ABSTRACT

LAMPE, WILLIAM RANDALL. Calpain and miR-21 regulation of MARCKS protein and Mucin Secretion in Airway Epithelial Cells. (Under the direction of Kenneth Adler).

Hypersecretion of mucus plays an important role in the pathology of many airway diseases, including asthma, chronic bronchitis, chronic obstructive pulmonary disorder (COPD), cystic fibrosis, and others. Hypersecretion can exacerbate the effects of inflammation and airway constriction by further blocking air flow. Myristoylated alanine-rich C-kinase substrate (MARCKS) protein, specifically the N-terminal region, has been shown to have an important role in airway mucin secretion, as mucin secretion from airway epithelial cells is inhibited by peptides analogous to the N terminus of MARCKS *in vitro* and *in vivo*.

It was investigated whether calpain, a protease, which cleaves MARCKS and the microRNA mir-21, which targets MARCKS mRNA, have an affect on mucin secretion in airway epithelial cells. It was also examined whether acute exposure to ozone had an effect on MARCKS function and secretion. Finally, it was examined whether patients with airway disease had increased level of MARCKS protein, which may contribute to hypersecretion in airway diseases. For these studies, three cell types were used: primary normal human bronchial epithelial (NHBE) cells, the virally-transformed HBE1 cell line, and the mucoepidermoid pulmonary carcinoma cell line, NCI-H292.

We investigated a potential role for the protease calpain in the MARCKS-related mucin secretion mechanism. Calpain is a calcium dependant cysteine protease that does not have a specific primary sequence for cleavage. It is thought to recognize its cleavage site based on tertiary structure. Calpain has been shown to

cleave MARCKS protein near the Phospohorylation Site Domain and is theorized to cleave it near the N-terminal. We theorized that calpain might cleave MARCKS near the N-terminus, thereby allowing dissociation from the membrane.

Air liquid interface cultures were exposed to either 250 nM phorbol-12-myristate-13-acetate (PMA) to stimulate the Protein Kinase C (PKC) pathway of mucin secretion or 100μM adenosine triphosphate (ATP) to activate calpain by increasing intracellular calcium. Calpain activity within airway epithelial cells was measured using a commercially available fluorescent cleavage assay.

Two commercially available calapin inhibitors Z-Leu-Leu-aldehyde (Z-LL-CHO) and Z-Leu-Leu-Tyr-flouromethylketone Z-LLY-FMK were used at 20μM to assess calpain function in these studies. Calpain activity was increased by treatment with PMA and the inhibtors Z-LL-CHO and Z-LLY-FMK reduced this activity. Pretreatment of the cells with Z-LL-CHO or Z-LLY-FMK reduced mucin secretion after they were stimulated by PMA. Undifferentiated HBE1 cells were transfected using the FuGENE®6 Transfection Reagent (Roche) with DNA constructs encoding for a MARCKS-YFP fusion protein to determine approximate locations of cleavage sites within the MARCKS protein, particularly at the predicted N-terminal cleavage site, which would be difficult to determine using unmodified MARCKS protein. We identified fragments of the MARCKS-YFP constructs that had been cleaved near the N-terminus. The amount of cleavage product increased when calpain was activated by ATP treatment. The cleavage of MARCKS protein by calpain may have an important role in mucus secretion.

The micro RNA miR-21 has been shown to be important in immune response. It also has been shown to directly target MARCKS RNA. There is a commercially available inhibitor or miR21, a chemically modified nucleic acid oligomer

complementary to a portion of the mir-21 RNA and an activator that mimics the mir-21 strand, which has also been chemically modified. The activator of miR21 increases the amount of miR21 and decreases the amount of MARCKS RNA. The inhibitor has the opposite effect; it decreases the amount of miR21 and increases the amount of MARCKS RNA. The activator also decreases mucus secretion and the inhibitor increases secretion, indicating that mir-21 is regulating MARCKS protein and its role in secretion.

Ozone has been declared a priority pollutant by the EPA and has been shown to exacerbate asthma, bronchitis and emphysema. Although extensive research has linked ozone exposure to inflammation and reduced airway function, the effects on the airway mucous layer remain unknown. Protein Kinase C is an important signaling molecule in the inflammatory response. As PKC is activated, it translocates from the cytosol to the plasma membrane. PKC alpha, delta and epsilon have been shown to phosphorylate MARCKS, causing MARCKS to translocate from the plasma membrane to the cytoplasm and participate in mucus secretion

NHBE cells and HBE1 cell line were grown at air liquid interface to enhance differentiation. These cultures were exposed to 0.8ppm ozone for 30 to 240 minutes. Cytosolic and membrane protein extracts showed no significant difference in the localization of PKC alpha delta and epsilon in Western Blots. Mucin secretion was measured by ELISA using a pan-mucin antibody (17Q2). After 60 minutes of exposure to ozone, mucus secretion was increased, but not by a statistically significant amount.

