

***EpOMEs and DiHOMEs require TRPA1 and/or
TRPV1 to induce calcium flux in human airway
smooth muscle***

By

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ABSTRACT

Introduction

Oxylipins are bioactive lipid mediators found throughout the lung and can act on Airway Smooth Muscle (ASM). Well-characterized species such as thromboxanes and leukotrienes elevate cytoplasmic calcium (Ca^{2+}) in ASM, initiate ASM contraction, and induce airway narrowing. Oxylipin profiles are changed in individuals who are exposed to pollution, allergens, and who live with asthma. However, many oxylipins, including EpOMEs and DiHOMEs have unknown functions related to Ca^{2+} flux and contraction in ASM. I propose that EpOMEs and DiHOMEs are capable of elevating Ca^{2+} flux in ASM cells and may be detrimental in obstructive diseases like asthma.

Methods

Senescence-resistant human ASM cells were cultured and loaded with Ca^{2+} indicator dye Fura-2 or Fluo-4. ASM were exposed to $10\mu\text{M}$ of 9,10 EpOME, 12,13 EpOME, 9,10 DiHOME, or 12,13 DiHOME and Ca^{2+} data was captured using fluorescent microscopy. ASM cells were loaded with inhibitors for cation channels Transient Receptor Potential Ankyrin 1 (TRPA1) (HC-030031), Transient Receptor Potential Vanilloid 1 (TRPV1) (AMG 517), and Gadolinium (GD^{3+}). ASM were then exposed to individual oxylipins at $10\mu\text{M}$, and fluorescent data was captured.

Results

9,10 EpOME, 12,13 EpOME, 12,13 DiHOME induced a significant increase in intracellular calcium ($[\text{Ca}^{2+}]_i$) compared to vehicle. 9,10 DiHOME was capable of inducing an increase in $[\text{Ca}^{2+}]_i$ but did not reach threshold for significance. HC-030031 caused a significant 66% reduction in 9,10 EpOME's Ca^{2+} response. AMG 517 was capable of significantly reducing 12,13 EpOME's response by 80% while HC-030031 significantly augmented 12,13 EpOME's response by 61%. AMG 517 and HC-030031 reduced 9,10 DiHOME's response significantly by 79% and 46%, respectively. HC-030031 was capable of significantly reducing 12,13 DiHOME's response by 65%.

Conclusion

These results show for the first time that EpOMEs and DiHOMEs are capable of increasing $[\text{Ca}^{2+}]_i$ in ASM cells. Additionally, EpOMEs and DiHOMEs require TRPA1 and/or TRPV1 to induce Ca^{2+} flux in ASM. EpOMEs and DiHOMEs are abundant in the lung and may initiate ASM contraction and airway narrowing through Ca^{2+} mechanisms. Future studies are required to understand the contractile abilities of EpOMEs and DiHOMEs and determine if they are detrimental in human airways, especially in obstructive disease like asthma.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACH	Acetylcholine
AHR	Airway Hyperresponsiveness
AITC	Allyl isothiocyanate
ALA	α -linoleic acid
AM	Acetoxymethyl
APS	Ammonium persulfate
ARDS	Acute Respiratory Distress Syndrome
ASM	Airway Smooth Muscle
ATP	Adenosine Triphosphate
B2AR	β 2-Adrenergic Receptor
BALF	Bronchoalveolar lavage fluid
BSA	Bovine Serum Albumin
BW	Bronchial Wash
CAMP	Cyclic Adenosine Monophosphate
CHO	Chinese hamster ovary cells
COPD	Chronic Obstructive Pulmonary Disease
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CYP	Cytochrome P450s
CYP450	Cytochrome P450
CystLTR₁	Cysteinyl Leukotriene Receptor
DGLA	Dihomo γ -linoleic acid
DHA	Docosahexaenoic acid
DiHOME	Dihydroxy-9Z-octadecenoic acid
DRG	Dorsal Root Ganglion
EBC	Exhaled Breath Condensate
EPA	Eicosapentaenoic acid
EPAC	Exchange Protein directly Activated by cAMP
EpOME	Epoxy-9Z-octadecenoic acid
FeNO	Fractional exhaled Nitric Oxide
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GPCR	G-Protein Coupled Receptors
HBSS	Hanks' Balanced Salt Solution
HETE	Hydroxyeicosatetraenoic acids
ICS	Inhaled corticosteroids
IgE	Immunoglobulin E
IL-13	Interleukin-13
IL-1β	Interleukin 1-Beta

kDa	Kilodalton
LA	Linoleic acid
LABA	Long-acting Beta agonists
LOX	Lipoxygenases
LTC₄	Leukotriene C4
LTD₄	Leukotriene D4
LTE₄	Leukotriene E4
M₂	Muscarinic 2
M₃	Muscarinic 3
MLC20	Myosin Light Chain 20
MLCK	Myosin Light Chain Kinase
MLCP	MLC phosphatase
NKA	Neurokinin A
PBS	Phosphate-Buffer Saline
PGE₂	Prostaglandin E ₂
PGI₂	Prostaglandin I ₂
PKA	Protein Kinase A
PKC	Protein Kinase C
PLA₂	Phospholipase A2
PUFA	Polyunsaturated Fatty Acid
SABA	Short Acting Beta Agonist
SN2	Substitution, Nucleophilic, Bimolecular
sPLA₂	Secretory Phospholipases
TEMED	tetramethyl ethylenediamine
TGF-β₁	Transforming growth factor-beta 1
TNFα	Tumor Necrosis Factor Alpha
TRPA1	Transient Receptor Potential Ankyrin 1
TRPC	Transient Receptor Potential Cation Channel
TRPV1	Transient Receptor Potential Vanilloid 1
TXA₂	Thromboxane A2

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CHAPTER 1: INTRODUCTION

Asthma

General Information and Prevalence

Within Canada roughly 3.8 million individuals lived with asthma in 2021, and the most recent 'Asthma Canada' update from 2023 now estimates there are 4.6 million individuals.^{1,2} Monitoring in Canada has also revealed that roughly 900,000 children under the age of 19 have asthma. It is estimated that 80,000 hospitalizations per year are due to asthma related symptoms and will cost the Canadian healthcare system 4.2 billion dollars annually by 2033.¹

Asthma has many clinical features such as wheezing, breathlessness, chest tightness, and cough which can sometimes manifest into acute fatal exacerbations. Asthma is a highly heterogenous disease that includes distinct and multiple endotypes, including allergic asthma, neutrophilic asthma, steroid-insensitive asthma, and more.³ Many therapeutics exist such as systemic or inhaled corticosteroids (ICS), bronchodilators, and the relatively new biologic therapies. Nonetheless, these therapeutics fail in some cases and remind us that continuing research is necessary to understand asthma pathophysiology to help develop novel therapies.

Features/Characteristics

Airway Narrowing

Airway narrowing is a reduction in the diameter of the conducting airways and is the major culprit that puts individuals into the emergency room. Airway diameter is largely controlled by Airway Smooth Muscle (ASM), a structural and important cell type that exists in the airway walls in a helical fashion from the trachea to the alveolar ducts.⁴ The physical shortening in the length of ASM leads to an inward narrowing force on the airways that reduces airflow to the respiratory zone for gas exchange. This normal physiological function of the airways is compromised in individuals with asthma, where exaggerated or premature narrowing is observed. This leads to increased resistance to airflow, gas trapping in the affected airways, and greater residual volumes that are associated with increased exacerbations in severe pediatric and adult asthma.⁵⁻⁷ This is especially evident in individuals with asthma who display small airway disease (50-60% of asthmatics), where structural and functional issues become even more apparent and is likely the major culprit in gas trapping.^{8,9} Airway narrowing in

asthma is usually caused by various environmental triggers such as allergens, pollution, viruses, and more. Signaling mediators released from immune cells act on ASM to induce cell shortening and narrowing. Relieving this narrowing via bronchodilators is the most significant pharmacologic treatment for patients and is briefly touched on in section 'Treatments'.

Airway Remodeling

Airway remodeling is a morphological change in the structure of the airways and has been argued to be either deleterious or bronchoprotective. The problem with remodeling seems to be when it pushes the scale too far in one direction leading to hyperresponsive airways and airflow resistance. This remodeling is observed in histological lung specimens from mild asthmatics, severe asthmatics, and fatal asthmatics and exhibits interesting differences from healthy individuals. This is reviewed well in van den Bosch et al. where in fatal asthma, remodeling displayed goblet cell hyperplasia, basement membrane thickening, increased mucus production, epithelial thickening, and ASM layer thickening.^{8,10,11} Additionally, remodeling of the extracellular matrix is observed, where structural proteins like collagen, fibronectin and elastic fiber are increased. This remodeling leads to the potential disruption of the forces and loads on the airways. This lung remodeling, whether developed early in life or throughout the disease, leads to tissue encroachment within the conducting space, restricted airflow, increased gas trapping (increased residual volume), and is correlated with disease severity.^{8,12} This is evidenced by the decrease in the FEV1/FVC ratio of asthmatics, especially in severe cases.^{13–15}

While ASM thickening is frequently associated with asthmatic airway remodeling it is unclear whether this is due to ASM hyperplasia, hypertrophy or both.^{8,10,16} For example, in a small study, non-fatal and fatal asthma patients displayed hypertrophy in the large airways. Contrastingly, fatal asthmatics displayed hyperplasia in all airway sizes demonstrating that hyperplasia and hypertrophy exist together or separately.¹⁰ With ASM being a major controller of airway tone, it is believed that this increased layer/mass could lead to a greater contractile force on the airways and increased airway wall stiffness. Additionally, encroachment of the inner wall from ASM mass will increase airway resistance. This is explained well in Paré et al, where a theoretical increase in airway wall thickness by 40% leads to an 80-fold increase in airway resistance.¹⁷ This is one reason why targeting and reducing ASM mass within the airway walls has been of particular interest for treatment in patients with asthma (i.e., bronchial thermoplasty).

Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR), a major feature of asthma, is a state where airways when challenged with triggers exhibit an excessive degree of narrowing. The amount of narrowing when challenged is greatly reduced in non-diseased individuals or has little to no effect.¹⁸ The exact reasons for this observed AHR are complex, yet many factors are implicated including viruses, airway inflammation (i.e., cytokines), increased diversity in airway microbiota, upregulation of contractile proteins in ASM, ASM mass increase, and infiltration of mediator releasing cells (i.e., mast cells).^{19–22} The production and release of contractile mediators that target ASM (i.e., various oxylipins, histamine, acetylcholine, etc.) could increase AHR and worsen airflow in asthma, especially during exacerbations.

Inflammation

As mentioned, asthma is a heterogenous disease with various endotypes incorporating both the innate and adaptive branches of the immune response. This immune response within individuals with asthma appears to be typically uncontrolled and potentially life threatening.²³ Triggers that would otherwise not significantly affect healthy individuals (i.e., allergens, pollution, viruses, cold air) activate many inflammatory processes across the lung. For example, antigen presentation from dendritic cells to T-cells and subsequent Th2 cell proliferation, release of various pro/anti-inflammatory cytokines and lipid mediators, B-cell activation and immunoglobulin E synthesis, recruitment of immune cells, mucus production, and structural cell alteration.^{3,23,24} One important factor of this inflammatory process includes the activation of eosinophils, mast cells, ASM, and bronchial epithelium which release many bioactive mediators such as the cysteinyl leukotriene, leukotriene D₄ (LTD₄), and the prostaglandin, thromboxane A₂ (TXA₂), both potent contractile effectors in ASM.^{3,25–27} Similarly, inflammation and damage to the surrounding tissues leads to release of substances capable of triggering airway narrowing. This was observed in rat lung slices where laser induced damage of the epithelium caused airway narrowing.²⁸ The bombardment of these inflammatory processes on surrounding tissue and the release of contractile mediators leads to reduced airflow, acute exacerbations, chronic remodeling, and paradoxical persistent inflammation.

Diagnosis

Symptoms like persistent wheezing, breathlessness, coughing, and chest tightness are all signs of a potential diagnosis for asthma. These symptoms exist in many other diseases like COPD and viral

infections. It then makes it important that proper diagnosis is performed to initiate proper treatment in these individuals, this is especially difficult in pediatric populations.^{29,30} Looking at historical patient data such as wheezing patterns or airflow obstruction problems is also helpful in determining potential cases of asthma but is not used alone to diagnose.

The non-invasive spirometry is a gold standard for diagnosis in populations older than 5 years old. Lung volumes are measured through spirometry and can give indications of asthma. One example includes using the forced expiratory volume in 1 second (FEV1) where lower FEV1 values indicate the severity of asthma.³¹ Another metric is the ratio of FEV1 over forced vital capacity (FVC) (i.e., FEV1/FVC). It is also recommended that a bronchodilator reversibility test be performed along with spirometry. This includes administering a short acting beta agonist (SABA) to see if FEV1/FVC ratio can be improved by more than 12%, if not, then asthma is likely.^{29,30,32} In children younger than 5, it is recommended that history and a therapeutic trial (3 months of SABA and/or ICS) are used to determine asthma.³² If these first line investigative tests cannot determine a case of asthma, the European Respiratory society suggests the use of a methacholine challenge.³⁰ Asthma is considered confirmed if a provocative dose of methacholine (≤ 8 mg/mL) causes a subsequent 20% drop in FEV1.

Treatment

The backbone of asthma therapy is presently inhaled corticosteroids (ICS)/ β -agonists and the newer biologics. Inhaled β -agonists such as SABAs and Long-acting Beta agonists (LABAs) are bronchodilators. SABAs are considered a rescue medication and all individuals including mild cases of asthma are recommended to have one.³² Inhaled β -agonists act on G Protein-Coupled Receptors (GPCRs) known as Beta-2 adrenergic receptors (B2AR) to initiate cyclic adenosine monophosphate (cAMP) production which leads to subsequent relaxing pathways in ASM and opening of the airways. Another important therapy is ICS, these are recommended for all cases of asthma and should be used alongside SABAs.³² ICS are highly important in disease control and the use of SABAs without ICS is a risk factor for increased mortality in asthma.³³ ICS acts by reducing inflammation, this includes the reduction of infiltrating immune cells like macrophages and eosinophils, reduction in inflammatory cytokines, upregulation of anti-inflammatory cytokines, and the upregulation of B2AR in ASM.³⁴

Other therapies include disrupting the actions of lipid mediators. This includes the pharmacologics Montelukast, Zafirlukast, and Pranlukast which act to block the action of leukotrienes

(LTD₄, LTE₄, LTC₄) at the cysteinyl leukotriene receptor (CysLTR₁). These cysteinyl leukotriene receptor antagonists reduce airway hyperresponsiveness, eosinophils in sputum and serum, and ASM contractility.^{35–37} Targeting oxylipins, their receptors, and their downstream processes is a potential route to alleviate symptoms in asthma. However, many oxylipins within the lung have no potential drug candidates, mostly due to the lack of knowledge on many of their functions.

Airway Smooth Muscle

What is Airway Smooth Muscle

ASM, are long spindle-like cells that are the structural and contractile unit of the airways. These cells are important regulators of airflow and do so through controlling the diameter of the airways. Particularly, shortening (contraction) of the ASM leads to a reduction in airway lumen diameter while lengthening (relaxation) leads to an increase in lumen diameter. Compared to skeletal muscle, ASM is capable of generating more force per cell even though it contains significantly less contractile machinery proteins (i.e., Myosin).³⁸

ASM mass is increased in obstructive lung diseases like severe asthma, the result of both hypertrophy and/or hyperplasia.^{39,40} The change in ASM mass and their intrinsic contractile properties contributes to airway hyperresponsiveness, and the reduced lung function commonly seen in individuals with asthma.^{13–15} ASM cells are capable of being modulated in disease states and exhibit changes in their functions, especially contractility. They are stiffer, more resistant to mechanical oscillations imparted by breathing, and generate more force upon contraction. It is believed that cytokines released from various immune cells within the ASM layer contribute to this modulation. For example, the Tumor Necrosis Factor Alpha (TNF α) influences ASM to have a greater Ca²⁺ signaling response after stimulation through receptor channels like GPCR and Transient Receptor Potential (TRP) C channels.^{41,42} Other cytokines like IL-13, interferon- γ , and IL-1 β exhibit similar functions. This likely leads ASM to exhibit an increased ability to contract than normal and may be a potential reason for AHR seen in individuals with asthma.

ASM also play a secretory role in the lungs and can release various cytokines and mediators (i.e., oxylipins) whether it be inflammatory, proliferative, or contractile/relaxing activators. These bioactive mediators are capable of acting through autocrine and paracrine pathways. For example, TGF- β ₁

production in ASM may act on other cells (paracrine) like immune cells for recruitment and proliferation but can also act in an autocrine fashion to induce its own proliferation and hypertrophy.⁴³ Similarly, inhibition of the enzyme diacylglycerol kinase in ASM leads to intracellular production of prostaglandin E₂ (PGE₂) production via the cyclooxygenase enzymes 1 and 2 (COX-1 and COX-2). PGE₂ can then act in an autocrine fashion to induce relaxation in the ASM cell.⁴⁴ Recently, oxylipin profiling in exclusively ASM cultures revealed countless oxylipins⁴⁵. This shows that ASM are not just structural but also secretory in nature. Many of these oxylipins remain unknown in the context of their functions in ASM physiology.

Contraction/Relaxation pathways

ASM is capable of contraction through various receptor-ligand interactions. Agonists like histamine, acetylcholine (ACH), toxins, and oxylipins (e.g., leukotrienes) activate a wide variety of protein receptors across the cellular membrane. Examples include GPCRs, ion channels like TRP channels, voltage-gated channels, Ca²⁺-dependent Potassium (K⁺) channels, and K⁺ channels. These protein channels activate downstream events to induce intracellular Ca²⁺ ([Ca²⁺]_i) release from organelles like the sarcoplasmic reticulum and extracellular Ca²⁺ intrusion through membrane bound channels.⁴⁶ This Ca²⁺ response can be transient or sustained through renewing Ca²⁺ oscillations. The increase in [Ca²⁺]_i concentration favors the binding of Ca²⁺ to the Ca²⁺-binding protein calmodulin. This Ca²⁺-calmodulin complex activates Myosin Light Chain Kinase (MLCK) which is responsible for phosphorylating the serine 19 residue of the 20 kDa myosin light chain (P-MLC20) within the contractile apparatus.⁴⁷ This allows for myosin-actin cross bridge formation and force generation.^{48,49} This leads to shortening of the ASM cell that then imparts a squeezing force capable of narrowing the airways.

Relaxation of ASM requires the use of relaxant mediators that use primarily GPCRs. For example, albuterol, the active drug commonly used in SABA bronchodilators, targets the GPCR B2AR. There exists anywhere from 30,000-40,000 B2ARs on any given ASM cell.⁵⁰ Once a mediator like albuterol binds the B2AR, the bound g-protein activates the enzyme adenylate cyclase to convert ATP to cAMP. cAMP activates various signaling pathways (i.e., Exchange Protein directly Activated by cAMP (Epac), Ca²⁺-activated K⁺ channels) with one being predominantly the activation of protein kinase A (PKA). PKA has many responses that deactivate the cellular cascades important for contraction, this includes the deactivation of MLCK through phosphorylation and the activation of MLC phosphatase (MLCP) which both lead to reduced activity of MLC20 phosphorylation.^{51,52} This leads to less cross-bridge cycling, the

subsequent reduction in generated force, the lengthening of ASM and the promotion of airway relaxation.

Calcium signaling

Ca^{2+} plays a huge role in cellular signaling physiology. From regulating the contraction of cardiomyocytes, fertilization of the ovum, activation of immune cells, to the contraction of ASM.^{53,54} A crucial step in ASM contraction is the induction and increase in the $[\text{Ca}^{2+}]_i$ concentration. Ca^{2+} has various routes of entry into the ASM cell cytoplasm. This includes extracellular membrane bound channels like voltage-gated Ca^{2+} channels, GPCRs, stretch activated ion channels, Ca^{2+} - dependent K^{+} -channels, and notably TRP channels. Activation of these protein channels by various agonists or mechanical stretch quickly initiates the intrusion of Ca^{2+} into ASM, significantly increasing $[\text{Ca}^{2+}]_i$ concentration. Another mechanism to increase $[\text{Ca}^{2+}]_i$ concentration is through intracellular stores, largely from the sarcoplasmic reticulum (SR).⁴⁶ This release is usually after activation of extracellular receptors that mediate downstream mechanisms capable of activating SR receptors to release Ca^{2+} . The SR quickly releases Ca^{2+} that either propagates the continued release of Ca^{2+} or the reuptake of Ca^{2+} . Transient or sustained/oscillatory Ca^{2+} responses in ASM have long been studied and vary differently on the stimulus. Nonetheless, a large spike in $[\text{Ca}^{2+}]_i$ and a subsequent return back to basal levels usually indicates reuptake of Ca^{2+} into the SR. Contrastingly, ASM show responses where there is a large Ca^{2+} spike followed by waves or oscillations that are above basal levels. To sustain this response a phenomenon called store-operated Ca^{2+} entry (SOCE) is activated and allows for the maintenance of continued Ca^{2+} release.⁵⁵ This is due to the depletion of the SR which initiates outer membrane Ca^{2+} channel activation that maintains intracellular Ca^{2+} levels. This SOCE mechanism is important in maintaining a continuous contractile response in ASM.^{56,57} Another Ca^{2+} signaling mechanism is the sustained $[\text{Ca}^{2+}]_i$ response in ASM where a slow steady increase in concentration is observed. The sustained $[\text{Ca}^{2+}]_i$ response seems to be through extracellular channels and interestingly TRP channels may be implicated. This was observed in Human ASM where activation of Transient Receptor Potential Ankyrin 1 (TRPA1) via cigarette smoke maintained a Ca^{2+} response significantly above basal levels for more than 10 minutes.⁵⁸

Numerous Ca^{2+} indicator dyes exist today with the first being developed in the late 1970's and early 1980's.⁵⁹ The first imaging of Ca^{2+} changes were seen in mouse thymocytes and pig node

lymphocytes using the ratiometric dye called 'Quin 2'.⁶⁰ The large molecule Quin 2 is capable of moving through cellular membranes via incubation with acetoxymethyl (AM) esters rendering it lipophilic and is highly selective for free Ca^{2+} ions. This is similar to the more utilized Ca^{2+} dye 'Fura-2 AM' which has various advantages over Quin 2. Quin 2's greater affinity for Ca^{2+} seemed to be worrisome in that it was believed that it would pull Ca^{2+} out of the intracellular stores. This led to the idea that potentially the cytoplasmic Ca^{2+} concentration values were far greater than the real values.⁶¹ It became apparent in the late 1980's that Fura-2 had a greater ability at measuring Ca^{2+} concentrations more accurately, had superior fluorescence intensity, and less bleaching issues.⁵⁹

For the ratiometric Fura-2 AM, once it enters into the cell, cytosolic enzymes known as esterases hydrolyze and remove the AM ester. This establishes two potential forms of Fura-2, where Fura-2 is bound to cytosolic Ca^{2+} ions (Fura-2- Ca^{2+}) or is free/unbound Fura-2.⁶² These 2 forms of Fura-2 exhibit two different excitation spectra, for example Fura-2- Ca^{2+} is excited at 340nm while unbound Fura-2 is excited at 380nm. Both forms of Fura-2 display an emission spectrum captured at 510nm. The ratios (hence the name ratiometric dye) 510nm/340nm and 510nm/380nm are first captured giving values for bound and unbound-Fura 2. This is further converted using the ratio 340nm/380nm to give a value that can be used to determine the Ca^{2+} concentration using the following equation.

$$[\text{Ca}^{2+}] = \frac{Sf2}{Sb2} * \frac{K_d(R - R_{min})}{(R_{max} - R)}$$

$[\text{Ca}^{2+}]$	Cytoplasmic Ca^{2+} concentration
K_d	Ca^{2+} dissociation constant of Fura-2
R	Ratio of intensities measured at 340 and 380 excitation
R_{max}	Ratio values measured under conditions where Ca^{2+} is present at saturating levels
R_{min}	Ratio values measured under conditions where no Ca^{2+} is present
$Sf2$	Fluorescence excited by the 380nm wavelength under conditions where no Ca^{2+} is present
$Sb2$	Fluorescence excited by the 380nm wavelength where Ca^{2+} is present at saturating levels

Another useful Ca^{2+} indicator dye is Fluo-4. Fluo-4 is a large chelator molecule bound to a fluorophore and can have an AM ester attached for cellular uptake.⁶³ This dye is much easier to work with in the sense of preparation and loading of cells but has some qualitative disadvantages compared to Fura-2. Fluo-4 is not a ratiometric dye in that it only captures fluorescence from one wavelength. For example, it only has one excitable state unlike fura-2, and this is when it is bound to Ca^{2+} . Fluo-4 bound to Ca^{2+} is excited at a wavelength of 494nm and emits at a wavelength of 516nm. Because of Fluo-4's non-ratiometric nature it therefore cannot measure Ca^{2+} concentration and can only infer Ca^{2+} flux in cells, an important differentiation between Fura-2 and Fluo-4 and a potential disadvantage depending on the experiment. Fluo-4 seems to be beneficial for measuring large Ca^{2+} changes and discovering potential activators of Ca^{2+} flux in cells. However, for more precise measurements, the use of Fura-2 is likely to lead to better experimental results as it has a greater ability to measure more miniscule Ca^{2+} changes like oscillations or sparks.

