclear, certain PUFA alterations that lead to increased production of AA, which is a precursor for a variety of oxygenated lipid mediators that can modulate inflammation, may provide a possible mechanistic explanation. Other PUFA abnormalities include a decrease in linoleic acid (LA) and docosahexaenoic acid (DHA). Studies have shown that these alterations in CF correlate with disease severity, and favor the increased metabolism of LA to AA. Furthermore, the literature demonstrates that there is an imbalance in the production of AA-derived inflammatory mediators. We hypothesize that inherent alterations in PUFA metabolism contribute to the inflammatory response and disease phenotype in CF via dysregulated eicosanoid production. This project will test the aforementioned hypothesis by examining the production of AA-derived eicosanoids and its relationship with PUFA metabolism.

CHAPTER II

INTERACTIONS OF LINOLEIC AND ALPHA-LINOLENIC ACIDS IN THE DEVELOPMENT OF FATTY ACID ALTERATIONS IN CYSTIC FIBROSIS

Introduction

Among the many phenotypic manifestations of this disease is pancreatic insufficiency, leading to malnutrition. As a result, CF patients require significant nutritional support to maintain body mass. Consensus recommendations suggest a high-calorie, high-fat diet [115]. However, they are not specific as to the sources or types of dietary fat to be consumed. Consequently, there is increasing concern over the sources and types of fat in the typical CF diet [116].

CF patients exhibit alterations in tissue and plasma levels of polyunsaturated fatty acids (PUFA)[117,118]. These changes are independent of the effects of malabsorption, as they persist in patients receiving pancreatic enzyme replacement therapy, as is the standard of care [119]. These results have been replicated in mouse[120,121] and cultured cell [122] models of the disease. The major alterations seen are decreases in linoleic acid (LA; 18:2n-6) and docosahexaenoic acid (DHA; 22:6n-3) and increases in palmitoleic acid (16:1n-7) and Mead acid (20:3n-9). Increased arachidonic (AA; 20:4n-6) is reported in some, but not all, studies. Multiple lines of evidence suggest a

connection between these abnormalities and CF pathophysiology. The magnitude of PUFA alterations correlate with disease severity [123-126], although it is unclear whether this represents cause or effect. However, correction of the abnormalities by dietary DHA supplementation, given as free fatty acids or glycerophospholipid liposomes, reversed CF-related pathology in mouse models [120,121]. Some have theorized that elevated AA levels contribute to CF by increasing production of pro-inflammatory eicosanoids [118].

Recent work from our laboratory has demonstrated that the PUFA abnormalities observed in the CF cultured cell model are due to changes in fatty acid metabolism. The n-3 and n-6 PUFA are metabolized via parallel pathways that share common desaturase and elongase enzymes[127]. Compared with wild-type (WT) cells, CF cells exhibit increased expression and activity of fatty acid metabolic enzymes, including Δ5-, Δ6-, and Δ9-desaturases, and elongase 6 causing increased production of AA, palmitoleic acid, and Mead acid [72,128]. We further demonstrated that DHA and eicosapentaenoic acid (EPA; 20:5n-3) suppress the expression of both Δ5- and Δ6-desaturases and normalize LA and AA levels [129]. These studies also showed some differences between the n-3 and n-6 metabolic pathways. Although they share the same enzymes, there was a clear preference for n-3 substrates, especially in CF cells.

In the setting of increased AA production through the n-6 pathway, it is possible that variations in substrate (*i.e.*, LA) concentrations play a significant role in the phenotypic manifestations of CF. Indeed, a recent study showed

that LA supplementation in cell culture and mouse CF models increased AA levels as well as the production of prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, and IL-8, leading to increased airway inflammation [130]. This is of particular concern in CF patients consuming a high-fat diet, given that the typical Western diet is high in LA, but low in alpha-linolenic acid (LNA; 18:3n-3), its counterpart in the n-3 pathway [131].

In the current study we investigated the impact of varying concentrations of the initial fatty acids in the n-3 and n-6 pathways on the development of the classic fatty acid alterations in CF. Using a well-characterized cell culture model of CF, we demonstrated that these alterations are strongly influenced by the input concentrations of both LA and LNA, and that LNA supplementation can markedly decrease production of AA, the fatty acid precursor of many pro-inflammatory eicosanoids. These results may have implications for nutritional management of patients with CF.

Materials and Methods

Materials:

[1-^{14}C]LA (55 mCi/mmol) and [1-^{14}C]LNA (55 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Fatty acid methyl ester (FAME) standards for HPLC were purchased from NuCheck Prep (Elysian, MN) or Larodan Fine Chemicals (Malmö, Sweden). HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA). Liquid scintillation cocktail (FlowLogic U) was purchased from LabLogic Systems,

Inc. (Brandon, FL). The different horse sera described in Table 1 were purchased from three different companies: “Serum 1” from Atlanta Biologicals (Lawrenceville, GA), lot #C1010; “Serum 2” from Invitrogen (Carlsbad, CA), lot #8131206; “Serum 3” from Omega Scientific (Tarzana, CA), lot #169101.

Cell culture:

Human bronchial epithelial cells (16HBEo−) expressing CFTR (WT) or not expressing CFTR (CF) were a kind gift from Dr. Pamela Davis (Case Western Reserve University School of Medicine, Cleveland, OH). 16HBE cells are immortalized human bronchial epithelial cells stably transfected with a segment of the *CFTR* gene in the sense or antisense orientation [132] that have been used widely to study PUFA alterations in CF [72,117,122,128,129,133]. Cells with the sense gene (WT cells) have normal CFTR expression and activity, while those with the antisense gene (CF cells) lack both [122,132]. Tissue culture flasks were coated with LHC basal media (Invitrogen) that contains 1 mg/mL BSA (Sigma-Aldrich, St. Louis, MO), 10 µg/mL human fibronectin (Sigma-Aldrich), and 3 µg/mL vitrogen (Angiotech Biomaterials, Palo Alto, CA). The cells were grown in MEM + glutamax (Invitrogen) with 10% horse serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) and maintained at 37^{o}C in 5% CO_2. The media was changed three times per week.

Fatty acid supplementation:

WT and CF cells were seeded at $3x10^5$ and $1x10^5$ cells/ well, respectively, onto 6-well plates and were grown for 7 days. On day 7, the cells were washed and fed medium containing 10% reduced-lipid FBS and supplemented with 0, 5,10, or 20 μM of LA or LNA for 4 hours. Supplementation media was prepared by adding media to free fatty acids (LA or LNA) that were dried under nitrogen in glass conical tubes followed by vortexing and sonicating three times for 5 seconds each to allow fatty acid binding to serum albumin.

Fatty acid composition analysis:

Cells ready for analysis were washed twice with ice-cold PBS then harvested by scraping on ice with a rubber policeman and pelleted by centrifugation at 100xg for 8 min. The cell pellet was resuspended in 0.5 mL PBS and 10 μg of heptadecanoic acid (17:0) was added as an internal standard before extracting lipids according to the modified Folch method [134]. Briefly, six volumes of chloroform-methanol (2:1 v/v) were added to the samples, after which they were incubated on ice for 10 min, vortexed, and centrifuged at 1100xg for 10 min. The resulting lower phase was transferred to a new tube and dried completely under nitrogen. Fatty acids were then methylated using boron trifluoride (BF_3)-methanol and a methanolic-base reagent. After adding an aliquot of 0.5 mL 0.5 N methanolic NaOH to samples, they were vortexed and heated at 100^oC for 3 minutes. This was followed by

the addition of 0.5 mL BF_3 and heating at 100^{o}C for 1 minute. The resulting fatty acid methyl esters (FAMEs) were extracted using 1 mL hexane followed by 6.5 ml of saturated NaCl solution. FAMEs in the hexane layer were identified and quantified by gas chromatography (GC) using an Agilent 7980A GC system (Agilent Technologies, Santa Clara, CA) equipped with a Supelcowax SP-10 capillary column (Supelco, Bellefonte, PA) coupled to an Agilent 5975C mass spectrometer (MS). FAME mass was determined by comparing areas of unknown FAMEs to that of the 17:0 internal standard. Results were reported as the molar percentage (mol%) of each FAME relative to the total mass of the sample, as previously described [133].

Fatty acid radiolabeling experiments:

On day 7, cells were incubated with media containing 10% horse serum (serum 3) supplemented with 0, 5, 10, or 20 μM of LA or LNA in addition to 4.1 μM of either [1-^{14}C]LNA or [1-^{14}C]LA for 4 hours. Radiolabeled LNA was added to media supplemented with unlabeled LA, whereas radiolabeled LA was added to media supplemented with cold LNA. Lipids were extracted as described above. However, in these studies, the final hexane layer was dried under nitrogen and the FAMEs resuspended in 50 μL acetonitrile for HPLC analysis. FAMEs were separated by reverse-phase high performance liquid chromatography (RP-HPLC) using a binary solvent system on an Agilent 1200 series instrument equipped with a (4.6 x 250 mm, 5 μm) Agilent zorbax Eclipse XDB-C18 column and a (4.6 x 12.5 mm, 5 μm) column guard. Solvent

A consisted of H_2O + 0.02% H_2PO_4 and solvent B was 100% acetonitrile. The solvent program was 58% solvent B for 25 min, 58-61% B for 2 min, 61% B for 8 min, 61-100% B for 15 min, 100% B for 20 min, and finally reconstitution to original conditions. Peaks were identified by UV detection at 205 nm and comparison with the retention times of unlabeled standards. Radiolabeled peaks were quantified using an IN/US β-RAM Model 4 scintillation detector coupled to the HPLC instrument. Results were reported as percentage of total measured counts in a sample.

Statistical Analysis:

All results are representative of two or more identical experiments. Relative distributions of fatty acids under various conditions in CF and WT cells were compared using two-way analysis of variance (ANOVA). Comparison of CF and WT cells under each condition was performed using a Bonferroni post-test. Slopes of lines representing change in labeled EPA and AA versus concentration of added LA or LNA were calculated and compared using linear regression. All statistical analysis was performed using GraphPad Prism v5.0 software (GraphPad Software, La Jolla, CA).

Results

Fatty Acid Composition of Different Horse Sera

Prior studies investigating PUFA abnormalities in CF cell culture models have used media containing horse serum because its relatively high content of LA accentuates n-6 PUFA abnormalities in this model[135]. However, over time we noted significant variation in these abnormalities when different lots of horse serum were used. To test whether these changes were due to differences in the fatty acid content of the sera, we cultured WT and CF cells in media containing three different horse sera, the fatty acid composition of which is shown in Table 1. The major fatty acids included the saturated fatty acids palmitic acid (16:0) and stearic acid (18:0), the monounsaturated fatty acid oleic acid (18:1n-9), and the PUFAs LA and LNA. Of these, there were significant lot-to-lot variations in LA, LNA, and oleic acid (Table 2). Other measured fatty acids individually represented <5% of the total fatty acid mass.

Table 2: Fatty Acid Composition of Different Horse Sera

Fatty Acid	Fatty Acid Composition (mg/mL)[a]			
	Horse Serum 1	Horse Serum 2	Horse Serum 3	*P* value[b]
Saturated Fatty Acids				
14:0	9.2±0.4	7.2±0.4	7.1±0.1	n.s.
15:0	1.6±0.1	1.7±0.1	2.0±0.1	0.04
16:0	200.0±6.2	183.6±9.3	164.8±4.2	n.s.
18:0	206.1±7.5	184.4±10.2	194.0±5.5	n.s.
20:0	4.1±0.1	4.5±0.2	3.9±0.1	n.s.
22:0	0.3±0.0	0.4±0.0	0.4±0.0	n.s.
n-3 Unsaturated Fatty Acids				
18:3	52.3±1.6	86.9±4.8	32.4±0.8	0.03
20:3	2.3±0.1	4.1±0.2	2.7±0.0	0.03
20:5	3.9±0.1	5.5±0.3	2.2±0.1	0.03
22:5	3.8±0.1	3.8±0.2	2.9±0.0	n.s.
22:6	3.1±0.6	2.3±0.7	1.6±0.2	n.s.
n-6 Unsaturated Fatty Acids				
18:2	387.7±13.1	417.1±20.6	465.8±18.4	0.05
18:3	4.2±0.1	4.4±0.2	4.2±0.1	n.s.
20:3	4.5±0.2	5.0±0.3	3.6±0.1	0.05
20:4	13.4±0.4	12.2±0.7	14.4±0.3	n.s.
n-7 Unsaturated Fatty Acids				
16:1	27.4±0.7	18.3±0.9	17.3±0.5	0.05
18:1	21.8±1.4	16.3±0.7	18.8±0.4	0.03
n-9 Unsaturated Fatty Acids				
18:1	303.8±9.3	184.6±9.9	132.9±7.5	0.03
20:1	5.4±0.1	3.8±0.2	3.7±0.1	n.s.
LA:LNA	7.4±0.1	4.8±0.0	14.4±0.3	0.03

[a]Mean±S.E.M. for three measurements; [b]One-way analysis of variance (ANOVA) by the Kruskal-Wallis non-parametric test.

Relationship of Cellular LA and LNA to Serum LA and LNA Levels

To determine the impact of the different sera on PUFA metabolism in CF, we incubated WT and CF cells in media containing the different lots of horse serum for 7 days. We then measured the relative PUFA distributions of the cells (Fig. 7). There was no significant difference in relative LA levels between WT and CF cells incubated in medium containing serum 1 (Fig. 7a). However, WT cells incubated in sera 2 and 3 had higher LA levels than those of CF cells. To determine whether the serum concentrations of LA were responsible for these differences, the relative cellular LA levels were plotted against those of serum LA (Fig. 7c). There was a clear positive correlation between serum LA content and cellular LA composition. Furthermore, the characteristic difference in LA levels between WT and CF cells was apparent only with serum containing moderate to high (≥36%) LA levels (sera 2 and 3).

Significant differences in cellular AA levels were only noted in cells incubated in serum 3, with levels higher in CF than WT cells (Fig. 7b). Prior studies [128,130] indicated that AA levels should rise in proportion with increasing LA inputs and that this increase would be greater in CF versus WT cells. On the contrary, there was no apparent correlation between relative serum LA and cellular AA levels (Fig. 7d). Rather, AA levels were lowest in the cells grown in the serum with medium LA content (serum 2). Noting that serum 2 also contained the highest concentrations of LNA, we sought to determine whether serum LNA also influenced AA levels. Accordingly, we plotted cellular LA and AA levels against the serum LA:LNA ratio (Fig. 7e,f). While there was no

apparent relationship between LA:LNA and LA levels (Fig. 7e), there was a positive correlation between LA:LNA and AA levels, particularly in CF cells (Fig. 7). In fact, the AA increase characteristic of CF was apparent only at the highest LA:LNA ratio. These findings suggest that production of AA is influenced by LNA, the initial substrate of the parallel n-3 pathway, such that AA production is higher when LNA is low and vice versa.

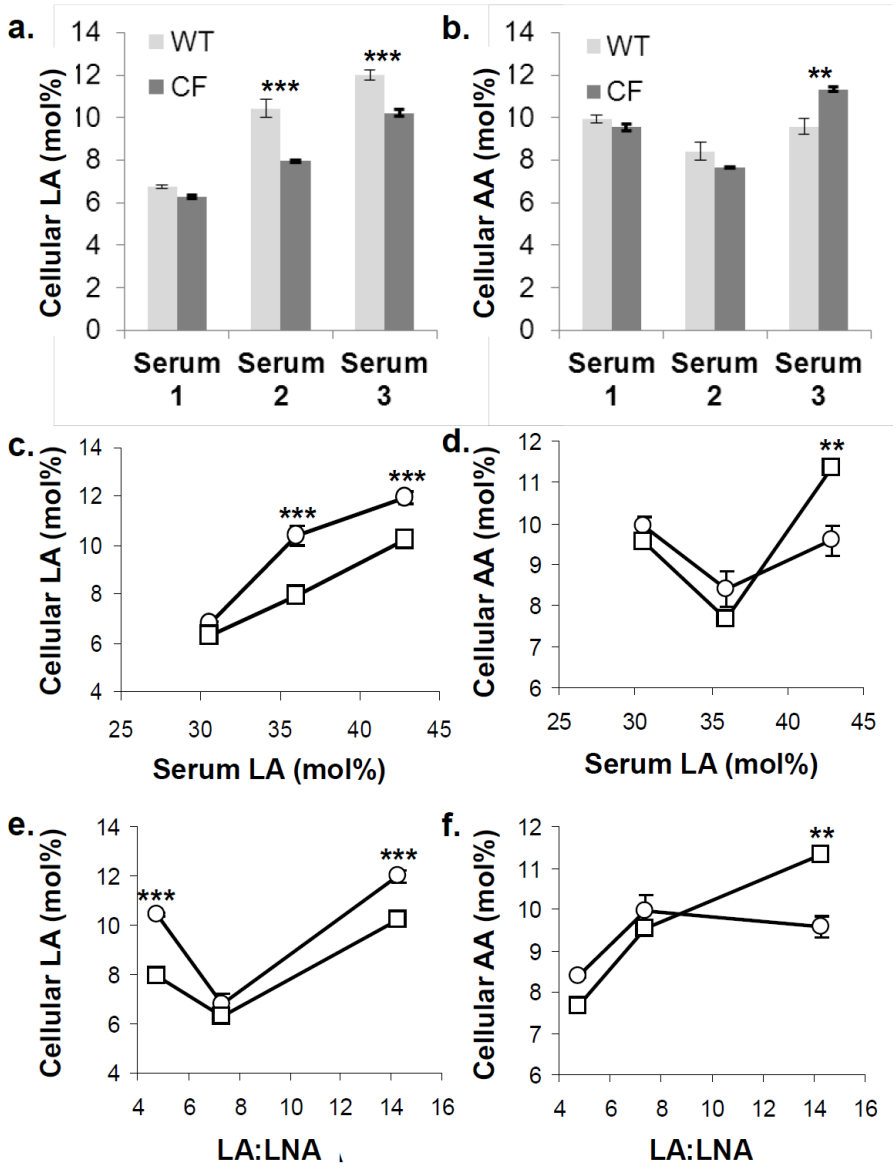

Figure 7:
Relationship of cellular LA and LNA concentrations to serum LA and LNA levels. Relative cellular LA (a,c,e) and AA (b,d,f) concentrations were measured by gas chromatography/mass spectroscopy (GC-MS) from extracts of WT and CF cells cultured for 7 days in media containing 10% horse serum from different lots (see Table 2 and Materials and Methods). Data are plotted as mean mole percent of each lipid (±S.E.M.; n=3) for cells treated with each of the three sera (a,b) or versus LA concentration (c,d) or LA:LNA ratio (e,f) in serum on the x-axes. WT cells are indicated by open circles (○) and CF cells by open squares in the line graphs (□). Statistical analysis performed using two-way ANOVA with pairwise comparisons calculated using Bonferroni post-test (see Materials and Methods). ***, $P<0.001$; **, $P<0.01$.

Relationship of Cellular EPA and DHA Concentrations to Serum LA and LNA Levels

In light of the metabolic relationship between the n-3 and n-6 pathways, we sought to determine whether LA levels might also influence LNA metabolism in CF. Cellular EPA and DHA levels varied significantly between cells incubated with different sera (Fig. 8a,b). EPA levels were consistently higher and DHA level consistently lower in CF compared to WT cells as shown previously [128,129]. Levels of cellular EPA and DHA, products of LNA metabolism, were plotted against serum LNA levels and serum LA:LNA ratios. In contrast with LA metabolism to AA in the n-6 pathway (Fig. 8), there was a strong positive relationship between serum LNA concentrations and cellular EPA and DHA levels in both WT and CF cells (Fig. 8,d), such that the low levels of DHA characteristic of CF were much more apparent in cells incubated in serum with higher concentrations of LNA. Accordingly, cellular EPA and DHA levels fell as the serum LA:LNA ratios increased (Fig. 8e,f).