To determine a link between MARCKS protein function and airway disease, the levels of MARCKS protein was examined in a normal donor, a non-diseased

smoker, an asthmatic and an individual with COPD. MARCKS was present in higher quantities in an individual with COPD than both a non-diseased smoker and a healthy donor. Tumor Necrosis Factor alpha (TNFα) is an important cytokine in immune response and injury repair. It is secreted primarily by Macrophages but also by T helper cells and Natural killer cells. When cells from diseased and non-diseased individuals were exposed to 100nM TNFα, the cells from the asthmatic patient showed a marked increase in MARCKS protein after three hours. The heightened levels of MARCKS in the COPD cells remained constant.

Calpain and miR-21 Regulation of MARCKS Protein and Mucin Secretion in Airway Epithelial Cells

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DEDICATION

To my parents, Warren and Jean Lampe

For their constant love and support

BIOGRAPHY

William Randall Lampe was born 1977. He is the son of Warren and Jean Lampe and has one older sister Julie Lampe. Randall lived in upstate New York until 1987 then moved to Oak Island, North Carolina. He attended South Brunswick middle and high school before attending North Carolina School of Science and Math. After graduating high school in 1995 Randall attended the University of North Carolina at Chapel Hill for undergraduate studies. After receiving his B.S. in Biology in 2000, he began working for the Cystic Fibrosis Center at UNC, focusing on culturing airway epithelial cells in an air liquid interface and studying the mechanisms of senescence in these cell cultures. Later he worked at Duke University in the Department of Pediatrics studying the use of ethyl nitrite as an inhaled anti-inflammatory drug for premature infants. Then he began work with the Human Vaccine Institute at Duke University studying the use of adjuvants in potential DNA vaccines for HIV. Randall began graduate school at North Carolina State University in 2006 as a Masters student in Toxicology before applying to transfer to the doctoral program in Toxicology with a concentration in Molecular and Cellular Toxicology. He began his dissertation research in Kenneth Adler's lab in the summer of 2007. His interests include photography, motorcycling, travelling, hiking and camping.

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CHAPTER 1: LITERATURE REVIEW

A. The Respiratory System

The lungs, a pair of vital organs positioned in the upper chest, are the site of gas exchange where oxygen enters the blood stream and carbon dioxide and other waste gases are excreted. Air enters through the nose or mouth and proceeds down the trachea. The trachea splits into two bronchi, each leading into one of the two lungs. Once inside the lung tissue itself (the parenchyma) the bronchi continue to split into progressively smaller bronchi and even smaller bronchioles, terminal bronchioles and alveolar ducts before ending at the alveoli, the site of gas exchange.

Lungs are uniquely susceptible to injury because they have a very large surface area and are directly exposed to inhaled gases, particles and pathogens that are present in the air. Other epithelial surfaces have innate barriers, either physical such as the stratum corneum, the keratin rich layers of dead cells protecting the skin, or chemical, such as the acidity of the stomach in which few organisms can survive (1). Yet, because of its role in transporting gases into and out of the lungs, the epithelium that lines the airways has developed a unique battery of defense mechanisms to counter inhaled microbes, particulates and gases. These defense mechanisms are discussed below.

The airway epithelium is made up of several different cell types, mainly ciliated, goblet, basal and also dendritic, neuroendocrine, intermediate, etc. In smaller bronchi and bronchioles and more distally, Clara cells (now referred to as "club cells") predominate. The epithelium is referred to as a "pseudostratified

ciliated columnar epithelium" as it appears to be layered when cross sections are examined, but in reality every cell in the epithelium touches the basement membrane (2, 3). Most of the cells lining the lumen in healthy individuals are ciliated cells. The coordinated beating of the cilia causes mucus, produced by goblet cells and submucosal glands, to be transported up and out of the airways to be expelled or swallowed.

The goblet cells, which are the major airway cell type examined in this thesis, are specialized to produce and secrete mucins, large polymeric glycopropteins, into the airway lumen. They are so named because, in cross section, they are seen to resemble a wine "goblet". They are present in the trachea and larger bronchi, but in disease states such as asthma can be found in smaller airways. Mucus produced by these cells is important in hydrating and lubricating the airways, and also plays a crucial function in clearing inhaled particles or pathogens out of the lungs in conjunction with the ciliated cells via the mucociliary clearance system. Ciliated cells contain apical projections called cilia, which contain the well-characterized 9 + 2 pattern of microtublules along with specific contractile proteins, especially dyneins, that regulate their coordinated beating which propels the mucus in a cephalad direction. It is thought that goblet and ciliated cells are terminally – differentiated cells, although there is some controversy as to this point, especially related to the Clara, or club cells, discussed below, which are theorized to act as progenitor cells for goblet cells (4). Thus, Clara or club cells may fulfill the role of precursor to other cell types in the distal airways (5). These cells secrete Clara cell secretory protein (CCSP), a surfactant, and also contain enzymes such as

cytochrome p450 for the detoxification of exogenous chemicals that have entered the airways. Basal cells directly attach to a supportive membrane made of collagen and other connective tissues yet do not have direct contact with the airway lumen. As mentioned above, these cells are thought to be the progenitors of most cells in the airway epithelium. They divide and differentiate into both ciliated and goblet cells allowing for cellular turnover on the epithelial surface (5). These cells are somewhat protected from environmental insults by the cells on the surface of the airway.