Cell models

Various cell models exist to determine contractile and relaxant pathways within the ASM field. The first discovering stages begin with using in vitro models using primary or senescence resistant ASM derived from donors. ASM in vitro gives the potential to study quantitatively and qualitatively the changes in phenomena related to the contractile and relaxing pathways. This includes changes in Ca^{2+} , P-MLC, PKA, PKC, MLCK, MLCP and measurements of cellular force vectors.

It is believed that ASM exists in two phenotypically different forms in vitro and in vivo.⁶⁴ This includes either the synthetic form of ASM or the contractile form, and both exist simultaneously but can be forced into one phenotype depending on environmental conditions. In cultures, ASM, when seeded at a low density along with various mitogens, exhibits first a synthetic phenotype. This is marked by circular morphology, an increased number of organelles like mitochondria, and a decreased abundance of contractile related proteins.⁶⁵ Additionally, these ASM have a reduced ability to contract. To push ASM into a contractile or 'mature' state, cultures are grown in serum deprived (SD) media (lacking mitogens for proliferation). ASM in this contractile state exhibit an elongated spindle-like shape and are abundant in contractile related proteins like smooth muscle α -actin and smooth muscle myosin.^{66,67} These ASM exhibit a much greater ability to contract than their synthetic counterparts. It is suggested then that studies looking at ASM contractility in vitro should be SD.

Oxylipins

What are they?/ Synthesis

Oxylipins are biologically active fatty acids that have countless functions across various tissues in the body. Their production starts from within the cellular membranes where physiological stimuli (i.e., allergens) mobilize the cleavage of phospholipids via enzymes known as phospholipase A2 (PLA₂) and secretory phospholipases (sPLA₂).⁶⁸ PLA₂ action is via an SN₂ reaction that releases a polyunsaturated fatty acid (PUFA). Many different PUFA's exist, examples include arachidonic acid (AA), linoleic acid (LA), α -linoleic acid (ALA), dihomo γ -linoleic acid (DGLA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), to name a few.⁶⁹ These PUFA's can be oxidized via cyclooxygenases (COX), lipoxygenases (LOX), cytochrome p450s (CYP), or non-enzymatic processes into downstream mediators known as oxylipins (**Figure 1**).⁷⁰ These short lived bioactive oxylipins can leave cells via carriers or by diffusion and can act on various tissues even at low concentrations (pM-nM ranges).⁷¹⁻⁷⁴

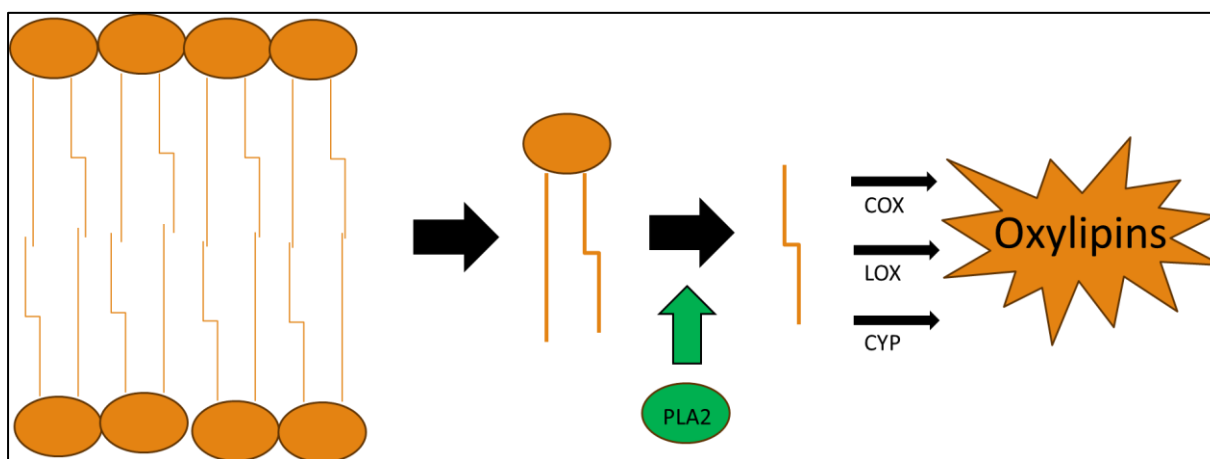


Figure 1. Synthesis of Oxylipins from cellular membranes. PLA₂, Phospholipase A2; COX, cyclooxygenases; LOX, lipoxygenases; Cytochrome p450s, CYP.

Types

There is an abundant variety of oxylipins in the human body. The first accounts of an unknown substance capable of producing relaxation and contraction in several vascular tissue types were discovered in the 1940's.⁷⁵ This unknown substance was found to be from the prostaglandin family which are derived from the PUFA arachidonic acid via COX oxidization. Many prostaglandins exist and all have various functions in lung physiology. For example, PGI₂ or prostacyclin is generally considered a

bronchodilator and is a mainstay drug for potent vasodilator effects in pulmonary hypertension.⁷⁶ Contrastingly, TXA₂, a downstream mediator from prostaglandin metabolism, is a potent bronchoconstrictor and is involved in the pathogenesis of asthma insomuch that therapies have been produced to combat TXA₂ effects.^{77,78} Another family of oxylipins derived from AA via the 5-LOX enzyme are the leukotrienes and are implicated in bronchoconstriction in asthma. Leukotrienes were initially called 'slow-reacting substance of anaphylaxis' due to their potent smooth muscle activation and prominent increase in allergic reactions. Various leukotrienes exist with the most important being LTB₄, LTC₄, LTD₄, and LTE₄ which are also known as cysteinyl leukotrienes (CysLTs). Leukotrienes are implicated in chemotaxis of various immune cells and play a role in airway hyperresponsiveness.⁷⁹ Similarly to TXA₂, various therapies have been developed to combat the actions of leukotrienes including Merck's 'Montelukast' and AstraZeneca's 'Zafirlukast'.

Within the lung, many structural and immune cells are capable of producing oxylipins. For example, bronchial epithelium and ASM release PGE₂, macrophages and neutrophils release LTB₄, platelets release thromboxane A₂, eosinophils release 15-HETE, and neutrophils release EpOMEs.^{27,80–83} These oxylipins can exert inflammatory, proliferative, migratory, and contracting/relaxing properties through paracrine or autocrine pathways. For example, bronchial epithelium releases PGE₂ during lipopolysaccharide challenge. PGE₂ can act on the bronchial epithelium receptors to enhance the endothelial barrier (autocrine) or move to ASM (paracrine) to promote bronchodilation, as well as reducing migration and proliferation.^{27,84,85}

Between the 3 major groups, prostaglandins, leukotrienes, and thromboxanes have had numerous studies describing their effects and will be considered the 'classical oxylipins' for this thesis. However, there exists countless 'non-classical' oxylipins that are only a fraction of what is found in the lungs, as pictured in **Figure 2**. Many of these oxylipins are unknown compared to the classical oxylipins, especially in the context of ASM physiology.

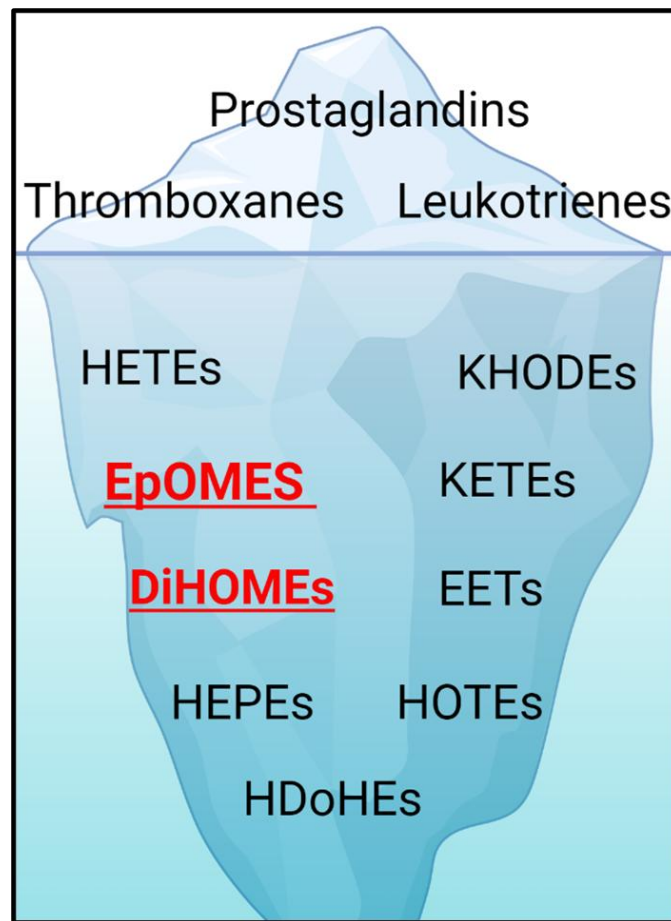


Figure 2. Iceberg analogy to show that the vast majority of oxylipins are undescribed in lung physiology and ASM contractility. The *EpOMES* and *DiHOMEs* are in red to show their importance for this thesis.

Receptors

Oxylipins use a variety of receptors on countless cell types across the human body and especially within the lung. Some examples include GPCRs, peroxisome proliferator-activated receptors (PPAR), nuclear receptors, voltage-gated ion channels, and cationic channels including the Transient Receptor Protein (TRP) family.^{69,86} The 3 major classical lipid mediators have had extensive research done on their ligand-receptor interactions in ASM. Prostaglandins, including TXA₂, use a variety of GPCRs annotated as the ‘prostanoid receptor subfamily’. Activation of these GPCRs and their corresponding G-protein (i.e., G_α, G_i, G_q, G_s) leads to many downstream effects. This can be cAMP production (i.e., PGI₂, PGD₂, PGE₂) via the membrane bound adenylate cyclase enzyme, decreased CAMP production (PGD₂, TXA₂), or the mobilization of intracellular Ca²⁺ (PGF2a, PGE₂).

Many oxylipin-receptor interactions are still being discovered especially in the context of non-classical oxylipins. Some interactions have been described in the lungs and sensory tissues; however, many still remain unknown. Examples like the HETEs, have many potential receptors including the thromboxane receptor, GPCRs, PPAR γ , Prostacyclin Receptor, TRP channels, and recently the Oxoeicosanoid receptor 1.^{72,87,88} In ASM, some oxylipins are capable of activating voltage gated-ion channels to induce relaxation. For example, in pre-contracted guinea pig bronchial strips, 8,9 EET caused relaxation through Ca^{2+} -activated K^+ channels (K_{Ca} channels).⁸⁹ Another oxylipin, 15-KETE, is believed to target smooth muscle directly in rat carotid arteries to produce a vasoconstriction response through voltage-gated potassium (Kv) channels.⁹⁰ Linoleic acid derived oxylipins like the HODEs (9-HODE and 13-HODE) and their metabolites KODEs (9-KODE and 13-KODE) activate Transient Receptor Potential Vanilloid 1 (TRPV1) receptors to induce transient Ca^{2+} flux in rat trigeminal (TG) neurons.⁹¹ Other oxylipins like 5-HETE and 15-HETE have also been implicated in TRPV1 activation in rat sensory neurons and HEK cells transfected with TRPV1.⁸⁷ Other potential receptors like Free fatty acid receptors (FFAR) may be activated by oxylipins. Yet the literature only suggests that free fatty acids activate FFAR. No data has looked at oxylipin activation of FFARs.⁹² Oxylipins are capable of activating various receptors in many tissue types. However, many oxylipin-receptor interactions remain unknown, especially in ASM physiology.

EpOMEs and DiHOMEs

The PUFA linoleic acid is converted via Cytochrome P450 enzymes (CYP2J2, CRP1A1, CYP2C8, CYP2C9) to two separate oxylipins known as 9,10-epoxy-12Z-octadecenoic acid (9, 10 EpOME) and 12(13) epoxy-9Z-octadecenoic acid (12, 13 EpOME).⁹³ These oxylipins can be further metabolized into 9,10-dihydroxy-12Z-octadecenoic acid (9, 10 DiHOME) and 12,13-dihydroxy-9Z-octadecenoic acid (12, 13 DiHOME) via the enzyme Soluble Epoxide Hydrolase (SEH) (**Figure 3**).

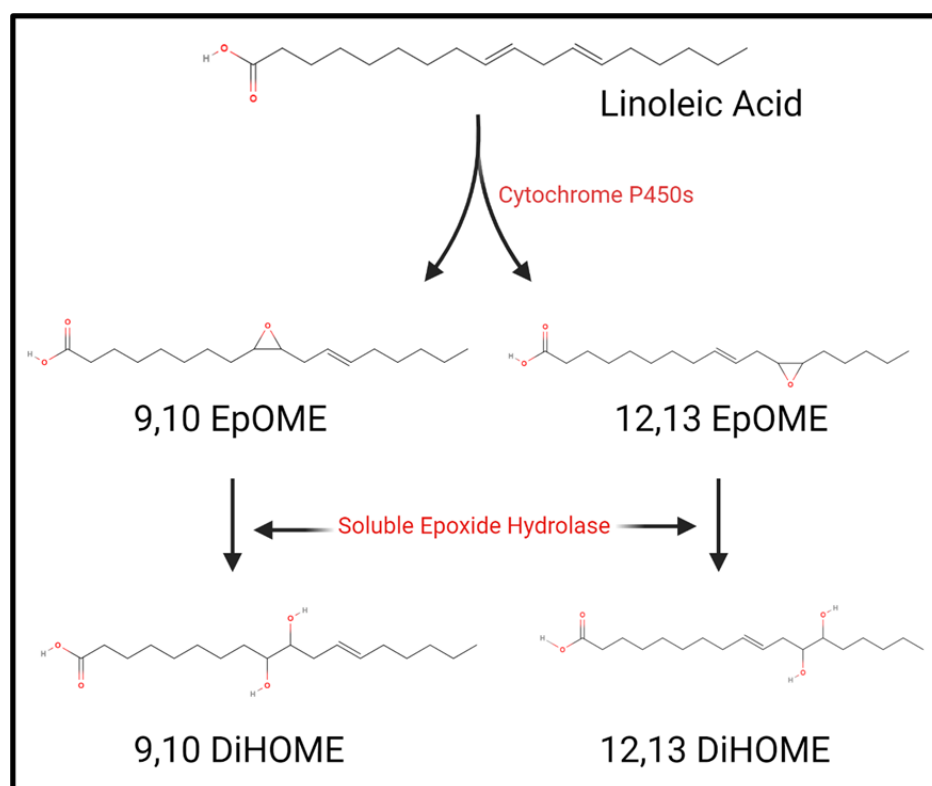


Figure 3. The Synthesis of EpOMEs and DiHOMEs.

9, 10 EpOME and 12, 13 EpOME in literature were first known as leukotoxin and isoleukotoxin, respectively while 9, 10 DiHOME and 12, 13 DiHOME were known as leukotoxin diol and isoleukotoxin-diol, respectively. These oxylipins are derived from various tissues and are not exclusive to just one physiological/anatomical system. These lipids have been observed in human circulation, neuronal tissues, feces, white adipose tissue and lungs.^{94–101} It is likely that these oxylipins are ubiquitous across the human body owing to the fact that CYP enzymes are in all tissues.¹⁰² Additionally, expression of the

SEH enzyme is said to be throughout numerous tissues, so it is likely that conversion of the EpOMEs occurs throughout tissues.^{93,103}

Studies in the 1980's were interested in what was termed "late deaths" from severe burn injuries where patients did not die from the wounds but from secondary issues like shock and infections. The observations of extensive damage to the skin and surrounding tissues suggested the release of toxic substances that entered the systemic circulation.^{104–106} This unknown substance was suggested to be of fatty acid nature and was later confirmed to be 9,10 EpOME.¹⁰⁴ 9, 10 EpOME was later shown to be elevated in patients with acute respiratory distress syndrome (ARDS) and curiously, burn patients commonly develop ARDS, potentially a consequence of 9, 10 EpOME.^{107,108} In rats, intravenous injections of 9, 10 EpOME caused marked damage to epithelial and alveolar space, and pulmonary edema, a common complication in ARDS.¹⁰⁹ Likewise, 9, 10 EpOME's metabolite 9, 10 DiHOME also caused edema in various lung tissues leading to respiratory distress and death in mice.¹¹⁰ These oxylipins in the 1980's and 90's were believed so toxic that authors suggested the removal of them during agricultural manufacturing to avoid consumption. Only until recently 12, 13 DiHOME has been considered a potential therapy for metabolic diseases and rescuing cardiac function.^{111,112}

In murine asthma models, 12, 13 DiHOME is implicated in amplifying inflammatory processes. Mice treated with 12,13 DiHOME and then challenged to allergen exhibit greater serum levels of IgE, cytokines IL-1 β , IL-1 α , TNF, and immune cells like CD3+ T cells, monocytes, and neutrophils compared to controls.⁹⁷ This increase in neutrophils is especially interesting since neutrophils, when activated, produce DiHOMEs which are capable of recruiting more neutrophils.⁸³ This positive feedback situation may lead to persistent inflammation seen in asthma. 12, 13 DiHOME also induces extensive damage to the bronchial and vascular spaces with marked increases in inflammatory infiltrates. In two human cohorts of neonates, asthma and atopy were more likely to develop in 2- and 4-year-olds when fecal 12, 13 DiHOME concentrations were elevated.⁹⁷ Nonetheless, it seems that 12, 13 DiHOME may be important in allergic asthma.

EpOMEs and DiHOMEs also play a role in pain sensation. In mice, zymosan induced inflammation caused a marked increase in the production of 12, 13 DiHOME in dorsal root ganglion (DRG) sensory neurons after 24 hours.¹¹³ Similarly, in the spinal dorsal horn, 9, 10 DiHOME and 12, 13 DiHOME was significantly increased after 6 and 24 hours. Both these lipids were then tested to see their effects on

sensory neurons. Both DiHOMEs (10 μ M) activated transient increases in [Ca²⁺] but only 12,13 DiHOME was significant in this response. This response could be abolished after the second addition of 12,13 DiHOME and in Ca²⁺-free medium indicating a potential membrane bound channel. To confirm this, a TRPV1 antagonist (AMG 9810) and a TRPA1 antagonist (HC-030031) were used to pinpoint the extracellular channel. AMG 9810 blocked 12, 13 DiHOME induced Ca²⁺ flux in sensory neurons while HC-030031 did not, indicating TRPV1 as the culprit receptor. Another interesting finding was the potentiation of TRPV1 when 12, 13 DiHOME was incubated with DRG neurons at low doses (100nM-1000nM). Significant Ca²⁺ flux were observed when TRPV1 agonist 'Capsaicin' (200nM) was added and this could be seen at various incubation concentrations of 12, 13 DiHOME (250nM, 500nM and 1000nM). In a similar rat model, burn injury induced significant increases in both EpOMEs and DiHOMEs.¹¹⁴ To understand their role in sensory neurons both EpOMEs and DiHOMEs were tested in a whole cell electrophysiology application. All EpOMEs and DiHOMEs were capable of inducing currents in sensory neurons with 9, 10 EpOME having a roughly 4x greater efficacy than 9,10 DiHOME and 12,13 EpOME having an 8x greater efficacy than 12, 13 DiHOME. To find the receptor, CHO cells expressing various TRP channels were stimulated by each EpOME and DiHOME. Activation was determined to be through TRPA1 and TRPV1 for all oxylipins and with 12, 13 EpOME and 12, 13 DiHOME showing some activity in TRPV3.

In DRG neurons from mice, fluorescent imaging confirmed 9,10 EpOME (10 μ M) was capable of inducing Ca²⁺ flux.¹¹⁵ In Ca²⁺-free medium, secondary additions of 9,10 EpOME were not capable of inducing a response, indicating an extracellular origin of the Ca²⁺. To determine the potential extracellular receptors, TRPV1 and TRPA1 inhibitors AMG9810 and HC-030031, respectively, were used. AMG9810 (1 μ M) was capable of significantly blocking Ca²⁺ mobilization in DRG neurons but this was not seen with HC-030031 (20 μ M), revealing 9, 10 EpOMEs requirement of TRPV1. Interestingly, and similarly to 12, 13 DiHOME, 9,10 EpOME incubated with neurons can sensitize TRPV1 channels. Again, the addition of TRPV1 agonist Capsaicin (200nM) was capable of significantly potentiating Ca²⁺ induced responses and this could be seen at various incubation concentrations of 9,10 EpOME (0.25 μ M- 2 μ M). 9, 10 EpOME and 12, 13 DiHOME seem to exhibit a 'double threat' where it can activate TRPV1 channels and also potentiate its ability to mobilize Ca²⁺.

Interestingly, no data has been found on EpOMEs and DiHOMEs contractile abilities in ASM. In other contractile tissues 9, 10 EpOME (10 μ M and 100 μ M) is capable of inducing vasodilation in rat

pulmonary vascular tissue with marked increases in cGMP.¹¹⁶ 9, 10 EpOME acts differently in neuronal tissue compared to smooth muscle and signifies the diverse functions of these oxylipins. In murine cardiomyocytes, 12, 13 DiHOME is capable of inducing changes related to contractile functions.¹¹² Specifically, 12, 13 DiHOME increased maximal cardiomyocyte shortening and velocity and induced greater maximal Ca^{2+} responses compared to controls. Similarly, in cat carotid arteries, 9, 10 EpOME and 12, 13 EpOME both significantly induced vasoconstriction in a dose-dependent fashion with minimal contraction at 2.5ug/ml (8.4 μM) and maximal contraction at 25ug/ml (85 μM).¹¹⁷ EpOMEs and DiHOMEs exhibit diverse effects on tissues and signifies the importance of understanding their functions on different cell types. If these EpOMEs and DiHOMEs are capable of inducing contraction in other tissues, it may be possible that they are capable of doing this in ASM. EpOMEs and DiHOMEs may be potential contractile agents in the lungs that could play a role in obstructive diseases like asthma.

Oxylipins and the Lung

Detection of Oxylipins in the Lung

There are several ways one may collect oxylipins from human lungs. Exhaled breath condensate (EBC) is one way to collect organic matter in the expired breath of an individual. By cooling the exhaled breath, organic matter such as DNA, RNA, proteins, and metabolites like oxylipins can be collected. It is an ideal and quick method to quantify samples in diseases like asthma regardless of where it is derived in the lung.^{118–122} Another technique is to extract bronchoalveolar lavage fluid (BALF). This is a substantially more invasive technique compared to EBC. Saline fluid is instilled into a given area of the lung like the smaller airways, alveoli, and the surrounding parenchyma which is then suctioned back up. This allows for the collection of cells, pathogens, proteins (i.e., cytokines), lipids such as oxylipins, and more.^{123–125} Bronchial wash (BW) extracts organic material similarly to BALF but from the larger airways like trachea, bronchi, and some smaller airways.¹²⁶ It is less invasive than BALF, quicker than BALF, and instills a lesser volume of saline into the lungs which avoids greater degrees of inflammation.^{126,127} BW along with BALF are useful techniques for understanding and characterizing the aspects affecting the proximal airways (i.e., BW) versus the peripheral airways (i.e., BALF).