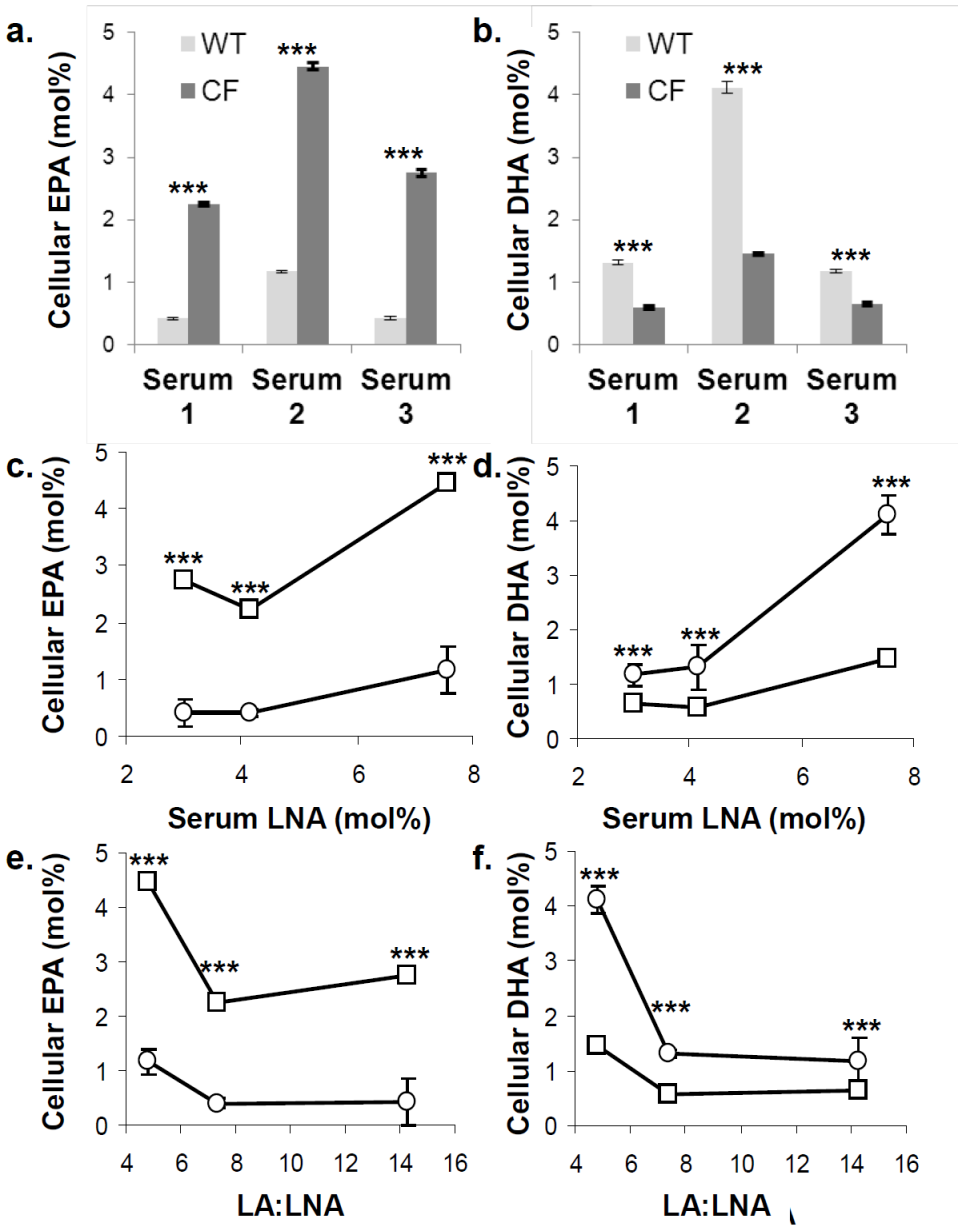

Figure 8:

Relationship of cellular EPA and DHA concentrations to serum LA and LNA levels. Relative cellular EPA (a,c,e) and DHA (b,d,f) concentrations were measured by gas chromatography/mass spectroscopy (GC-MS) from extracts of WT and CF cells cultured for 7 days in media containing 10% horse serum from different lots (see Table 2 and Materials and Methods). Data are plotted as mean mole percent of each lipid (±S.E.M.; n=3) for cells treated with each of the three sera (a,b) or versus LNA concentration (c,d) or LA:LNA ratio (e,f) in serum on the x-axes. WT cells are indicated by open circles (○) and CF cells by open squares in the line graphs (□). Statistical analysis performed using two-way ANOVA with pairwise comparisons calculated using Bonferroni post-test (see Materials and Methods). ***, $P<0.001$.

There are likely other differences besides fatty acid concentrations in the various sera that could potentially account for these changes. Thus, to confirm the impact of LNA on LA to AA metabolism, we incubated CF and WT cells in serum 3, which had the highest LA:LNA ratio, supplemented with LNA sufficient to reduce the ratio from 14 to 10 and 5 (Fig. 9). As serum LNA concentrations increased, relative cellular LA levels rose (Fig. 9a) and AA levels fell (Fig. 9b). AA:LA ratios, roughly indicative of desaturase activity, fell significantly with increasing LNA in both CF (2.01±0.03, 1.40±0.04, and 0.72±0.01, respectively; $P<0.001$) and WT cells (1.29±0.02, 1.03±0.02, and 0.53±0.01, respectively; $P<0.001$).

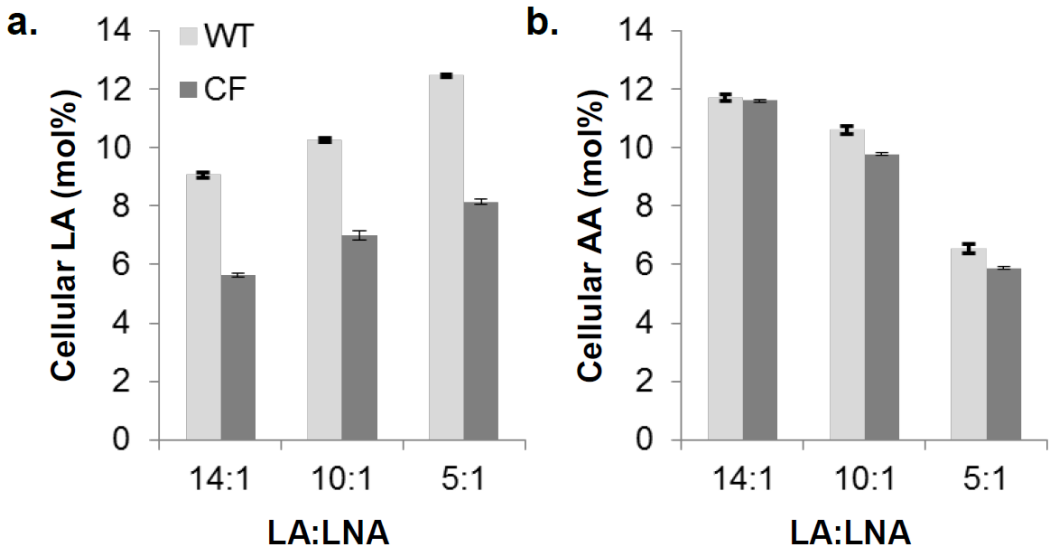

Figure 9:
Effect of increasing concentrations of LNA on n-6 PUFA metabolism. WT and CF cells were cultured for 7 days as described in Materials and Methods in medium containing 10% serum 3 (see Table 2) with a LA:LNA ratio of 14.4:1 supplemented with either no LNA, or additional LNA sufficient to lower the LA:LNA ratio to 10:1 or 5:1. Relative cellular LA (a) and AA (b) concentrations were measured by gas chromatography/mass spectroscopy (GC-MS) from cell extracts as described in Materials and Methods. Data are plotted as mean mole percent of each lipid (±S.E.M.; n=3). Statistical analysis performed using two-way ANOVA with pairwise comparisons calculated using Bonferroni post-test (see Materials and Methods). ***, $P<0.001$.

Metabolism of n-3 and n-6 Precursors Is Suppressed by Addition of Exogenous Precursors from the Opposite Pathway

To directly measured the effect of LNA on LA to AA metabolism, we incubated cells with radiolabeled LA in the presence of increasing concentrations of unlabeled LNA for four hours and then measure labeled LA and AA (Fig. 10a,b). As has been reported previously [128,133], there was greater conversion of LA to AA in CF versus WT cells, as indicated by lower levels of labeled LA and higher levels of labeled AA in CF cells. Addition of unlabeled LNA decreased metabolism of LA to AA, reflected in dose-dependent small increases in labeled LA and large decreases in labeled AA. These changes were greater in the CF cells, such that the differences in LA and AA levels between CF and WT cells completely disappeared as LNA levels were increased.

LA had a similar effect on labeled LNA metabolism (Fig. 10c,d). As has been noted previously[97,136], conversion of LNA to EPA was significantly higher than the conversion of LA to AA, especially in CF cells, with labeled LNA-derived EPA comprising nearly 50% of total counts (Fig. 10d), while labeled LA-derived AA represented less than 20% of total counts in CF cells (Fig. 10b). Nevertheless, there was a dose dependent decrease in EPA production from LNA with increasing concentrations of unlabeled LA. As with LA metabolism, this effect was larger in CF than in WT cells. In this case, the differences between the CF and WT cells persisted even at the highest concentrations of LA.

To compare the effects of LA on LNA metabolism with those of LNA on LA metabolism, we compared slopes of calculated linear regression lines for the data in Figures 10b and 10d. The calculated rates of decline were similar between LA and LNA for both CF (0.71±0.17% AA/μM LNA versus 1.00±0.05% EPA/μM LA; P=0.17) and WT cells (0.39±0.17% AA/μM LNA versus 0.31±0.04% EPA/μM LA; P=0.67).

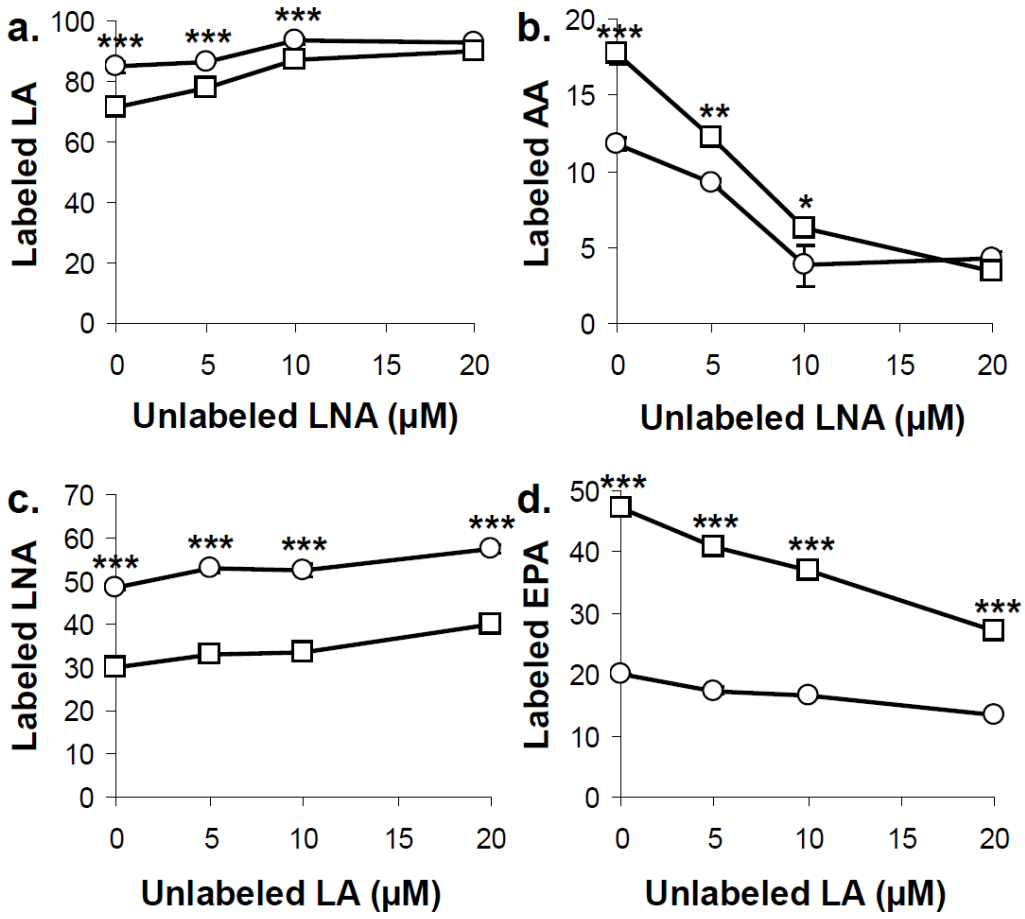

Figure 10:
Metabolism of n-3 and n-6 precursors is suppressed by addition of exogenous precursors from the opposite pathway. WT (○) and CF (□) cells were cultured for 7 days in medium containing 10% serum 3 as described in Materials and Methods. On day 7, the medium was replaced with reduced-lipid medium containing 4.1 μM [1-^{14}C]LA (a,b) or [1-^{14}C]LNA (c,d) and 0, 5, 10, or 20 μM unlabeled LNA (a,b) or LA (c,d). After labeling for 4 hours, cells were washed and refed complete medium. After culturing for an additional 20 hours, cells were harvested and levels of labeled LA (a), AA, (b), LNA (c), and EPA (d) were determined by HPLC and scintillation counting as described in Materials and Methods. Data are expressed as percent of total counts and points represent the mean ± S.E.M (n=3). Statistical analysis performed using two-way ANOVA with pairwise comparisons calculated using Bonferroni post-test (see Materials and Methods). ***, *P*<0.001.

Discussion

These results highlight the interaction between substrates of the n-3 and n-6 PUFA metabolic pathways, demonstrating that the principal initial substrate of one pathway can suppress metabolism in the other pathway. The most obvious explanation for this effect is substrate competition for the common metabolic enzymes. However, effects on the expression of these enzymes cannot be ruled out as a cause. In fact, our group has previously demonstrated that LNA can reduce Δ5- and Δ6-desaturase expression [129]. It is likely that the observed changes are due to a combination of these and other effects.

Another possible explanation for the reduction of AA production by LNA is that increased abundance of one fatty acid reduces the relative abundance of others by dilution. While this may be true to some extent, it cannot account for all of the results. For example, if dilution accounted for the reduction of AA concentrations with addition of LNA (Fig. 9), one would expect that LA levels would also be reduced by dilution. In fact, the opposite is true. In addition, dilution effects cannot account for the results of Figure 13, as the data is presented as a percentage of total counts from labelled fatty acids, while the potential diluent is unlabelled.

Several results suggest differences in these effects between CF and WT cells. In Figure 10f, AA production in CF cells appears to be more responsive to the LA:LNA ratio than WT cells, such the AA level are

significantly higher in CF cells at the highest ratio. Similarly, LA to AA metabolism appears to be more sensitive to LNA levels in CF than WT cells, such that the difference AA production (higher in CF cells) at baseline declines and then disappears as LNA levels rise.

The observations are consistent with the clear differences in PUFA metabolism that have been described in CF cells [128,129,133]. However, differences in oxidation and uptake could also play a role in the metabolic differences of fatty acids between CF and WT cells. Further studies are required to enumerate the relative contribution of this various pathways.

In the experiments detailed in Figures 7 and 8, it appears that the serum ratio of LA to LNA has a greater impact on LA to AA metabolism than on LNA to EPA metabolism. Prior studies have demonstrated that the common metabolic pathway favors LNA to EPA metabolism over that of LA to AA [137-139], a result that has been confirmed by our group [128,129]. Thus, LNA may compete more effectively for the common metabolic enzymes than LA. A second consideration is that the cellular concentration of LA is at least an order of magnitude higher than that of LNA in these cells (data not shown). Thus, exogenous addition of LNA has a greater fractional impact on the cellular LNA concentration than exogenous LA has on cellular LA concentrations, which may also account for some differences. It is important to note, however, that there was no statistical difference between the impact of LNA on labeled LA metabolism and the effect of LA on labeled LNA metabolism in the experiments detailed in Figure 10.

These findings have implications for the experimental conditions used to study CF metabolism in animal and cell culture models. Our group has previously shown that the PUFA abnormalities characteristic of CF are observed in cell culture models when the cells are incubated in horse serum with higher concentrations of LA, but not in fetal bovine serum that has low concentrations of LA [122]. Looking at LA levels alone, the current study seems to indicate that at least 36% serum LA is required to show differences in cellular LA concentrations between WT and CF cells, and higher levels are required to observe differences in AA. However, LA levels in plasma of CF patients with typical tissue PUFA abnormalities only range between 19-26% [140]. But as these results show, differences in AA are dependent on both LA and LNA input concentrations, with higher LA and lower LNA favoring the appearance of increased AA production. In this light, it is important to note that typical plasma LA:LNA ratios are greater than 100:1 [141]. In addition, these technical issues may explain why occasional studies have failed to detect the characteristic PUFA abnormalities in CF models[71,72]. It should be noted that LA and LNA were not the only PUFAs whose levels differed between the tested sera (Table 2). However, they were the most abundant of these fatty acids in the sera by far, and thus, expected to have the greatest impact on metabolism.

These results may have important implications for nutritional therapy of CF. Nutritional recommendations for CF patients include a high-calorie, high-fat diet to counteract the effects of malabsorption [115]. However, there are no

recommendations regarding the particular fatty acid content. The modern western diet has an LA:LNA ratio that is significantly higher than historic norms, and this ratio has increased substantially over the past century [142,143]. The data presented here suggest that these higher ratios may accentuate the metabolic abnormalities that are thought to contribute to the pathophysiology of CF. Of particular concern is increased production of AA, which is thought to play a role in CF-related inflammation [118]. Indeed, Zaman *et al.* have demonstrated that increased LA consumption increased AA production in both CF cells and *CFTR* knockout mice, leading to increases in inflammatory mediators and enhanced airway inflammation [130]. These data suggest that attention should be paid to the sources of fat in the CF diet. However, the current report is restricted to a cell culture model lacking *CFTR* expression and further study is needed to assess the impact of these dietary factors in clinical outcomes of patients with more typical *CFTR* mutations.

Of particular interest is the role that LNA might be playing in the metabolism of n-6 fatty acids. Dietary LNA has significant beneficial health effects, particularly in the prevention of cardiovascular disease that may be due, in part, to its anti-inflammatory effects[144,145]. While this may be due to conversion of LNA to the known anti-inflammatory metabolites of DHA, the conversion from LNA to DHA is extremely limited [146]. The data described here support older data that LNA may exert its beneficial effect by reducing metabolism of LA to the more pro-inflammatory AA [147] and that this is due to a

greater affinity for LNA than LA by desaturase enzymes [148,149]. Therefore, this may be more relevant than its role as a substrate for DHA production.

In summary, we demonstrate the role that LA and LNA play in the development of PUFA abnormalities characteristic of CF both as precursors in their own metabolic pathways and as modulators of the parallel pathways in PUFA metabolism. Delineating these relationships may have important implications for understanding CF pathophysiology as well as nutritional therapy of this disease.

CHAPTER III

INCREASED UPTAKE AND RELEASE OF ARACHIDONIC ACID IN CULTURED BRONCHIAL EPITHELIAL CELLS WITH A CYSTIC FIBROSIS PHENOTYPE

As shown in chapter II, there is an increased production of AA in CF cells when its substrate LA is readily available. However, the cause behind the increased AA production was not delineated. Here we will demonstrate that this may be due to an increased demand in CF cells for this biologically active fatty acid.

Introduction

The increased inflammation in CF has been shown to involve a variety of mediators that include cytokines and oxygenated products of fatty acids [85,94]. Eicosanoids are oxygenated products derived from 20-carbon fatty acids, especially AA, and play an important role in inflammation in CF. The release of AA from membrane phospholipids is an important rate-limiting step in the production of AA-derived pro-inflammatory eicosanoids, and AA release is reported to be increased in CF. Carlstedt-Duke *et al*. [81] and Strandvik *et al*. [150] reported that defective regulation of AA release is an important defect in the pathology of CF. Miele *et al*. showed that AA release is increased in human pancreatic epithelial cells that express mutated CFTR in response to A23187 [82].

In this study we utilized a human bronchial epithelial cell line (16HBEo−) sense or wild type (S or WT) and antisense (AS, CF) to study the uptake of radiolabeled AA by cells. We also characterized the calcium-dependent release of AA in these cells in response to calcium ionophore A23187 and by late phase filtrate from *Pseudomonas aeruginosa* that is commonly found in infected CF lungs. CF cells exhibited both higher AA uptake and higher AA release, in association with increased expression of cytosolic phospholipase A_2 (cPLA$_2$α). Stimulation with late phase *Pseudomonas aeruginosa* filtrate caused a significant amplification of this response.