Submucosal glands, found beneath the epithelial layer, are complex structures, which can produce large amounts of mucus. Their secretion can result from signaling from the nervous system (6). In the lungs of healthy people, it has been suggested that submucosal gland secretions make up the majority of airway mucus (7). Glands are distributed from the trachea down through the bronchial branches until the airways are reduced to only 1-2 mm in diameter. In the trachea, the submucosal glands are spaced about one per square millimeter (8). These glands are structured so that many tubules come together into a collecting duct. These tubules are surrounded by mucous cells close to the collecting duct and serous cells deeper in the tubules (9). Approximately 40% of the volume of the gland is mucous cells and 60% serous cells. The mucous cells secrete mucin and the serous cells secrete water, electrolytes, and other substances, which function as antimicrobials, antiinflammatories, and antioxidants, such as lysozyme and defensins (10, 11). The glands also contain Enterochromaffin cells which connect the nervous system to the

submucosal glands; when stimulated by the nervous system they release serotonin (5-HT), which causes both serous and mucous cells to secrete (12).

The alveoli are the small air sacs at the end of the airways. They are the sites of gas exchange in the lungs. In order to facilitate gas exchange, the epithelial layer must be quite thin, and the thin membranes of the alveoli are made up of Type I cells, which constitute approximately 95% of the alveolar surface. The alveoli are coated by a thin layer of liquid; If this liquid were simply water, the surface tension at the air liquid interface would cause the alveoli to collapse, so the coating fluid is a lipid – laden surfactant(s) which decrease the surface tension of the alveolar surface liquid (ASL).

Surfactants lower the surface tension of the interface between liquid and air. Pulmonary surfactant, a lipid detergent, usually forms as a lipid monolayer lining the alveoli in the air/liquid interface(13). The concept of a pulmonary surfactant system was first proposed by von Neergaard in 1929 (14). It was recognized that a film within the lungs, which reduced surface tension at the air liquid interface, was needed for lungs to function (14). After birth, pulmonary surfactant is absolutely necessary for proper lung function and the regulation of alveolar surface integrity. Pulmonary surfactants are secreted by type II alveolar epithelial cells, which are cuboidal cells that make up approximately 5% of the alveolar cell population. These cells can proliferate in some lung diseases, such as fibrosis, and are filled with lamellar bodies that appear to contain packaged surfactant stored within the cells (15). Surfactants A, B, C and D have been described and are discussed in more detail below.

The ability of pulmonary surfactant to lower surface tension on the alveoli plays an important part in lung physiology. It increases pulmonary compliance, regulates the surface liquid in the alveoli, and prevents pulmonary edema. Without proper surfactant, a condition known as atelectasis can occur in which the alveoli are deflated. Once they have collapsed it takes a great deal of force to reopen them because of the surface tension of the liquid on the epithelial layer. In addition to losing the ability for gas exchange, a collapsed portion of the lung would be more susceptible to disease. For example, if pneumonia starts in the collapsed portion of the lung, it could lead to chronic inflammation, which can lead in turn to bronchiectasis and scarring.

Atelectasis can be determined clinically by measuring the lungs' compliance. Pulmonary compliance, a measure of the force required to inflate the lungs, is one measurement in respiratory physiology that clinicians use to gauge lung function. Changes in compliance occur in pulmonary disease: for example, in pulmonary fibrosis there is a decrease in pulmonary compliance, since connective tissue makes it harder to expand the lungs. Increased compliance is also associated with pulmonary diseases such as COPD and emphysema, which are characterized by a loss of alveoli and decreased lung elasticity.

Also, limiting surface tension lowers the forces drawing fluid from the capillaries to the alveolar spaces, so surfactant can reduce the level of fluid accumulation regulating the liquid layer in the alveoli. The importance of surfactant in the airway is observable in premature infants that have insufficient surfactant, which leads to collapse of the alveoli and development of Infant Respiratory

Distress Syndrome (IRDS), which is the leading cause of death in preterm infants (16).

During the respiratory cycle, the surface area of the alveoli varies. As the lungs empty, the alveoli compress and this causes surfactant to rise in concentration. The surface tension of the alveolar surface liquid is very low at this point, which allows the alveoli to maintain their structure. When the lungs expand, so do the alveoli. This effectively lowers the concentration of surfactant and increases the surface tension. It is only at this point that new molecules of surfactant can be incorporated into the air liquid interface.