Prostaglandins/Leukotrienes

Classical oxylipins like prostaglandins and leukotrienes (i.e., CysLTs) have long been known to be within the human lungs and multiple studies have detected them in BW, BALF, and EBC.^{99–101,128–130}

Changes between healthy and diseased states demonstrate that oxylipin profiles can differ. For example, CysLTs in healthy individuals are distinctly lower than individuals with asthma.¹³⁰ Similarly, children with exercise induced bronchoconstriction and asthma show greater amounts of CysLTs compared to children without asthma.¹³¹ The prostaglandins also exhibit changes in abundance across disease and exposure. Individuals with mild asthma show a significant increase in PGE₂ compared to healthy individuals.¹⁰¹ Aspirin-induced asthma shows increased amounts of PGE₂ and TXB₂ compared to non-sensitive individuals with asthma.¹³² Additionally, individuals exposed to allergens like pollen have increased PGE₂ compared to healthy individuals.¹⁰⁰ These classical lipids are detectable in the lungs and are changed in disease and exposure to allergens.

Non-classical Oxylipins in the lung

Although classical oxylipins are important in disease, countless other oxylipins have been found in the lungs. I found 8 studies that looked at various classical and non-classical oxylipins.^{99–101,128,129,133–135} This included oxylipins like HETEs, DiHETEs, EETs, KETEs, HODEs, KODEs, TriHOMEs, EpOMEs, DiHOMEs, and many more. 63 different oxylipins were detected while only 14 were considered classical oxylipins (**Figure 4**). This coincides with one study where classical oxylipins accounted for 10-20% in individuals with asthma while non-classical oxylipins accounted for 70-80% of the oxylipin profile.¹⁰⁰ Some of these oxylipins have the ability to contract or relax ASM. For example, HETEs like 5-HETE are elevated in moderate to severe asthmatic and is a known contractile mediator in human bronchial tissue.¹³⁶ Similarly, 15-HETE is capable of contracting human bronchial smooth muscle in a dose-dependant fashion. EETs on the other hand are ASM relaxing agents in humans and have been therapeutically intriguing for diseases related to the heart, CNS, and especially the lungs.^{89,137} Many of these non-classical oxylipins exist in the lung yet many remain unknown in the context of ASM contractility, including the EpOMEs and DiHOMEs. This indicates a substantial gap in knowledge and is made clear in **Figure 4**.

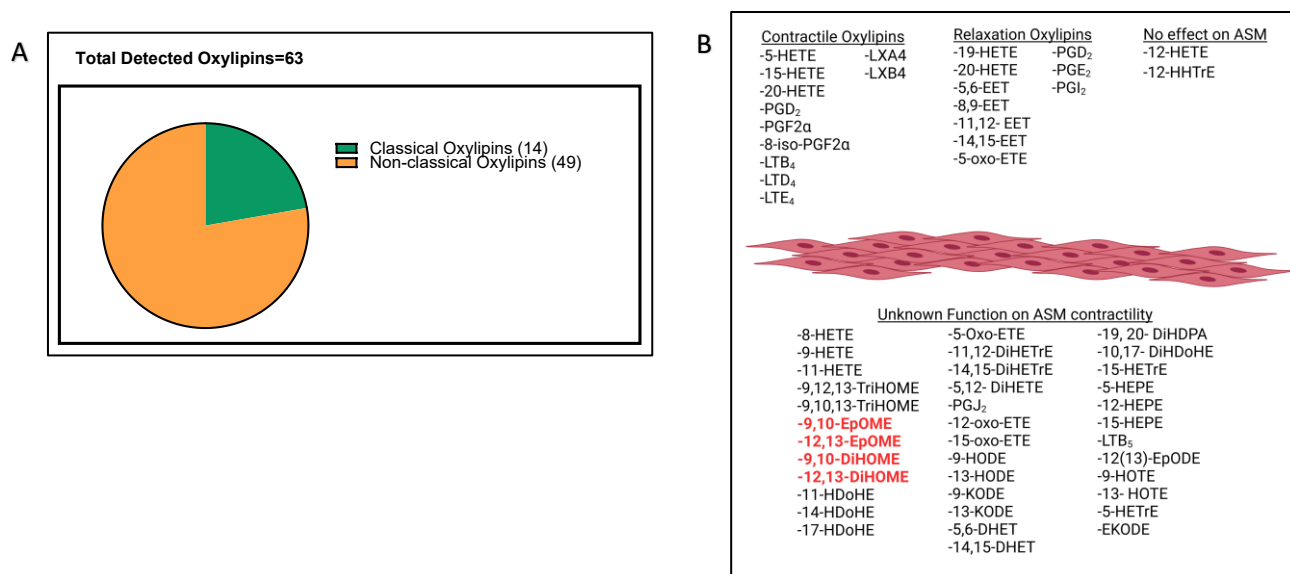


Figure 4. Oxylipins found in the human lung. (A) The amount of classical vs. non-classical oxylipins detected in the lungs across 8 studies.^{99–101,128,129,133–135} (B) Many lung derived oxylipins remain unknown in the context of ASM contractility.

EpOMEs and DiHOMEs in lungs

Looking only at samples taken from humans (i.e., BALF, BW, and EBC), I found 8 studies that found classical oxylipins as well as non-classical in the lungs. Interestingly, 7 of the 8 studies indicated that EpOMEs and DiHOMEs were detectable in the lungs. In 4 of the 7 studies, significant changes were seen between healthy and individuals with asthma as well as individuals exposed to allergen or pollution.

Birch pollen exposure to asthmatic individuals significantly increased 9, 10 EpOME and 9, 10 DiHOME concentrations.¹⁰⁰ Both sets of EpOMEs and DiHOMEs were the driving factor for the differences seen in oxylipin profiles between asthmatics provoked with birch pollen compared to controls. Interestingly, the EpOMEs and DiHOMEs were the most abundant oxylipins in BALF of both groups and far more abundant than the classical oxylipins like PGE₂ and TXB₂. This seems to be a common finding across other studies.^{99–101,135}

In an EBC study, 1 individual with mild asthma was subjected to allergen exposure. 5 oxylipins including 12, 13 DiHOME were increased after exposure when compared to baseline.¹³⁴ 12, 13 DiHOME was the only oxylipin that was significantly elevated after allergen exposure. indicating a potential

inflammatory oxylipin response. EBC was only collected from one individual here and lacks statistical power.

In an air pollution study, healthy individuals were exposed to either biodiesel exhaust or filtered air.¹³⁵ EpOMEs and DiHOMEs were both detected across both groups in BALF and BW, again at large amounts. Changes were seen in BALF fluid where 12, 13 DiHOME concentrations were significantly increased in individuals subjected to biodiesel exhaust. Even within healthy individuals oxylipin profiles can change when exposed to harmful particulate matter. This is similar to what is seen in healthy non-smokers and healthy smokers where EpOMEs and DiHOMEs are changed in healthy smokers. For example, 9, 10 DiHOME is increased in healthy males who smoke but not in males who have never smoked.¹³³ Likewise, the EpOMEs are elevated in healthy female smokers but not in females who have never smoked.

The abundance of these oxylipins have the potential to be altered in diseased states, and from allergen or pollutant exposure. It is thus important to understand their role in the lungs. This is especially important in the context of ASM muscle physiology. Are these oxylipins potentiating or causing contraction in ASM leading to airway narrowing? Are they potential relaxing agents and lead to opening of the airways? It is unknown whether they are benign or detrimental to ASM in diseased and even healthy individuals.

Transient Receptor Potential (TRP) Channels

What are they

TRP Channels are membrane spanning protein ion channels that are found across many various tissues in humans.^{138,139} There are 6 TRP families; TRPC, TRPV, TRPA, TRPM, TRPML, and TRPP that contains 28 subtypes. These all possess 6 transmembrane regions, a hydrophobic channel pore, and N- and C-termini situated in the cytoplasm. Most of these channels play a role in Ca^{2+} permeability but with varying selectivity. For example, in the TRPV family, TRPV1, TRPV2, TRPV3, and TRPV4 are non-selective cation channels while TRPV5 and TRPV6 are only permeable to Ca^{2+} ions.¹⁴⁰ For the purpose of this thesis, only a couple channels will be mentioned.

Transient Receptor Potential Ankyrin 1 (TRPA1)

TRPA1 is a well-studied non-selective cation channel and chemo-nociceptor that plays a role in sensing noxious stimuli, tissue damage, and pain.^{114,139,140} Numerous TRPA1 agonists have been discovered such as mustard oil, wasabi, cigarette smoke, pollutants, oxidized phosphatidylcholines, and various oxylipins.^{58,139,141} These channels are not exclusive to just neuronal tissue; they exist extensively throughout the lungs. For example, ASM, fibroblasts, and epithelium all show functional and detectable TRPA1.^{141–143}

Transient Receptor Potential Vanilloid 1 (TRPV1)

TRPV1 is a non-selective ion channel and is activated by compounds that possess vanillyl groups, examples include the chili pepper derived substance capsaicin and even jellyfish poison.¹⁴⁴ Noxious stimuli activate TRPV1 to relay a burning/pain sensation to the central nervous system. Similarly to TRPA1, TRPV1 is found throughout human tissues including sensory neurons, neurons that innervate the lung tissues (i.e., ASM, parenchyma, upper airways), bronchial epithelium of healthy and severe asthmatics, and ASM.^{145–147}

Knowledge Gap

There exists countless oxylipins within the lung. Oxylipin profiles in individuals are changed from pollution, allergen exposure, cigarette smoke, or asthma.^{99–101,128,129,133–135} Several oxylipins have known functions within the lungs and play a key role in ASM tone. As mentioned, prostaglandins like PGE₂ are considered relaxing lipid mediators while leukotrienes like LTD₄ produce a contractile response. Although these classical oxylipins have extensive understanding on ASM physiology, many of the non-classical oxylipins have no data on their actions on ASM contractility and are a complete mystery. Eight different studies looking at either EBC, BALF, and/or BW fluid showed that 63 oxylipins were detected in the lungs and 49 were non-classical and many have no known function on their ability to relax or contract ASM. This indicates a substantial land of discovery on whether these oxylipins are more contractile or relaxing in nature. Knowledge of oxylipin actions on ASM will help to determine potential detrimental mediators in the lungs that may influence or potentiate airway narrowing and exacerbations in individuals with asthma. Additionally, potential therapeutics to target various oxylipins and downstream pathways may be of benefit to individuals with asthma as seen with Montelukast and Zafirlukast. However, we first need to understand what they do.

I selected 4 oxylipins that were present across 7 of the 8 studies. These were 9, 10 EpOME, 12, 13 EpOME, 9, 10 DiHOME and 12, 13 DiHOME. These 4 interrelated oxylipins are present in the lungs in large amounts compared to other oxylipins. Additionally, these oxylipins exhibit changes in abundance following exposure to allergens, pollution, or cigarette smoke and are changed in individuals with asthma. I believe these oxylipins are important to study because (1) they are present and abundant within the lungs, (2) they change in abundance due to exposures or disease, (3) no data exists on their ability to contract or relax ASM, and (4) are potential TRPV1 and TRPA1 activators.

Rationale

The main goal was to determine if these oxylipins cause an increase in $[Ca^{2+}]_i$ in ASM to understand if they are potentially detrimental in the context of asthma. To tackle this, I first set out to look at the role these oxylipins have in ASM Ca^{2+} signaling, an important and crucial process to generate force and cell shortening in ASM. I used inductive reasoning from Ca^{2+} studies in neurons to guide the project.

1. The oxylipins EpOMEs and DiHOMEs induce $[Ca^{2+}]_i$ flux and ion currents in neuronal tissue through TRPV1 and TRPA1 Ca^{2+} channels.^{113–115}
2. Airway Smooth Muscle cells possess functional TRPA1 and TRPV1 Ca^{2+} channels.^{141,147}
3. Therefore, EpOMEs and DiHOMEs will induce $[Ca^{2+}]_i$ flux in Airway Smooth Muscle.

Hypothesis 1

Oxylipins 9,10 EpOME, 12, 13 EpOME, 9,10 DiHOME, and 12, 13 DiHOME cause calcium flux in Airway Smooth Muscle

Objective 1: Determine if 9,10 EpOME, 12, 13 EpOME, 9,10 DiHOME, and 12, 13 DiHOME cause $[Ca^{2+}]_i$ flux in Airway Smooth Muscle.

Hypothesis 2

Oxylipins 9,10 EpOME, 12, 13 EpOME, 9,10 DiHOME, and 12, 13 DiHOME require receptor channels TRPV1 and/or TRPA1 in Airway Smooth Muscle

Objective 2: Determine if 9,10 EpOME, 12, 13 EpOME, 9,10 DiHOME, and 12, 13 DiHOME require TRPV1 and TRPA1 channels to induce $[Ca^{2+}]_i$ flux in Airway Smooth Muscle.

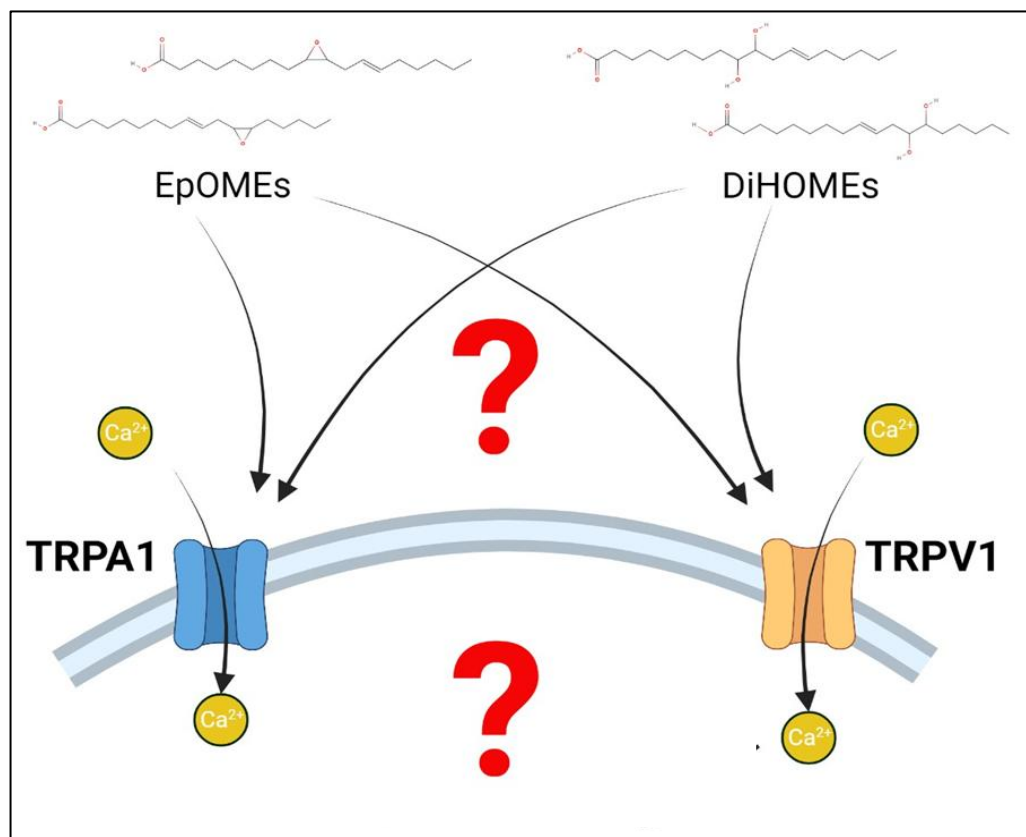


Figure 5. Representation of overarching Hypothesis. Do EpOMEs and DiHOMEs use TRPA1 and/or TRPV1 to induce Ca^{2+} flux in Airway Smooth muscle?

CHAPTER 2: METHODS

Senescence Resistant Human Airway Smooth Muscle culture

Primary ASM were retrieved from patients without asthma and were altered to be senescence resistant using a previously described method.^{148,149} Briefly, individuals who were deemed to have normal lung functions had a biopsy performed to extract healthy tissue, then ASM were isolated and cultured. The human telomerase reverse transcriptase gene (hTERT) along with a neomycin resistant gene (HygB) was cloned and packaged into Moloney murine leukemia retrovirus. ASM were then infected with retrovirus to incorporate the hTERT and HygB genes. GeneticinTM (GibcoTM), an antibiotic to target cells that cannot produce neomycin was added to ASM (400ug/ml). This was used to select for senescence resistant ASM containing the hTERT and HygB genes. hTERT ASM were grown in Dulbecco's modified Eagle's medium (DMEM) containing low glucose (1g/mL), 5% fetal bovine serum (FBS), and 1% Penicillin-Streptomycin (10,000 units/mL penicillin-10,000 µg/mL Streptomycin) unless stated otherwise.

Chemicals

9, 10 EpOME, 12, 13 EpOME, 9, 10 DiHOME, and 12, 13 DiHOME were purchased from Cayman Chemical. Oxylipins were stored in methyl acetate at -80°C. A glass 'Hamilton' syringe was used to extract oxylipins in methyl acetate and then placed in a glass vial. Pure oxylipins were retrieved by evaporating off methyl acetate via a gentle stream of nitrogen. Oxylipins were resuspended with either HBSS, Ca²⁺-free HBSS or SD media. TRPA1 inhibitor HC-030031 (Cat# H4415) and TRPV1 inhibitor AMG-517 (Cat# SML2717) were purchased from Sigma-Aldrich. Inhibitors were resuspended in DMSO at a stock concentration of 1mM and stored at -20°C. Inhibitors were thawed on day of experiment and mixed with SD media to a final concentration of 10µM. Gadolinium Chloride (GD³⁺) (011287-06) a non-specific extracellular Ca²⁺ channel blocker was purchased from Thermo Scientific Chemicals and made fresh on days of experimentation. GD³⁺ was made in HEPES-buffered Hanks' balanced salt solution (HBSS) and added to cells with a final concentration of 500uM. For Ca²⁺ experiments I used HBSS from GibcoTM (14025092) which contained Ca²⁺ (CaCl) along with added HEPES (20mM). For HBSS without Ca²⁺ I prepared our own buffer with 5.33 mM KCl, 0.441 mM KH₂PO₄, 0.407 mM MgSO₄-7H₂O, 0.493 mM MgCl₂-6H₂O, 137.93 mM NaCl, 4.17 mM NaHCO₃, 0.338 mM Na₂HPO₄ [anhydrous], 5.56 mM dextrose, and 20 mM HEPES, and at a pH of 7.4.

Fluo-4 Calcium Reading

hTERT ASM were seeded at 10,000 cells/well in 96-well black polystyrene plates (Costar®) until 80% confluency. Cells were SD for 24 hours in DMEM containing low glucose (1g/mL), 0% FBS and 1% Penicillin-Streptomycin. SD media contained Insulin-Transferrin-Selenium (ITS) (1X) from Gibco™. ASM should be SD for 7 days to induce a more contractile phenotype.⁶⁶ However, I observed issues with ASM cultures (i.e., cell death, detaching from well) when SD for longer than 24 hours especially in 96-well plates.

Ca²⁺ imaging was performed using the protocol from 'Fluo-4 Direct™ Calcium Assay Kit' (ThermoFisher Scientific). Fluo-4 was prepared directly from protocol and contained probenecid (5mM) and buffer containing Ca²⁺ (provided with kit). For Fluo-4 experiments looking at Ca²⁺-free responses, I used Ca²⁺-free HBSS to prepare fluo-4. Inhibitors were prepared in SD media containing ITS (1X) and then added to a same volume of Fluo-4 (2X). This produced a 1x concentration of Fluo-4 as required in protocol kit. SD media was removed from 96-well and Fluo-4 and inhibitor mixture was added to cells. Cells were incubated in dark for both 30 minutes at 37°C and then for 30 minutes at room temperature. It is suggested from some Fluo-4 dye kits that it is best to incubate at 37°C (30 minutes), then room temperature (30 minutes) and then to perform experiment at room temperature. Our experiments were performed at room temperature as well (≈23°C). It is also suggested to incubate for a total time of at least 1 hour to allow for de-esterification of the AM esters bound to Fluo-4. This allows the Fluo-4 molecule to bind Ca²⁺ and remain in the cytoplasm. Fluorescent reads were then performed on 'Cytation 5' (Agilent Technologies, inc.). Baseline fluorescent reading of ASM was measured for 5 seconds, then stimulants were injected, and reading was captured ever 1 second over a period of 50 seconds. ACH was used as a positive control. Fluorescence was measured via excitation at 494 nm and emission at 516 nm.

Fura-2 Calcium imaging

hTERT ASM were seeded at 80,000 cells/well in 4 well chambered coverglass wells from NUNC (0.16 - 0.19 mm thick coverglass). ASM were grown until 70% confluency and then SD for 5-7 days in DMEM containing low glucose (1g/mL), 0% FBS and 1% Penicillin-Streptomycin. SD media contained Insulin-Transferrin-Selenium (ITS) (1X) from Gibco™.

Cells were loaded with Ca^{2+} -sensitive 'Fura-2, AM, cell permeant' dye from (Invitrogen™). Briefly, 50uL of DMSO was added to Fura-2 dye. Fura-2 dye in DMSO was then mixed with 5ml of HEPES-buffered HBSS (Gibco™) containing CaCl, 0.1% BSA, and 5ul 20% Pluronic acid (in DMSO). SD media was removed, and cells were washed 2 times with HBSS. Fura-2 mixture was then loaded to each well at a final concentration of 5μM (400uL/well) and incubated for 1 hour at 37°C. Fura-2 dye was then removed and cells were washed four times each with HBSS. Cells were then incubated with HBSS for 30 minutes at room temperature (~23°C) to allow for the cleavage of the AM esters from Fura-2. Ca^{2+} - free experiments were also performed. HBSS without Ca^{2+} was prepared and was used to prepare Fura-2 such that when ASM were incubated there was no extracellular Ca^{2+} . Fluorescent imaging of cells was via an Olympus LX-70 inverted epifluorescence microscope linked to a Nikon camera. A Sutter Instruments Lambda 10-2 filter wheel that was capable of alternating between excitation wavelengths of 340 and 380nm was used and emission was captured at 510nm. Capturing of real-time Ca^{2+} flux was via the NIS imaging software. Baseline was measured for 20 seconds, then oxylinpin treatment was injected manually, and reading was performed for 60 seconds. I retrieved images ever 3-4 seconds due to the lambda filter wheels ability to switch between the excitation wavelengths. Excitation wavelengths of 340nm and 380nm and emission of 510nm were converted real-time to directly measure the $[\text{Ca}^{2+}]_i$ concentrations. To obtain Ca^{2+} concentration directly, a Ca^{2+} calibration kit was used to generate a calibration curve (i.e., Invitrogen 'Fura-2 Calcium Imaging Calibration Kit'). This was done before experiments were performed. Histamine was used as a positive control. Data was captured as Ca^{2+} concentration (nM) and analysed using excel and GraphPad.

Airway Smooth Muscle Protein extraction

To extract protein from ASM, cells were grown to confluence in T75 flasks. ASM were SD for 24 hours. All media was removed, and 3 ml of cold PBS was added to cells while on ice. 180uL of RIPA lysis buffer containing 1x protease inhibitor, 3x phosphatase inhibitor, and 1x phenylmethanesulfonylfluoride (PMSF) was added to the cells and then put in -80°C for 30 minutes. Cells were then scraped, and the subsequent cell lysate was then stored at -80°C for 24 hours. Cell lysates were vortexed (3 times with 5-minute intervals), sonicated (2 times for 10 seconds each), and centrifuged for 5 mins at 4°C at 13350 RPM (13709 RCF). Protein quantification was performed using protocol from 'Pierce™ BCA Protein Assay kit' (Thermo Scientific). Protein was prepared in 4x Laemmli Sample Buffer (Bio-Rad) and RIPA lysis buffer. Proteins were warmed at 95°C for 5 minutes, then stored at -20°C.