Materials and Methods

Materials:

[1-^{3}H]AA (200 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). HPLC standards for arachidonic acid, PGE_2, PGD_2 and $PGF_{2\alpha}$ were purchased from NuCheck Prep (Elysian, MN) or Cayman Chemicals (Ann Arbor, MI). HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA). Liquid scintillation cocktail (FlowLogic U) was purchased from LabLogic Systems, Inc. (Brandon, FL). Liquid scintillation cocktail (BioSafe II) was purchased from (RPI, Mount Prospect, IL).

Cell Culture:

Human bronchial epithelial cells (16HBEo−) expressing CFTR (S, sense; WT) or not expressing CFTR (AS, antisense; CF) were a kind gift from Dr. Pamela Davis (Case Western Reserve University School of Medicine, Cleveland, OH). Cells with the sense gene (WT cells) have normal CFTR expression and activity, while those with the antisense gene (CF cells) lack both [122,132] The cells were grown in MEM + glutamax (Invitrogen) with 10% horse serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) and maintained at 37^{o}C in 5% CO_2. Cells were seeded in 6-well plates and grown for 7 days and the medium was changed every 2 days.

Quantitative Real-Time PCR:

Total RNA was isolated from cells using TRIzol (Life Technologies, Carlsbad, CA) and contaminating DNA was removed from samples by DNA-free (Ambion, Austin, TX). cDNA was synthesized from 1 µg of total RNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR was performed in 10 µL reactions containing 50 ng cDNA, TaqMan primer-probes sets and TaqMan Universal PCR Master Mix in 96-well plates (Life Technologies, Carlsbad, CA). Each reaction was performed in triplicate. C_t values were determined using the CFX96 Real Time PCR Detection System with CFX Manager software (Bio-Rad, Hercules, CA). Relative mRNA levels were calculated using the comparative C_t method while RPLP0 was used as a reference gene.

siRNA Knockdown:

Cytoplasmic phospholipase A_2 ($cPLA_2\alpha$) siRNA or negative control were purchased from Life Technologies (Carlsbad, CA). Cells were seeded and allowed to attach overnight. After 24 hours, 40-60% confluent cells were transfected using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions, using 50 nmol of total siRNA. Transfections were repeated on days 4 and 6. On day 7, RNA was harvested and analyzed by qRT-PCR to determine the efficiency of the knockdown.

Pseudomonas aeruginosa Filtrate (PaF) Stimulation

Late phase filtrate (PaF) was prepared from a stable mucoid *Pseudomonas aeruginosa* isolate from the lungs of a cystic fibrosis patient as described previously [151]. Briefly, *Pseudomonas* was grown in 4% peptone (BD Diagnostics) at 37^{o}C for 72 hours with shaking at 270 RPM. The filtrate was then collected and centrifuged at 10,000 RPM for 1 hour at 4^{o}C followed by filtration through a 0.22 µm filter (Nalgene). The filtrate was heat inactivated at 95^{o}C for 10 minutes prior to use.

Arachidonic Acid Uptake:

On day 7, cells were washed twice with PBS + (5 mg/ml) BSA, then incubated with medium containing 10% reduced lipid FCS and supplemented with 0.5 nM [1-^{3}H]AA for 5, 15, 30, or 60 minutes. After the incubation, the medium was aspirated and two 500 µL aliquots were counted in a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter (LSC) as a control.

Subsequently, the cells were washed three times with PBS + (5 mg/ml) BSA, then lysed with 1N NaOH and two 500 µL aliquots were counted by LSC to quantitate AA uptake.

Arachidonic Acid Release:

On day 7, cells were washed twice with PBS + (5 mg/ml) BSA, then incubated for 24 hours with medium that contained 10% reduced lipid FCS and supplemented with 0.5 nM [1-^{3}H]AA. After the incubation, the medium was aspirated and two 500 µL aliquots were counted by LSC. Subsequently, the cells were washed three times with PBS + (5 mg/ml) BSA, then incubated for 5, 10, 15, or 30 minutes with medium that contained 2 mg/ml BSA and varying concentrations of A23187 in the presence or absence of PaF or cPLA2α siRNA. After the stimulation, the medium was aspirated and cells were washed three times with PBS + (5 mg/ml) BSA, then lysed with 1N NaOH and two 500 µL aliquots of both release medium and lysates were counted by LSC. The percent AA release was calculated from dpm counts as [Release Medium / (Release Medium + Cell Lysate)] x 100.

HPLC Analysis:

The Release medium was extracted and analyzed by reverse phase high performance liquid chromatography (RP-HPLC) followed by quantitation of radioactivity in a liquid scintillation detector. Release medium collected after A23187 stimulation for 15 minutes, as described above, was acidified to pH 3

using 1 N HCl. The samples were then extracted using 100 mg C18 Restek SPE Cartridges (Bellefonte, PA). Eicosanoids were eluted from the SPE cartridges with ethyl acetate followed by methanol. Eluted samples were dried under nitrogen and then reconstituted in 50 µL solvent A for HPLC analysis. RP-HPLC separation was performed on a 1200 series Agilent HPLC system equipped with a (4.6 x 250 mm, 5 µm) Agilent Zorbax Eclipse XDB-C18 column and a (4.6 x 12.5 mm, 5 µm) column guard. The separation system was set as 15 minutes of 100% solvent A acetonitrile/H_2O/acetic acid (38:62:0.01 by vol), followed by 15 minutes of 100% solvent B acetonitrile/H_2O/acetic acid (70:30:0.01 by vol), and finally 15 minutes of 100% acetonitrile. Radiolabeled peaks were identified by comparing their retention times to the retention times of unlabeled standards identified by UV detection at 205. Quantification of the radioactivity in individual peaks was with an IN/US β-RAM Model 4 scintillation detector coupled to the HPLC instrument.

Statistical Analysis:

Data comparison was performed using *t* test or two-way ANOVA with Bonferroni post-test for pairwise comparisons as described in Figure Legends using Prism 6 (GraphPad Software, La Jolla, CA). $P<0.05$ was considered statistically significant.

Results

Arachidonic Acid Uptake Is Increased in CF Cells

Previous work from our group showed that there is increased metabolism from LA to AA in CF cells [71]. Due to the importance of AA as a precursor for pro-inflammatory eicosanoids, we were interested in investigating whether there is an increased demand for AA in CF cells. To that end, we examined the uptake of AA by WT and CF cells by incubating confluent cells with 0.5 nM [1-^{3}H]AA for several time points. After radiolabeled AA incubation, the medium was removed and radioactivity quantified by LSC. CF cells showed increased uptake of [1-^{3}H]AA at 15, 30, and 60 minutes (Figure 11). This indicates that CF cells have higher demand for AA, which may be due to increased utilization of this fatty acid by those cells.

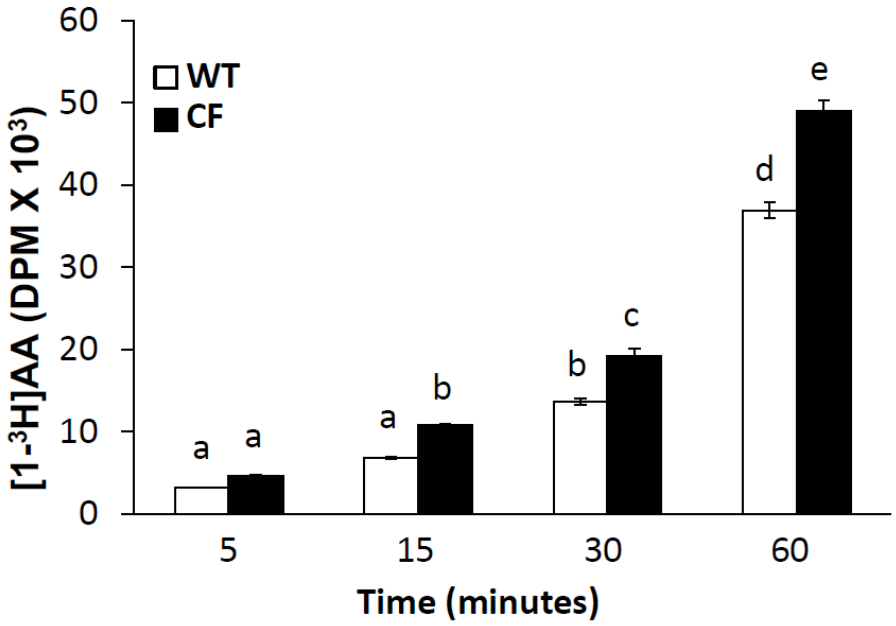

Figure 11:
Uptake of [1-^{3}H]AA is increased in CF cells. 16HBEo− (S, WT) and (AS, CF) cells were grown for 7 days. On day 7, cells were incubated with 0.5 nM [1-^{3}H]AA for 5, 15, 30, or 60 minutes. After the incubation, cells were lysed with 1N NaOH, and two 500 µL aliquots of the cell lysate were counted by LSC to measure uptake as described in Materials and Methods. Bars represent mean ± SEM (n=3). These results represent one of two independent experiments. Unlike letters indicate significant pairwise comparison differences with P at least <0.05.

Calcium-dependent Arachidonic Acid Release Is Increased in CF Cells

In our next set of experiments, we examined the relative amount of AA release in both WT and CF cells. The incorporation of radiolabeled AA was similar between WT and CF cells after 24 hours of incubation (Figure 12a). For this reason, the cells were incubated with radiolabeled for 24 hours in AA release studies. After a 24-hour incubation with 0.5 nM [1-^{3}H]AA, confluent cells were stimulated for 15 minutes with 1 μM calcium ionophore A23187. After stimulation, the medium was removed and radioactivity was measured by LSC. We saw that increasing cytosolic Ca^{2+} concentrations by stimulating the cells with A23187 for 15 minutes caused a significantly greater increase in AA release from CF cells than WT cells (Figure 12b). To make certain that the measured radioactivity was mainly [1-^{3}H]AA, we extracted and analyzed the release medium by RP-HPLC connected to a LSC. The main radiolabeled peak that we detected corresponded to the peak of free AA (Figure 12c). Additionally, AA release was significantly higher in CF cells after 15 or 30 minutes stimulation with 1 μM of A23187 (Figure 13a). Increasing A23187 concentration from 2.5 to 10 μM amplified AA release from both cell lines, but release was always higher in CF cells (Figure 13b).

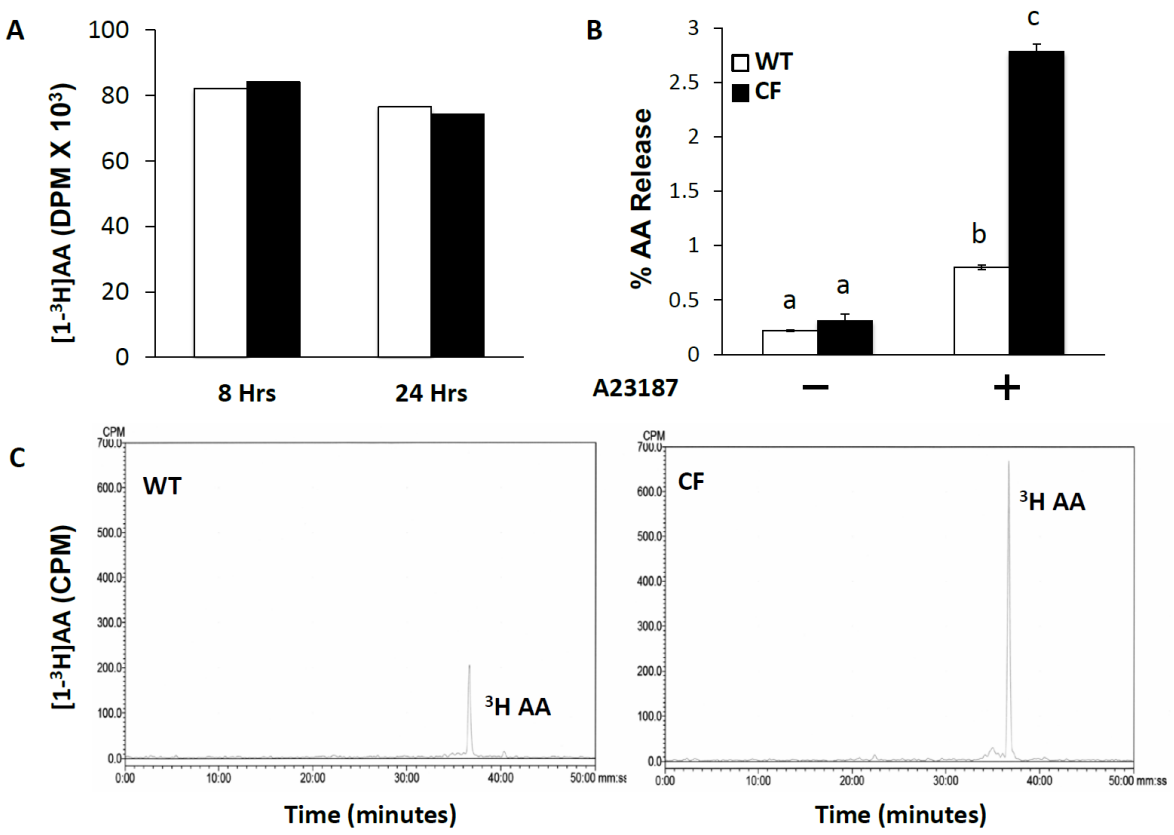

Figure 12:

A23187-induced AA release is increased in CF cells. 16HBEo− (S, WT) and (AS, CF) cells were grown for 7 days. (A) On day 7, cells were incubated with 0.5 nM [1-^{3}H]AA for 8 or 24 hours. After the incubation, cells were lysed with 1N NaOH, and two 500 µL aliquots of the cell lysate were counted by LSC to measure uptake as described in Materials and Methods. Alternatively, cells were incubated with 0.5 nM [1-^{3}H]AA overnight. After 24 hours, cells were incubated for 15 minutes with either 1 µM of A23187 or DMSO. After the stimulation, the medium was collected (Release Medium) (B) Cells were lysed with 1N NaOH, and two 500 µL aliquots of both Release Medium and cell lysate were counted by LSC. The percent AA release was calculated from dpm as [Release Medium / (Release Medium + Cell Lysate)] x 100 as described in Materials and Methods; (C) (Release medium) was extracted for eicosanoid measurement, then analyzed by RP-HPLC connected to a liquid scintillation counter as described in Materials and Methods. Bars represent mean ± SEM (n=3). These results represent one of two independent experiments. Unlike letters indicate significant pairwise comparison differences with P at least <0.05.

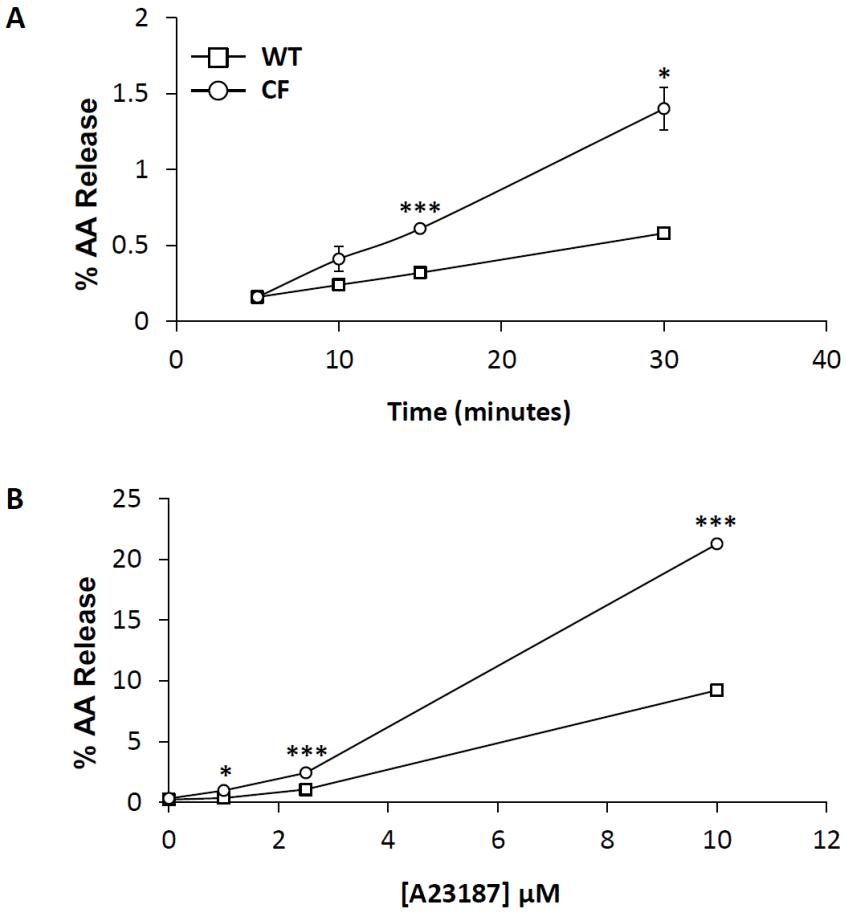

Figure 13:
Increase in calcium-induced AA release in CF cells. 16HBEo− (S, WT) and (AS, CF) cells were grown for 7 days. On day 7, cells were incubated with 0.5 nM [1-^{3}H]AA overnight. After 24 hours, cells were incubated (A) with 1 µM of A23187 for 5, 10, 15, or 30 minutes; or (B) for 15 minutes with 1 µM, 2.5 µM, 10 µM of A23187 or DMSO. After the stimulation, medium was collected (Release Medium), cells were lysed with 1N NaOH, and two 500 µL aliquots of both Release Medium and cell lysate were counted by LSC. The percent AA release was calculated from dpm as [Release Medium / (Release Medium + Cell Lysate)] x 100, as described in Materials and Methods. Open circles (○, CF) and open squares (□, WT) represent mean ± SEM (n=3). These results represent one of two independent experiments. ***P<0.001, *P<0.05.

Cytosolic Phospholipase A_2 ($cPLA_2\alpha$) Plays an Important Role in the Increased Calcium-induced Arachidonic Acid Release in CF Cells

Cytosolic phospholipase A_2 is a calcium-dependent enzyme that frees AA from the sn-2 position in membrane phospholipids. It is thought that the free AA released by this enzyme is mostly destined for eicosanoid production. Therefore, elucidating its role in CF may shed light on the role of AA in the inflammatory response in this disease. We first compared mRNA expression levels of both $cPLA_2\alpha$ and $cPLA_2\beta$ between confluent WT and CF cells. We found that the mRNA expression of both enzymes was higher in CF cells (Figure 14a). To determine the contribution of $cPLA_2\alpha$ in the increased Ca^{2+}-induced AA release in CF cells we performed siRNA knockdown of the gene in both cell lines. The knockdown caused 80% and 86% decrease in mRNA expression of $cPLA_2\alpha$ in WT and CF cells, respectively (Figure 14b). Importantly, the knockdown in CF cells was accompanied by a 36% decrease in AA release in response to stimulation for 15 minutes with 2.5 μM of A23187, while it remained the same in WT cells (Figure 14c).