Pulmonary surfactant is composed of 40% Dipalmitoylphosphatidylcholine (DPPC), and 40% of other phospholipids such as Phosphatidylcholine and Phosphatidylglycerol. It also contains cholesterol, with only about 5% of surfactant made up of protein. SP-A and SP-D are oligomeric glycoproteins which bind to phospholipids, including DPPC (17). In addition, they play a role in the innate immune system, as they bind to pathogens and direct other cells to phagocytose them or release cytokines and/or reactive oxygen species (18). Surfactant Proteins SP-B and SP-C are necessary to allow spreading of the phospholipids over the surface of the liquid. These are extremely hydrophobic proteins. They bind to anionic lipids such as Phosphatidylcholine and Phosphatidylglycerol. SP-C is made from a much larger (21kDa) precursor protein than the active form, which is only 4.2 kDa. This protein must be cleaved to become active. SP-C contains a highly hydrophobic region of 34 amino acids. This domain contains many, often adjacent, valine residues. This region, coupled to the lipids bound by SP-C, acts as a

surfactant (19). Humans and mammals without a functional Surfactant protein c gene, SFTPC, often develop progressive interstitial pneumonitis (20), indicating the need for a fully functional surfactant system to maintain the health of the lung.

Mucus

Mucus structure and function

Mucus is present on all mucous membranes, including the gastrointestinal tract, reproductive system, eyes, and respiratory system. Mucus generally forms a protective layer, protecting the surfaces from contaminants. In airways, mucus is necessary to clear inhaled particles, fungi, bacteria and viruses. It also serves to maintain humidity in the airways. It is composed of transudate, glycoproteins (mucins), enzymes, proteins, macroglobulins and immunoglobulins. The airway mucus layer also may contain different immune and inflammatory cells immersed in the mucus itself (21).

Mucus in the airways is organized into two distinct layers. The upper layer is a gel that is high in viscosity and contains most of the glycoproteins; under normal conditions, inhaled particles and microbes remain trapped in the mucus and are cleared out of the airways via the mucociliary system. A pericilliary layer, which is lower in viscosity, allows the cilia to beat, moving mucus and trapped material in the upper layer out of the airways. The dynamics of the pericilliary layer, and exactly how the beating cilia contact the upper layer to provide optimal mucociliary transport, has been and continues to be the subject of numerous studies over the last few decades, but exact mechanisms of how these layers interact remain

unknown.

Mucus in the mammalian airways is a colloidal suspension of mostly water. The sputum of healthy lungs contains around 2-4% solids (22). A large portion of the solid matter is made of mucin gycoproteins, that is, proteins that are chemically bound to many carbohydrates. In many pulmonary diseases, goblet cells and submucosal glands undergo severe alteration, be it increasing in size (hypertrophy), increasing in number where they are normally found (hyperplasia) or appearing in places, such as goblet cells in smaller airways, where they are not normally seen (metaplasia). The end result of these processes is that the airways contain more mucus than normal, leading to airway obstruction, which characterizes asthma, chronic bronchitis, and other inflammatory airway diseases (1).

Mucins

Mucins are gycoproteins, proteins that are chemically bonded to many carbohydrates. Mucins are also extensively modified post-translationally by myristoylation, glycosylation and/or phosphorylation. A MUC protein backbone typically consists of an NH_2-terminal domain, one or more central domain(s) with a high number of tandem repeat (TR) domains, and a COOH-terminal domain. Many O-glycans attach to threonine or serine residues in the TR domains. O-glycans can vary widely in size and distribution. Their carbohydrate content may account for between 50-90% of their total mass. It is mucins that give mucus its viscosity and elasticity (23). At least 20 different human mucins have been identified, and they are present in the respiratory, gastrointestinal, ocular and reproductive systems. They

exist as either membrane-tethered or secreted. In the respiratory system, 11 different mucin genes have been identified via protein or mRNA expression. The mucins MUC5AC, MUC5B, MUC19 and MUC2 are secreted by both goblet cells and submucosal glands, with the cysteine – rich MUC5AC and MUC5B predominating. The most important and heavily studied mucins in the airways are MUC5AC MUC5B and MUC2. It was thought that MUC5AC is secreted primarily by goblet cells while MUC5B is mainly secreted by submucosal glands, but that remains controversial.

The amount of mucus in the airways is important. Insufficient mucus can leave the lungs susceptible to disease and chemical exposure, while an excess of mucus or inability to clear mucus can lead to obstructive disease. There is both increased production and secretion in inflammatory airway diseases including chronic bronchitis, asthma and Cystic Fibrosis. Airways must allow air to pass deep into the lungs through increasingly narrow pathways. The contribution of mucus to airway obstruction in these diseases can be modeled mathetmatically. The Hagan-Poiseuille's equation of fluid dynamics states that the amount of airflow is proportional to the airway radius to the fourth power. Reducing the diameter of an airway by half only allows one sixteenth of the amount of airflow. Excess airway mucus effectively reduces the diameter of the airway, reducing airflow and amplifying the restriction caused by contracted airways as seen in asthma and chronic bronchitis (24).