Immunoblotting TRPA1 and TRPV1

8% Gels were made using distilled H₂O, 30% acrylamide mix (Bio-Rad), 0.5M Tris (pH=8.8), 10% SDS, 10% APS, and TEMED. Protein samples were thawed from -20°C and vortex. 40ug of protein was loaded from each of the six human ASM donors and then SDS-PAGE was performed. Proteins were transferred onto PDVF membranes via electrotransfer for 1:15 hours at 4°C. Membranes were blocked with 5% milk in TBST for 1 hour at RT. Mouse Anti-TRPA1 (Santa Cruz; sc-376495) was prepared to 1% milk in TBST (TRPA1; 1:500), and Rabbit anti-TRPV1 (Invitrogen; PA1-748) was prepared in 0.5% BSA in TBST (TRPV1; 1:1000). Primary antibodies were added to membranes for 24 hours at 4°C. Membranes were incubated with secondary antibody for TRPA1 (1:5000) (Novus Biologics; NBP1-75116), and TRPV1 (1:5000) (Novus Biologics; NBP1-75283) for 1 hour at RT. Membranes were exposed to ECL-max for 1 minute and imaged on ChemiDoc imager (Bio-Rad). α -Tubulin was used as loading control for both TRPV1 and TRPA1 blots.

Data analysis

All data are presented as (mean $\pm$ SEM) where applicable. All experiments are from 2-5 donors with each 'n' representing ASM originating from independent flasks. To obtain Fura-2 data I used NIS imaging software where boundaries were drawn around ASM cells in focus and data was then extracted and put into excel. For any given experiment there was >15 cells per field of view. Cells that exhibited circular morphology and were not similar to ASM were not selected. For Fura-2 data, maximum changes in intracellular Calcium (Max $\Delta[Ca^{2+}]_i$) were calculated as $[Ca^{2+}]_i$ average baseline subtracted from the max $[Ca^{2+}]_i$. For fluo-4 data acquisition, selection of cells is not possible as I did not select 'Cytation 5's' imaging capability. Thus, fluorescent data is captured from the same area per treatment well. For fluo-4 data, fluorescence is presented as $\Delta F/F_0$ (i.e., ('fluorescence value'-'average baseline fluorescence')/average baseline fluorescence). Data was analyzed using either one-way ANOVA, two-way ANOVA, paired t-test, and when residuals were not normally distributed as determined by Shapiro-Wilk test and QQ-plot a Wilcoxon signed rank test was used and noted in figure caption where applicable. Statistical significance was set at a P value of ≤ 0.05 .

CHAPTER 3: RESULTS

Oxylipin 12, 13 EpOME induces a dose-dependent intracellular increase in fluorescence in Airway Smooth Muscle

Confluent and SD (7 days) healthy human ASM were loaded with the Ca^{2+} indicator dye Fluo-4 and treated cells with the oxylipin 12, 13 EpOME at 2 different concentrations or vehicle (DMSO). ASM cells demonstrated a marked increase in fluorescence after addition of both concentrations of 12, 13 EpOME (**Figure 6A**). The lowest concentration (0.1 μM) exhibited a sharp increase and transient fluorescent signature 65 seconds post injection. Contrastingly, the highest concentration [10 μM] produced a sharp increase but a sustained fluorescent signature showing a kinetic difference between the two doses (**Figure 6A**). Positive control ACH showed a sharp increase and transient fluorescent curve at the highest concentration [10 μM] but no response at the lowest concentration [0.1 μM]. This observation may be due to the impaired function of M3 receptors in SD/ non-contractile ASM which has been described in canine ASM cell cultures.¹⁵⁰

To quantitatively describe the kinetics of the curves, I took the max fluorescence at 10 seconds post treatment. 12, 13 EpOME [0.1 μM] exhibited a 4-fold increase in max fluorescence (0.850 ± 0.341) compared to vehicle (0.218 ± 0.211) 10 seconds post injection but was not significant (**Figure 6B**). Contrastingly, 12, 13 EpOME [10 μM] showed a significant 4-fold increase (0.841 ± 0.179) in max fluorescence compared to vehicle (0.218 ± 0.211) (**Figure 6B**). I also took the max fluorescence at 56 seconds post treatment to understand the sustained or transient nature of the 12, 13 EpOME's response. 12, 13 EpOME [0.1 μM] exhibited a 3-fold max increase in fluorescence (0.290 ± 0.149) compared to vehicle (0.097 ± 0.074) (**Figure 6C**). Interestingly, 12, 13 EpOME [10 μM] showed a significant sustained max fluorescence signature that was 9.5-fold (0.864 ± 0.297) greater than vehicle (0.097 ± 0.074) at 56 seconds (**Figure 6C**). This data shows that different concentrations of 12, 13 EpOME have varying kinetic responses.

The positive control ACH a known ASM contractile mediator that produces intracellular Ca^{2+} mobilization was used to compare 12, 13 EpOME's response. At 10 seconds post injection I took the max fluorescent values and compared 12, 13 EpOME [0.1 μM] and [10 μM] to ACH [10 μM]. 12, 13 EpOME [10 μM] showed a 1.75-fold increase (0.841 ± 0.179) in fluorescence compared to ACH (0.480 ± 0.260) but was not significant (**Figure 6D**). At 56 seconds post injection 12, 13 EpOME [10 μM] diverged and showed a significant 29-fold increase (0.864 ± 0.297) from ACH (0.029 ± 0.096) indicating different kinetics (**Figure 6E**).

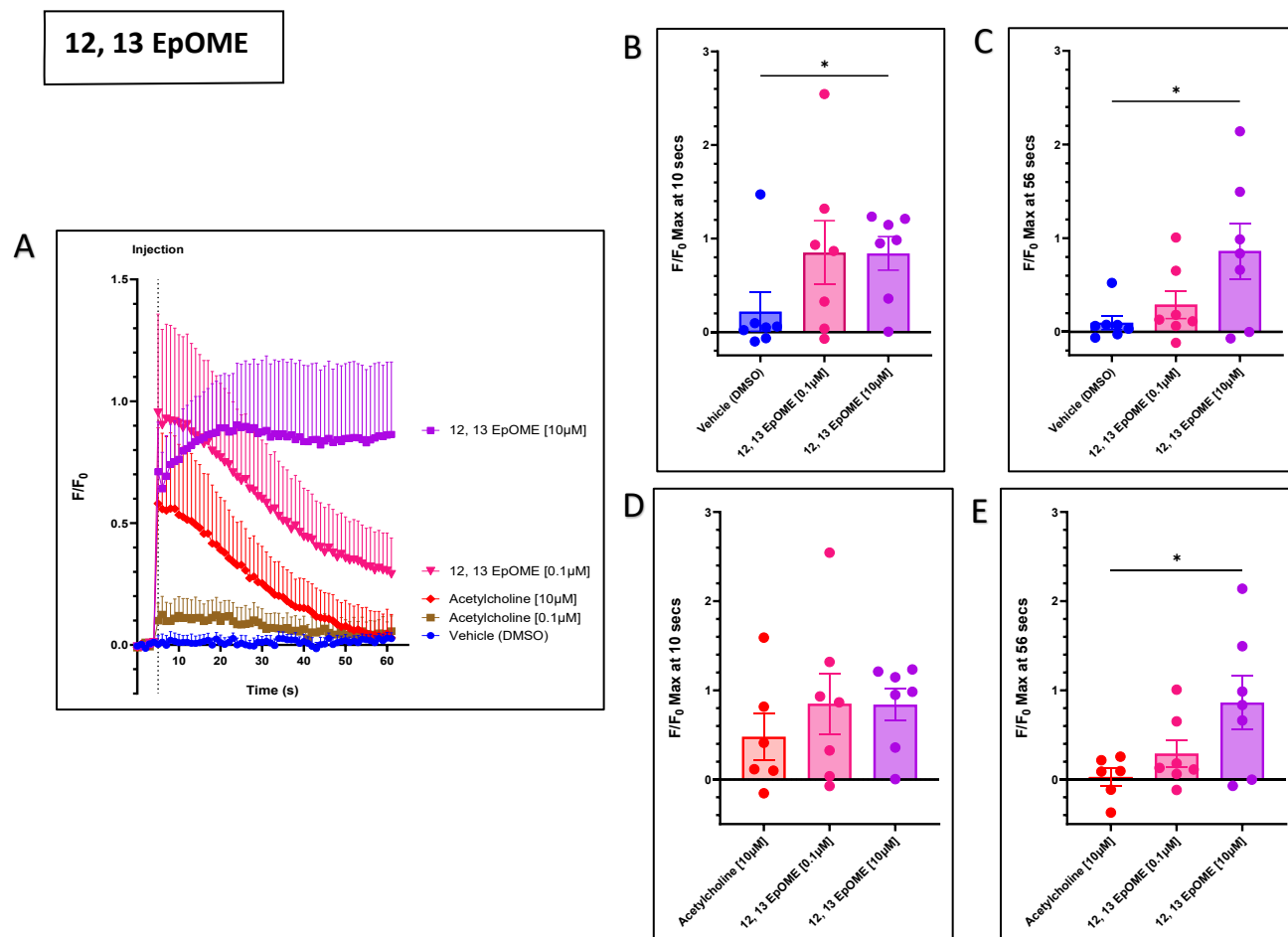


Figure 6. 12, 13 EpOME produces an Increase in Intracellular Fluorescence in Human ASM. ((A) Representative Fluorescent curves in response to ACH and 12, 13 EpOME in human ASM. ACH (0.1µM; n=3, 3 donors or 10µM; n=6, 4 donors) , 12, 13 EpOME (0.1µM; n=7, 4 donors or 10µM; n=7, 4 donors). Max Fluorescent data of 12, 13 EpOME at (B) 10 seconds post-injection and at (C) 56 seconds post-injection. Max Fluorescent data of ACH vs. 12, 13 EpOME at (D) 10 seconds post-injection and (E) at 56 seconds post-injection. Data was analyzed via one-way ANOVA and Fisher's LSD post-hoc test (*P≤0.05).

Linoleic-derived Oxylipins induce a rise in intracellular Ca^{2+} in Airway Smooth Muscle

Once I had observed that 12, 13 EpOME induced significant fluorescent fluxes in ASM I wanted to see whether oxylipins related to 12, 13 EpOME could produce similar responses. Instead of Fluo-4 I used the Ca^{2+} indicator dye Fura-2. This provided us the ability to measure directly the Ca^{2+} concentration in ASM, obtain greater resolution, and to look at individual cell responses. To study the $[\text{Ca}^{2+}]_i$ response in ASM, a concentration threshold value of 30nM $[\text{Ca}^{2+}]_i$ where any response above threshold was considered a cell that responded to treatment. This threshold was based on the max $[\text{Ca}^{2+}]_i$ observed in the vehicle average curve of all experiments. All concentrations were equimolar [10 μ M] including the positive control histamine.

Oxylipins 9, 10 EpOME and 12, 13 DiHOME showed a sharp spike in $[\text{Ca}^{2+}]_i$ which was similar to positive control histamine [10 μ M] post-injection (**Figure 7B & Figure 10B**). This Ca^{2+} response was sustained over the 70 seconds compared to the transient response in histamine. Contrastingly, 12, 13 EpOME and 9, 10 DiHOME showed a slow rise in $[\text{Ca}^{2+}]_i$ concentration post-injection and was also sustained over 70 seconds (**Figure 8B & Figure 9B**). These results indicate that these oxylipins may use an alternative mechanism for the rise in $[\text{Ca}^{2+}]_i$ when compared to histamine which is a known GPCR activator. To quantify the responses, I selected the max $[\text{Ca}^{2+}]_i$ from each experiment where oxylipin treatment produced a response above threshold. Firstly, 9, 10 EpOME induced a significant increase in $[\text{Ca}^{2+}]_i$ ($310.4 \pm 118.1\text{nM}$) compared to vehicle ($72.06 \pm 24.64\text{nM}$) (**Figure 7C**). This response was comparable to positive control histamine ($259.7 \pm 67.26\text{nM}$). Secondly, 12, 13 EpOME, the sister oxylipin of 9, 10 EpOME induced a significant increase in $[\text{Ca}^{2+}]_i$ ($328.4 \pm 106.6\text{nM}$) compared to vehicle ($62.14 \pm 23.37\text{nM}$) (**Figure 8C**). Thirdly, 9, 10 DiHOME, the daughter oxylipin of 9, 10 EpOME induced an increase in $[\text{Ca}^{2+}]_i$ ($165.8 \pm 51.37\text{nM}$) when compared to vehicle ($62.14 \pm 23.37\text{nM}$) but was not significant ($P=0.055$) (**Figure 9C**). Finally, 12, 13 DiHOME, the daughter oxylipin of 12, 13 EpOME induced a significant increase in $[\text{Ca}^{2+}]_i$ ($311.50 \pm 121.73\text{nM}$) compared to vehicle ($41.40 \pm 17.27\text{nM}$) (**Figure 10C**).

9, 10 EpOME

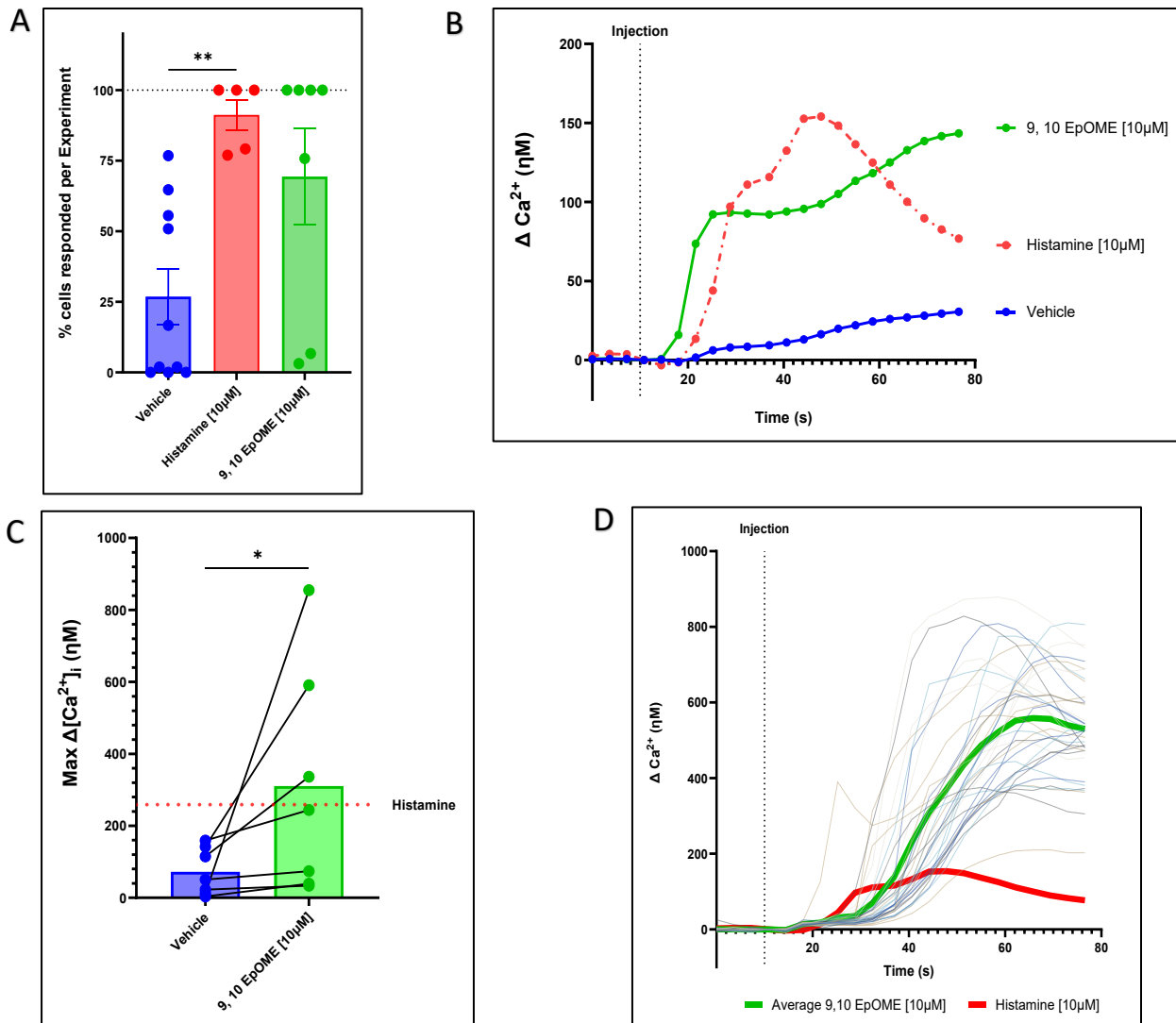


Figure 7. 9, 10 EpOME triggers an increase in Intracellular Calcium concentration in Human ASM.

(A) Percentage Of Human ASM Cells That Exhibited an Intracellular Rise in Calcium in a Given Experiment. 9, 10 EpOME (10μM; n=7 from 3 donors). ASM cells that exhibited an increase in $[\text{Ca}^{2+}]_i$ above 30nM were considered a cell that had a response. The percentage of cells that were above 30nM in a given experiment were then plotted. Histamine was used as a positive control. Vehicle was methyl acetate in HBSS. **(B)** Averaged $[\text{Ca}^{2+}]_i$ Curves in Response to 9, 10 EpOME in Human ASM. Each data point represents an average of at least 15 cells from each of the 7 experiments across 3 donors. **(C)** Max $[\text{Ca}^{2+}]_i$ response in ASM after 9, 10 EpOME treatment. 9, 10 EpOME (10μM; n=7, 3 donors) and vehicle (n=7, 3 donors). Histamine (10μM) comparison is shown as a red dashed line. **(D)** Representative $[\text{Ca}^{2+}]_i$ traces of individual cells after 9, 10 EpOME treatment. Averaged 9, 10 EpOME from all ASM is represented in green. Averaged histamine from all experiments is represented in red for comparison. Data is from 1 donor with 36 individual ASM cells shown in transparent curves. Data was analyzed via a **(A)** one-way ANOVA and Fisher's LSD post-hoc test (**P≤0.01) and **(C)** Wilcoxon signed rank test (*P≤0.05).

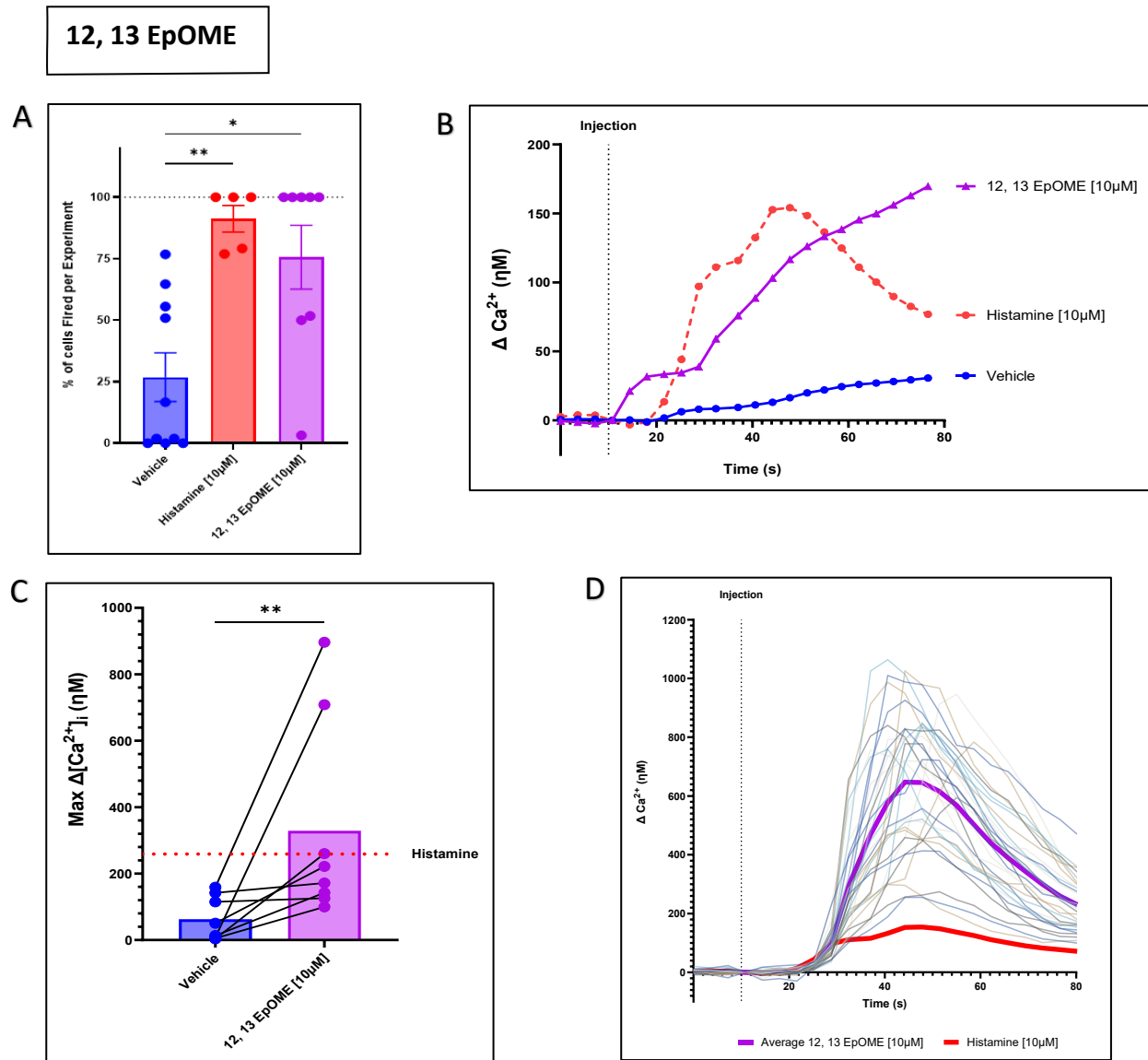


Figure 8. 12, 13 EpOME triggers an increase in Intracellular Calcium concentration in Human ASM.

(A) Percentage of Human ASM Cells that Exhibited an Intracellular Rise in Calcium in a Given Experiment. 12, 13 EpOME (10μM; n=8 from 3 donors). ASM cells that exhibited an increase in $[\text{Ca}^{2+}]_i$ above 30nM were considered a cell that had a response. The percentage of cells that were above 30nM in a given experiment were then plotted. Histamine was used as a positive control. Vehicle was methyl acetate in HBSS. **(B)** Averaged $[\text{Ca}^{2+}]_i$ Curves in Response to 12, 13 EpOME in Human ASM. Each data point represents an average of at least 15 cells from each of the 8 experiments across 3 donors. **(C)** Max $[\text{Ca}^{2+}]_i$ in ASM after 12, 13 EpOME treatment. 12, 13 EpOME (10μM; n=8, 3 donors) and vehicle (n=8, 3 donors). Histamine comparison is shown as a red dashed line. **(D)** Representative $[\text{Ca}^{2+}]_i$ traces of individual cells after 12, 13 EpOME treatment. Averaged 12, 13 EpOME from all ASM is represented in purple. Averaged histamine from all experiments is represented in red for comparison. Data is from 1 donor with 31 individual ASM cells shown in transparent curves. Data was analyzed via a **(A)** one-way ANOVA and Fisher's LSD post-hoc test (* $P \leq 0.05$, ** $P \leq 0.01$) and **(C)** Wilcoxon signed rank test (** $P \leq 0.01$).