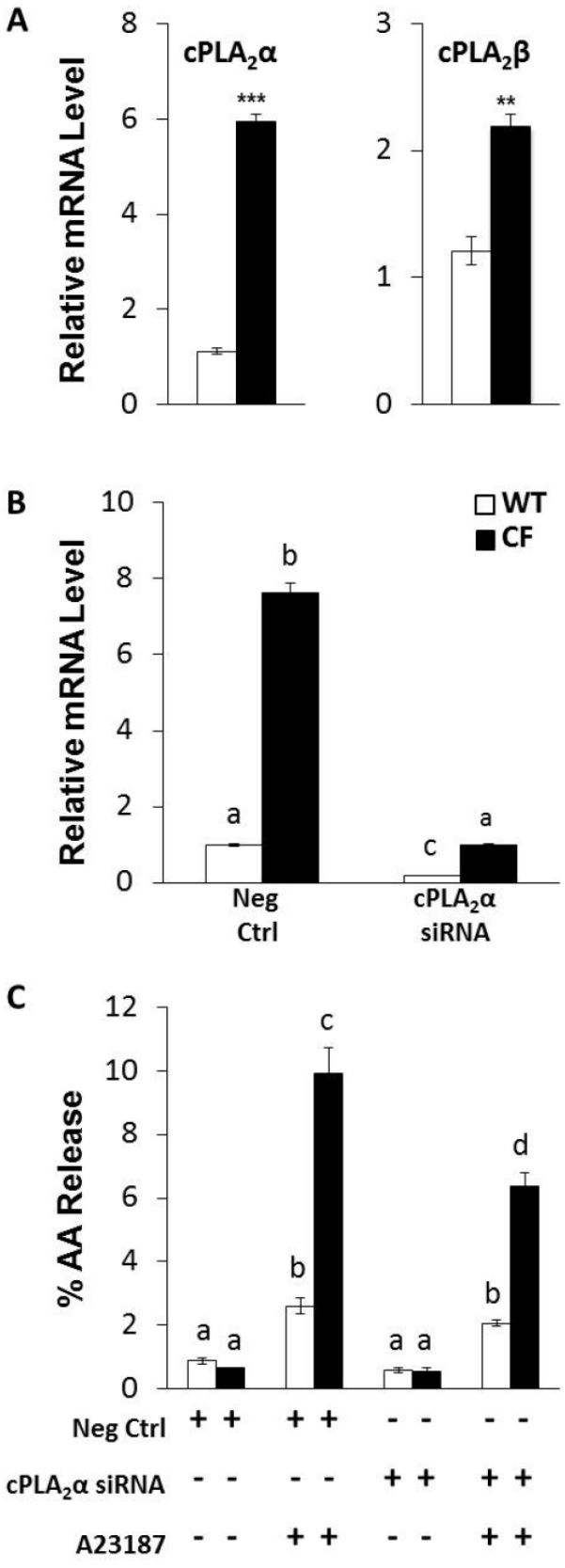
A
Relative mRNA Level
cPLA2α
cPLA2β
***
**
B
WT
CF
Relative mRNA Level
a
b
c
a
Neg Ctrl
cPLA2α siRNA
C
% AA Release
a a b c a a b d
Neg Ctrl + + + + - - - -
cPLA2α siRNA - - - - + + + +
A23187 - - + + - - + +

Figure 14:

Cytosolic Phospholipase A2 contributes to AA release. 16HBEo− (S, WT) and (AS, CF) cells were grown for 7 days. (A) On day 7, mRNA was extracted, cDNA was synthesized, and primers for the mRNA sequences for cytosolic phospholipase A2α ($cPLA_2\alpha$) and $cPLA_2\beta$ were used for qRT-PCR analysis as described in Materials and Methods. (B) cPLA2α siRNA knockdown decreased the calcium-induced AA release in CF cells. 16HBEo− (S, WT) and (AS, CF) cells were transfected using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions and grown for 7 days. On day 7, mRNA was extracted, cDNA was synthesized, and primers for the mRNA sequences for cytosolic phospholipase A2α (cPLA2α) was used for qRT-PCR analysis as described in Materials and Methods. Alternatively (C), cells were incubated with 0.5 nM [1-^{3}H]AA overnight. After 24 hours, cells were incubated for 15 minutes with either 2.5 μM of A23187 or DMSO. After the stimulation, medium was collected (Release Medium), cells were lysed with 1N NaOH, and two 500 μL aliquots of both Release Medium and cell lysate were counted by LSC. The percent AA release was calculated from dpm as [Release Medium / (Release Medium + Cell Lysate)] x 100 as described in Materials and Methods. Bars represent mean ± SEM (n=3). These results represent one of two independent experiments. ***P<0.001, **P<0.01. Unlike letters indicate significant pairwise comparison differences with P at least <0.05.

Inflammatory Stimulation with Pseudomonas aeruginosa Late Phase Filtrate Amplifies the Calcium-induced Arachidonic Acid Release in CF Cells

Assessing whether AA release is influenced by inflammatory stimulation may be important in identifying the role of AA in the increased inflammatory response in CF. To that end, we used late phase *Pseudomonas aeruginosa* filtrate (PaF) as an inflammatory stimulus in addition to A23187, and measured AA release from confluent WT and CF cells by LSC. We found that AA release was higher in CF cells after stimulation for 15 minutes with 2.5 μM of A23187. AA release was amplified significantly when the cells were stimulated by both A23187 and PaF for 15 minutes and was higher in CF cells relative to WT cells (Figure 15). These findings suggest that AA release may be contributory to the excessive inflammatory response in CF by increasing the availability of AA as a substrate for the production of pro-inflammatory eicosanoids.

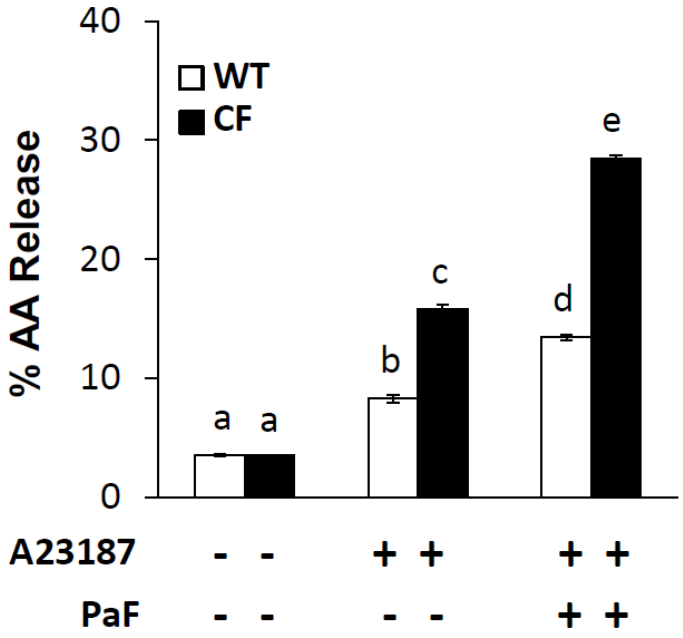

Figure 16:
Stimulation with late-phase *Pseudomonas* filtrate (PaF) enhances the calcium-induced AA release in CF cells. 16HBEo− (S, WT) and (AS, CF) cells were grown for 7 days. On day 7, cells were incubated with 0.5 nM [1-^{3}H]AA overnight. After 24 hours, cells were incubated for 15 minutes with 2.5 μM A23187, PaF + 2.5 μM A23187 or DMSO. After the stimulation, medium was collected (Release Medium), cells were lysed with 1N NaOH, and two 500 μL aliquots of both Release Medium and cell lysate were counted by LSC. The percent AA release was calculated from dpm counts as [Release Medium / (Release Medium + Cell Lysate)] x 100 as described in Materials and Methods. Bars represent mean ± SEM (n=3). These results represent one of two independent experiments. Unlike letters indicate significant pairwise comparison differences with P at least <0.05.

Discussion

In this study, we showed that there is an increased uptake of AA and AA release in CF cells compared to their WT counterparts. Moreover, we demonstrated that cytosolic phospholipase A2 plays an important role in AA release. It was also shown that stimulation by late phase *Pseudomonas aeruginosa* filtrate was able to amplify the calcium-induced AA release in CF cells. These results provide a link from the observed increase in LA to AA metabolism and lung inflammation in CF. These findings also suggest new approaches for dietary management in CF.

The observed increased uptake of AA after 15 minutes and up to 60 minutes in CF cells compared to WT cells in this study is in agreement with a previous report that showed there was 30% increase in AA uptake by CF cells after 10 minute pulse experiments [82]. The increased uptake of AA by CF cells may indicate higher demand for this fatty acid. This could be related to the apparently increased consumption of AA for the production of pro-inflammatory eicosanoids.

Levistre et al. investigated the release of AA in tracheal epithelial cells in response to bradykinin and found that CF cells exhibited higher AA release that is related to $cPLA_2$ [152]. Similarly, in human bronchial epithelial cells (16HBEo−), we found that AA release was higher in CF cells than in WT cells in response to calcium ionophore A23187. We demonstrated that AA release

was apparently dependent on intracellular Ca^{2+} concentrations as AA release increased with longer exposure times as well as higher doses of A23187.

A study by Wu et al. showed that cytosolic phospholipase A_2 ($cPLA_2\alpha$) inhibition or knockout in CF mice was able to reduce LPS-induced airway constriction [153]. Another study by the same group reported that the activity of $cPLA_2\alpha$ was associated with mucus overproduction in the lungs of CF mice in response to LPS stimulation [154]. In our study, we saw that the expression of both calcium dependent cytosolic phospholipases $cPLA_2\alpha$ and $cPLA_2\beta$ was higher in CF compared to WT cells. This appeared to play a role in the elevated AA release observed in CF cells since siRNA knockdown of $cPLA_2\alpha$ caused a significant decrease in the A23187-dependent AA release. The knockdown did not lower AA release in CF cells to WT levels. One possible explanation is that the mRNA expression in CF cells for $cPLA_2\alpha$ remained 5-fold higher compared to WT cells after the knockdown. Another possible explanation is there is a contribution of $cPLA_2\beta$, which is also elevated in CF cells, in the release of AA. We also measured the mRNA expression of calcium-dependent secretory phospholipase A_2 ($sPLA_2$-IIA) and found that there was no difference in expression between WT and CF cells (data not shown). This does not rule out the involvement of $sPLA_2$-IIA completely as activity of this enzyme may account for a portion of the increased AA release in CF cells.

Arachidonic acid release from membrane phospholipids is an important rate-limiting step in the production of AA-derived pro-inflammatory

eicosanoids. The cyclooxygenase-2 (COX-2) and 5-lipoxygensae (5-LOX) pathways are two of the main pathways in inflammation-related eicosanoid production. While all products of the COX-2 and 5-LOX pathways play an essential role, prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) are especially important in the inflammatory response. LTB_4 is a potent neutrophil chemoattractant, while PGE_2 affects many cellular processes via cAMP and/or Ca^{2+} signaling [43]. *Pseudomonas aeruginosa* (Pa) is responsible for the majority of lung infections in CF patients at an early age, and their infections lead to tissue destruction and subsequently to mortality. The above mentioned AA-derived eicosanoids are thought to be important participants in the CF inflammatory response that leads to neutrophil accumulation and the ensuing tissue destruction [155], as many COX-2 and 5-LOX products have been shown to be increased in patients and various models of CF [71,83-87,89,90]. Therefore, the study of AA release in response to Pa inflammatory stimulation is important in understanding the contribution of AA and its metabolites in the pathology of CF. To study this, we used late phase *Pseudomonas aeruginosa* filtrate (PaF) in addition to A23187 to assess the role of inflammatory stimulation in calcium-induced AA release. PaF stimulation caused a significant increase in the AA release in CF cells compared to WT cells. This adds further evidence that the increased AA release may contribute to the inflammatory response in CF by increasing the availability of AA for the production of AA-derived eicosanoids.

The results in this report demonstrate a possible link between the observed PUFA alterations and the inflammatory response in CF. Previous work from our group showed that LA levels were lower, while AA levels were higher, in CF compared to WT cells. This was accompanied by an increase in the expression and activity of Δ6- and Δ5- desaturase that led to increased metabolism of LA to AA in the n-6 pathway [71]. CF cells may require higher amounts of AA to produce larger amounts of pro-inflammatory eicosanoids, which may lead to the observed increased metabolism from LA to AA and elevated uptake of AA. Other published work by our group has shown that n-3 fatty acids, namely (LNA, 18:3n-3) and (DHA, 22:6n-3), are able to decrease the levels of AA in CF cells by direct competition for or inhibition of the metabolic enzymes shared by the n-3 and n-6 pathways [97,156]. These findings suggest the need for a change in the dietary recommendation for CF patients, in which n-6 fatty acid intake is decreased and n-3 fatty acids is increased, in order to decrease the levels of AA.

In summary, the data reported in this study demonstrate that there is dysregulation in AA utilization in CF bronchial epithelial cells compared with WT controls that leads to increased uptake and release of this fatty acid. The dysregulation of AA uptake and release may be highly contributory to the inflammatory response in CF.

CHAPTER IV

PRODUCTS OF THE 5-LIPOXYGENASE PATHWAY MEDIATE CYCLOOXYGENASE-2 AND CYTOKINE OVEREXPRESSION IN RESPONSE TO *PSEUDOMONAS AERUGINOSA* IN AN AIRWAY EPITHELIAL CELL MODEL OF CYSTIC FIBROSIS

The work presented in chapter III establishes that CF cells uptake more AA and release more of this fatty acid in response to stimulation. Here we will show that this dysregulation in AA utilization leads to the elevated inflammatory response through products of the 5-LOX pathway.

Introduction

Mutations in the *CFTR* gene cause varying degrees of channel dysfunction, affecting multiple tissues. However, most morbidity and mortality in CF are due to progressive pulmonary failure [7]. In the lungs, defective channel function compromises mucociliary clearance, causing obstruction of respiratory passages by thick, viscid mucus [11]. This establishes a vicious cycle of obstruction, infection, and inflammation, which leads to tissue destruction [157]. In particular, enhanced inflammation appears to play a significant role in CF pathophysiology. Infected CF airways exhibit marked inflammation, characterized by profuse neutrophil infiltration and production of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and IL-8 [158]. This inflammation appears to be disproportionate to levels of infection [26]. In fact, there is evidence to suggest that inflammation may be increased even in the

absence of infection [159-161]. These findings imply that *CFTR* mutations may establish a uniquely pro-inflammatory state, although the mechanisms are poorly understood.

Eicosanoids are among the mediators that could be responsible for enhanced inflammation in CF. There is considerable evidence for abnormal eicosanoid biosynthesis in CF. Production of prostaglandins and leukotrienes is increased in CF patients [94,162-168], while lipoxin production appears to be reduced [169,170]. These changes are associated with increased expression of COX-2 and 5-LOX in CF patient tissues [91,171] and in epithelial cell culture models of CF [128].

As a major source for the production of pro-inflammatory eicosanoids, AA availability is a limiting factor for the production of these mediators. However, a number of studies have demonstrated increased metabolism of AA from its precursor fatty acid, linoleic acid (LA; 18:2n-6) in CF cells [120-122,133,172]. This is due to increased expression of Δ5- (Δ5D) and Δ6-desaturases (Δ6D) in CF cells [128,129] and accounts for the plasma PUFA alterations consistently observed in CF patients [reviewed in [117,118]]. Dietary studies in animal models suggest that this altered PUFA metabolism may have an impact on inflammation. Lungs of *CFTR*$^{-/-}$ knockout mice supplemented with LA show increased AA levels and markedly increased neutrophil production in response to an inflammatory stimulus [130]. On the other hand, *CFTR*$^{-/-}$ knockout mice supplemented with docosahexaenoic acid (DHA; 22:6n-3), which is known to suppress expression of the fatty acid desaturases

[129], exhibit reduced AA levels and decreased neutrophil production in the lungs [120].

While these studies are suggestive, the relationships between abnormalities in PUFA metabolism, eicosanoid production, and inflammation in CF are incompletely understood. In this report, we test the hypothesis that alterations in PUFA and eicosanoid biosynthesis play a significant role in the disproportionate and excessive inflammatory response characteristic of CF airways. Utilizing a human airway epithelial cell culture model, we found marked over-expression of PUFA and eicosanoid biosynthesis enzymes in response to an inflammatory stimulus in CF compared with control cells. This resulted in significantly increased production of pro-inflammatory cytokines that is predominantly mediated by eicosanoids of the 5-LOX pathway. These findings suggest a prominent role for PUFA and eicosanoid metabolism in the altered inflammatory response in CF.

Materials and Methods

Materials:

NS-398 was obtained from Tocris Bioscience (Minneapolis, MN), AA-861 from Santa Cruz (Santa Cruz, CA), docosahexaenoic acid from NuChek Prep (Elysian, MN), and Bacto Peptone from BD Diagnostics (Sparks, MD). MK-886, d_4-LTB_4, and rabbit monoclonal antibody for detection of human COX-2 were obtained from Cayman Chemicals (Ann Arbor, MI). SC-57461-A and mouse monoclonal antibody for detection of human β-actin were from

Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibody for detection of human mPGES-1 and polyclonal goat anti-rabbit secondary antibody were from Abcam (Cambridge, MA). Rabbit monoclonal antibody for detection of human IL-6 was from Cell Signaling Technology (Danvers, MA) and polyclonal sheep anti-mouse secondary antibody was from GE Healthcare Life Sciences (Pittsburgh, PA). Fatty acid methyl ester (FAME) standards for HPLC were purchased from NuCheck Prep (Elysian, MN). HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA). Liquid scintillation cocktail (FlowLogic U) was purchased from LabLogic Systems, Inc. (Brandon, FL).

CF Mice:

All experiments were carried out under supervision of the Vanderbilt Division of Animal Care using protocols approved by the Institutional Animal Care and Use Committee. $CFTR^{tm1/UNC}$ heterozygous mice ($CFTR^{+/-}$) on a C57BL/6J genetic background (B6.129P2-Cftrtm1Unc/J, stock number 002196) were purchased from Jackson Laboratories (Bar Harbor, ME). These mice carry one copy of *CFTR* lacking exon 10 that encodes the first transmembrane domain of CFTR ($CFTR^{TM1UNC}$) [173]. The mice were housed within a specific pathogen-free barrier facility with a 12 hour light/dark cycle. Homozygous knockout (CF; $CFTR^{-/-}$) and wild-type (WT; $CFTR^{+/+}$) mice were generated by mating male and female heterozygotes. Tail-clip samples from 14-day old mice were used for genotyping by PCR [174]. WT and CF mice were weaned at approximately day 21 and subsequently maintained on a water and

liquid Peptamen (Nestle HealthCare Nutrition, Inc, Florham, NJ) diet for 14 additional days. The liquid diet was used to prevent intestinal obstruction. Mice were euthanized with carbon dioxide and lung tissue was collected and cell suspension enriched for epithelial cells was generated as previously described[120].

Cell Culture:

Human airway epithelial cells (HAEC) CuFi-1 and NuLi-1 were a kind gift from Dr. Simon Rousseau (McGill University, Montreal, Quebec, Canada). NuLi-1 (control) cells were derived from the normal airway of a 36-year-old healthy subject, while CuFi-1 (CF) cells were derived from the airway of a 14-year-old patient with cystic fibrosis due to homozygous ΔF508 mutation in the *CFTR* gene [175]. The cells were grown in serum-free Bronchial Epithelial Growth Media (BEGM) that was supplemented with BEGM Bullet Kit (Lonza), linoleic acid (L9530) (Sigma), and 50 µg/mL G-418 (Life Technologies), and maintained at 37^{o}C in 5% CO_2. The medium was changed every other day until 2-days post-confluence (7 days).

Sense and antisense human bronchial epithelial cells (16HBEo^{-}) were a kind gift from Dr. Pamela Davis (Case Western Reserve University School of Medicine, Cleveland, OH). These cells are immortalized human bronchial epithelial cells stably transfected with a segment of the CFTR gene in the sense (control) or antisense (CF) orientation [132]. Control cells have normal CFTR expression and activity, while CF cells lack both [122,132]. These cells

were grown in MEM + glutamax (Life Technologies) with 10% horse serum (Omega Scientific), penicillin (100 U/mL), and streptomycin (100 µg/mL), and maintained at 37^{o}C in 5% CO_2. Medium was changed every other day.

Fatty Acid Supplementation:

Docosahexaenoic acid (DHA) was added to BEGM medium that has been supplemented with bovine serum albumin (BSA) at a 2.5:1 BSA:DHA molar ratio. Control medium contained BSA only. CuFi-1 and NuLi-1 cells were set up on day 0 and allowed to attach for 24 hours in BEGM serum-free medium as described above. On day 1, cells were washed with PBS and fed DHA-supplemented or control medium that was changed every other day until confluence.

Pseudomonas aeruginosa Filtrate (PaF) Stimulation:

PaF was prepared from a stable mucoid *P. aeruginosa* isolate obtained from the lungs of a cystic fibrosis patient as described previously [176-178]. Briefly, the bacteria were grown in 4% peptone (BD Diagnostics) at 37^{o}C for 72 hours with shaking at 270 RPM. The culture was then collected and centrifuged at 10,000 RPM for 1 hour at 4^{o}C followed by filtration through a 0.22 µm filter (Nalgene). The resulting filtrate was heat-inactivated at 95^{o}C for 10 minutes prior to use. Cells were stimulated with 20 µL PaF (equivalent to 15 µg PaF protein) per mL of culture medium for 12 hours in the presence or absence of various treatments (NS-398, AA-861, MK-886, or SC-57461A).