Respiratory Disease

Asthma

Asthma is a chronic inflammatory disease of the airways characterized by increased bronchoconstriction and airways hyperreactivity, declines in lung function, and airway remodeling reflected by enhanced smooth muscle hyperplasia and hypertrophy, basement membrane thickening, and goblet cell hyper- and metaplasia (25-27). Asthma is a common disease, affecting 1 in 12 people, and its prevalence is rising. A genetic predisposition to develop asthma is now well recognized (28). Asthma is caused by activation of the innate immune system in a way to produce a Th2 response. Th2-related cytokines, especially IL-4, IL-5, and IL-13 are in large part responsible for the increases in eosinophil influx into the airway and airway inflammation (29, 30). The pathology of asthma involves many cytokines and chemokines (31, 32). Some are also present in other diseases that involve inflammation, and include IL-1, IL-6 and TNF-alpha. These are present in COPD, inflammatory bowel disease, rheumatoid arthritis etc. Some cytokines are more specific for allergic-type inflammation such IL-4, IL-5, IL-9 and IL-13. These cytokines are secreted mainly by Th2–type lymphocytes, while Th1 cells produce and secrete IL-2 and Interferon gamma. (33). This signaling causes influx of many cells of the immune system, including eosinophils, macrophages, lymphocytes, and mast cells into the airways (34). The cytokines secreted by these leukocytes cause allergic airway inflammation, goblet cell metaplasia, increased mucus secretion, smooth muscle hyperplasia, and airway hyperresponsiveness. There are significant changes in pro-inflammatory gene and protein expression in target cells of Th2

cytokines (35, 36), and these cytokines contribute both directly and indirectly to changes in structure and function of the airways. Chronic asthma leads to goblet cell hyperplasia, which in turns leads to a greater amount of secreted mucus. Asthmatics also have increased luminal mucus and a higher ratio of MUC5B to MUC5AC (37) . One of the most significant structural changes includes abnormal deposition of collagen in the subepithelium (36). Ultimately, there is a thickening of the walls of the airways, both large and small, and this remodeling contributes greatly to reduced lung function in combination with contraction of smooth muscle, mucus obstruction, and pulmonary edema (36). Airway remodeling in patients with asthma makes it more likely they will have exacerbations often, requiring hospitalization and possibly death if the airway obstruction is severe (38). Excessive mucus also decreases the ability to clear pathogens from the airway, leading to the possibility of infection. Asthma is typically treated with beta-agonist and corticosteroids. Corticosteroids reduce inflammation and can also reduce mucus cell metaplasia. Beta-agonists reduce smooth muscle contraction and also may stimulate ciliary transport (39, 40).

COPD

While the precise definition of Chronic Obstructive Pulmonary Disease (COPD) is still a matter of debate (41), clinically the diagnosis groups people who would need similar treatment. Epidemiologically, a quantitative measure of lung function such as forced expiratory volume in 1 second (FEV1) provides an objective means of assessing disease severity. COPD is characterized by chronic bronchitis

and emphysema, and often, chronic asthma is considered a characteristic of this disease. In COPD there is hypertrophy of submucosal glands (42) and goblet cell hyperplasia and metaplasia, which contribute to an increase in MUC5AC in airways (43). Although asthma and COPD share a number of symptoms, there are differences in the mucus produced. COPD mucus is less viscous, lacks significant plasma exudate, the ratio of MUC5AC to MUC5B is often reduced, and mucins are fully released into the lumen, not tethered to the epithelial layer. In asthma, mucus is more viscous, has significant plasma exudate, has a higher ratio of MUC5AC to MUC5B, and there is tethering of the mucus to the epithelial layer (44), 52). In COPD, the inflammation of the airways is primarily neutrophilic, but 20-40% people with COPD also have eosinophilic airway inflammation and an associated increase in IL-5 levels in their mucus (45).

Once activated, eosinophils produce a number of products: reactive oxygen species, cationic proteins, and inflammatory mediators such as leukotrienes and prostaglandins(46, 47). They also produce cytokines such as interleukins, TNFα and TGFß (both a growth factor and pro-fibrotic factor) (47-50).

Cystic Fibrosis

Cystic Fibrosis is an autosomal recessive trait caused by a mutated gene for the Cystic Fibrosis Transmembrane Regulator (CFTR) chloride channel. Approximately one in 30 Caucasians are carriers for the recessive mutation. Cystic fibrosis can result from a variety of mutation in the CFTR gene, but 70% of patients have the delta-f508 mutation. The mutation occurs when three nucleotides are

deleted from the gene causing the deletion of a phenyalanine (F) at position 508 (51). Mutation frequency varies among groups; for example amongst people with an Ashkenazi Jewish background, M1282X is the most common mutation, occurring in 48% of cases. The delta-f508 mutation causes an alteration in post-translational processing of CFTR. Since the cell has mechanisms to recognize and destroy improperly processed proteins with the delta-f508 mutation, the cell detects an error in protein folding while the protein is still in the endoplasmic reticulum. Ubiquitin binds to the dysfunctional CFTR molecules and causes them to be degraded in the proteosome before reaching the plasma membrane (52).