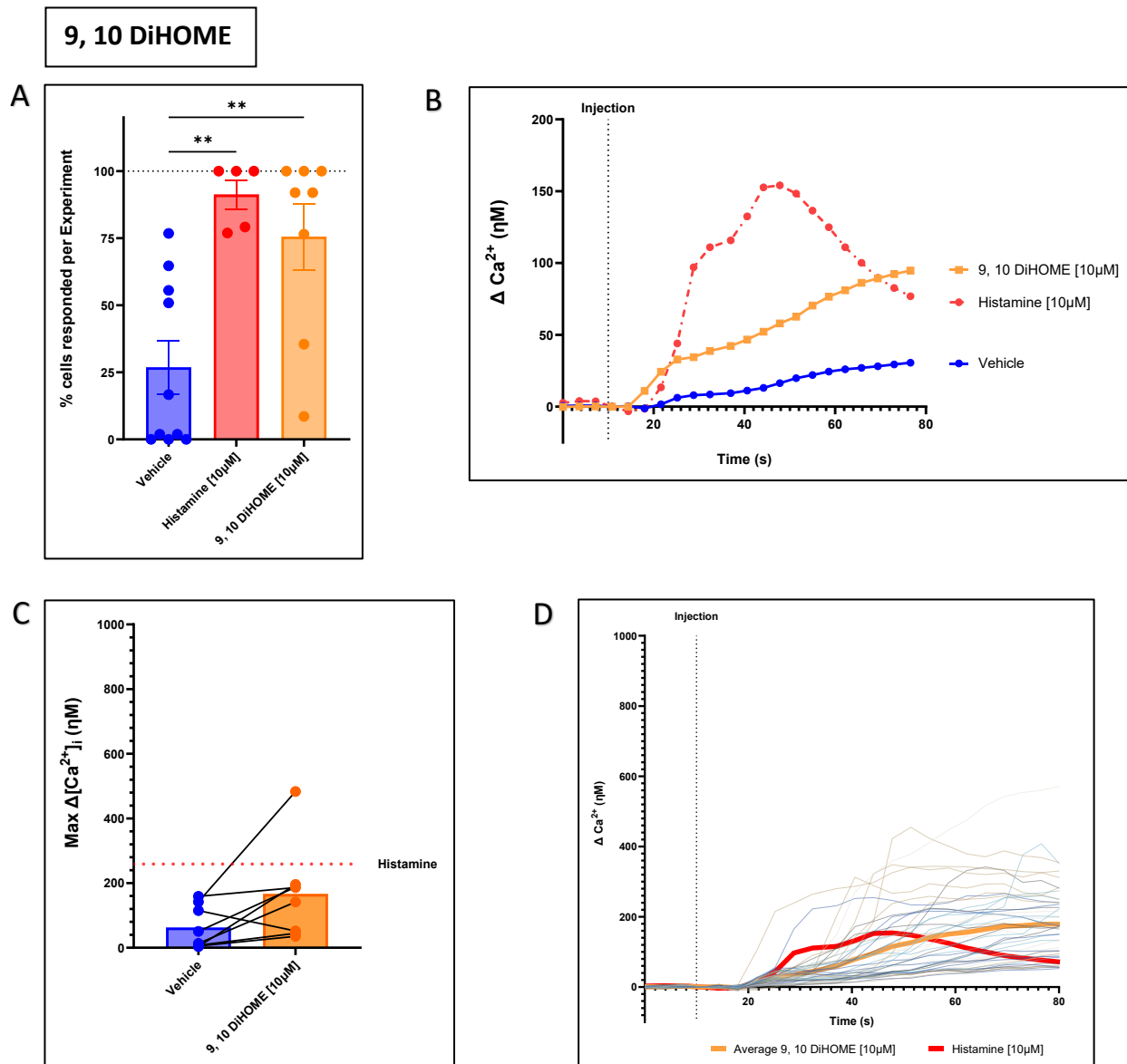


Figure 9. 9, 10 DiHOME triggers an increase in Intracellular Calcium concentration in Human ASM.

(A) Percentage of Human ASM Cells That Exhibited an Intracellular Rise in Calcium in a Given Experiment. 9, 10 DiHOME (10µM; n=8 across 3 donors). ASM cells that exhibited an increase in $[\text{Ca}^{2+}]_i$ above 30nM were considered a cell that had a response. The percentage of cells that were above 30nM in a given experiment were then plotted. Histamine was used as a positive control. Vehicle was methyl acetate in HBSS. **(B)** Averaged $[\text{Ca}^{2+}]_i$ Curves in Response to 9, 10 DiHOME in Human ASM. Each data point represents an average of at least 15 cells from each of the 8 experiments across 3 donors. **(C)** Max $[\text{Ca}^{2+}]_i$ in ASM after 9, 10 DiHOME treatment. 9, 10 DiHOME (10µM; n=8) and vehicle (n=8). Histamine comparison is shown as a red dashed line. **(D)** Representative Calcium traces of individual cells after 9, 10 DiHOME treatment. Averaged 9, 10 DiHOME from all ASM is represented in orange. Averaged histamine from all experiments is represented in red for comparison. Data is from 1 donor with 45 individual ASM cells shown in transparent curves. Data was analyzed via a **(A)** one-way ANOVA (**P≤0.01) and Fisher's LSD post-hoc test **(C)** Wilcoxon signed rank test.

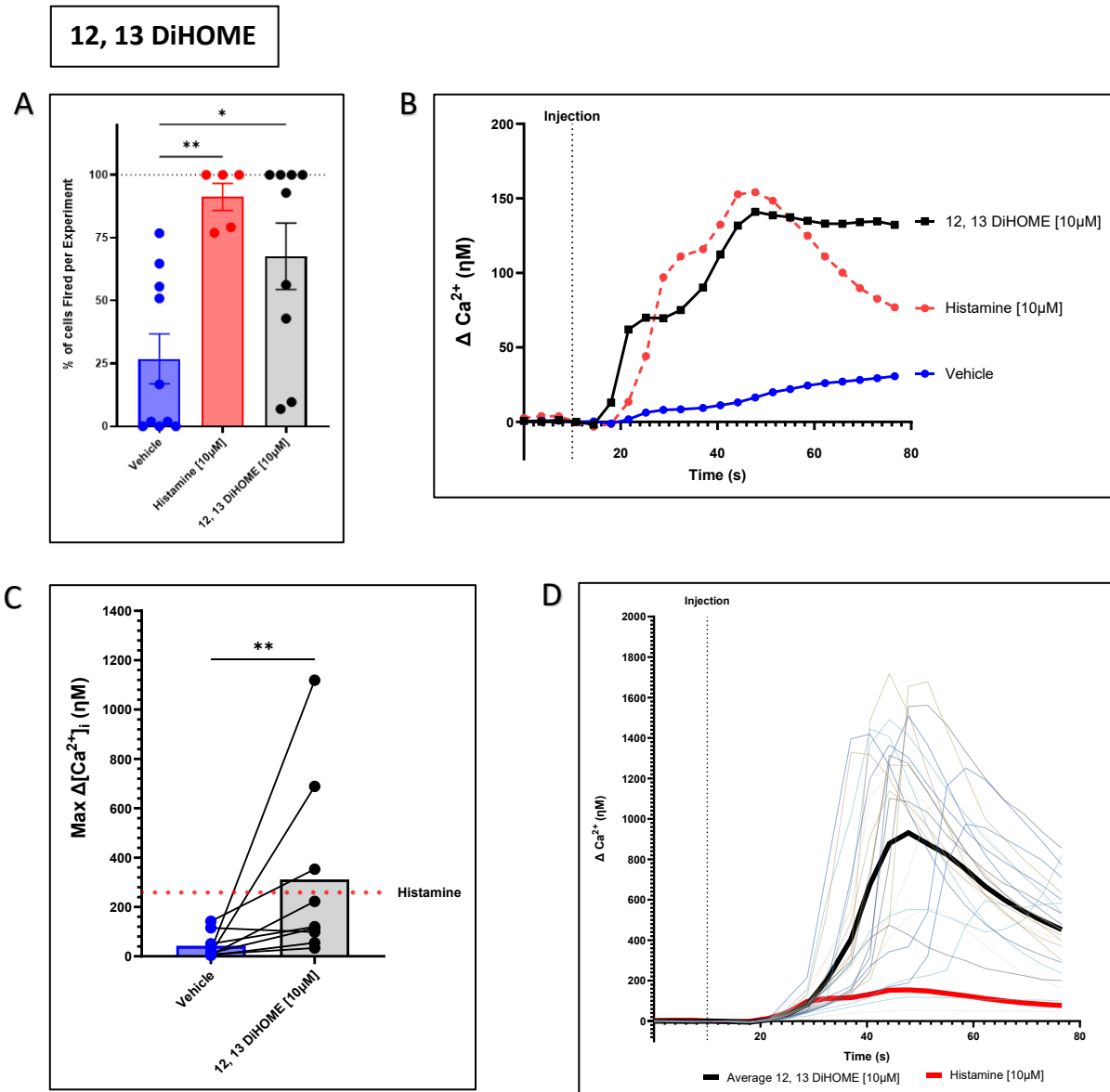


Figure 10. 12, 13 DiHOME triggers an increase in Intracellular Calcium concentration in Human ASM. **(A)** Percentage Of Human ASM Cells that Exhibited an Intracellular Rise in Calcium in a Given Experiment. 12, 13 DiHOME (10 μ M; n=9 across 3 donors). ASM cells that exhibited an intracellular increase in Ca^{2+} above 30nM were considered a cell that had a response. The percentage of cells that were above 30nM in a given experiment were then plotted. Histamine was used as a positive control. **(B)** Averaged $[\text{Ca}^{2+}]_i$ Curves in Response to 12, 13 DiHOME in Human ASM. Each data point represents an average of at least 15 cells from each of the 9 experiments across 3 donors. **(C)** Max $[\text{Ca}^{2+}]_i$ in ASM after 12, 13 DiHOME treatment. 12, 13 DiHOME (10 μ M; n=8), vehicle (n=8). Histamine comparison is shown as a red dashed line. **(D)** Representative $[\text{Ca}^{2+}]_i$ traces of individual cells after 12, 13 DiHOME treatment. Averaged 12, 13 DiHOME from all ASM is represented in black. Averaged histamine from all experiments is represented in red for comparison. Data is from 1 donor with 24 individual ASM cells shown in transparent curves. Data was analyzed via a **(A)** one-way ANOVA (* $P \leq 0.05$, ** $P \leq 0.01$) and Fisher's LSD post-hoc test **(C)** Wilcoxon signed rank test (** $P \leq 0.01$).

Oxylipins require extracellular Ca^{2+} to induce a rise in intracellular Ca^{2+} in Airway Smooth Muscle

Following the observation that the 4 oxylipins caused a rise in $[\text{Ca}^{2+}]_i$ in ASM. I wanted to determine how this rise in $[\text{Ca}^{2+}]_i$ occurred. The most efficient way to uncover the mechanism was to incubate ASM with Ca^{2+} -Free HBSS (0mM Ca^{2+}) 30 mins post-treatment. This allowed us the ability to infer whether Ca^{2+} was originating from the extracellular space, or the intracellular space and the potential receptors involved. These experiments were compared to the Fura-2 experiments done in Ca^{2+} containing HBSS. It should be noted that these experiments were not matched with the Ca^{2+} containing HBSS. I performed a set of experiments to confirm this response in a different technique (Fluo-4) with the oxylipin 9, 10 EpOME. This set of experiments had matched Ca^{2+} vs. Ca^{2+} -Free HBSS treatment groups thus offering more statistical power.

Firstly, 9, 10 EpOME showed a reduction in $[\text{Ca}^{2+}]_i$ across the 70 seconds post injection and exhibited a slow and steady $[\text{Ca}^{2+}]_i$ rise compared to the sharp $[\text{Ca}^{2+}]_i$ increase when ASM are in Ca^{2+} HBSS (**Figure 11A**). In the Ca^{2+} -free group, the max $[\text{Ca}^{2+}]_i$ response was reduced 2.6-fold ($117.83 \pm 118.13\text{nM}$) compared to ASM in Ca^{2+} HBSS ($310.43 \pm 118.13\text{nM}$) (**Figure 11E**). This indicates that the rise in $[\text{Ca}^{2+}]_i$ by 9, 10 EpOME is dependant on extracellular Ca^{2+} . For the Fluo-4 experiment, I observed a similar reduction in the response of 9, 10 EpOME in the Ca^{2+} -free group (**Figure 12**). I observed a significant 4-fold reduction in the max fluorescent response in the Ca^{2+} -free group (0.107 ± 0.063) compared to the Ca^{2+} group (0.429 ± 0.135) (**Figure 12B**). This further confirms through an alternative method that extracellular Ca^{2+} is required for 9, 10 EpOME's action in ASM.

Secondly, ASM treated with 12, 13 EpOME had a reduced rise in $[\text{Ca}^{2+}]_i$ across 70 seconds compared to ASM in Ca^{2+} HBSS (**Figure 11B**). In the Ca^{2+} -free group the max $[\text{Ca}^{2+}]_i$ response was reduced 2.4-fold ($136.16 \pm 39.86\text{ nM}$) compared to ASM in Ca^{2+} HBSS ($328.4 \pm 106.6\text{nM}$) (**Figure 11E**). This indicates that the rise in $[\text{Ca}^{2+}]_i$ by 12, 13 EpOME is dependent on extracellular Ca^{2+} .

Thirdly, exposing ASM to 9, 10 DiHOME showed a reduction in $[\text{Ca}^{2+}]_i$ across 70 seconds post injection compared to ASM in Ca^{2+} HBSS (**Figure 11C**). In the Ca^{2+} -free group the max $[\text{Ca}^{2+}]_i$ response was reduced 2.7-fold ($61.81 \pm 23.609\text{nM}$) compared to ASM in Ca^{2+} HBSS ($165.77 \pm 51.37\text{nM}$) (**Figure 11E**).

Finally, ASM exposed to 12, 13 DiHOME exhibited an almost complete ablation of the response when in Ca^{2+} -Free HBSS (**Figure 11D**). In the Ca^{2+} -free group the max $[\text{Ca}^{2+}]_i$ response was reduced 6.4-fold (48.56

$\pm 27.67\text{nM}$) compared to ASM in Ca^{2+} HBSS ($311.50 \pm 121.73\text{nM}$) (**Figure 11E**). Interestingly, this data indicates that 12, 13 DiHOME requires completely the use of extracellular Ca^{2+} to produce its response in ASM.

Post-hoc analysis via Two-way ANOVA reveals that Ca^{2+} is a significant factor for all lipids and is necessary for the Ca^{2+} response (**Figure 11E**). However, there was no significant difference between Ca^{2+} vs. Ca^{2+} -free responses when looking at oxylipins individually. This is likely due to the variability and the nature of the analysis as the Ca^{2+} vs. Ca^{2+} -free experiments were not paired. Future studies, similar to what is shown in **Figure 12** will need to be carried out such that there is paired analysis between the groups.

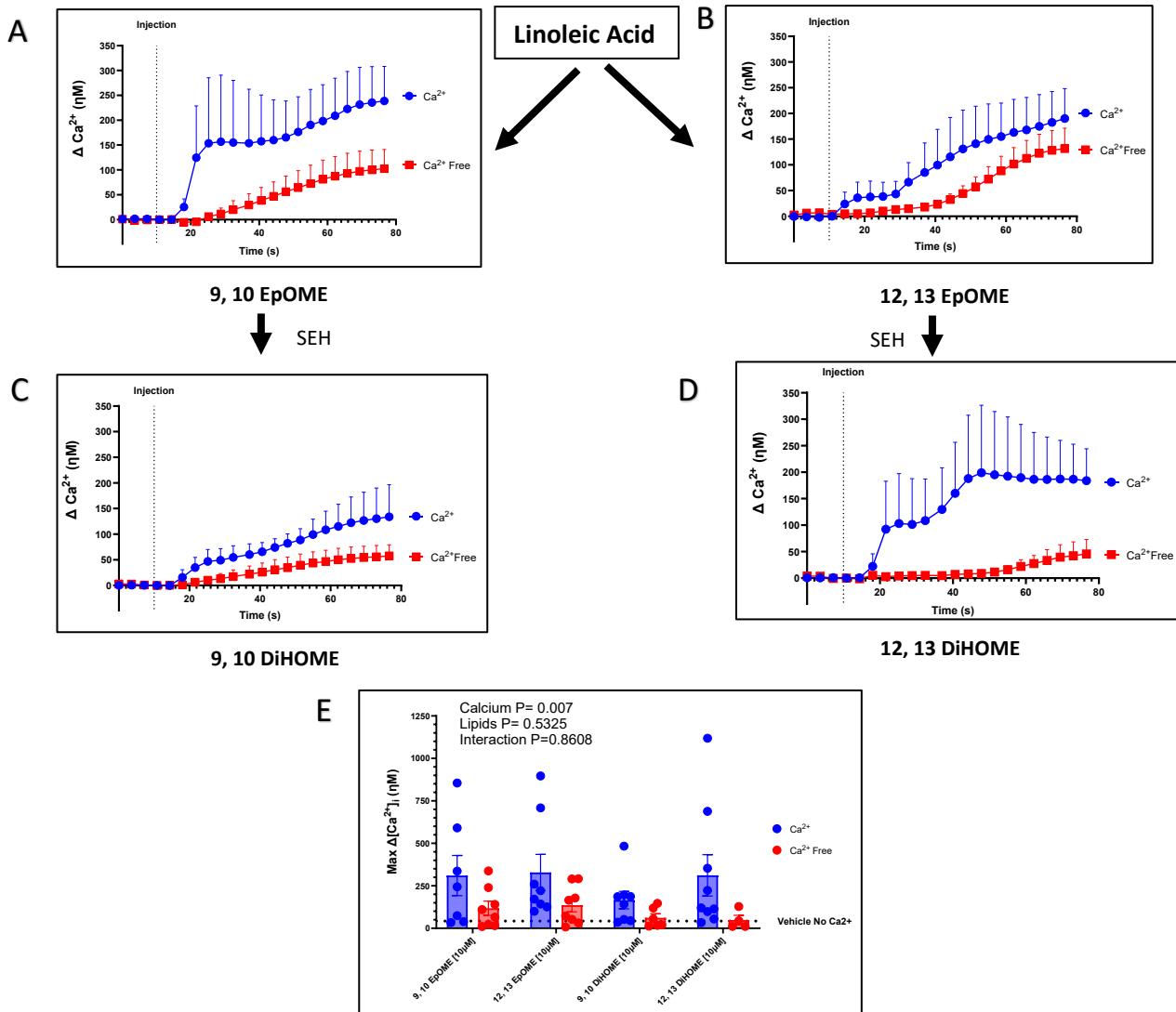


Figure 11. Post-hoc analysis; Oxylipins require Extracellular Calcium to provoke Intracellular Calcium flux in human ASM. (A) 9, 10 EpOME requires extracellular Calcium to induce $[Ca^{2+}]_i$ flux in ASM. 9, 10 EpOME's response in Ca^{2+} HBSS (blue) (n=7 across 2 donors), 9, 10 EpOME's response in Ca^{2+} -free HBSS (red) (n=8, across 2 donors). (B) 12, 13 EpOME requires extracellular Calcium to induce $[Ca^{2+}]_i$ flux in ASM. 12, 13 EpOME's response in Ca^{2+} HBSS (blue) (n=8 across 3 donors), 12, 13 EpOME's response in Ca^{2+} -free HBSS (red) (n=8, across 2 donors). (C) 9, 10 DiHOME requires extracellular Calcium to induce $[Ca^{2+}]_i$ flux in ASM. 9, 10 DiHOME's response in Ca^{2+} HBSS (blue) (n=8 across 3 donors), 9, 10 DiHOME's response in Ca^{2+} -free HBSS (red) (n=6, across 2 donors). (D) 12, 13 DiHOME requires extracellular Calcium to induce $[Ca^{2+}]_i$ flux in ASM. 12, 13 DiHOME's response in Ca^{2+} HBSS (blue) (n=9 across 3 donors), 12, 13 DiHOME's response in Ca^{2+} -free HBSS (red) (n=4, across 2 donors). (E) Maximum $[Ca^{2+}]_i$ responses induced by all oxylipins in ASM in Ca^{2+} HBSS or Ca^{2+} -Free HBSS. Vehicle response in Calcium-Free HBSS is shown for comparison in dotted black line. Data was analyzed via two-way ANOVA and Fisher's LSD post-hoc test. Interactions for Ca^{2+} HBSS or Ca^{2+} -Free HBSS data was analyzed using Lipids and Ca^{2+} as factors.

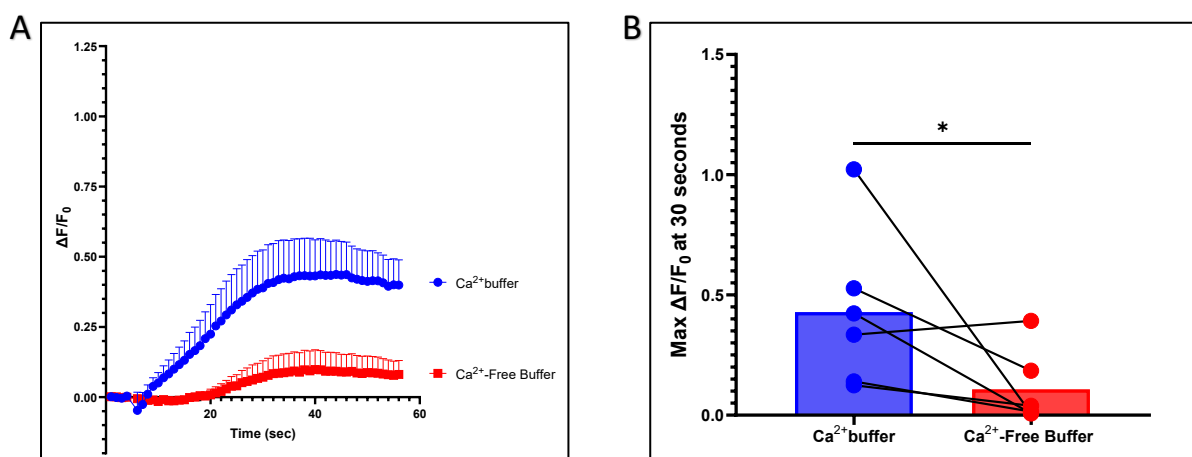


Figure 12. 9, 10 EpOME requires Extracellular Calcium to induce fluorescence in ASM. (A) Average representative fluorescent curves in response to 9, 10 EpOME in Ca^{2+} -media or Ca^{2+} -free media. 9, 10 EpOME (10 μ M; n=6, from 4 donors). **(B)** Max Fluorescent data of 9, 10 EpOME at 30 seconds post-injection in Ca^{2+} -media or Ca^{2+} -free media. Data was analyzed via a ratio paired t-test (* $P \leq 0.05$).

Functional expression of TRPA1 and TRPV1 in Human ASM

I used published data of EpOMEs and DiHOMEs activating TRPA1 and TRPV1 channels in neuronal tissues to assess whether this was true in ASM.^{113–115} I wanted to show that TRPA1 and TRPV1 were expressed in our human ASM cultures and were functionally active. I observed expression of TRPA1 in all of the donors at varying expression levels and at the expected weight (~127 kDa) (**Figure 13C**). Once I had observed TRPA1 in ASM I set out to see if they were functionally active. I used a potent and selective TRPA1 agonist known as JT-010. ASM were loaded with Fluo-4 and then treated with JT-010. JT-010 showed a large increase in max fluorescence (1.627 ± 0.4818) (**Figure 13B**). To validate that JT-010 was targeting TRPA1 channels I used a selective TRPA1 inhibitor known as HC-030031. HC-030031 was capable of significantly reducing JT-010's ability to induce fluorescence in ASM by 2-fold (0.757 ± 0.367) (**Figure 13C**). This data suggests that the inhibitor HC-030031 was functional, and that JT-010 was capable of inducing fluorescence, thus indicating that TRPA1 are functional in human ASM.