After 12 hours of stimulation, the media was used for eicosanoid or cytokine analysis while the cells were harvested for protein or mRNA analysis. The 12 hour time was selected because time course studies indicated maximal stimulation at this point (data not shown).

Fatty acid composition analysis:

After growing for 7 days, cells were scraped on ice with a rubber policeman and ice-cold PBS then centrifuged at 100xg for 8 min. Pelleted cells were resuspended with 500 µL ice-cold PBS and 10 µg of heptadecanoic acid (17:0) was added as an internal standard. Lipids were then extracted according to the modified Folch method [134]. Briefly, 3 mL of chloroform-methanol (2:1 v/v) were added to resuspended cells and vortexed heavily. Samples were incubated on ice for 10 min, vortexed, then centrifuged at 1100xg for 10 min. The resulting lower phase was dried completely under nitrogen in preparation for fatty acid methylation using boron trifluoride (BF_3)-methanol and a methanolic-base reagent. Briefly, 500 µL of 0.5 N methanolic then 500 µL NaOH BF_3-methanol were added to samples. Each step was followed by heating at 100^{o}C for 3 minutes then 1 minute, respectively. The resulting fatty acid methyl esters (FAMEs) were extracted with 1 mL hexane followed by 6.5 ml of saturated NaCl solution. FAMEs were identified using an Agilent 7980A gas chromatography (GC) system (Agilent Technologies, Santa Clara, CA) equipped with a Supelcowax SP-10 capillary column (Supelco, Bellefonte, PA) coupled to an Agilent 5975C mass spectrometer (MS). FAME

mass was determined by comparing areas of unknown FAMEs to that of the 17:0 internal standard. Results were reported as the molar percentage (mol%) of each FAME relative to the total mass of the sample, as previously described [133].

Fatty acid radiolabeling experiments:

On day 7, cells were incubated with media containing 10% reduced lipid-FCS and [1-^{14}C]LA for 4 hours. Lipids were extracted and fatty acids were methylated as described above. FAMEs in the final hexane layer were resuspended in 50 µL acetonitrile after hexane was dried under nitrogen in preparation for HPLC analysis. Radiolabeled FAMEs were separated by reverse-phase high performance liquid chromatography (RP-HPLC) using a binary solvent system on an Agilent 1200 series instrument equipped with a (4.6 x 250 mm, 5 µm) Agilent Zorbax Eclipse XDB-C18 column and a (4.6 x 12.5 mm, 5 µm) column guard. Solvent A consisted of H_2O + 0.02% H_2PO_4 and solvent B was 100% acetonitrile. The solvent program was 58% solvent B for 25 min, 58-61% B for 2 min, 61% B for 8 min, 61-100% B for 15 min, 100% B for 20 min, and finally reconstitution to original conditions. UV detection at 205 nm and comparison with the retention times of unlabeled standards was used for identification of radiolabeled peaks. Quantification of the peaks was done by using an IN/US β-RAM Model 4 scintillation detector coupled to the HPLC instrument. Results were reported as percentage of total measured counts in a sample.

Eicosanoid Analysis:

Cell culture media were collected from different treatment conditions as described above, and were acidified to pH 3 using 1 N HCl followed by the addition of 10 ng internal standard d_4-LTB_4. The samples were then extracted using 100 mg C18 Restek SPE Cartridges (Bellefonte, PA). Eicosanoids were eluted from the SPE cartridges with ethyl acetate followed by methanol. Eluted samples were dried under nitrogen and derivatized with *N*-(4-aminomethylphenyl)pyridium (AMPP) that was synthesized as described previously [179]. Derivatization was initiated by the addition of 10 µL of ice-cold acetonitrile/dimethylformamide (4:1 by volume) to the dried samples, then 10 µL of ice-cold 640 mM 1-ethyl-3-(e-dimethylaminopropyl)carbodiimide. Next, 20 µL of a freshly-prepared solution of 5 mM N-hydroxylbenzotriazole and 15 mM AMPP dissolved in acetonitrile was added to the samples. The samples were vortexed, incubated for 30 minutes at 60^{o}C, transferred to analysis vials, and kept on ice for LC-MS/MS analysis. Samples (20 µL) were analyzed on a Thermo LTQ mass spectrometer that was interfaced to an Acquity liquid chromatograph using a C18 column. The mobile phase consisted of solvent A (water:acetonitrile, 95:5 and 0.1% formic acid) and solvent B (water:acetonitrile 5:95 0.1% formic acid). The elution program was a linear gradient from A to B over 10 minutes. The electrospray needle was maintained at 5.03 kV and ion transfer tube operated at 31.8 V and 280^{o}C. Transitions for 5-HETE, LTB_4, d_4-LTB_4, and PGE_2 were recorded in the selected reaction

monitoring (SRM) mode as *m/z* 487 to 283, *m/z* 503.5 to 322.9, *m/z* 507 to 325, and *m/z* 519 to 239, respectively. Since there were no significant differences in cellular protein concentrations, eicosanoid levels in cell culture media were reported as ng/mL.

Cytokine Analysis:

IL-6 and IL-8 in culture media were measured in triplicates by MILLIPLEX (EMD MILLIPORE, Billerica, MA) at the Vanderbilt Hormone Assay and Analytical Services Core.

Quantitative Real-Time PCR:

Total RNA was isolated from cells using TRIzol (Life Technologies, Carlsbad, CA) and contaminating DNA was removed from samples using DNA-free (Ambion, Austin, TX). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR (qRT-PCR) was performed in 10 µL reactions containing 50 ng cDNA, TaqMan primer-probes sets and TaqMan Universal PCR Master Mix in 96-well plates (Life Technologies, Carlsbad, CA). Each reaction was performed in duplicate or triplicate. C_t values were determined using the CFX96 Real Time PCR Detection System with CFX Manager software (Bio-Rad, Hercules, CA). Relative mRNA levels were calculated using the comparative C_t method using *RPLP0* or *GAPDH* as a reference gene in cultured cells and mice tissue, respectively.

Immunoblot Analysis:

Whole-cell lysates were prepared with RIPA lysis buffer (Sigma-Adrich) and 2X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA). Protein concentration was determined using BCA assay (Thermo Scientific). Protein samples were mixed 1:1 with 2X Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) and stored in aliquots at -80^{o}C. For electrophoresis, 15 µg of protein were run on pre-cast 4-20% polyacrylamide gels (Bio-Rad) and transferred to Immobilon-P PVDF membranes (EMD Millipore). Membranes were blocked using 5% (w/v) Blotting Grade Blocker (Bio-Rad) in TBS-Tween (Sigma-Aldrich). To visualize protein bands after primary and secondary antibody incubation, SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used and membranes were exposed to Amersham Hyperfilm ECL film (GE Healthcare). β-actin was used as a loading control.

Statistical Analysis:

Data was compared using *t* test or two-way ANOVA with Bonferroni post-test for pairwise comparisons as appropriate (see Figure Legends) using Prism 6 (GraphPad Software, La Jolla, CA). $P<0.05$ was considered statistically significant.

Results

Increased Expression of Key Eicosanoid Biosynthetic Enzymes In CF Mice

CFTR$^{-/-}$ knockout (CF) mice exhibit an increased inflammatory response compared to wild-type (WT) mice [120]. To determine whether eicosanoid biosynthesis might play a role in this heightened response, the expression of key enzymes in the eicosanoid synthesis pathways was measured in the lungs of CF and WT mice. Indeed, mRNA expression of COX-2, 5-LOX, and mPGES-1 were all significantly elevated in CF compared to WT mice at baseline (Fig. 16).

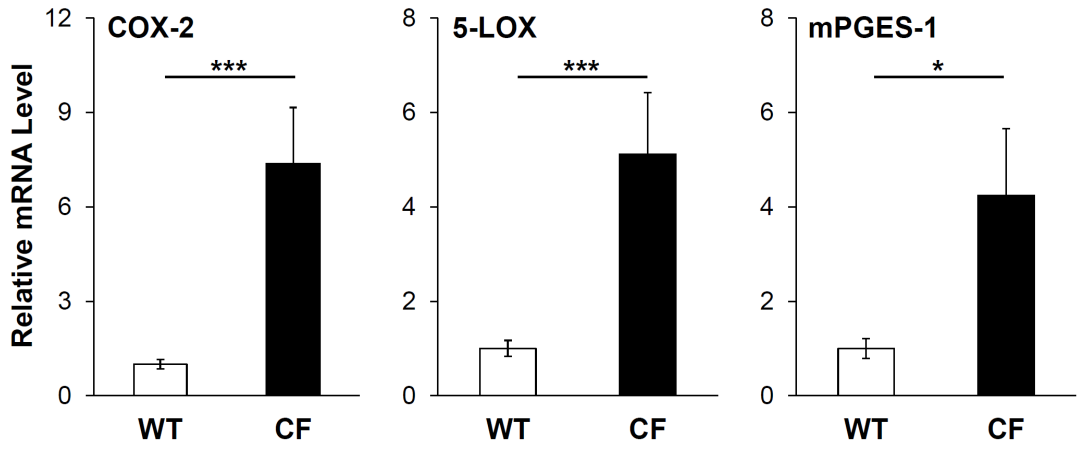

FIGURE 16:
Relative mRNA expression levels of eicosanoid metabolic enzymes in lung epithelial cells from CFTR$^{-/-}$ knockout (CF) and wild-type (WT) mice. Cell suspension enriched for epithelial cells was collected from lung tissue of CF and WT mice and mRNA was extracted as described in Experimental Procedures. Relative mRNA sequences for cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), and microsomal PGE_2 synthase-1 (mPGES-1) were measured by qRT-PCR analysis. Relative expression was determined using the $\Delta\Delta C_t$ method using GAPDH as an invariant control. Bars represent mean ± SEM (n=7). *, $P<0.05$; ***, $P<0.001$.

Increased Eicosanoid Biosynthesis in CF Cells

To determine the impact of eicosanoid biosynthesis on inflammation in CF, we focused on a cell culture model, using the CF HAEC line, CuFi-1, and the corresponding control line, NuLi-1. Inflammation was stimulated with PaF, a filtrate of medium in which a clinical isolate of *Pseudomonas aeruginosa* from a CF patient was cultured [176-178]. In untreated CuFi-1 cells, the mRNA expression of Δ6D, $cPLA_2α$, and mPGES-1 was significantly higher than in NuLi-1 cells (Fig. 17A). PaF stimulated a further increase in mRNA expression of these genes as well as COX-2 in CuFi-1 cells, while there was little or no change in expression in NuLi-1 cells (Fig. 17A). Corresponding protein expression of COX-2 was undetectable in both NuLi-1 and CuFi-1 cells at baseline, but there was a significant increase in CuFi-1 cells only after incubation with PaF. Expression of mPGES-1 protein was also significantly higher in CuFi-1 cells compared with NuLi-1 cells, although there was no apparent stimulation by PaF (Fig. 17B). To determine the effect of these changes on PGE_2 production, PGE_2 levels were measured in culture medium from these cells. PGE_2 levels were significantly higher in medium from CuFi-1 cells compared with that from NuLi-1 cells both at baseline and after PaF stimulation (Fig. 17C). PaF appeared to slightly increase PGE_2 levels in CuFi-1 cells, but the difference was not statistically significant.

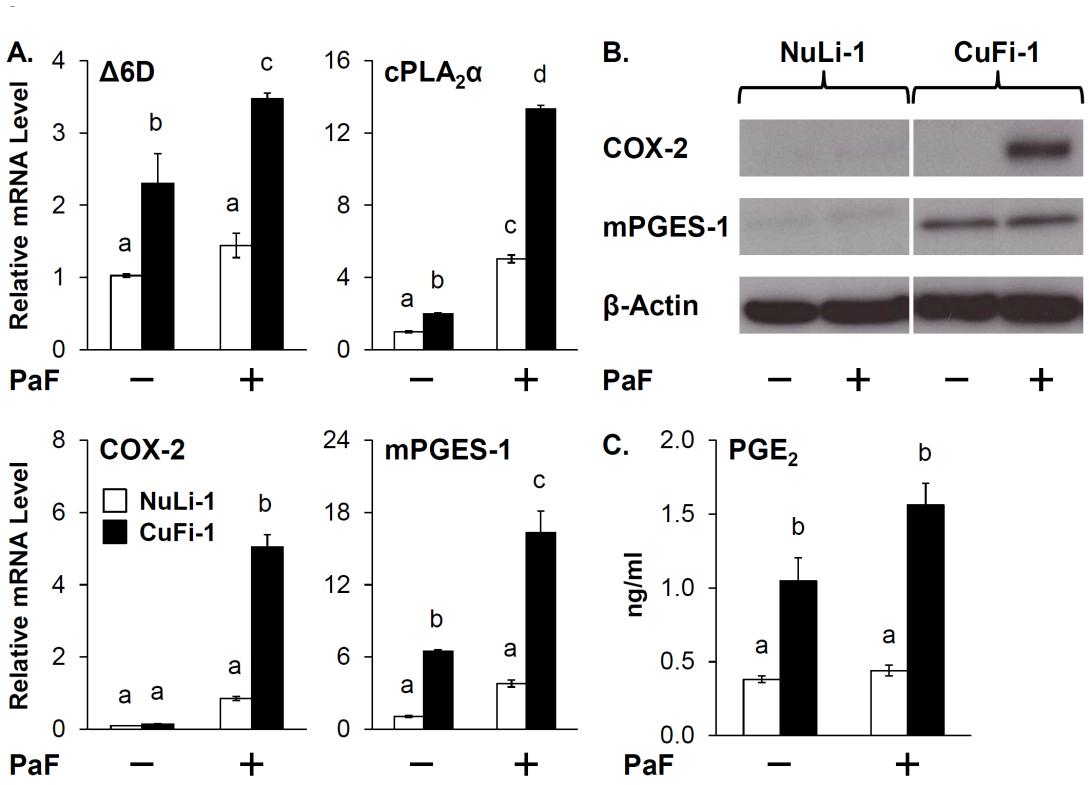

FIGURE 17:

Eicosanoid Biosynthesis in NuLi-1 (control) and CuFi-1 (CF) cells. A. Relative mRNA expression of fatty acid and eicosanoid metabolic enzymes in NuLi-1 (control) and CuFi-1 (CF) cells. NuLi-1 and CuFi-1 cells were cultured for 7 days as described in Experimental Procedures. On day 7, the cells were stimulated with *Pseudomonas aeruginosa* filtrate (PaF) for 12 hours. After stimulation, cells were harvested and mRNA extracted. Relative mRNA levels for Δ6-desaturase (Δ6-D), cytosolic phospholipase A2α (cPLA$_2$α), cyclooxygenase-2 (COX-2), and microsomal PGE$_2$ synthase-1 (mPGES-1) were measured by qRT-PCR. Relative expression was calculated by the $\Delta\Delta C_t$ method using RPLP0 as an invariant control. B. Protein expression of COX-2 and mPGES-1 in NuLi-1 and CuFi-1 cells. Cells were cultured and stimulated with PaF as above. After stimulation, whole cell lysates were subjected to SDS-PAGE and immunoblotting was performed as described in Experimental Procedures using antibodies against COX-2 and mPGES-1. Anti-β-actin antibodies were used as a loading control. C. PGE$_2$ production in NuLi-1 and CuFi-1 cells. Cells were cultured and stimulated as above. After stimulation, culture media was collected, extracted, and derivatized as described in Experimental Procedures. PGE$_2$ levels were measured by LC-MS/MS. Bars represent mean ± SEM (n=3). These results are representative of at least two independent experiments. Unlike letters indicate significant differences with any pair of values with at least $P<0.05$ by two-way ANOVA with Bonferroni post-test for pair-wise comparisons.

Expression of 5-LOX mRNA was also significantly higher in CuFi-1 cells compared with NuLi-1. PaF stimulation reduced 5-LOX expression in both cell types, although expression remained higher in the CuFi-1 cells (Fig. 18A). Accordingly, LTB_4 levels in culture medium were higher in CuFi-1 cells at baseline, and declined with PaF stimulation (Fig. 18B).

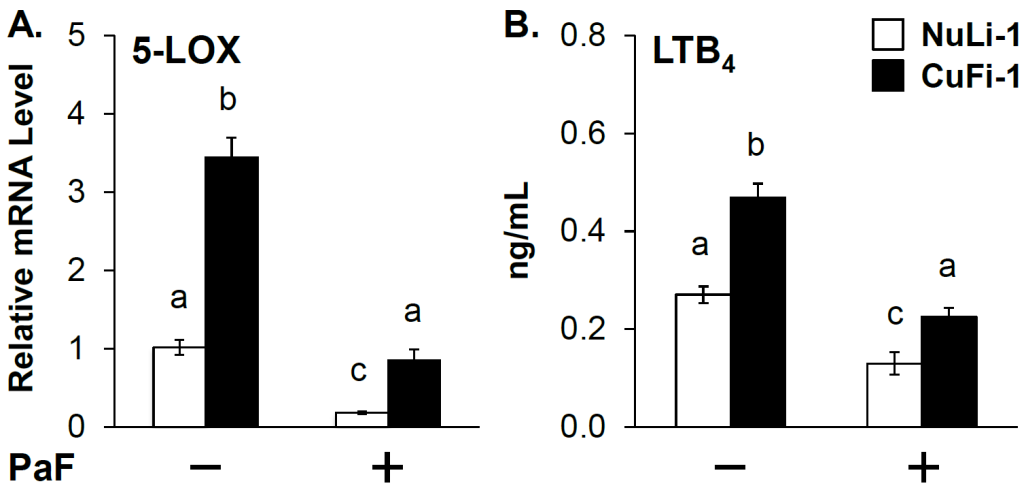

FIGURE 18:
Relative mRNA expression of 5-lipoxygenase (5-LOX) in NuLi-1 (control) and CuFi-1 (CF) cells. Cells were cultured for 7 days as described in Experimental Procedures. On day 7, the cells were stimulated with or without *Pseudomonas aeruginosa* filtrate (PaF) for 12 hours. After stimulation, cells were harvested and mRNA extracted. Relative mRNA levels for 5-LOX were measured by qRT-PCR. Relative expression was calculated by the $\Delta\Delta C_t$ method using RPLP0 as an invariant control. (B) LTB_4 production in NuLi-1 and (CF) cells. Cells were cultured and stimulated as above. After stimulation, culture media was collected, extracted, and derivatized as described in Experimental Procedures. LTB_4 levels were measured by LC-MS/MS. Bars represent mean ± SEM (n=3). These results are representative of at least two independent experiments. Unlike letters indicate significant differences with any pair of values with at least $P<0.05$ by two-way ANOVA with Bonferroni post-test for pair-wise comparisons.

Cytokine Production Is Higher in CF Cells

Interleukins IL-6 and IL-8 are thought to play an important role in pulmonary inflammation in CF [158,177]. To determine the effect of PaF stimulation on the production of these cytokines, culture medium was collected from both cell types before and after 12 hours of PaF stimulation. IL-6 and IL-8 levels, measured by MILLIPLEX multianalyte panel assays, were almost undetectable at baseline, but showed a marked increase in CuFi-1 cell cultures with PaF stimulation (Fig. 19A). Corresponding increases in NuLi-1 cell cultures were much smaller and not statistically different from baseline. Similar changes were seen in IL-6 and IL-8 mRNA levels (Fig. 19B) and IL-6 protein levels (Fig. 19C), indicating that these assays could be used as surrogates to measure production of cytokines.