In cystic fibrosis, mucus is thicker and has much poorer clearance than in healthy individuals. Also, the mucus' pH is more acidic and pH regulation is disrupted. Airway epithelial cells from patients with cystic fibrosis are unable to adapt to an acid challenge like normal cells due to the lack of a CFTR dependent HC03- channel (53). Airway surface liquid (ASL) with lower pH inhibits antimicrobial activity within the ASL and diminishes bacterial killing; it is interesting that increasing ASL pH had a beneficial effect in a pig model of CF. (54). Airway surface liquid from the nasal and tracheal epithelium has a greater concentration of Cl^- ions The overall change in ion concentrations inhibits the function of both the innate and adaptive immune systems in their response to pathogens.

Pulmonary infections are the leading cause of mortality in cystic fibrosis. *Pseudomonas aeruginosa* is the most common opportunistic infection in the lungs, with *Burkholderia cepacia* and *Staphylococcus aureus* also common. The mucus in

cystic fibrosis patients is produced in greater amounts and has poorer clearance properties than mucus in healthy patients. Higher levels of NaCl in the airway surface liquid limit the ability of the immune system to kill *P. aeruginosa* (55). The changes in the airways allow for easier initial infection and some strains of *P. aeruginosa* form a protective slime layer within the mucus. This layer hinders the movement of both leukocytes and antibodies (56), contributing to susceptibility to infection.

Innate Immune System

The innate immune system contains various Pattern-Recognition Receptors (PRRs). These recognize signature molecules that are conserved over a large number of pathogens. There are several classes of PRRs including Toll-Like Receptors (TLRs), Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (57-60). TLRs are evolutionarily conserved innate receptors expressed in various immune and non-immune cells of the mammalian host. TLRs play a crucial role in defending against pathogenic microbial infection through the induction of inflammatory cytokines and type I interferons upon ligand binding. Additionally, TLRs also trigger the adaptive immune system to create a pathogen specific response. T and B cells create a "memory" of the pathogen and maintain immunity over time (61, 62). TLRs will recognize different types of pathogen-associated microbial patterns (PAMPs); for example, TLR 1, 2, and 6 recognize lipoproteins and glycolipids. Double stranded RNA is found in retroviruses and is a ligand for TLR3. TLR4

recognizes Lipopolysaccharide (LPS) a component of the cell wall of Gram-negative bateria, while TLR5 identifies flagellin, a component of flagellum in many types of bacteria. TLR7 and TLR8 will bind single stranded RNA. TLR9 recognizes unmethylated CpG DNA strands. TLR11 identifies Profilin, which regulates the dynamics of the actin in cytoskeleton. TLRs will also bind to many molecules endogenous to the host.

Immunoglobulin A (IgA) is very important to the immune system of the lungs and other mucous membranes. IgA secreted onto mucosal surfaces accounts for approximately 70% of all immunoglobulins produced in the body. IgA is produced by B-cells and is actively transported from outside the basal side across the epithelial cells to the apical side where it is cleaved from the membrane and allowed to diffuse into the mucus (63). In mice, IgA is the important immunoglobulin in the upper respiratory tract while IgG appears to be more prominent in the parenchyma of the lungs (64). IgA is capable of neutralizing pathogens and exotoxins, such as cholera toxin. Typically the neutralization takes place in the airway lumen. However, neutralization can occur as IgA is being transported through the epithelial layer (65-67).

Another defense mechanism in the airways is the production of defensins. Defensins are small (2-6kDa) proteins that are cationic and have three disulfide bonds which give the proteins their higher order structure. They have an anti-microbial function. Defensins will polymerize and then can create pores in the membranes of pathogens. They are capable of defending against a wide spectrum of pathogens, including Gram negative and positive bacteria and some enveloped

viruses (68). Defensins are thought to be important in the innate immune response to Lipopolysaccharide (LPS) in a way that is dependant on CD14 (69).

MARCKS Protein

Structure and function

Myristoylated alanine-rich kinase C substrate (MARCKS) consists of 323 amino acids in humans. Its expected molecular weight based on the number of amino acids is 40kDa, but it is detected near 85kDa by Western blot analysis due to its shape and extensive post-translational modifications. MARCKS is ubiquitously expressed in all eukaryotic cell types. It is a well-studied substrate of Protein Kinase C (PKC). MARCKS has important roles in many cellular functions such as neurosecretion, exocytosis, cell motility, membrane trafficking and mitogenesis through regulation of cytoskeletal structures (70-73).

Amongst mammalian species, there is some variation in the amino acid sequence of MARCKS. However, there are three domains that are highly conserved. These are the myristoylated N-terminus, the Multiple Homology 2 domain (MH2), and the Phosphorylation Site domain (PSD). The MH2 domain can bind phospho-serine residues. It contains a central β-sandwich with a conserved loop-helix region that can bind phosphorylated serine residues. The MH2 domain is also present on Smad proteins, which are important for regulating TGF-beta (74). Presently, the exact function of the MH2 domain on MARCKS is unknown. The PSD region contains three serine residues which are targets of phosphorylation by PKC and perhaps other kinases: Ser152, Ser 156 and Ser163 (75-77). This domain is highly