Similarly, using 6 non-asthmatic human donors I performed immunoblotting to find expression of TRPV1. I observed expression of TRPV1 across all donors. I observed bands at a weight of 75 kDa which is not the expected weight (95 kDa) (**Figure 14C**). However, immunoblotting with vascular smooth muscle and HEK293 cells have observed bands at a similar molecular weight of 75 kDa.^{151,152} Once I had observed that TRPV1 were present in our ASM cultures I used Fluo-4 to see if TRPV1 was functional. I used a selective TRPV1 agonist known as capsaicin. Capsaicin was capable of inducing a large fluorescent response in human ASM (0.4667 ± 0.1654) (**Figure 14B**). To confirm that capsaicin was targeting TRPV1 I used a potent selective TRPV1 inhibitor known as AMG517. AMG 517 was capable of significantly reducing capsaicin's response by 7.5-fold (0.06275 ± 0.03402) (**Figure 14B**). Interestingly, I saw a decrease in capsaicin's response in the presence of HC-030031 (**Figure 14A & B**). Strangely, this response was not expected as HC-030031 is selective for TRPA1 inhibition only, this has not been confirmed in ASM cultures and may be targeting TRPV1 channels. This data suggests that the inhibitor AMG 517 was functional, and that capsaicin was capable of inducing fluorescence, thus indicating that TRPV1 are functional in human ASM.

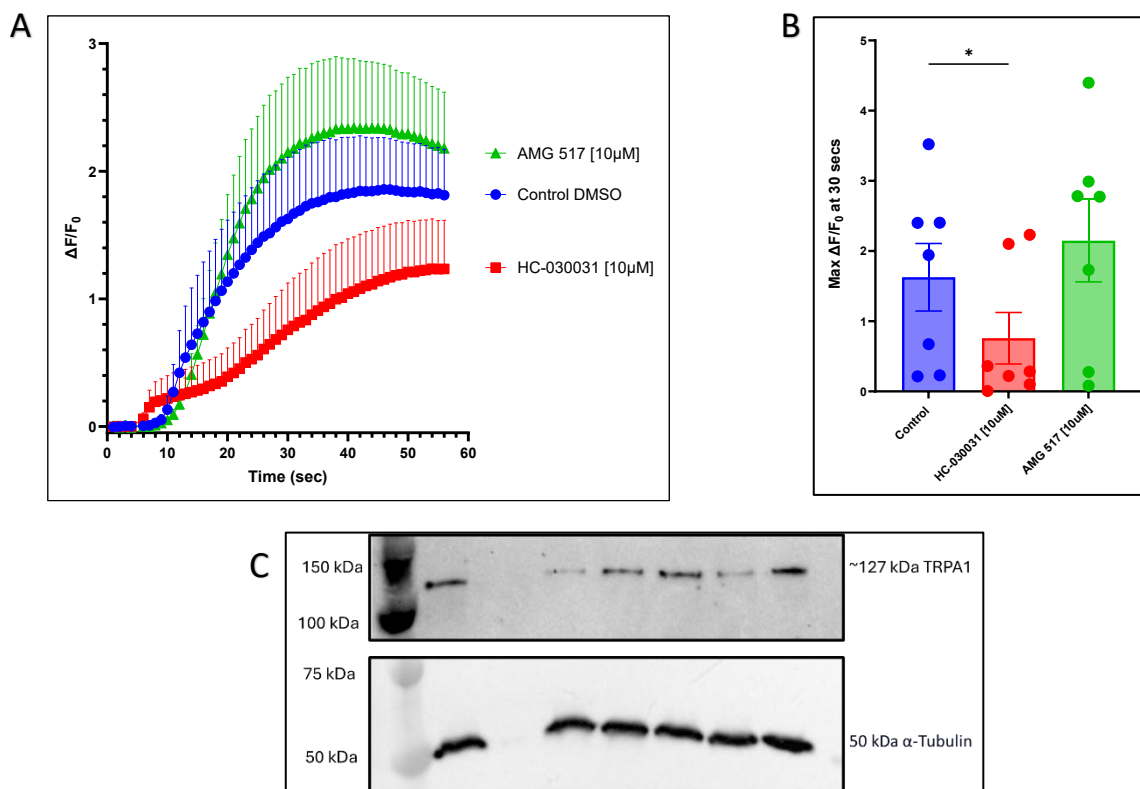


Figure 13. TRPA1 Expression and Functionality in Human ASM. **(A)** Average representative fluorescent curves in response to TRPA1 agonist JT-010. JT-010 (0.33μM; n=7, from 5 donors). ASM were incubated with HC-030031 (10μM) or AMG 517 (10μM) for 1 hour. **(B)** Max Fluorescent data of JT-010 at 30 seconds post-injection. Data was analyzed via a one-way ANOVA and Fisher's LSD post-hoc test (*P≤0.05). **(C)** Immunoblot of TRPA1 Expression in Human ASM. Human ASM from 6 donors. TRPA1 was located at its expected weight (~127 kDa). The housekeeping gene α-Tubulin was used as loading control (50 kDa). Lane 3 had no protein loaded due to issues with broken well.

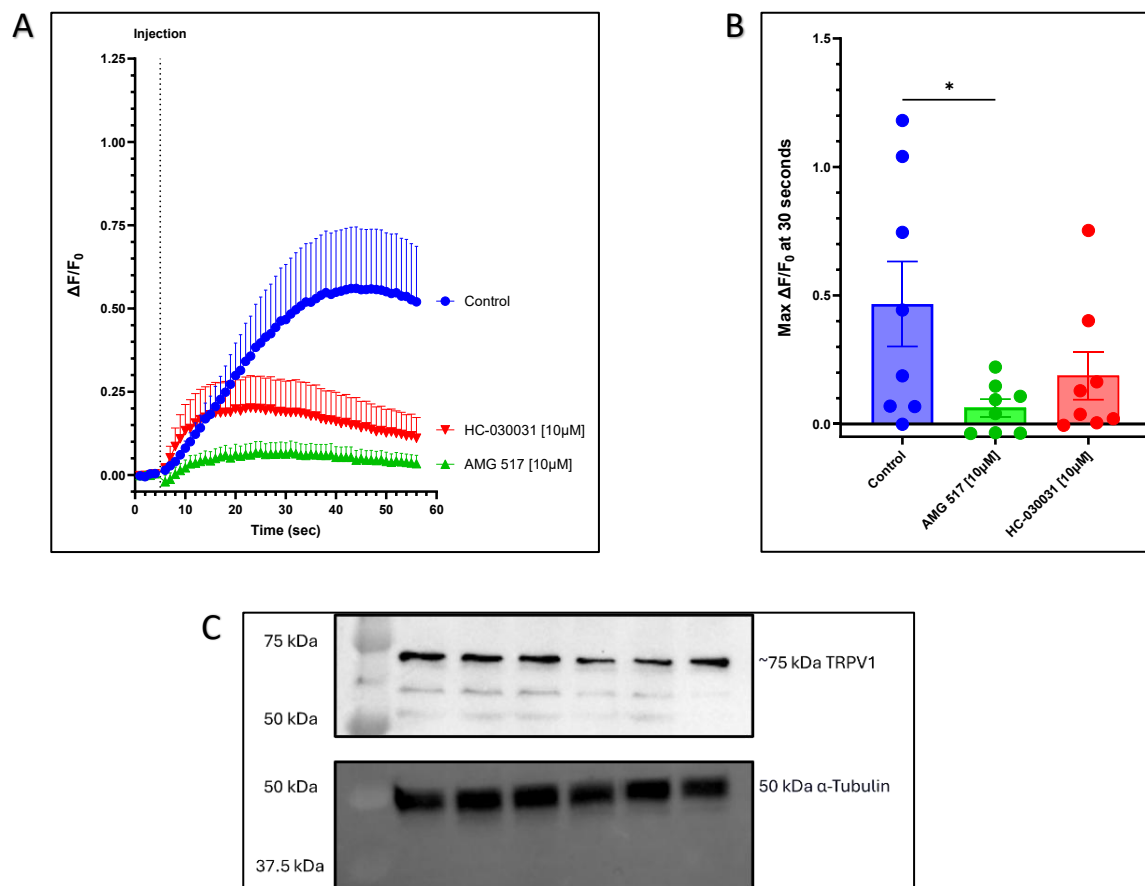


Figure 14. TRPV1 Expression and Functionality in Human ASM. **(A)** Average representative fluorescent curves in response to TRPV1 agonist Capsaicin. Capsaicin (33 μ M; n=8, from 5 donors). ASM were incubated with HC-030031 (10 μ M) or AMG 517 (10 μ M) for 1 hour. **(B)** Max Fluorescent data of Capsaicin at 30 seconds post-injection. Data was analyzed via a one-way ANOVA and Fisher's LSD post-hoc test (*P $\leq$ 0.05). **(C)** Immunoblot of TRPV1 Expression in Human ASM. Human ASM from 6 donors. TRPV1 was located at the 75kDa location while the expected weight is ~95kDa. Observations of a TRPV1 band at 75 kDa have been shown in recent literature. The housekeeping gene α -Tubulin was used as loading control (50 kDa).

Oxylipins require extracellular Ca^{2+} channels TRPA1 and/or TRPV1 to induce fluorescence in Human ASM

I observed that extracellular Ca^{2+} was necessary for the rise in $[\text{Ca}^{2+}]_i$ when ASM were exposed to oxylipins. I therefore was interested in the potential extracellular membrane receptors that may produce this Ca^{2+} response. Our only potential lead based on the existing literature was to target the TRPA1 and TRPV1 receptor channels. I first profiled our ASM cell lines and confirmed that TRPA1 and TRPV1 are expressed and are functional. I then loaded ASM with Ca^{2+} indicator dye Fluo-4 and either TRPA1 inhibitor (HC-030031) or TRPV1 inhibitor (AMG 517). I then treated cells with equimolar amounts of Oxylipins [10 μM].

Firstly, the control group 9, 10 EpOME showed a substantial increase in fluorescence from baseline post injection (**Figure 15A**). This fluorescence response could be reduced via the TRPA1 inhibitor HC-030031. This is evidenced by the max fluorescent data where HC-030031 caused a significant 66% reduction in 9, 10 EpOME's ability to elicit a fluorescent response in ASM (**Figure 15B**). GD^{3+} and AMG 517 were not capable of reducing fluorescence significantly. This data indicates that TRPA1 is required for 9, 10 EpOME's response in ASM.

Secondly, the exposure of ASM to 12, 13 EpOME elicited a large increase in fluorescence intensity (**Figure 16A**). This response could be virtually ablated via the TRPV1 inhibitor AMG 517. AMG 517 was capable of significantly reducing 12, 13 EpOME's max fluorescence by roughly 80% (**Figure 16B**). Similarly, GD^{3+} was capable of significantly reducing 12, 13 EpOME's response by 70%. Interestingly, the TRPA1 inhibitor HC-030031 significantly potentiated 12, 13 EpOME's response by 61% (**Figure 16B**). This data indicates that 12, 13 EpOME requires TRPV1 to induce fluorescence and that TRPA1 inhibition increases the ability of 12, 13 EpOME to induce fluorescence in ASM.

Thirdly, 9, 10 DiHOME caused roughly a 5-fold increase in fluorescence from baseline (**Figure 17A**). This response could be reduced by both the TRPV1 inhibitor and the TRPA1 inhibitor. Specifically, max fluorescent data reveals that 9, 10 DiHOME's response could be significantly reduced by 79% when AMG 517 is present, while HC-030031 reduced the response by 46% (**Figure 17B**). GD^{3+} was capable of completely ablating the fluorescent response of 9, 10 DiHOME (**Figure 17A & B**). This data indicates that 9, 10 DiHOME requires both TRPA1 and TRPV1 channels for its fluorescent response.

Finally, 12, 13 DiHOME produced a 4-fold increase in fluorescence from baseline (**Figure 18A**). Max fluorescent data revealed that TRPA1 inhibitor HC-030031 was capable of significantly reducing 12, 13 DiHOME's response by 65% (**Figure 18B**). A similar response was observed in the GD³⁺ group. TRPV1 inhibitor AMG 517 showed some reduction in 12, 13 DiHOME's fluorescent response but was not significant (**Figure 18B**). This indicates that 12, 13 DiHOME requires TRPA1 to induce fluorescence in ASM.

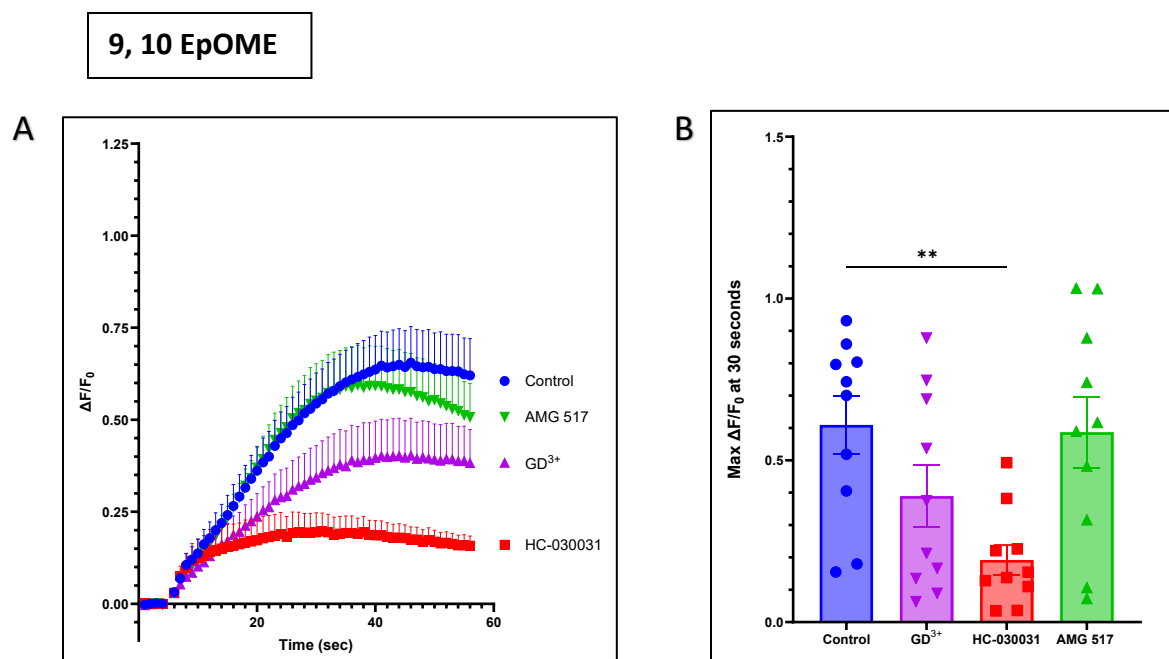


Figure 15. 9, 10 EpOME requires TRPA1 channels to induce fluorescence in Human ASM. (A) Average representative fluorescent curves in response to 9, 10 EpOME (10 μ M; n=10, from 4 donors). ASM were incubated with HC-030031 (10 μ M), AMG 517 (10 μ M) or GD³⁺ (500 μ M) for 1 hour. **(B)** Max fluorescent data of 9, 10 EpOME and inhibitors at 30 seconds post-injection. Data was analyzed via a one-way ANOVA and Fisher's LSD post-hoc test (**P $\leq$ 0.01).

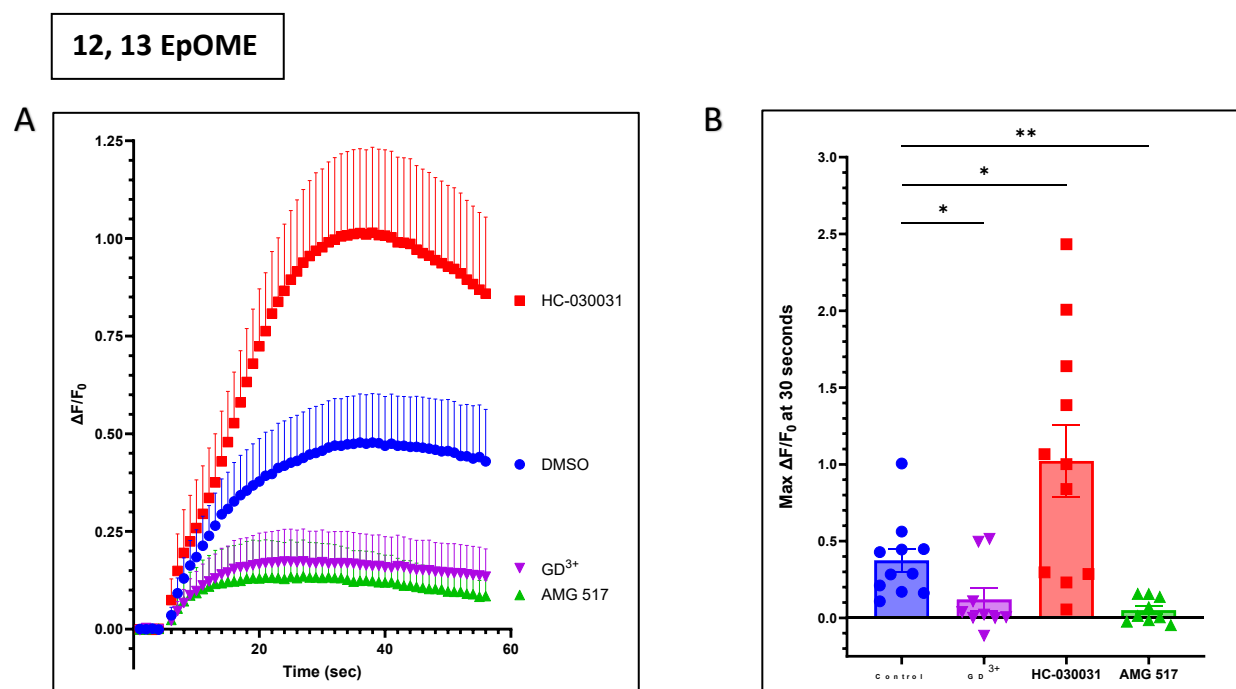


Figure 16. 12, 13 EpOME requires TRPV1 channels to induce fluorescence in Human ASM. (A) Average representative fluorescent curves in response to 12, 13 EpOME. 12, 13 EpOME (10 μ M; n=11, from 4 donors). ASM were incubated with HC-030031 (10 μ M), AMG 517 (10 μ M) or GD³⁺ (500 μ M) for 1 hour. **(B)** Max Fluorescent data of 12, 13 EpOME and inhibitors at 30 seconds post-injection. Data was analyzed via a one-way ANOVA and Fisher's LSD post-hoc test (*P $\leq$ 0.05, **P $\leq$ 0.01).

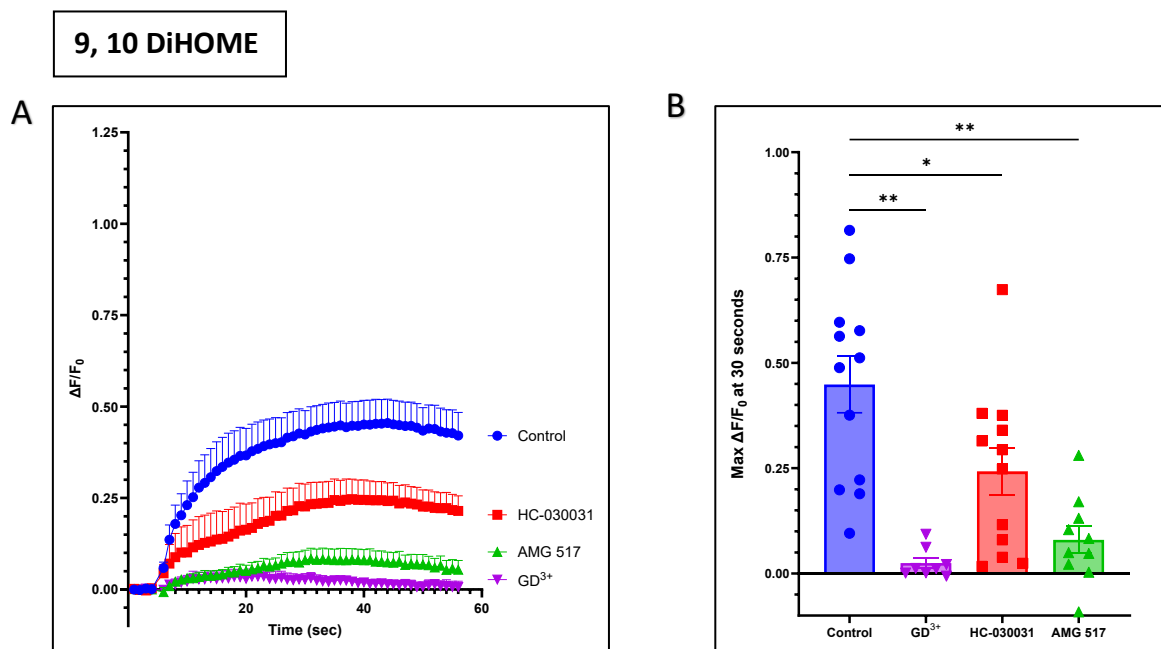


Figure 17. 9, 10 DiHOME requires TRPV1 and TRPA1 channels to induce fluorescence in Human ASM. (A) Average representative fluorescent curves in response to 9, 10 DiHOME. 9, 10 EpOME (10 μ M; n=12, from 5 donors). ASM were incubated with HC-030031 (10 μ M), AMG 517 (10 μ M) or GD³⁺ (500 μ M) for 1 hour. **(B)** Max Fluorescent data of 9, 10 DiHOME and inhibitors at 30 seconds post-injection. Data was analyzed via a one-way ANOVA and Fisher's LSD post-hoc test (*P $\leq$ 0.01, **P $\leq$ 0.01).

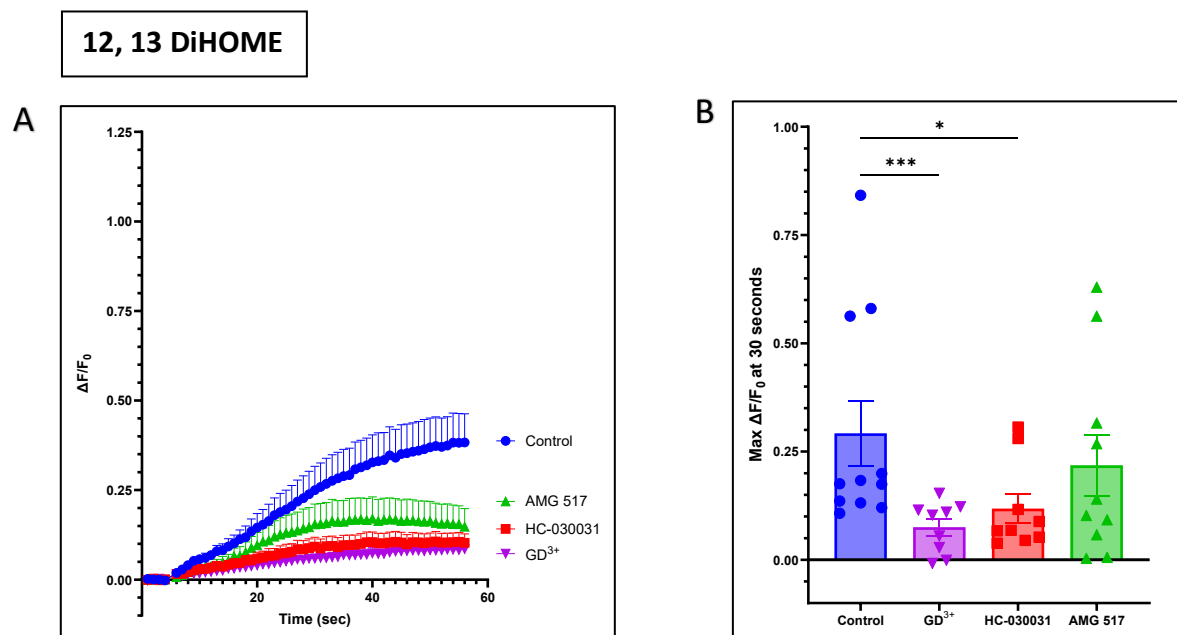


Figure 18. 12, 13 DiHOME requires TRPA1 channels to induce fluorescence in Human ASM. **(A)** Average representative fluorescent curves in response to 12, 13 DiHOME (10 μ M; n=10, 5 donors). ASM were incubated with HC-030031 (10 μ M), AMG 517 (10 μ M) or GD³⁺ (500 μ M) for 1 hour. **(B)** Max Fluorescent data of 12, 13 DiHOME and inhibitors at 30 seconds post-injection. Data was analyzed via a one-way ANOVA and Fisher's LSD post-hoc test (*P $\leq$ 0.01, ***P $\leq$ 0.001).