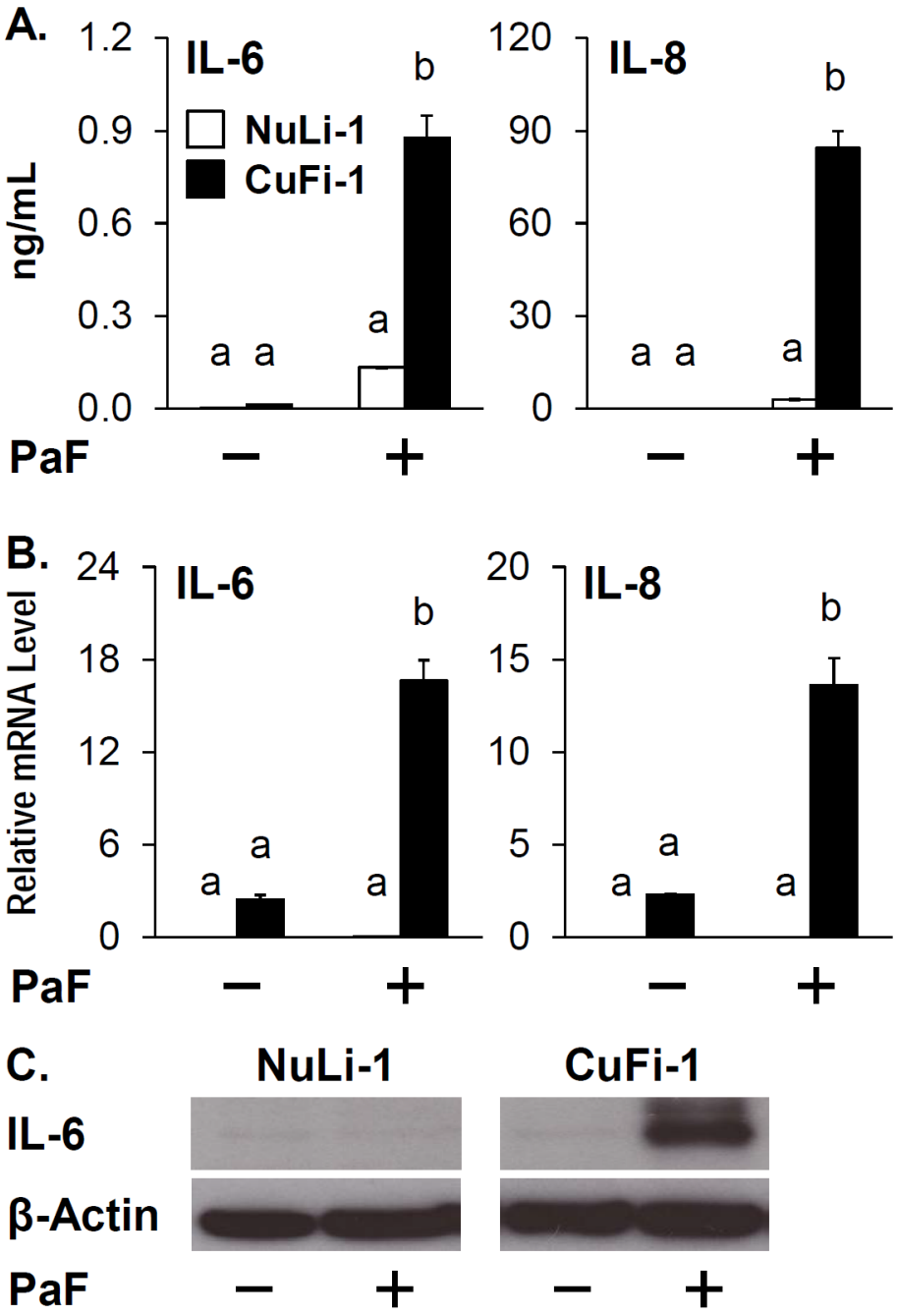
A.
IL-6
IL-8
NuLi-1
CuFi-1
ng/mL
1.2
0.9
0.6
0.3
0.0
120
90
60
30
0
a a
a
b
PaF
–
+
B.
Relative mRNA Level
24
18
12
6
0
20
15
10
5
C.
NuLi-1
CuFi-1
IL-6
β-Actin

FIGURE 19:

Cytokine levels in NuLi-1 (control) and CuFi-1 (CF) cells. A. Cytokine production in NuLi-1 (control) and CuFi-1 (CF) cells. Cells were cultured for 7 days as described in Experimental Procedures. On day 7, the cells were stimulated with or without *Pseudomonas aeruginosa* filtrate (PaF) for 12 hours. After stimulation, culture media was collected and IL-6 and IL-8 concentrations were measured by MILLIPLEX as described in Experimental Procedures. B. IL-6 and IL-8 mRNA expression in NuLi-1 and CuFi-1 cells. Cells were cultured and stimulated as above. After stimulation, mRNA was extracted and cDNA was synthesized as described in Experimental Procedures. Relative mRNA levels for IL-6 and IL-8 were measured by qRT-PCR. Relative expression was calculated by the $\Delta\Delta C_t$ method using RPLP0 as an invariant control. C. IL-6 protein expression in NuLi-1 and CuFi-1 cells. Cells were cultured and stimulated as above. After stimulation, whole cell lysates were subjected to SDS-PAGE and immunoblotting was performed as described in Experimental Procedures using antibodies against IL-6. Anti-β-actin antibodies were used as a loading control. Bars represent mean ± SEM (n=3). These results are representative of at least two independent experiments. Unlike letters indicate significant differences with any pair of values with at least $P<0.05$ by two-way ANOVA with Bonferroni post-test for pair-wise comparisons.

Increased Expression of Eicosanoid Biosynthetic Enzymes in Another Cell Model

To confirm that the changes observed in eicosanoid biosynthesis and cytokine production are due to the defect in CFTR, the PaF stimulation experiments were repeated in another respiratory epithelial cell line, 16HBEo$^-$. In these cells, CFTR expression is blocked by stable transfection of plasmid expressing CFTR mRNA in the antisense orientation (antisense cells) [132]. Control cells express the same mRNA, but in the sense orientation (sense cells), such that CFTR expression is unchanged. Similar to the results seen in CuFi-1 and NuLi-1 cells, mPGES-1, cPLA$_2$α, and 5-LOX all showed significantly greater expression in antisense versus sense cells at baseline (Fig. 20B-D). COX-2 mRNA was also higher at baseline in these antisense cells (Fig. 20A). Incubation with PaF further stimulated COX-2 expression in antisense cells only (Fig. 20A), as well as mRNAs for IL-6 and IL-8 (Fig. 20E-F). Similar to the CuFi-1 and NuLi-1 cells, PaF significantly reduced 5-LOX expression in antisense cells, although it remained higher than in sense cells.

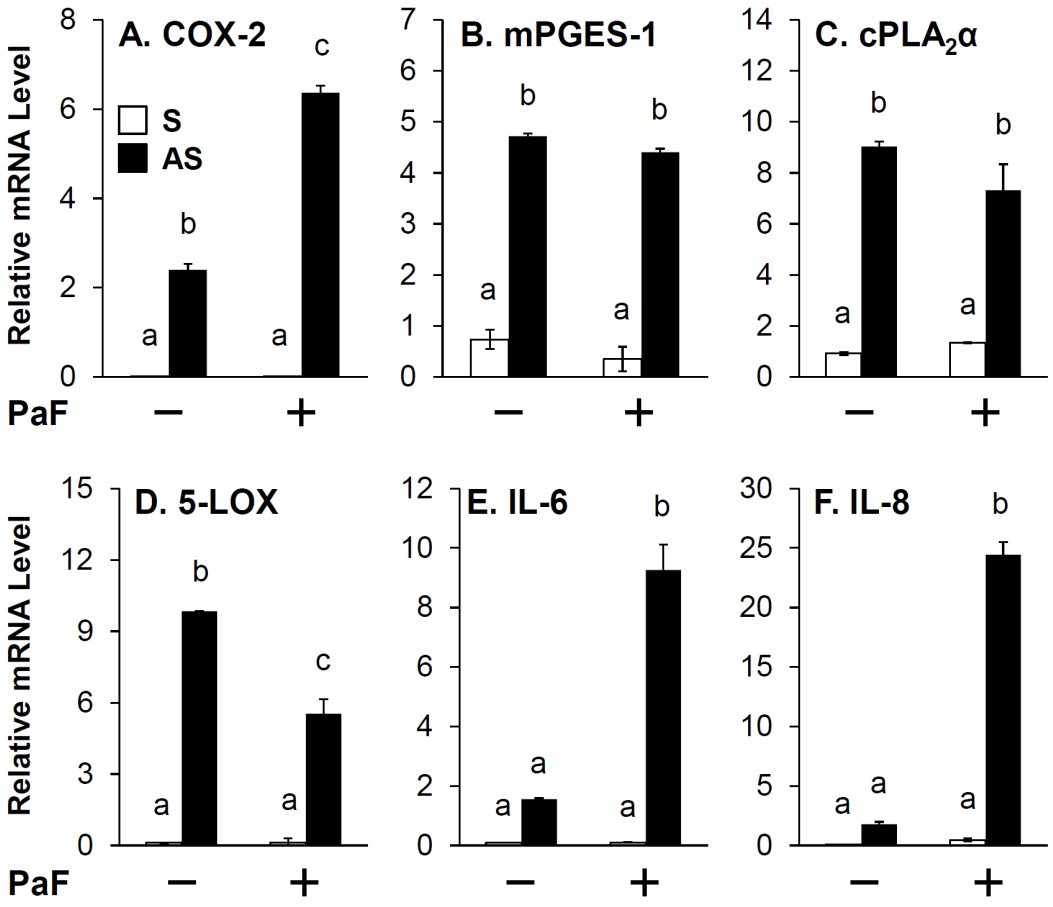

FIGURE 20:

Relative mRNA expression of eicosanoid metabolic enzymes and cytokines in 16HBEo$^-$ sense (S) and antisense (AS) cells. S cells express CFTR (control), while AS cells lack CFTR expression (CF). Cells were grown in complete medium for 7 days as described in Experimental Procedures. On day 7, the cells were stimulated with *Pseudomonas aeruginosa* filtrate (PaF) for 12 hours. After stimulation, mRNA was extracted and cDNA was synthesized as described in Experimental Procedures. Relative mRNA levels for cyclooxygenase-2 (COX-2), microsomal PGE_2 synthase-1 (mPGES-1), cytosolic phospholipase A2α (cPLA$_2$α), 5-lipoxygenase (5-LOX), IL-6, and IL-8 were measured by qRT-PCR. Relative expression was calculated by the $\Delta\Delta C_t$ method using RPLP0 as an invariant control. Bars represent mean ± SEM (n=3). These results are representative of at least two independent experiments. Unlike letters indicate significant differences with any pair of values with at least $P<0.05$ by two-way ANOVA with Bonferroni post-test for pair-wise comparisons.

Inhibition of Eicosanoid Biosynthesis Blocks Paf-Stimulated Cytokine Production

To test the hypothesis that eicosanoids drive PaF-stimulated cytokine production from CF cells, we stimulated CuFi-1 cells in the presence or absence of the COX-2 specific inhibitor NS-398 [180] or the 5-LOX inhibitor AA-861 [181]. Incubation with 10 μM NS-398 eliminated PGE_2 production by the cells (Fig. 21). While inhibition of COX-2 by NS-398 had no effect on COX-2 mRNA expression, it increased 5-LOX expression at baseline without changing the inhibitory effect of PaF on 5-LOX (Fig. 21). In addition, there was no change in IL-6 expression, but there was a small, but statistically significant decrease in PaF-stimulated IL-8 expression.

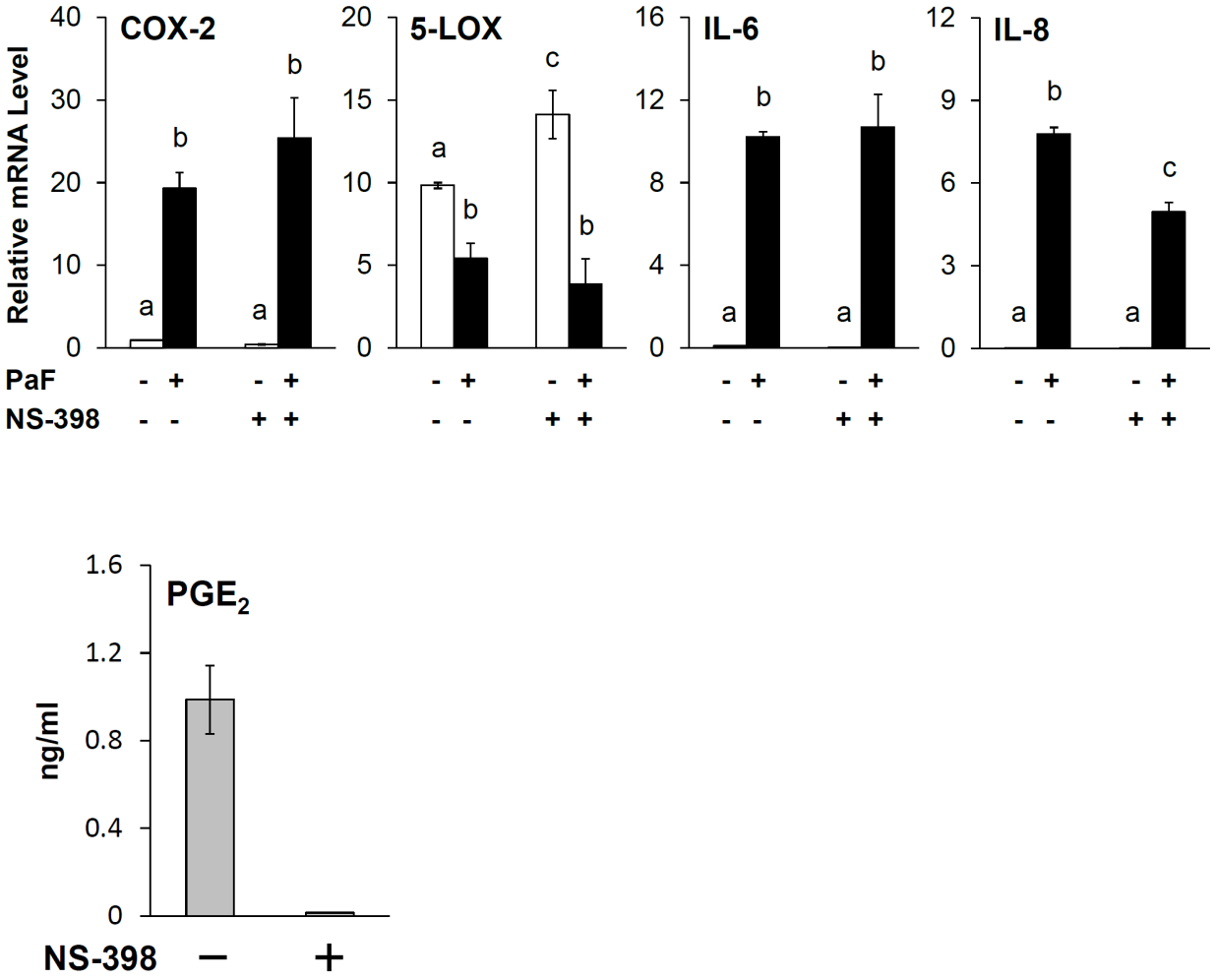

FIGURE 21:
Effect of cyclooxygenase-2 (COX-2) inhibition by NS-398 on relative mRNA expression of eicosanoid metabolic enzymes and cytokines in CuFi-1 (CF) cells. Cells were cultured for 7 days as described in Experimental Procedures. On day 7, the cells were stimulated with or without *Pseudomonas aeruginosa* filtrate (PaF) for 12 hours in the presence of 10 μM NS-398 or equal volumes of vehicle (DMSO). After stimulation, mRNA was extracted and cDNA was synthesized as described in Experimental Procedures. Relative mRNA levels for COX-2, 5-lipoxygenase (5-LOX), IL-6, and IL-8 were measured by qRT-PCR. Relative expression was calculated by the $\Delta\Delta C_t$ method using RPLP0 as an invariant control. Bars represent mean ± SEM (n=2). These results are representative of at least two independent experiments. Unlike letters indicate significant differences with any pair of values with at least $P<0.05$ by two-way ANOVA with Bonferroni post-test for pair-wise comparisons.

Incubation with 10 μM AA-861 reduced LTB_4 in the culture medium to undetectable levels (Figure 22). Inhibition of 5-LOX by AA-861 completely blocked the PaF-stimulated increase in COX-2 mRNA and protein expression (Fig. 22). Baseline 5-LOX mRNA was also reduced to levels seen after PaF stimulation. In addition, incubation with AA-861 completely blocked the PaF-stimulated increase in IL-6 and IL-8 mRNA and IL-6 protein (Fig. 22).

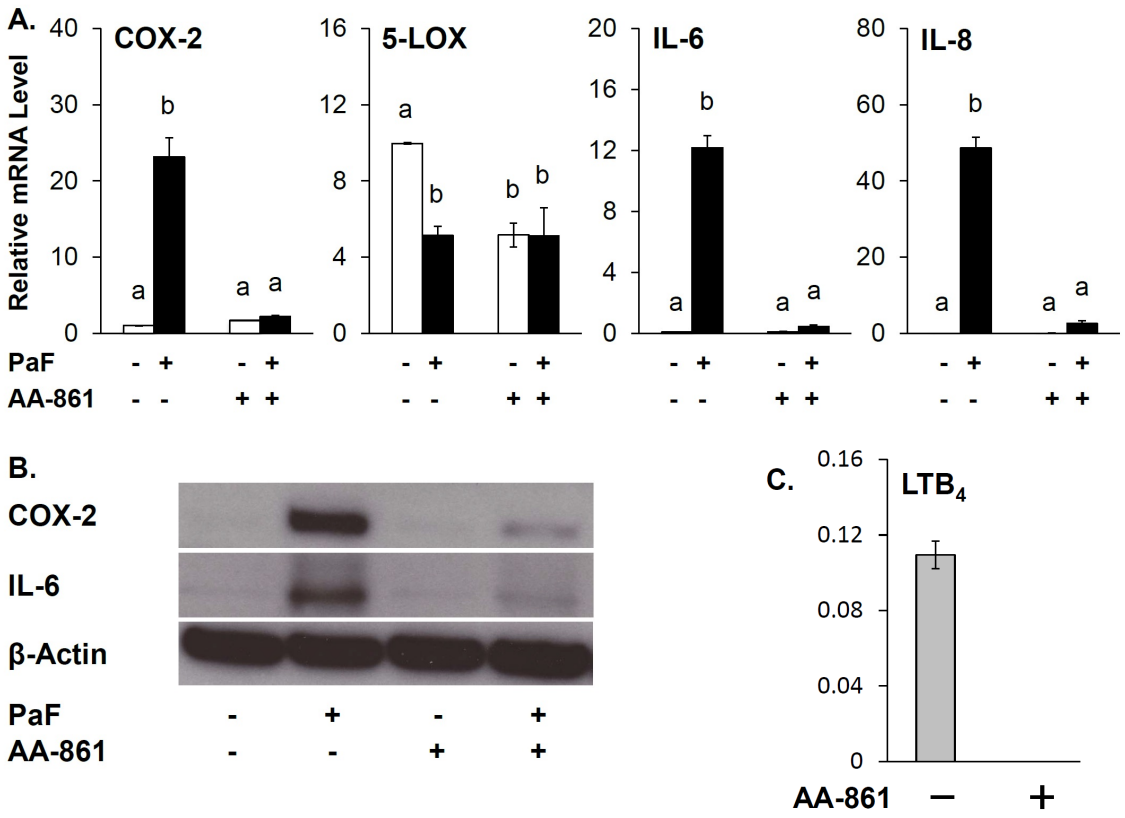

FIGURE 22:

A. Effect of 5-lipoxygenase (5-LOX) inhibition by AA-861 on relative mRNA expression of eicosanoid metabolic enzymes and cytokines in CuFi-1 cells. Cells were cultured for 7 days as described in Experimental Procedures. On day 7, the cells were stimulated with or without *Pseudomonas aeruginosa* filtrate (PaF) for 12 hours in the presence of 10 μM AA-861 or equal volumes of vehicle (ethanol). After stimulation, mRNA was extracted and cDNA was synthesized as described in Experimental Procedures. Relative mRNA levels for cyclooxygenase-2 (COX-2), 5-LOX, IL-6, and IL-8 were measured by qRT-PCR. Relative expression was calculated by the $\Delta\Delta C_t$ method using RPLP0 as an invariant control. Bars represent mean ± SEM (n=2). Unlike letters indicate significant differences with any pair of values with at least $P<0.05$ by two-way ANOVA with Bonferroni post-test for pair-wise comparisons. B. Effect of 5-LIOX inhibition by AA-861 on protein expression of COX-2 and IL-6 in CuFi-1 cells. Cells were cultured and stimulated with PaF in the presence of 10 μM AA-861 or vehicle (ethanol) as above. After stimulation, whole cell lysates were subjected to SDS-PAGE and immunoblotting was performed as described in Experimental Procedures using antibodies against COX-2 and IL-6. Anti-β-actin antibodies were used as a loading control. These results are representative of at least two independent experiments.