basic and consists of about 25 amino acids; it is often referred to as the effector domain since it is the site on MARCKS which is phosphorylated by PKC, and also is the site that binds and crosslinks actin filaments as well as being the site for binding of calcium/calmodulin. In fact, MARCKS appears to have at least two sites for binding actin, located on either end of the PSD. These are thought to bind F-actin, crosslinking two filaments. When MARCKS is phosphorylated, it undergoes a change in conformation. When this happens, one of the actin binding sites becomes blocked from binding to actin. When MARCKS is dephosphorylated, it allows actin to bind to the site once again (78-80). The PSD site is highly basic because it contains 12 or 13 positively charged Lysine and Arginine residues. These positively charged residues allows the PSD in cellular MARCKS to bind to the inner face of the plasma membrane electrostatically via ionic interactions with the head groups of phospholipids in the membrane bilayer (81-83). Finally, the The N-terminal region is myristoylated and the hydrophobic myristic acid intercalates into the plasma membrane and aids in securing MARCKS to the plasma membrane (75, 84). The N-terminus of MARCKS is considered essential for membrane binding.

MARCKS and Secretion

MARCKS has been shown to be an important molecule regulating cellular secretion, and the highly conserved N-terminal region is critical to airway mucin secretion. A myristoylated peptide analogous to the first 24 amino acids of MARCKS (called the "MANS" peptide) attenuates mucin secretion by airway epithelial cells both *in vitro* (72, 85, 86) and *in vivo* (73, 87, 88). This peptide has also

been shown to affect other functions of MARCKS: It inhibits migration of neutrophils, (89) macrophages, (90) and mesenchymal stem cells (91). A control missense peptide consisting of the same amino acids in random order does not produce the same results.

The role of MARCKS in secretion often is secondary to its phosphorylation by PKC at the PSD site (70, 92-94). MARCKS is a prominent PKC substrate. Members of both the conventional and novel classes of PKC can phosphorylate MARCKS. There are various PKC isoforms, which require different activation mechanisms: conventional PKCs (α, βI, βII and γ) require both DAG and Ca2+, novel PKCs (δ, ε, ζ, ε), require only DAG; and atypical PKCs (η, λ, ζ) do not require DAG or Ca2+ for activation (95). The relative efficiency of PKC isoforms in phosphorylating MARCKS is PKC delta >epsilon> alpha (96). Additionally, PKC beta and PKC zeta will phosphorylate MARCKS (93, 97-99). PKC delta has been shown to be involved in exocytosis in a number of other cell types. It is involved in secretion of mucin by airway epithelial cells (75-77), neurotransmitters in the brain,(100) insulin in islet cells (101, 102) gastric peptides from goblet cells (103), inflammatory cell degranulation (104-106), and release of platelet dense granules (107).

When a secretagogoue, such as UTP, comes into contact with a Gq-protein coupled receptor (GqCPR) or a Receptor Tyrosine Kinase (RTK), The Gq receptor will activate Phospholipase C, which will then cleave phosphatidylinositol 4,5-bisphosphate (PIP2) into two molecules, diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP3). DAG is lipophilic and will remain in the plasma membrane while IP3 is lipophobic causing it to translocate to the cytoplasm. Once in the

cytoplasm, IP3 can bind to receptors on the endoplasmic reticulum (ER) and open calcium channels, causing calcium to flow into the cytosol from the ER and intracellular calcium levels to rise. Increased intracellular calcium can have a wide spectrum of actions within cells, mostly related to stimulation of cell functions. DAG in turn can go on to further activate PKC and enhance phosphorylation of PKC substrates, including MARCKS, which also can contribute to increased cell function.

Ozone

Ozone is one of the six types of air pollution that are covered under the National Ambient Air Quality Standards as a result of the Clean Air act. These six pollutants are found throughout the United States in varying amounts. The others are particle matter (PM), nitrogen oxides, carbon monoxide, sulfur oxides and lead.

Ozone is a major constituent of the urban air pollution known as smog. Unlike ozone in the stratosphere, which protects us from the sun's harmful ultraviolet rays, ozone near the ground can cause significant health problems. Individuals suffering from airway diseases are the most sensitive. Ozone is composed of three oxygen molecules bound together instead of the two molecules that comprise the oxygen our bodies need. It is produced by the interaction of light and other forms of pollution such as hydrocarbons, nitrogen oxides and carbon monoxide. The primary sources of these chemicals are automobiles and fossil fuel power plants.

The EPA sets primary standards that are based upon human health effects. The agency also provides a set of secondary standards to prevent environmental and

property damage. The primary air quality standard for ozone was set to be 120 parts per billion (ppb) average for 1 hour (108). The standard is currently set at 75 ppb averaged over 8 hours. These levels are often exceeded in many urban areas in the US and around the world, especially in cities with dense populations and many cars and trucks. This increased production, coupled with a local climate that creates stagnant air such as a valley or depression, leads to the highest concentrations of surface ozone. For example, Los Angeles, California, which resides in a valley, had a maximum level of approximately 570 ppb of ozone in the 1970's(109). Since then, ozone levels have been decreasing in Los Angeles. Mexico City is one of the largest cities in the world with nearly 9 million people living within city limits and over 21 million inhabitants living in the metropolitan area. It is located within a valley with limited airflow and is approximately 8000 feet in elevation. As a result, 8-hour ozone levels are between 100 and 200 ppb year round. During the worst seasons, fall and winter, ambient levels of 300 ppb are not uncommon (110, 111).