CHAPTER 4: DISCUSSION

Asthma is a disease that is still being understood to this very day. The pathophysiology of the disease is complex and presents many characteristics such as airway remodeling, hyperresponsiveness, and inflammation. An important feature of asthma is the production of bioactive oxylipins from immune cells whether infiltrating or residential and the structural cells of the lungs. Various triggers including allergens, pollution, and viruses initiate inflammatory processes that drive the production of these oxylipins. Many are synthesized, transported, and delivered to cell types including epithelium, fibroblasts, immune, and especially ASM. This leads to the initiation of processes like proliferation, migration, and notably ASM contraction or relaxation. Oxylipins are also capable of initiating and elevating cytoplasmic Ca^{2+} . The importance of Ca^{2+} in regulating ASM contraction is well known. Increases in $[\text{Ca}^{2+}]_i$ are necessary to initiate Ca^{2+} /calmodulin complexes to activate MLCK such that phosphorylation of the MLC20 can occur, an important step in cross-bridge cycling and force generation.

Many oxylipins within the lung are undescribed in the context of ASM contractility and Ca^{2+} physiology. Additionally, between healthy and individuals with asthma oxylipin profiles can differ in response to pollution and allergens. Specifically, the oxylipins known as the EpOMEs and DiHOMEs fall into this category. However, only little data exists for EpOMEs and DiHOMEs in the lung especially in the context of ASM physiology. In this thesis, the main objective was to determine whether the understudied oxylipins 9, 10 EpOME, 12, 13 EpOME, 9, 10 DiHOME, and 12, 13 DiHOME were capable of inducing cytoplasmic Ca^{2+} flux in human ASM and what receptors mediated this response.

12, 13 EpOME induces an increase in fluorescence in Human ASM

Our first set of studies were to determine if the oxylipin 12, 13 EpOME was capable of inducing Ca^{2+} flux within human ASM. I used the Ca^{2+} indicator dye Fluo-4 which was capable of measuring and inferring Ca^{2+} flux through fluorescence. I observed a large spike in fluorescence with the lowest concentration of 12, 13 EpOME (0.1 μM) that was similar to the known ASM Ca^{2+} mobilizer ACH at its highest concentration (10 μM). This response was transient and slowly returned back towards baseline over 55 seconds. The similarities in the curves of 12, 13 EpOME and ACH suggested that 12, 13 EpOME may use a similar receptor. It is well known that ACH targets the GPCR receptors known as the Muscarinic 2 (M_2) and 3 (M_3) receptors to initiate the release of Ca^{2+} from the SR.¹⁵³ It is unlikely that 12, 13 EpOME targets this receptor as no evidence exists that oxylipins target these receptors. It may be activating other ASM receptors that have similar functions to the M_2 and M_3 receptors like the GPCRs known as the Free Fatty Acid Receptors (FFAR) 1 and 4. Although fatty acids are not oxygenated like

oxylipins there may be activation of FFARs by these similar types of lipids. FFARs activation in ASM produce similar fluorescent curves to what I observed with 12, 13 EpOME.⁹² Similarly to M₂ and M₃ activation, FFAR produces IP₃ and induces Ca²⁺ release from the SR. It may be possible that 12, 13 EpOME uses a similar receptor, but this is unknown.

The highest dose of 12, 13 EpOME (10μM) induced a large fluorescent increase that was sustained over the reading period of 55 seconds. When comparing to ACH at equimolar ranges I saw similar maximal fluorescent increases 10 seconds post injection, indicating that initially they had similar responses. This similarity diverged at 56 seconds post injection where 12, 13 EpOME showed a significant increase in maximal fluorescence compared to ACH, indicating different kinetic profiles between ACH. 12, 13 EpOME at higher concentrations seems to induce a sustained response compared to lower concentrations. The reason for this is unknown and requires more in-depth studies to understand this mechanism. Speculatively speaking, the high amounts and presence of 12, 13 EpOME are potentially maintaining the fluorescence response. This is similar to ASM that are in continuous presence of agonists like histamine or bradykinin where only washing or chelation of Ca²⁺ could return levels back to basal levels.¹⁵⁴ Likewise, activation through the TRPA1 receptor shows evidence of maintaining a fluorescent response in human ASM exposed to cigarette smoke.⁵⁸ This maintenance was probably not a SOCE mechanism as the SOCE activator cyclopiazonic acid was used. This was used to deplete the SR and activate SOCE, then cigarette smoke was added to ASM, and a response was still seen indicating other mechanisms were activated rather than SOCE alone. It was concluded that potentially SOCE was still implicated as cyclopiazonic acid did not fully activate SOCE, or, that other channels such as other TRP channels were allowing continuous Ca²⁺ flux, however this was not confirmed. Similarly, 12, 13 EpOME may be targeting other TRP channels that are pushed into an open conformation rather than a renewing SOCE mechanism.

EpOMEs and DiHOMEs increase intracellular Ca²⁺ in Human ASM

Once I determined that 12, 13 EpOME was capable of causing Ca²⁺ flux in ASM, I assessed whether oxylipins related to 12, 13 EpOME, such as 9, 10 EpOME, 9, 10 DiHOME, and 12, 13 DiHOME could do the same. Human ASM when exposed to 9, 10 EpOME (10μM) showed a sharp rise in [Ca²⁺]_i that was similar to the known Ca²⁺ mobilizer histamine. 9, 10 EpOME showed a similar maximal Ca²⁺ response to histamine at equimolar quantities indicating comparable efficacy. Time course data reveals that 9, 10 EpOME and histamine curves diverge where histamine shows a curve returning back to

towards baseline while 9, 10 EpOME shows a sustained and increasing $[Ca^{2+}]_i$ response, indicating different mechanisms. The reason for the different kinetics is unknown but may be similar to what was observed in the 12, 13 EpOME fluo-4 data. It may be that the presence of high amounts of 9, 10 EpOME are maintaining the $[Ca^{2+}]_i$ through SOCE or through other TRP channel activation. Other oxylipins like EKODE show similar Ca^{2+} kinetics in rat glomerulosa cells where there is a large spike in Ca^{2+} and a maintenance phase.^{155,156} EKODE is suggested to work through an IP_3 mechanism but the reason for the sustained response was unknown. Likewise, to our findings, 9, 10 EpOME is capable of inducing $[Ca^{2+}]_i$ flux and ion currents in mice DRG neurons and sensory neurons, respectively.^{114,115} I show for the first time that the oxylipin 9, 10 EpOME is capable of inducing Ca^{2+} flux in ASM.

For 12, 13 EpOME, I observed a slow and steady increase in the $[Ca^{2+}]_i$ response. The maintenance of Ca^{2+} aligns with the fluo-4 data, however, I did not observe a large and quick spike as seen in the Fura-2 data. Interestingly, 9, 10 EpOME and 12, 13 EpOME exhibited different curves even though they are chemically similar (i.e., regioisomers). Even small chemical changes can have completely different Ca^{2+} kinetics in ASM. 12, 13 EpOME showed a divergence in its curve compared to histamine, where 12, 13 EpOME continued to increase in $[Ca^{2+}]_i$ while histamine indicated a return back to basal $[Ca^{2+}]_i$ levels. Again, I observed a sustained response with this oxylipin that was similar to 9, 10 EpOME and may be through activation of SOCE mechanisms or extracellular channels allowing for the continuous renewal of Ca^{2+} entry. Our results were similar to rat sensory neurons where 12, 13 EpOME was capable of inducing a slow rise in ion currents that is sustained for 20 seconds and then returns slowly back to baseline over 50-60 seconds.¹¹⁴ This mechanism was concluded to be through TRP channels like TRPA1, TRPV1, and to a lesser extent TRPV3.

I then tested the metabolite of 9, 10 EpOME, 9, 10 DiHOME. 9, 10 DiHOME exhibited a slow rise in $[Ca^{2+}]_i$ that was much smaller than both histamine and the EpOMEs. It seemed that 9, 10 DiHOME was not as efficacious as the EpOMEs to induce $[Ca^{2+}]_i$ increase but nonetheless produced a response greater than vehicle. Within the tissues as well as the lungs, Soluble Epoxide Hydrolase (SEH) converts 9, 10 EpOME to 9, 10 DiHOME. Based on our data this conversion leads to the decreased efficacy in 9, 10 DiHOME's ability to induce a large Ca^{2+} flux. This may lead to a decreased magnitude of the contractile ability of 9, 10 DiHOME compared to 9, 10 EpOME. It seems that this conversion would be beneficial in the context of ASM, however more data is required to understand their airway narrowing abilities before a conclusion can be made. 9, 10 DiHOME had no initial spike in $[Ca^{2+}]_i$ but presented a slow and steady

increase in $[Ca^{2+}]_i$. A similar response is seen in brown and white adipocytes where exposure to 9, 10 DiHOME had a slow increasing flux of Ca^{2+} .¹⁵⁷ No experiments were done to see the receptors or how this Ca^{2+} response was sustained. Additionally, and contrastingly to our results 9, 10 DiHOME is capable of inducing a sharp increase in ion currents through TRPA1 and TRPV1 channels that quickly returns back to baseline, this was observed in rat sensory neurons.¹¹⁴

Finally, I tested 12, 13 DiHOME on ASM and observed a sharp increase in $[Ca^{2+}]_i$ albeit to a lesser extent than histamine. 12, 13 DiHOME exhibited a plateau phase in its response which was not observed in histamine. Similar to the other oxylipins some mechanism is propagating the maintenance of the $[Ca^{2+}]_i$ response and leads us to believe it is either through some SOCE mechanism or TRP channels. In other tissues such as mice sensory neurons, 12, 13 DiHOME is capable of inducing ion currents through TRP channels, TRPA1 and TRPV1.¹¹⁴ Additionally, in mice spinal dorsal horns, 12, 13 DiHOME induced transient increases in $[Ca^{2+}]_i$ through a TRPV1 mechanism.¹¹³ Our results showed that conversion of 12, 13 EpOME to 12, 13 DiHOME leads to greater efficacy in inducing maximal changes in $[Ca^{2+}]_i$. From a therapeutic standpoint an SEH inhibitor could be beneficial to avoid 12, 13 DiHOME production if this is true. SEH inhibitors have shown promise in pre-clinical models of allergic asthma and are capable of reducing inflammation, remodelling, and airway hypersensitivity.^{158–160} Future studies are necessary to understand the contractile differences between the oxylipins before SEH can be considered. Our results along with other published data leads us to believe that 12, 13 DiHOME may be a potential contractile agent in ASM.

The data presented shows that these 4 oxylipins are capable of inducing an increase in $[Ca^{2+}]_i$ in ASM and exhibit varying kinetic profiles. Based on the Fura-2 data 12, 13 EpOME has the highest efficacy in elevating cytoplasmic Ca^{2+} , followed by 9, 10 EpOME, 12, 13 DiHOME, and finally 9, 10 DiHOME. In asthma, triggers initiate inflammatory processes where production of oxylipins act on various cell types and cause various responses including intracellular Ca^{2+} mobilization. Prime examples, like the leukotrienes and thromboxanes do this very process and are potent contractile mediators in ASM. So, this raises the question, are these EpOMEs and DiHOMEs working in a similar fashion? These oxylipins are found across the airway tree in humans and are produced by structural cells like airway epithelium, fibroblasts, and ASM.^{45,99–101} For example, the oxylipin 9, 10 DiHOME is produced by fibroblasts.⁴⁵ Additionally, human neutrophils and various leukocytes were the first cells to show production of 9, 10 EpOME and 12, 13 EpOME, respectively.^{83,105,106} In asthma, infiltrating neutrophils and resident

leukocytes triggered after allergen exposure may synthesize EpOMEs. This was observed in the birch pollen study where 9, 10 EpOME was elevated compared to controls after exposure to pollen.¹⁰⁰ These elevated levels of oxylipins may target ASM and induce increases in $[Ca^{2+}]_i$ that could potentially lead to contraction. Additionally, the sustained responses I observed from all oxylipins may be important in the contractile response of ASM. Renewing propagation of a $[Ca^{2+}]_i$ response whether in the form of oscillations or sustained responses is important in the degree that an airway contracts.^{56,57,161} Based on the Ca^{2+} data, EpOMEs and DiHOMEs are plausible contractile mediators in ASM and warrant further study to determine if they are detrimental in obstructive diseases like asthma.

EpOMEs and DiHOMEs require extracellular Ca^{2+} to induce rises in cytoplasmic Ca^{2+}

To understand the potential routes of cytoplasmic Ca^{2+} entry I removed Ca^{2+} from the buffer and exposed ASM to each oxylipin. In the 9, 10 EpOME exposure, the sharp rise in $[Ca^{2+}]_i$ was lost but still included a slow and increasing response. This showed that extracellular Ca^{2+} is required for 9, 10 EpOME's response and for the initial sharp increase. This is interesting because when Ca^{2+} is removed from the extracellular environment what is usually lost is the sustained or oscillatory response not the sharp rise.^{141,162} The response I observed is not well described in the literature. The potential slow increase in $[Ca^{2+}]_i$ may be through some mechanism allowing slow leakage from the SR or other Ca^{2+} stores. I also performed this experiment using another Ca^{2+} -indicator dye further confirming the quick rise in $[Ca^{2+}]_i$ caused by 9, 10 EpOME. There was also a sustained response in the Ca^{2+} buffer likely through some SOCE mechanism that is renewing cytoplasmic Ca^{2+} . In Ca^{2+} -free media I could abolish this quick rise and to a certain degree the maintenance phase. Nonetheless, this data gives evidence that 9, 10 EpOME requires extracellular Ca^{2+} to induce a sharp rise in $[Ca^{2+}]_i$. For 12, 13 EpOME, in Ca^{2+} -free media I saw a reduction in the $[Ca^{2+}]_i$ response compared to Ca^{2+} -containing buffer, but the curves had similar kinetics. It seems that removal of Ca^{2+} is capable of attenuating the 12, 13 EpOME response but cannot remove the slow rise in $[Ca^{2+}]_i$. This reveals the likelihood that 12, 13 EpOME requires some extracellular receptor and that there exists some intracellular store aiding in the response. This was also observed in the 9, 10 DiHOME group in Ca^{2+} -free buffer. On the other hand, when I exposed ASM in Ca^{2+} -free buffer to 12, 13 DiHOME I saw a complete ablation of the Ca^{2+} response. There was a loss of the quick rise in $[Ca^{2+}]_i$ as well as a complete loss in the maintenance phase indicating that 12, 13

DiHOME requires completely extracellular Ca^{2+} . This led us to believe that these oxylipins use mostly extracellular receptors and to a lesser extent intracellular receptors to induce $[\text{Ca}^{2+}]_i$ increases in ASM.

Human ASM possess functional TRPA1 and TRPV1

Once I understood that extracellular Ca^{2+} was required for the oxylipin responses I wanted to determine the potential receptors they were acting through. For this thesis I was interested in extracellular receptors only. Within the literature, the only receptors EpOMEs and DiHOMEs use are TRPA1, TRPV1, and to some extent TRPV3. This has mostly been observed in ion currents in neuronal tissue and TRP channel expressing CHO cells.¹¹⁴ I did not look at TRPV3 as it had only little influence in inducing ion currents when compared to TRPA1 and TRPV1. I first set out to determine if TRPA1 and TRPV1 were present in our ASM cell lines and whether they were functional. I observed TRPA1 expression across all donors and I confirmed their functionality using TRPA1 agonist JT-010. Inhibitor data also showed that TRPA1 inhibitor HC-030031 at 10 μM had specific inhibition, however, I could not wipe out the response completely and may be why one study used 50 μM for ASM cultures.¹⁴¹ Nonetheless, I used 10 μM for downstream experiments and may be one limitation of our results. Similarly, I observed TRPV1 expression as well as functional activity using the TRPV1 agonist capsaicin. Here I showed that TRPV1 inhibitor AMG 517 at 10 μM had specific inhibition for TRPV1 and was also used for later experiments. These results aligned with previous evidence that ASM possess functional TRPA1 and TRPV1.^{141,147}

EpOMEs and DiHOMEs require TRPA1 and/or TRPV1 to elevate fluorescence in Human ASM

After confirming TRP channel expression and functionality, I set out to determine whether the EpOMEs and DiHOMEs required these channels to induce Ca^{2+} flux in ASM. Our data shows for the first time that 9, 10 EpOME requires TRPA1 to induce an increase in fluorescence in ASM. This is different from mice sensory neurons where 9, 10 EpOME requires both TRPA1 and TRPV1 whereas in CHO cells expressing TRP channels requires TRPV1 only.^{113,114} Strangely, GD^{3+} was not capable of removing the fluorescent response completely. This made us wonder how the blocking of TRPA1 ablated the fluorescent response but GD^{3+} did not. TRPA1 is an extracellular Ca^{2+} channel that should be blocked by GD^{3+} .¹⁶³ Either preparation of GD^{3+} was not sufficient for blocking 9, 10 EpOME's response or some compensatory mechanism is allowing Ca^{2+} release from intracellular stores. Potentially this mechanism

is non-functional specifically when TRPA1 is blocked. GD^{3+} and the TRPA1 inhibitor HC-030031 act in quite different ways and may be the reason why. For example, GD^{3+} competes against Ca^{2+} , mainly hijacking Ca^{2+} signalling while HC-030031 blocks TRPA1 activity through the transmembrane regions 4 and 5.^{164,165} It may be that TRPA1 via downstream mechanisms is still allowing Ca^{2+} release from intracellular stores even though GD^{3+} is blocking TRPA1 Ca^{2+} flow. Nonetheless this observation was interesting.

Many physiological processes in the lung are mediated through TRPA1, this includes the cough response, hyperreactive airways, inflammation, and recently airway narrowing.^{166–168} Some examples such as cigarette smoke, oxidized phosphatidylcholines (OXPCs), and ovalbumin are capable of inducing airway narrowing or upregulating proteins important in contraction like MLCP through TRPA1.^{58,141,169} In ASM cultures, OXPCs require TRPA1 to induce an increase in $[\text{Ca}^{2+}]_i$. This leads to MLC phosphorylation that subsequently generates the narrowing of the airways in mice. This example leads us to reason that through TRPA1, 9, 10 EpOME may be capable of inducing airway narrowing through a similar fashion to OXPCs.

For 12, 13 EpOME, I show for the first time that TRPV1 is required for the increase in cytoplasmic Ca^{2+} flux in ASM. Contrastingly to CHO cells expressing TRP channels, 12, 13 EpOME was capable of inducing ion currents through both TRPV1 and TRPA1 showing some difference between ASM.¹¹⁴ TRPV1 activation is considered to play a role in the pathophysiology of asthma for example airway hyperresponsiveness, chronic cough, and bronchoconstriction through some neuronal mechanisms.^{170–172} However, very few studies have looked at the direct implications of TRPV1 in ASM and whether activation by triggers leads to a more contractile state. In sensory neurons, TRPV1 activation releases neuropeptides like neurokinin A (NKA) that act directly on ASM and induce airway narrowing. This is not believed to be through direct TRPV1 activation in ASM.^{172–174} However, in whole guinea-pig bronchi TRPV1 agonists such as capsaicin induce dose-dependent contractions.¹⁷⁵ I believe that direct TRPV1 activation in ASM by 12, 13 EpOME may lead to contraction in a similar fashion.

Another interesting finding was the potentiation of 12, 13 EpOME's Ca^{2+} response when added to ASM pre-incubated with TRPA1 inhibitor HC-030031. The reason for this was unknown but was nonetheless reproducible. In neuronal cells, a comparable response is seen in TRPA1-negative/TRPV1-positive neurons.¹⁷⁶ In these neurons, capsaicin (TRPV1 agonist) induced a large increase in the ionic

current whereas TRPA1 agonist Allyl isothiocyanate (AITC) AITC did not induce any response. In TRPA1-positive/TRPV1-positive neurons capsaicin induced a smaller and less significant increase in current while AITC produced a normal response through TRPA1. It seems that inhibiting or loss of TRPA1 allows TRPV1 to compensate and produce an even larger Ca^{2+} flux/ ionic current compared to when they are both functioning. Potentially TRPA1 has some regulatory control on TRPV1 that determines the magnitude of Ca^{2+} entry into cells like ASM. Interestingly, TRPA1 and TRPV1 are known to form complexes in TRP expression CHO cells, DRG neurons, mouse trigeminal neurons, and transfected HEK293T cells.¹⁷⁷⁻¹⁷⁹ These interactions are mostly found on the plasma membrane. Thus, there may be some proximal interaction allowing one to either compensate or potentiate when one is inhibited. This may be what is occurring within our ASM cultures. However, complexes of TRPA1 and TRPV1 have not been observed in ASM yet. For our experiments I used HC-030031 due to its potential therapeutic benefit in pain and lung studies.^{180,181} HC-030031 has been used to reduce bronchoconstriction and hyperresponsiveness in guinea pigs and rats.^{181,182} However, HC-030031 has yet to be used in human trials and our data may show one possible reason for this. For example, I showed that HC-030031 potentiates increases in cytoplasmic Ca^{2+} after exposure of TRPV1 activator 12, 13 EpOME. If HC-030031 were to be used for bronchodilation in humans, there actually may be a favoring of airway narrowing through a TRPV1 potentiation effect.

Looking at 9, 10 DiHOME's response, fluorescence was reduced by both TRPA1 and TRPV1 inhibitors indicating the requirement of both receptors. However, I observed a preferential favouring of the TRPV1 receptor. In the HC-030031 group (TRPA1 inhibitor) I saw a slight reduction in the Ca^{2+} response even though it was assumed that TRPV1 was functional. This may indicate some crosstalk mechanism where TRPV1 desensitisation is occurring when TRPA1 is inhibited. Downstream mechanisms may be attenuating TRPV1's ability to induce Ca^{2+} , however this is unknown. As mentioned before, TRPA1 and TRPV1 complexes exist and modulation with each other is possible, additionally, individual TRPA1 channels are capable of modulating TRPV1 and the vice versa is true as well.^{178,183} For example, in HEK293T cells transfected with TRPA1 and TRPV1, TRPA1 activation was capable of sensitizing TRPV1 to have a greater $[\text{Ca}^{2+}]_i$ response.¹⁸³ The crosstalk between these two TRP channels is complex and elusive but exists and may be why I observed this response.