The 5-LOX accessory protein 5-lipoxygenase-activating protein (FLAP) physically interacts with 5-LOX and is essential to its enzymatic function [182]. Thus, to confirm the role of 5-LOX in the stimulation of cytokine production, cells were incubated with 10 μM MK-886, a specific small molecule inhibitor of FLAP [183]. Similar to the results seen with AA-861, incubation of CuFi-1 cells with MK-886 completely blocked the PaF-stimulated increases in COX-2, IL-6, and IL-8 mRNA expression (Fig. 23).

Based on its role in the initiation of acute inflammation, we hypothesized that LTB_4 was the most likely 5-LOX product to stimulate IL-6 and IL-8 production. LTA_4 hydrolase (LTA4H) catalyzes the conversion of LTA_4, the product of 5-LOX, to LTB_4 [184]. When CuFi-1 cells were incubated with 0.5 mM SC-57461A, a specific small molecular inhibitor of LTA4H [185], the PaF-stimulated increases in COX-2, IL-6, and IL-8 expression were completely abrogated (Fig. 23).

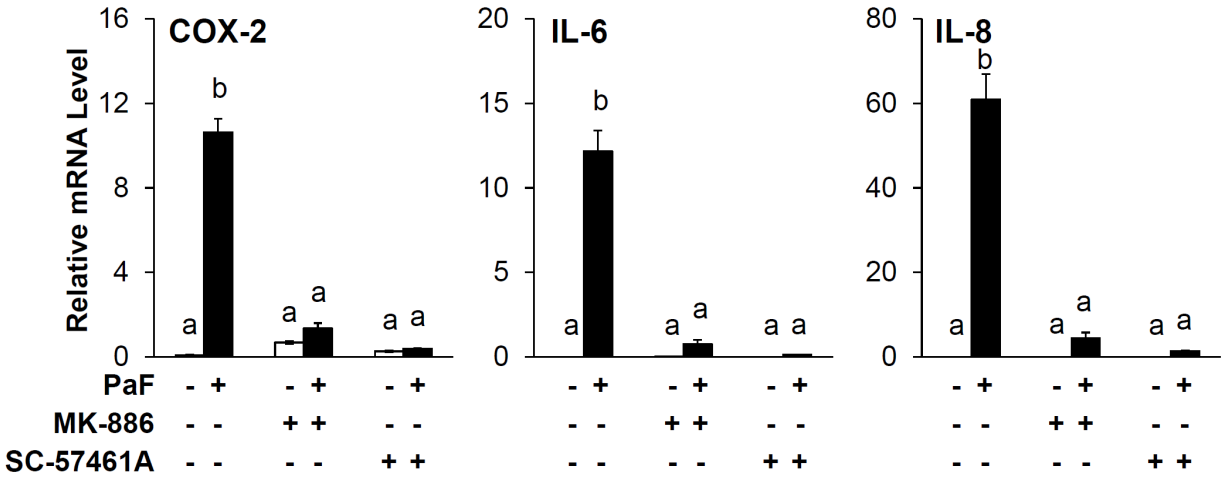

FIGURE 23:
Inhibitors of 5-lipoxygenase activating protein (FLAP) and LTA4 hydrolase (LTA4H) block PaF-stimulated increase COX-2 and cytokine expression in CuFi-1 cells. Cells were cultured for 7 days as described in Experimental Procedures. On day 7, the cells were stimulated with or without *Pseudomonas aeruginosa* filtrate (PaF) for 12 hours in the presence of either 10 μM MK-886 (FLAP inhibitor), 0.5 mM SC-57461-A (LTA4H inhibitor) or equal volumes of vehicle (DMSO). After stimulation, mRNA was extracted and cDNA was synthesized as described in Experimental Procedures. Relative mRNA levels for cyclooxygenase-2 (COX-2), IL-6, and IL-8 were measured by qRT-PCR. Relative expression was calculated by the $\Delta\Delta C_t$ method using RPLP0 as an invariant control. Bars represent mean ± SEM (n=2). These results are representative of at least two independent experiments. Unlike letters indicate significant differences with any pair of values with at least $P<0.05$ by two-way ANOVA with Bonferroni post-test for pair-wise comparisons.

DHA Attenuates the Paf-Stimulated Inflammatory Response

A prior study demonstrated that hyperreactive inflammatory response seen in the lungs of *CFTR*$^{-/-}$ knockout (CF) mice could be blunted by dietary supplementation with DHA [120]. To determine whether this effect was mediated through the eicosanoid pathway, CuFi-1 cells were cultured for 6 days in the presence or absence of 20 μM DHA prior to PaF stimulation. Cells treated with DHA showed a significant attenuation of the PaF-stimulated increases in COX-2, IL-6, and IL-8 mRNA (Figure 24A) and COX-2 and IL-6 protein expression (Fig. 24B). In addition, DHA pretreatment reduced baseline 5-LOX mRNA levels (Fig. 24A).

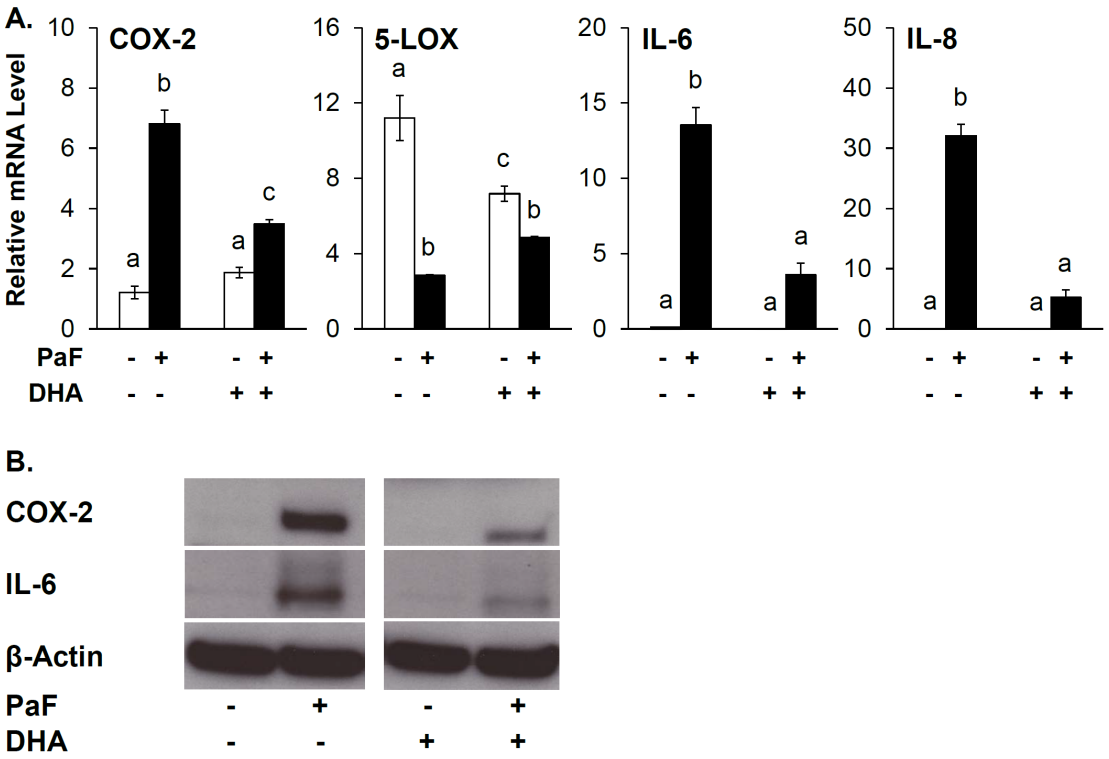

FIGURE 24:
Docosahexaenoic acid (DHA) supplementation blocks PaF-stimulated increase COX-2 and cytokine expression in CuFi-1 cells. A. Cells were cultured for 24 hours in standard medium as described in Experimental Procedures. After 24 hours, cells were cultured in medium supplemented with either 20 μM DHA in a BSA:DHA 2.5:1 molar ratio or equivalent BSA concentrations without DHA for a total of 7 days. On day 7, the cells were stimulated with *Pseudomonas aeruginosa* filtrate (PaF) for 12 hours without DHA. After stimulation, mRNA was extracted and cDNA was synthesized as described in Experimental Procedures. Relative mRNA levels for cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), IL-6, and IL-8 were measured by qRT-PCR. Relative expression was calculated by the $\Delta\Delta C_t$ method using RPLP0 as an invariant control. Bars represent mean ± SEM (n=2). Unlike letters indicate significant differences with any pair of values with at least $P<0.05$ by two-way ANOVA with Bonferroni post-test for pair-wise comparisons. B. Cells were cultured and stimulated with PaF in the presence of 10 μM AA-861 or vehicle (ethanol) as above. After stimulation, whole cell lysates were subjected to SDS-PAGE and immunoblotting was performed as described in Experimental Procedures using antibodies against COX-2 and IL-6. Anti-β-actin antibodies were used as a loading control. These results are representative of at least three independent experiments.

PUFA Alterations Observed in CF Cells

To confirm that the reported PUFA alterations are present in the HAEC cell line, we measured fatty acid levels by GC-MS in both CuFi-1 and NuLi-1 cells. CuFi-1 cells exhibited the hallmark changes in the n-7 and n-9 pathways, where levels of 16:1n-7, 18:1n-7, and 18:1n-9 were all higher in CuFi-1 (Fig. 25A). In the n-3 pathway, DHA levels were lower in CuFi-1 cells (Fig. 25B). Finally, in the n-6 pathway, CuFi-1 cells had lower levels of LA (Fig. 25C) and higher levels 18:3n-6, 20:3n-6, and 20:4n-6 (Fig. 25D), measured by GC-MS. The lower levels of LA and higher levels of its downstream products in the n-6 pathway, correlates with the increased expression of Δ6D we saw in these cells (Fig. 17). The increased metabolism from LA to AA was confirmed by radiolabeled studies (Fig. 26), measured by HPLC and radioactive counter.

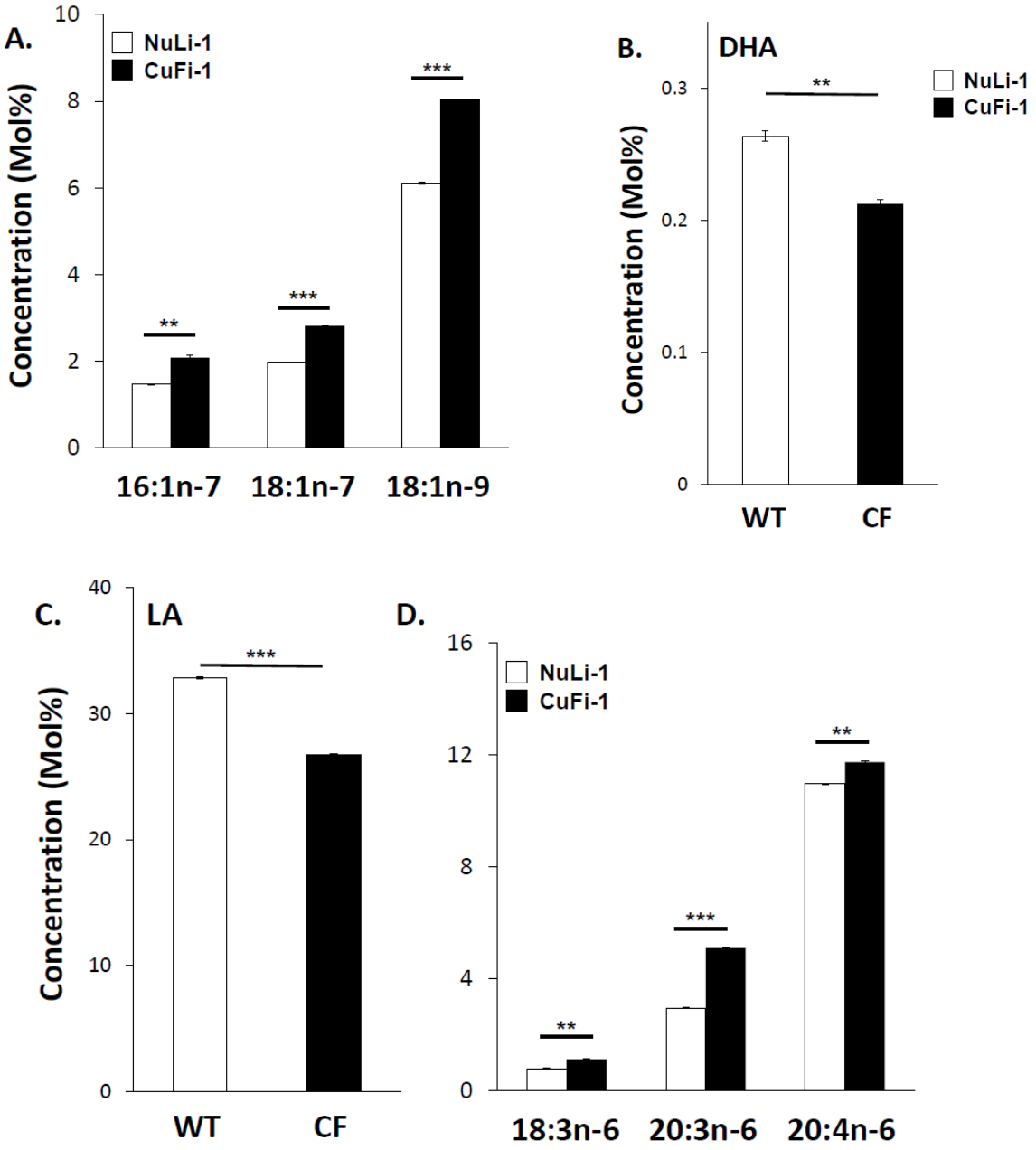

FIGURE 25:

Characteristic PUFA alterations are present in CuFi-1 cells. Fatty acid composition of HAECs NuLi-1 (WT) and CuFi-1 (CF) cells. WT and CF cells were grown in serum-free BEGM media supplemented with LA for 7 days. On day 7, total lipids were extracted and fatty acids were methylated. The resulting FAMEs were identified and quantified by GC-MS as described in Materials and Methods. Levels of (A.) common n-7 and n-9 fatty acids; (B.) 22:6n-3, DHA; (C.) 18:2n-6, LA; (D.) common n-6 Fatty acids. Data are presented as the molar percentage (mol%) of total mass of fatty acids. Bars represent mean ± SEM (n=2). These results represent two independent experiments. ***P<0.001, **P<0.01.

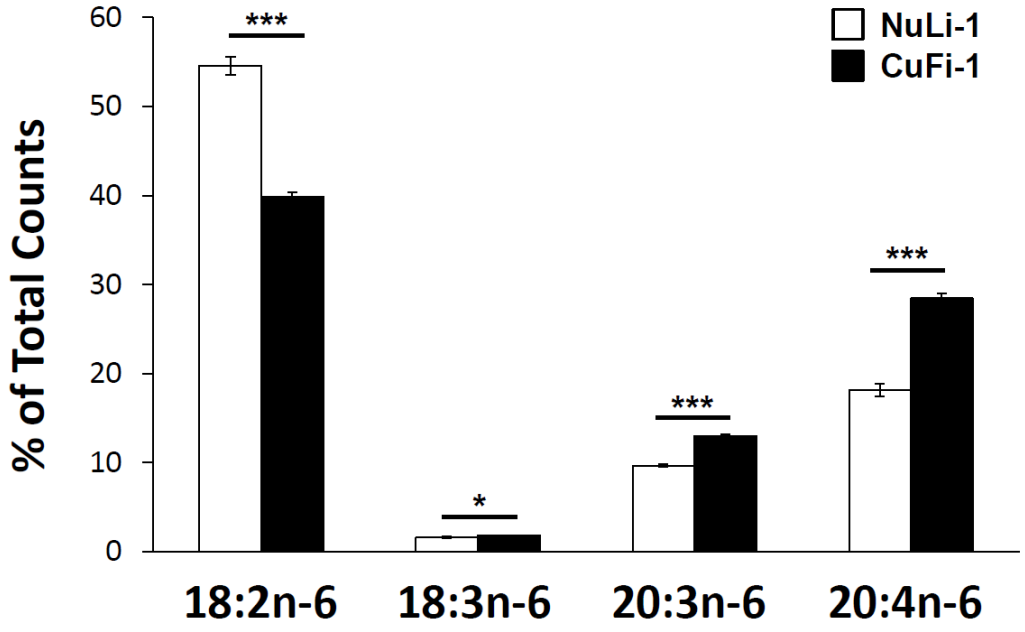

FIGURE 26:

Increased metabolism in the n-6 pathway in CuFi-1 cells. Metabolism of [1-^{14}C]LA through the n-6 pathway in HAECs NuLi-1 (WT) and CuFi-1 (CF) cells. WT and CF cells were grown in serum-free BEGM media supplemented with LA for 7 days. On day 7, cells were incubated for 4 hours with [1-^{14}C]LA then chased with fresh medium for 20 hours. After that, total lipids were extracted and fatty acids were methylated. The resulting radiolabeled FAMEs were identified and quantified by HPLC coupled with a radioactive detector and reported as percentage of total counts (dpm) as described in Materials and Methods. Bars represent mean ± SEM (n=2). These results represent two independent experiments. ***P<0.001, *P<0.05.

Discussion

The results described in this work draw a connection between aberrant fatty acid and eicosanoid biosynthesis and the hyperresponsive inflammatory response that is characteristic of CF pulmonary disease. First, the results demonstrate that CF airway epithelial cells over-express fatty acid and eicosanoid biosynthesis enzymes at baseline, including Δ6D, $cPLA_2α$, COX-2, 5-LOX, and mPGES-1, resulting in higher baseline production of PGE_2 and LTB_4 (Figs. 16,17). This expression is further induced by exposure to products of *Pseudomonas* in PaF (Figs. 17,20). These findings support an already abundant literature demonstrating increased expression of eicosanoid metabolic enzymes in CF patients and disease models [88,91,128,171,186] and elevated prostaglandin and leukotriene levels in patient specimens [162,163,167,168,187,188]. However, the mechanism of these changes and their impact on inflammation has not been previously elucidated.

Cytokines, especially IL-6 and IL-8, are important contributors to the inflammatory process in CF. In particular, airway epithelial cells are an important source of IL-8, which is the primary chemoattractant of neutrophils in the CF lung [26,158]. Accordingly, exposure of CF cells to PaF stimulates a marked increase in IL-6 and IL-8 production that is not seen in control cells (Figs. 19,20). The results in this report indicate that eicosanoids play a crucial role in the stimulated production of these cytokines. In particular, LTB_4, which is the primary product of 5-LOX/FLAP and LTA4H, appears to be the major

stimulator of IL-6 and IL-8 production as evidenced by the nearly complete reduction of PaF-stimulated cytokine production when cells are exposed to inhibitors of this pathway (Figs. 22,23). Other studies have shown that this increase in IL-6 expression in CF cells is due to over-activation of the MAP kinases p38 and ERK [177]. These findings are consistent in that the LTB_4 receptors BLT1 and BLT2 signal through the MAPK pathway [189-191].

Inhibition of 5-LOX/FLAP and LTA4H in CF cells also blocks stimulation of COX-2 expression by PaF (Figs. 22,23), indicating that induction of COX-2 in airway epithelial cells is also downstream of LTB_4. This may be due to activation of the NFκB pathway, which is activated by LTB_4 and induces COX-2 expression in CF cells [88,192]. This is consistent with other models of eicosanoid function in acute inflammation that exhibit sequential production of LTB_4 during initiation followed by COX-2 induction and PGE_2 production [193]. The models also describe an early peak of LTB_4 production, followed by a rapid decline [194]. This may account for the reduction of 5-LOX expression and LTB_4 production at 12 hours following PaF exposure (Fig. 18). It is important to note, however, that both 5-LOX expression and LTB_4 production remain higher in CF than control cells at this time point, suggesting that there may be prolonged stimulation in the CF cells. Future studies examining the time course of this response will be required to test this hypothesis.

PGE_2 also appears to play some role, albeit minor, in cytokine production, as inhibition of COX-2 by NS-398 causes a slight, but significant reduction in IL-8 expression (Fig. 21). These data agree with a previous study

in a homozygous ΔF508 epithelial cell line that showed increased IL-8 release in response to bradykinin stimulation [195]. In this model, IL-8 production was amplified by supplementation with either AA or PGE_2, and blunted by COX inhibitors indomethacin and NS-398.