Finally for 12, 13 DiHOME, HC-030031 was capable of blocking the Ca^{2+} response to a similar magnitude to GD^{3+} indicating the requirement of TRPA1. This ablation of the response via GD^{3+} or HC-

030031 shows the requirement of exclusively extracellular Ca^{2+} . This aligned with our Fura-2 data where removal of Ca^{2+} from the extracellular environment removed 12, 13 DiHOME's response completely. I observed a slight decrease in 12, 13 DiHOME's response in the TRPV1 inhibitor group (AMG 517) indicating some TRPV1 requirement. This data aligns with sensory neurons where ion currents induced by 12, 13 DiHOME favoured TRPA1 over TRPV1.¹¹⁴ It seemed that AMG 517 was capable of removing the sustained or maintenance phase of the response. Potentially 12, 13 DiHOME acts initially through TRPA1, initiating a slow rise in Ca^{2+} flux but is then ablated by some TRPV1 mechanism (due to TRPV1 inhibition) that inhibits further release from TRPA1 through a crosstalk mechanism. As mentioned, TRPA1 and TRPV1 seem to be modulating each other to a certain degree. I show for the first time that 12, 13 DiHOME requires TRPA1 to induce Ca^{2+} signalling within ASM. This leads us to believe that 12, 13 DiHOME is a potential contractile mediator in the lungs. This is based on evidence that TRPA1 activation mediates the influx of Ca^{2+} that is capable of initiating processes that are necessary for airway narrowing (i.e., phosphorylation of MLC, force generation, reduced airway diameter).^{58,141,169}

Within the lungs, EpOMEs and DiHOMEs are produced in varying abundance. BALF fluid shows EpOMEs to be much greater in abundance compared to the DiHOMEs both in healthy and asthmatic individuals.^{99–101,135} Our experiments specifically looked at the responses individually, yet these lipid mediators act on the surrounding tissues likely together or in proximity to each other. More specifically, these oxylipins are not acting alone. For example, synthesis of both 9, 10 EpOME and 12, 13 EpOME in the vicinity of ASM may induce greater Ca^{2+} responses. Potentially 9, 10 EpOME activates TRPA1 that may sensitize TRPV1, then 12, 13 EpOME activates TRPV1 to induce an even greater Ca^{2+} response compared to acting alone. This is similar to what is seen in DRG neurons and HEK293T cells using TRPA1 agonist AITC and TRPV1 agonist capsaicin.¹⁸³ In individuals with asthma, TRPV1 expression is increased and in mice, OVA sensitization leads to greater expression of TRPA1.^{184,185} The greater abundance of TRPA1 and TRPV1 in individuals with asthma may lead to greater oxylipin-receptor interactions that could lead to greater incidences of airway narrowing. Thus, it is important to understand these oxylipins in the broader context of lung physiology and whether they are benign or detrimental in obstructive diseases like asthma.

These oxylipins have been found and are changed in abundance in the lungs of healthy individuals and individuals with asthma. Additionally, changes are observed after exposure to pollutants like biodiesel and subway air or allergens like birch pollen. After exposure to a trigger, oxylipins may lead

to action on ASM that will induce increases in Ca^{2+} flux that could through downstream contractile mechanisms initiate the narrowing of the airways. This may be detrimental in obstructive lung diseases like asthma where oxylipins could potentiate and initiate bronchoconstriction, leading to sinister exacerbations. This thesis indicates that these oxylipins are potential contractile mediators through TRPA1 and/or TRPV1 mediated pathways. More research needs to be done to understand whether EpOMEs and DiHOMEs are capable of inducing bronchoconstriction. I believe that I have given evidence to support further the discovery of their actions in ASM physiology and contractility.

TRPA1 and TRPV1 in Disease

TRPA1 and TRPV1 have become research targets in the last few decades due to their potential implications in various diseases across the human body including the airways. Examples include chronic cough, pulmonary fibrosis, and airway inflammation and hyperresponsiveness related to COPD and asthma.^{142,186,187} The exact role of TRPA1 in these diseases seems to be largely unknown but various pharmacologic interventions have been performed mainly targeting TRPA1's inhibition. One study looked at the effects of particulate matter ($\text{PM}_{2.5}$) in air to see how TRPA1 modifies lung inflammation and AHR.¹⁶⁶ Mice pretreated with particulate matter ($\text{PM}_{2.5}$) and then exposed to varying doses of ACH exhibited a dose dependent increase in lung resistance and a left ward shift indicating AHR. This resistance could be significantly reduced with TRPA1 inhibitor A967079 and/or TRPV1 inhibitor AMG9810. Additionally, inflammatory cells (i.e., macrophages, lymphocytes, and neutrophils) in the BALF were reduced in mice pre-treated with A967079 and/or AMG9810. Similarly, histological lung changes showed lower inflammation when mice were pretreated with A967079 and/or AMG9810 compared to controls. It seems that TRPA1 is not just a simple ion channel that allows the influx of cations but is an important modulator of airway tone. An OVA-challenged model in various small mammals was interested in pharmacologic inhibition of TRPA1 to reduce inflammation and cough responses.¹⁶⁷ Cough responses in guinea pigs after OVA challenge were significantly reduced in animals given GDC-0334, an inhibitor that binds and prevents the conformational opening of TRPA1. Immune cells like eosinophils and neutrophils were also reduced significantly at differing doses of GDC-0334. This was different between rats and guinea pigs indicating that TRPA1 induced inflammation is diverse across species. Between guinea pig, rat, murine, and human cell types, TRPA1 is expressed differently at transcriptional and protein levels and may be the reason for this. For example, mouse ASM seem to have no functional TRPA1 and no detectable mRNA, while human ASM has functional TRPA1 and

detectable mRNA.^{141,167} This may be why some pre-clinical models using TRP inhibitors have not been translated to human studies in the lung field. In TRPA1 knockout mice (TRPA1^{-/-}) exposed to LPS, lung function measurements exhibited bronchial hyperreactivity compared to mice with intact TRPA1, suggesting that TRPA1 helps to mitigate bronchial hyperreactivity.¹⁶⁸ However, this observation may be inaccurate as Penh was used to measure bronchial reactivity.¹⁸⁸ Neutrophil activity was significantly greater in TRPA1^{-/-} mice compared to TRPA1 intact mice, likely indicating that TRPA1 facilitates reduced neutrophil activity during LPS challenge.

TRPV1 is similarly implicated in asthma and plays a role in airway hyperresponsiveness, chronic cough, and bronchoconstriction through neural mechanisms.^{170–172} For example, activation of TRPV1 in sensory neurons initiates the release of neuropeptides which act on ASM to induce contraction.^{172,173} In one study, OVA-challenged mice showed a reduction in airway resistance when treated with TRPV1 inhibitor capsazepine.¹⁸⁹ This reduction could be mirrored in mice treated with TRPV1 siRNA. Additionally, blocking TRPV1 was capable of significantly reducing immune cell abundance and cytokines, for example, eosinophils, macrophages, IL-4, IL-5, IL-13, IL-33, and TSLP were all lower compared to controls (OVA-challenged). Similarly, when OVA-challenged mice were exposed to formaldehyde and PM_{2.5}, TRPV1, substance P (SP), and Calcitonin Gene-Related Peptide (CGRP) expression could be significantly mitigated with capsazepine showing the role TRPV1 has in the allergic response as well as exposure to pollutants.

What do TRPA1 and TRPV1 do in ASM?

In ASM, TRPA1 and to a lesser extent TRPV1 have been implicated in Ca²⁺ mobilization and contraction in ASM. These two channels exist across the outer membrane (TRPA1 and TRPV1) and sarcoplasmic membrane (TRPV1) in ASM, it thus seems possible that the activation of these channels will lead to increased intracellular Ca²⁺ and potential downstream processes necessary for contraction. In ASM, fluorescent imaging reveals significant increases in Ca²⁺ flux by various TRPA1 agonists cinnamaldehyde, acrolein, and cigarette smoke extract (CSE).¹⁴² These responses could be significantly attenuated by TRPA1 antagonists HC-030031 or AP18. Interestingly, human primary ASM exposed to cigarette smoke (CS) induced a 2-fold increase in [Ca²⁺]_i.⁵⁸ This response was sustained for a length of time (>10 mins) and could be abolished in Ca²⁺-free media, thus indicating that extracellular Ca²⁺ is required for the response. To confirm the mode of extracellular Ca²⁺ influx, a TRPA1 inhibitor HC-030031 was used. Ca²⁺ responses were significantly blunted compared to control, indicating TRPA1 as the channel for CS/CSE

induced Ca^{2+} release. Furthermore, the activation of TRPA1 by CS extract led to significant increases in phosphorylated MLC, an indicator of ASM contraction. It seems that TRPA1 channels have the potential to activate contractile mechanisms. This is further confirmed by oxidized phosphatidylcholines that were capable of significantly inducing transient and oscillatory $[\text{Ca}^{2+}]_i$ release, increasing P-MLC in ASM, and decreasing airway lumen size.¹⁴¹ These responses could be attenuated via inhibition of TRPA1 via HC-030031, indicating again, TRPA1's role in ASM contractility.

TRPV1 has some significance in ASM contractility, although to a lesser extent than TRPA1 and likely through a neuronal mediated way.^{147,190} It is believed that TRPV1 is not implicated in ASM contractility directly.¹⁹¹ Firstly, this was based on evidence that there exists no detectable TRPV1 transcripts in ASM. This is conflicting as a more recent observation showed TRPV1 expression in ASM at the transcript and protein level.¹⁴⁷ Secondly, capsaicin (TRPV1 agonist) did not induce Ca^{2+} release in human ASM. This data was not shown, and the authors only used a small dose of Capsaicin (10uM). Thirdly, induction of guinea pig tracheal contraction via voltage-gated channel activation could not be inhibited when using TRPV1 inhibitor capazipine. Contrastingly, one study observed capsaicin induced contraction of guinea pig tracheal rings, but this response could be attenuated via Na^+ -channel inhibitors.¹⁴⁷ This suggests that intact nerves within the tracheal rings may be excreting potential contractile agents and capsaicin induced contraction is not through ASM TRPV1 channels directly. However, TRPV1 in ASM may be acting as potentiators of the sustained contractile tone. This is seen in ASM exposed to methacholine where Ca^{2+} oscillations are more frequent in controls compared to ASM incubated with TRPV1 inhibitor capazipine.¹⁴⁷ Thus, inhibiting TRPV1 may lead to reduced tonal maintenance through less frequent Ca^{2+} oscillations favoring ASM force reduction or airway relaxation.

Potential Oxylipins that activate TRPA1

Besides our findings, oxylipins 9,10 EpOME, 12, 13 EpOME, 9, 10 DiHOME, and 12, 13 DiHOME require TRPA1 for ion currents in CHO cells transfected with TRP channels.¹¹⁴ Oxylin 5,6-EET, an arachidonic acid derived mediator shows TRPA1 activity in murine sensory neurons.¹⁹² 5,6-EET showed a significant transient increase in Ca^{2+} with the maximal amount of responding cells at 100nM. This response could be significantly reduced when added to TRPA1^{-/-} neurons and when using TRPA1 inhibitor HC-030031. This is interesting as it would seem that if 5,6-EET activated TRPA1 in ASM it would potentially lead to a more contractile state, but this is not the case. In bovine and guinea pig ASM, 5,6-EET is a potent bronchorelaxant likely through K^+ channels and not TRPA1.^{193,194} Oxylipins play different

roles in different tissues and highlights the importance of understanding their roles in various tissues especially in developing novel therapies.

Potential Oxylipins that activate TRPV1

Besides our results, oxylipins 9,10 EpOME, 12, 13 EpOME, 9, 10 DiHOME, and 12, 13 DiHOME also require TRPV1 for ion currents in CHO cells transfected with TRP channels.¹¹⁴ 20-HETE a known bronchoconstrictor oxylipin, when added to guinea pig ASM is capable of eliciting potent force generation at an effective concentration of 1.5 μ M.⁷¹ This response could be completely abolished in ASM with TRPV1 antagonist capazipine, with maximal inhibition at 10 μ M. Another oxylipin, 15-HETE, causes slow and sustained contraction of guinea pig airway trachea likely via TRPV1.¹⁹⁵ This was confirmed by treating airways with a TRPV1 antagonist capazipine that abolished the tracheal reflex completely. However, this may be through some unknown neuronal mechanism as these were intact lungs, and some authors have suggested that capazipine may have a general bronchodilator effect not exclusively through TRPV1. Capazipine can slightly inhibit small airway contraction from other contractile agents like LTD₄, PGD₂, and histamine.¹⁹⁶ Other oxylipins including 9-HODE, 13-HODE, 9-KODE and 13-KODE are TRPV1 agonists in neuronal tissues.⁹¹ These oxylipins have not been studied in ASM physiology.

Limitations

Firstly, for this thesis I used ASM from healthy donors and not from individuals with asthma. Although EpOMEs and DiHOMEs are changed in asthmatics after exposure to allergen or pollution they still exist to a large degree in healthy individuals as well. Because no data exists, our project was a basic discovery on their potential functions in ASM, and we believed that understanding EpOMEs and DiHOMEs in healthy ASM was the first step to determining their actions. From a physiological standpoint, understanding actions first in healthy tissue helps to compare and see the differences in diseased tissue. Future studies should look at healthy vs asthmatic ASM to understand whether EpOMEs and DiHOMEs act differently in disease states (i.e., asthma).

Secondly, oxylipins seem to be very elusive molecules due to their lipid nature. Oxylipins were suspended in methyl acetate from the supplier and were used on cells. I became skeptical of this as I did not know what this vehicle was doing to ASM. Therefore, methyl acetate was evaporated off via nitrogen to allow for the precipitation of oxylipins. This led to issues with resuspension in solutions.

Solutions with oxylipins needed to be vortexed for extended periods of time (i.e., 5 mins) and used promptly. Similarly, I observed a loss of bioactivity within a month when oxylipins were in -20°C and prompted us to put the oxylipins in -80°C to ensure activity. Along with vortexing and proper storage I believe the oxylipins were then usable for experimentation. Nonetheless this was a limitation that was mitigated through required trial and error.

Thirdly, I believe that knocking out the channels TRPA1 and TRPV1 through silencing (siRNA) could offer supporting data for our results. This could help us to compare our results with our inhibitor data to see if our inhibitors were functioning and whether I had the correct concentrations. Similarly, I observed some specificity issues with our inhibitors (i.e., HC-030031) and this could help confirm that they were targeting their channels.

Fourthly, our Ca^{2+} reading duration (i.e., ≈ 1 minute) could have been longer. For our experiments I chose a smaller time capture due to time limitations with the Fura-2 microscope. Ca^{2+} responses are sometimes transient, oscillatory, sustained or exhibit quick sparks and these can last from milliseconds to minutes.^{56,58,174} EpOMEs and DiHOMEs all seemed to show sustained responses across our reading times. It would have been interesting to observe whether the responses remained sustained for a long period of time or returned back to baseline. Long sustained Ca^{2+} fluxes have been observed through TRPA1 activation via cigarette smoke and lasted for longer than 10 minutes.⁵⁸ Therefore, it may be beneficial to read each treatment for 5 minutes or longer to see the responses. Fortunately, our institution has received a brand new Fura-2 microscope capable of imaging for longer periods of time.

Finally, our Fura-2 Ca^{2+} vs. Ca^{2+} -free data was also a limitation. I first performed experiments in Ca^{2+} -buffer and then separately Ca^{2+} -free a few weeks later after I thought of the idea. Here I was unable to match exposures such that I could compare them together. Therefore, I performed a post-hoc analysis and suggest the data be interpreted as such. To mitigate this, I will repeat these experiments in a matched fashion using Fluo-4 as I have already done with 9, 10 EpOME. Our goal is to perform this for 12, 13 EpOME and the DiHOMEs. This will allow us to confirm the trends I observed in our Ca^{2+} vs. Ca^{2+} -free Fura-2 data.

Significance

The role oxylipins 9,10 EpOME, 12,13 EpOME, 9,10 DiHOME, and 12,13 DiHOME play in asthma pathophysiology is completely unknown, even though they have been identified in the airways of people

with asthma. I am the first to describe the role these molecules have in inducing Ca^{2+} flux in ASM and I believe they may be contractile mediators. Future research will be needed to look at the potential contractile abilities of these oxylipins and determine if they are detrimental in diseases like asthma. I have also discovered the potential receptors they require, and this will help us to explore whether these molecules and their associated signaling networks can be leveraged in the treatment of asthma. This may be through targeting TRPA1 and TRPV1 channels specific to ASM or second messengers downstream to these channels. Additionally, if these oxylipins mediate ASM contraction, then future studies could look at potentially inhibiting the SEH enzyme, or the upstream specific CYP450 enzymes. Already SEH inhibitors have been developed in pre-clinical models and human trials. Similarly, inhibitors that target specifically the enzymes CYP2J2, CYP2C8, and CYP2C9 necessary for EpOME synthesis could be inhibited to avoid both EpOME and DiHOME production. I show the importance of needing to understand what these oxylipins within the lung are doing. I believe that many oxylipins have unknown capabilities in ASM physiology and within the context of diseases like asthma. It is evident that further research is required, and I have only scratched the surface.

Future Directions

In this study, I discovered that oxylipins EpOMEs and DiHOMEs are capable of inducing Ca^{2+} flux in ASM through TRPA1 and TRPV1 mechanisms. Other lipid mediators and contractile agonists are capable inducing bronchoconstriction through Ca^{2+} mechanisms. Examples include leukotrienes, thromboxanes, prostaglandins, and some HETEs species. This leads us to believe that EpOMEs and DiHOMEs are likewise capable of inducing bronchoconstriction through Ca^{2+} mechanisms. Therefore, future studies looking at contractile mechanisms is our first goal.

Precision-cut lung slices (PCLS) are the gold standard of measuring airway narrowing ex-vivo. PCLS allows for the excision and measurement of animal and human airways where the structure of the airways is intact. ASM, fibroblasts, epithelium, cilia, the extracellular matrix, and more are all within a PCLS, thus providing physiological relevance. One goal would be to test our EpOMEs and DiHOMEs in healthy and allergic murine PCLS and determine if they are capable of inducing airway narrowing. Our main goal would be to do this in healthy and asthmatic human PCLS as our experiments were performed in human ASM. Our institution just received a PCLS machine from the company 'SCIREQ' capable of measuring narrowing in PCLS preparations.

If I observe narrowing in our PCLS, another important direction would be using TRPV1 and TRPA1 inhibitors along with the PCLS. Murine or human PCLS would be pre-incubated with TRPV1 and/or TRPA1 and then exposed to EpOMEs and DiHOMEs. This would determine whether TRPA1 and TRPV1 are necessary for airway narrowing and whether our inferences were correct. Additionally, using mice models I could silence or knockout TRPV1 and/or TRPA1 completely and determine if these oxylipins can narrow PCLS. Contrastingly, models exist that can lead to overexpression of TRPA1 in the airways (i.e., OVA-challenge).¹⁸⁵ This would be interesting to see if EpOMEs and DiHOMEs that target TRPA1 induce greater PCLS narrowing due to overexpression.

Nonetheless, the future of this project is to focus on the contractile capabilities of these oxylipins and to determine if they are benign or detrimental in human airways.

Conclusion

In this thesis, I hypothesize that oxylipins 9, 10 EpOME, 12, 13 EpOME, 9, 10 DiHOME, and 12, 13 DiHOME will cause an elevation in cytoplasmic Ca^{2+} in Airway Smooth Muscle. I believe I have gained evidence through in-vitro experimentation and real-time Ca^{2+} imaging to support this hypothesis. I have shown for the first time in ASM that both EpOMEs and DiHOMEs are capable of inducing $[\text{Ca}^{2+}]_i$ flux. 9, 10 EpOME had a rapid increase in $[\text{Ca}^{2+}]_i$ that remained sustained. 9, 10 EpOME had similar maximal $[\text{Ca}^{2+}]_i$ to histamine, a known $[\text{Ca}^{2+}]_i$ inducer and airway bronchoconstrictor. 12, 13 EpOME had a slow and increasing rise in $[\text{Ca}^{2+}]_i$ and had a comparable maximal $[\text{Ca}^{2+}]_i$ increase comparable to histamine. 9, 10 DiHOME had a slow but small increasing rise in $[\text{Ca}^{2+}]_i$ but did not have a similar maximal $[\text{Ca}^{2+}]_i$ to histamine. 12, 13 DiHOME had a large spike in $[\text{Ca}^{2+}]_i$ that remained sustained and had a maximal $[\text{Ca}^{2+}]_i$ similar to histamine. I also showed that extracellular Ca^{2+} is required for the EpOME's and DiHOME's Ca^{2+} responses. This indicated that these oxylipins potentially use extracellular channels in ASM to induce increases in $[\text{Ca}^{2+}]_i$. To confirm this, I targeted the extracellular Ca^{2+} channels TRPV1 and TRPA1 with inhibitors. I found that 9, 10 EpOME required TRPA1 to induce Ca^{2+} flux. 12, 13 EpOME showed a requirement of TRPV1 to induce Ca^{2+} flux and that inhibition of TRPA1 via HC-030031 potentiated 12, 13 EpOME's response to a greater amount compared to just 12, 13 EpOME alone. This indicated that there is some TRPA1 mechanism sensitizing TRPV1. 9, 10 DiHOME showed a requirement of TRPV1 and to a lesser extent TRPA1. The reduction in the Ca^{2+} flux in the TRPA1 inhibitor group indicated that there was some TRPA1 mediated mechanism that was desensitizing TRPV1. Finally, I observed that 12, 13 DiHOME requires TRPA1 and to a less significant extent TRPV1 to induce Ca^{2+} flux in ASM. These findings

show support for our second hypothesis where I show for the first time in human ASM that EpOMEs and DiHOMEs require TRPA1 and/or TRPV1 channels to induce Ca^{2+} flux.

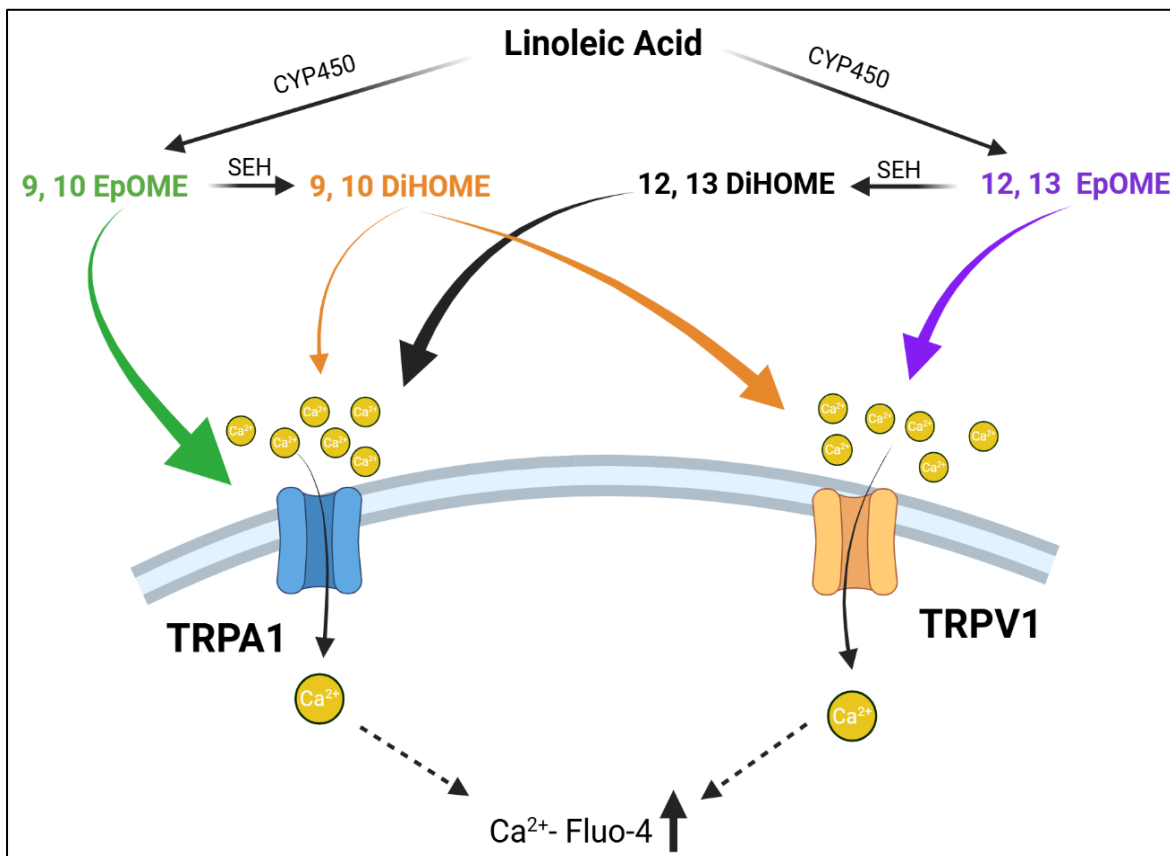


Figure 19. EpOMEs and DiHOMEs require TRPA1 and/or TRPV1 to induce calcium flux in Human ASM.

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