These results suggest a model for the excessive stimulation of inflammation by airway epithelial cells in CF (Fig. 27). At baseline, the fatty acid and eicosanoid metabolic pathways are primed for production of LTB_4. AA levels are higher in CF cells due to increased activity of Δ6D [128], and there is higher expression of $cPLA_2$, responsible for release of AA from phospholipids, and 5-LOX. Thus, upon stimulus, there is a burst of LTB_4, which stimulates COX-2, resulting in enhanced PGE_2 production, and together they stimulate the production of IL-6 and IL-8 and the initiation of acute inflammation.

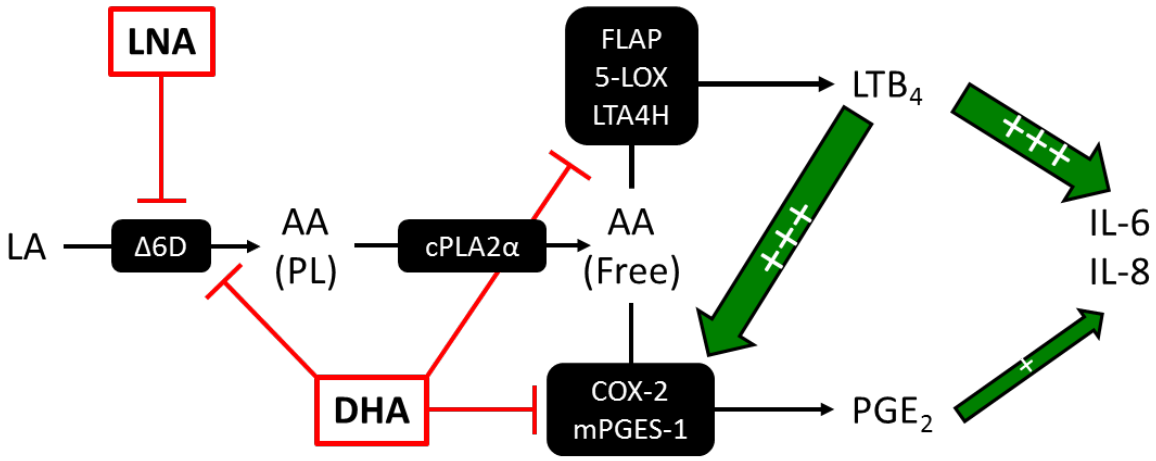

FIGURE 27:
Model illustrating the role of fatty acid and eicosanoid metabolism in cytokine production in CF cells. Black arrows indicate metabolic pathways. Stimulatory effects are indicated by gray arrows, the thickness of which indicates relative degree of simulation. Gray lines indicate inhibition of various enzymes by DHA. LA, linoleic acid; AA, arachidonic acid, DHA, docosahexaenoic acid; PL, phospholipid; Δ6D, Δ6-desaturase; FLAP, 5-lipoxygenase activating protein; 5-LOX, 5-lipoxygenase; LTA4H, LTA_4 hydrolase; COX-2, cyclooxygenase-2; mPGES-1, microsomal PGE_2 synthase.

These results highlight the importance of the FLAP/5-LOX/LTA4H pathway in the initiation and maintenance of the exaggerated inflammatory response in CF. Consequently, this pathway could be an attractive target for pharmacologic intervention in CF, where inhibition of LTB_4 production could potentially prevent the harmful effects of excessive inflammation. However, a clinical trial to test the effects of the leukotriene receptor BLT1 antagonist BIIL 284 on lung disease in CF patients was terminated early due to a significant increase in the number of pulmonary exacerbations in patients receiving the drug compared to those receiving placebo [196]. A follow-up study in mice showed that while BIIL 284 reduced pulmonary neutrophils, there was an increase in *Pseudomonas* bacteremia and associated inflammation [197]. Thus, complete inhibition of LTB_4 action might be detrimental on balance as it reduces the ability to combat infection.

An alternative approach could be to target the pathway at an earlier step. In this study, we demonstrate that pre-incubation of CF cells with DHA substantially reduces PaF-stimulated COX-2 expression, as well as IL-6 and IL-8 production (Fig. 24). Other studies in cultured airway epithelial cells show that DHA decreases AA production by inhibiting the CF-associated increase in fatty acid desaturase expression and activity [129]. In addition, DHA reduces expression of COX-2 and mPGES-1 through the free fatty acid receptor-4 [198]. Other studies support this approach. Freedman *et al.* [120] showed that dietary DHA supplementation in a CF mouse model normalized the pulmonary inflammatory response to *Pseudomonas* lipopolysaccharide. This was

accompanied by a significant decrease in eicosanoids, particularly the prostaglandins PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ [199]. In addition, several clinical trials in CF patients have shown that supplementation with n-3 fatty acids, including DHA, modulates leukotriene production[110,200,201]. Importantly, this supplementation did not result in significant adverse effects[202].

These results may also have dietary implications. Due to malabsorption, CF patients are treated with high-fat diets. Unfortunately, the modern western diet is enriched in n-6 fatty acids, such as LA, to a much greater extent than historical norms [143]. In CF cells, higher levels of LA input contribute to excessive AA production [156]. This contributes to increased eicosanoid and IL-8 production in LA-supplemented CF cells and increased pulmonary inflammation in CF mouse models [130]. Thus, a diet lower in n-6 and higher in n-3 fatty acids might be beneficial for CF patients.

This study has a few limitations. First, all comparisons were made between baseline and a single time point after stimulation. That time point was selected based on preliminary time course studies that indicated maximum COX-2 and cytokine stimulation (data not shown). However, inflammation is a dynamic process, so additional studies will be required to better understand the precise interaction between eicosanoids, cytokines, and the underlying molecular mechanisms that regulate them. Second, eicosanoid measurements were made in total cell culture medium. As they are expected to act in an autocrine and/or paracrine fashion, these data may not reflect the actual local concentrations at and between cells. Third, the primary cell culture model used

here involves cells from two different individuals, one with CF and one healthy [175]. Thus, it is possible that differences in genetic background could account for some of the changes observed. However, this concern is mitigated by confirmation of many of the observed differences in another cell culture model where the CF and control cells share the same genetic background (Fig. 20).

CHAPTER V

DISCUSSION AND FUTURE WORK

Summary

The studies presented in this document demonstrate our work on the role of PUFA alterations and eicosanoid production in the inflammatory response in CF. We have shown that there is increased uptake and release of AA in CF cells. Moreover, we have demonstrated that there are changes in eicosanoid biosynthesis in CF airway epithelial cells compared with normal controls resulting in increased production of at least LTB_4 and PGE_2. Furthermore, we have revealed that eicosanoid biosynthesis, in particular the LTB_4 pathway, plays a crucial role in the marked overproduction of pro-inflammatory cytokines in CF cells. Lastly, we have illustrated that inhibition of eicosanoid biosynthesis with small molecule inhibitors, but also with DHA, can abrogate this excessive cytokine production. These results not only shed light on the mechanisms of hyperactive inflammation in CF, but they also highlight potential therapeutic targets and approaches that could be used to better treat patients with CF.

Future Studies

Our work demonstrated that PUFA alterations are part of an intrinsic defect in CF that contributes to the inflammatory response by excessive production of AA and its oxygenated metabolites. Our results in chapter IV clearly showed that products of the 5-LOX pathway, particularly LTB_4, drive the exaggerated COX-2 pathway and cytokine production observed in CF airway epithelial cells. However, since inflammation is a dynamic process, further studies, both *in vitro* and *in vivo*, are required to elucidate the role of various cell types and their PUFA-derived oxygenated metabolites in the dysregulated inflammation in CF.

We hypothesize that the high LTB_4 production levels by CF airway epithelial cells in chapter IV leads to the amplified neutrophil recruitment observed in the CF airway. Thus, we anticipate neutrophils to show higher chemotaxis response to media collected from CF airway epithelial cells, when performing in vitro chemotaxis assays. While this will implicate the airway epithelium as a major source of LTB_4 that leads to neutrophil recruitment, there may be defects in CF neutrophils that lead to their excessive response. This concept can be tested in a variety of ways. First, neutrophil chemotaxis assays can be repeated with both CF and WT neutrophils, to measure any difference in the response of CF neutrophils to chemoattractant signals, such as LTB_4. There have been studies that suggest a defect in the inflammatory response and a possible dysregulation in oxygenated metabolite production in CF neutrophils. Bravo *et al*. reported that CF neutrophils have impaired PUFA

release[203], while Xiao *et al*. demonstrated that reconstitution with ΔF508 neutrophils in WT mice lead to more severe LPS-induced lung inflammation and injury[204]. To assess whether eicosanoid production plays a role in the dysregulated neutrophilic inflammatory response, studies that examine expression and activity of both COX-2 and 5-LOX pathways should be performed on CF neutrophils. These studies may show an increase in production of pro-inflammatory eicosanoids, especially LTB_4, in CF neutrophils. Moreover, they may also uncover other dysregulated pathways in these leukocytes, such as eicosanoid signaling. In particular, higher expression or signaling through LTB_4 receptors BLT_1 and BLT_2 in CF neutrophils may contribute to their increased recruitment in CF.

The interaction between airway epithelial cells and neutrophils affects eicosanoid production pathways in both cell types and drives several inflammatory events. In vitro production of some oxygenated metabolites, such as LXA_4, may only occur in a co-culture model that mimics this interaction. LXA_4 is a well-known pro-resolving mediator that can be produced via the combined action of two lipoxygenase enzymes derived from airway epithelial cells and neutrophils[205]. We hypothesize that the decrease in LXA_4, observed in CF[95], is due to defective "class-switching" events in the CF airway, which contributes to the unrelenting inflammatory response. Therefore, assessing this interaction in response to stimulation in a co-culture model of airway epithelial cells and neutrophils will further enhance our understanding of the role of PUFA-derived oxygenated metabolites in the pathophysiology of CF

lung disease. PGE_2 can effectively switch the production of AA-derived eicosanoids from pro-inflammatory to pro-resolving by modulating the expression of LOX enzymes to stop LTB_4 production while inducing LXA_4 production. If CF neutrophils have elevated LTB_4 production, it is plausible that PGE_2-signaling in these leukocytes may be defective. Therefore, CF neutrophils continue producing LTB_4 instead of switching to LXA_4, which leads to non-resolving inflammation phenotype. To elucidate this non-resolving phenotype, the production and signaling of n-3—derived pro-resolving mediators should be investigated in both neutrophils and airway epithelial cells in CF.

Even though the proposed studies above will aid in characterizing role of airway epithelial cells and neutrophils, inflammation is a complex process with many contributing factors that cannot be completely recapitulated *in vitro*. Therefore, an *in vivo* model is necessary to truly understand the role of PUFA-derived oxygenated metabolites in the dysregulated inflammatory response in CF. Mouse models are the most widely used animal models in CF. While these models have greatly helped in elucidating many facets of the disease, they are poor surrogates for pulmonary studies as they do not have pronounced lung disease[22]. A group from the University of Iowa has recently developed a *ΔF508* pig model of CF that develops features typical of human disease. In particular, CF pigs develop, very early in life, spontaneous lung disease that is characterized by airway obstruction, infection, and inflammation[206]. This will provide a valuable system to investigate the onset of

inflammation in the lungs of CF pigs and assess the role of PUFA-derived oxygenated metabolites in the development of lung disease.

We demonstrated in chapter IV that inhibition of the 5-LOX pathway was able to attenuate the inflammatory response in CF airway epithelial cells. The next step would be to evaluate the effect of this attenuation on neutrophil recruitment both *in vivo* and *in vitro*. To test this, neutrophil chemotaxis and co-culture assays should be repeated with inhibition of the 5-LOX pathway in airway epithelial cells, neutrophils, or both cell types. This will clarify whether the overly active 5-LOX pathway in airway epithelial cells is the main cause for the increased neutrophil recruitment, or that both cell types are involved in this dysregulation. The role of the 5-LOX pathway in the excessive inflammatory response can be further elucidated by pharmacologically inhibiting this pathway in a ΔF508 pig model, and monitoring the inflammatory response in these animals. Specifically, assessing neutrophil recruitment and measuring the generation of inflammatory mediators, such as cytokines and PUFA-derived metabolites. Alternatively, the development of a (*ALOX5*$^{-/-}$/*ΔF508*) pig model may elucidate the role of 5-LOX in CF more precisely.

Due to its potency as a neutrophil chemattractant, LTB_4 has been thought to play significant role in CF airway inflammation. A recent clinical study investigated this role by administering a specific BLT inhibitor (BIIL 284) to CF patients, and monitoring pulmonary function[207]. However, this study had to be terminated early due to increased pulmonary exacerbations. A follow-up study aiming to examine the cause of the increased exacerbations,

demonstrated a decrease in pulmonary neutrophil count and an increase in *Pseudomonas aeruginosa* bacterial load in the lungs and circulation of mice treated with BIIL 284 compared to their matched controls[208]. These studies demonstrate the significant role of LTB_4 in the inflammatory response and bacterial clearance. Moreover, the results suggest that inhibiting the 5-LOX pathway to a level that only decreases the inflammatory response rather than completely inhibiting that response may yield better therapeutic benefits for CF patients. Therefore, we hypothesize that normalizing LTB_4 production in CF airways, using lower doses of pharmacological inhibitors of the 5-LOX pathway, will result in an inflammatory response sufficient to combat bacterial infections but will not lead to excessive neutrophilic inflammation. This can be tested by performing dose response studies using inhibitors of the 5-LOX pathway, such as AA861, in the *ΔF508* CF pig model.

Our work showed that DHA supplementation had a significant inhibitory effect on the inflammatory response in CF airway epithelial cells. As demonstrated in chapter IV, DHA attenuated the expression of both COX-2 and 5-LOX enzymes in CF cells. DHA may also affect the inflammatory response through other mechanisms. It may decrease the production of pro-inflammatory AA-derived eicosanoids via inhibition of Δ6D[97], or by replacing AA in phospholipids. Alternatively, DHA may exert its effect through the conversion to its anti-inflammatory oxygenated metabolites[209]. The fact that DHA levels are low in CF, and that decreased resolvin levels have been associated with poorer pulmonary function in CF patients[210], may partly

explain the beneficial effects of DHA in CF. The effect of DHA should be evaluated on the production oxygenated metabolites, neutrophil function, and inflammatory response using both *in vitro* and *in vivo* studies, similar to the studies proposed for 5-LOX inhibition. It will be interesting to determine whether combining DHA with pharmacological inhibitors of the 5-LOX pathway will give the same beneficial effects, but with lower doses.

Significance

Mechanistic Connection of PUFA Alterations in CF to Disease Pathology

The work presented in this document provides a novel mechanism that connects the observed PUFA alterations in CF to disease pathophysiology. Herein we demonstrate that low levels of LA and increased production and availability of AA contribute to the excessive inflammatory response in CF via the production of pro-inflammatory eicosanoids. In particular, products of the 5-LOX pathway, namely LTB_4, are responsible for driving the excessive production of both PGE_2 and inflammatory cytokines IL-6 and IL-8. This suggests that these PUFA alterations are essential in driving the increased neutrophilic inflammation in the airways of CF patients by the increased production of the powerful chemoattractant LTB_4.

Nutritional Modification

To avoid malabsorption and malnutrition, it is recommended that CF patients intake a high-calorie, high-fat diet[12,14]. However, there are no

guidelines regarding the type of fat in CF diets. This leads to CF patients consuming large amounts of n-6 PUFA, LA in particular, due to the high n-6/n-3 ratio in typical western diets[36]. Previous work from our group has shown that there is an increased metabolism from LA to AA in CF cells[71]. Moreover, as we demonstrated in chapter II, the increased production of AA in CF cells is more pronounced when LA/LNA ratio is higher. This increase in AA levels can further increase inflammation in CF. In support of this, Zaman *et al*. demonstrated that LA supplementation in CF cells and mice caused a significant increase in AA-derived eicosanoids, and increased cytokine production and neutrophilic inflammation[136]. This identifies the need for modification of the nutritional recommendations to increase the ratio of n-6/n-3 PUFA by both decreasing n-6 PUFA intake while increasing n-3 PUFA supplementation.

Therapeutic Potential

Significant improvements in CF treatments over the past 3 decades have led to an increase in median survival age among patients. Treatments in CF mainly deal with nutritional and pulmonary management. Nutritional management is designed to combat pancreatic insufficiency and malabsorption; patients are given pancreatic enzyme replacements and fat-soluble vitamin supplements combined with recommendation of a high-calorie, high-fat diet[2,7,211]. Pulmonary management is based on antibiotic therapy to fight chronic infections, and chest physiotherapy to aid in mucus clearance[2].

The presence of excessive non-resolving inflammation leads to tissue destruction, and therefore is considered as a therapeutic target. To this end, the effects of high-dose ibuprofen have been studies in two large clinical trials. Konstan *et al*. demonstrated that when taken for 4 years, high-dose was able to decrease the rate of lung function decline without any adverse effects[212]. Another study showed that ibuprofen had no effect on forced expiratory volume in 1 second (FEV_1), although patients on ibuprofen spent fewer days in the hospital[213]. This is in line with our COX-2 inhibition findings in chapter IV, where inhibition of this pathway caused a slight decrease in IL-8 expression, and may explain the small benefits of ibuprofen in CF. Even though high-dose ibuprofen did not cause harmful effects in these studies, this non-selective COX inhibitor can potentially cause harmful side effects when taken for prolonged periods. This was demonstrated by a retrospective study, which demonstrated that many patients had to discontinue high-dose ibuprofen treatment due to GI side effects[214]. Therefore, there is a need for the development of safer, more potent anti-inflammatory treatments.

An important application of our work is the development an adjuvant therapy for CF lung disease, which can be tested in a clinical trial. The therapeutic basis of this trial is a combination of DHA supplementation and 5-LOX inhibition by AA-861. Doses of both treatments will be determined based on the results of our proposed *in vitro* and *in vivo* studies, outlined above. These studies will establish the lowest combined concentrations of DHA and AA-861 that can attenuate the excessive inflammatory response, but will not

hinder effective bacterial clearance. To avoid reaching inconclusive results, the proposed clinical study must follow the guidelines described next. A large number of CF patients must be recruited at an early age, prior to the decline in pulmonary function. Moreover, the study must be placebo controlled and long enough to assess the therapeutic effects on the development and progression of lung disease. In addition, special attention must be given to the nutritional aspect of the study, especially keeping n-6/n-3 ratio as close to 1:1 as possible. The outcome of the study can be determined by a variety of clinical markers and pulmonary function tests. For example, the levels of cytokines, PUFA, and PUFA-derived oxygenated metabolites may be used as markers of clinical status. These markers can be measured in various tissues, such as exhaled breath condensate, sputum, plasma, and urine.

Biomarker Development

Finally, CF patients are often faced with intermittent episodes of acute exacerbation of their pulmonary symptoms[215]. These episodes must be treated aggressively with antibiotic therapy, whilst increasing airway clearance techniques, and improving nutrition[7]. Even with these aggressive treatments, experiencing pulmonary exacerbations usually leads to a long-term decline in lung function and a decrease in survival rate[216]. Therefore, being able to identify the risk of having a pulmonary exacerbation flare prior to its occurrence, will lead to improved pulmonary outcomes and even extend life expectancy in CF patients[217]. A significant outcome of the proposed clinical

study, described above, could be the identification of new markers of pulmonary exacerbation. Measurement of the aforementioned clinical markers during the clinical study will provide enough data points to predict possible patterns of production that occur during the course of disease development, including exacerbations. In support of this, Wojewodka *et al*. demonstrated a correlation between certain inflammatory markers and the probability of re-exacerbation in CF patients[217]. In our study we may find that, for example, an increase in the ratio of LTB_4/LXA_4 or AA/DHA is predictive of a pulmonary exacerbation episode. This can be utilized to monitor the clinical status of patients, and prompt their hospitalization when these markers are elevated to prevent the development of a pulmonary exacerbation flare.

Closing

Cystic fibrosis remains a devastating disease that causes the death of young adults. Therefore, a better understanding of CF pathophysiology will lead to the development of better therapies and improvement in the quality of life of CF patients. These studies shed light onto the role of altered PUFA and eicosanoid production abnormalities in CF pathophysiology, particularly in the development of excessive airway inflammation.

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