Ozone is a very strong oxidizing agent (108). In fact, ozone is so reactive it may degrade before reaching the lung epithelium upon inhalation. It is thought to react with molecules in the mucus in airways and alveolar surface liquid, forming products that are more stable, and it is these that can interact with the epithelium (112). Airway fluids contain antioxidants to protect against oxidative stress such as urate, ascorbate (vitamin C), and GSH, which are present in high concentrations in mucus and ASL. These are very reactive with ozone and such antioxidants form relatively benign products when reacting with ozone, protecting against injury (113).

Lipid peroxides are highly toxic and damaging to biological systems (114);

Humans have evolved an antioxidant system to protect against oxidative stress—a state of imbalance between oxidant production and antioxidant defenses. Ozone and other inhaled toxins, air pollutants, and tobacco smoke cause oxidative stress (115-117) through direct generation of reactive oxygen species (ROS). Vitamin E (Alpha-tocopherol) acts as a further defense by scavenging peroxyl radicals produced by ozone interacting with fatty acids in the fluid (115).

A population at risk for inhalation of ozone consists of patients with underlying lung disease. There is consistency in the findings that relate the acute increases in urban air pollution (mainly particulate matter) and the short-term health effects (i.e. mortality and hospital admissions) in patients suffering from chronic obstructive pulmonary disease (COPD). Beyond the acute effects, a relevant public health and scientific question is to what extent chronic exposure to air pollution is related with lung function impairment and development of COPD. As early as the Great Smog of London in 1952, people began to recognize this association. The majority of deaths occurring during the Great Smog involved subjects diagnosed with bronchitis (118). From a study in Chicago, an association between air pollution and daily symptoms was observed during periods with lower levels of black smoke and sulfur dioxide.

In the United Kingdom during the 1950s, it was proven that air pollution not only caused acute exacerbation in COPD patients, but also that chronic bronchitis was more prevalent in postmen who worked in areas with higher pollution than in postmen working in less polluted areas (119). These results were validated by later studies (120). It has been shown that children who are actively exercising in areas

with elevated levels of ozone are more likely to develop asthma. Children who were actively exercising in areas with low levels of ozone were not any more likely to develop asthma than more sedentary children (121). Many risk factors are associated with ozone induced asthma hospitalization: low birth weight, age, socioeconomic status, and race. Blacks and Hispanics are almost twice as likely as whites to be admitted to a hospital for acute asthma symptoms. People living in areas of high poverty are also more likely to be hospitalized for acute asthma symptoms (122).

Breathing air with a high concentration of ozone causes a decrease in lung function and increases airway reactivity. It is linked to increased exacerbations of asthma (123). Exposure to high levels of ozone can bring about a greater sensitivity to allergens (124). Conversely, reactivity to ozone is increased with previous exposure to an allergen (125). There is a greater risk of developing asthma in children if the child has spent the first 8 years of life in heavy traffic areas (126). When epithelial cells are exposed to ozone they have an increase in production of pro-inflammatory cytokines, including IL-1, IL-6, TNF α, IL-8, and also an increase in intracellular oxidative stress (127, 128). Ozone is also thought affect lung development: In postnatal primates, exposure to ozone affects the pattern of alveolar development (129).

Interestingly, the sugar hyaluronan (HA) has been shown to mediate ozone induced airway hyperresponsiveness. Instilling mouse airways with HA causes airway hyperreactivity, and instillation of a peptide that binds HA had a protective effect on development of ozone - induced airway hyperreactivity. (130).

Hyaluronan is an anionic, nonsulfated glycosaminoglycan present in epithelium, connective and neural tissues. It is a polysaccharide found in body fluids of all vertebrates and also some bacteria. It is synthesized in the plasma membrane and can remain there, and it also can bind to extracellular matrix and exist as a free floating "pool" (131). Hyaldherins are proteins, which recognize HA and bind them to proteoglycans, stabilizing the structure of the extracellular matrix. Hyaldherins have been implicated in many important physiological processes such as lubrication, water homeostasis and filtering and altering plasma protein location (132). Interestingly, with respect to the effects of ozone described above, HA binding through α1 anti trypsin and CD44 are necessary for ozone induced AHR. In addition, it has been shown that TLR4 is necessary for HA to mediate hyperresponsiveness. TLR4 deficient mice are partially protected from ozone induced airway hyperreactivity. They are also protected against developing ozone AHR after intratracheal instillation of HA (133).

Asthmatics are particularly sensitive to air pollution. Ozone is one of the most important components of air pollution. The airway epithelium is directly exposed to ozone, which is highly unstable and likely does not penetrate beyond the epithelial layer. This would imply that the epithelial cells play a vital part in the reaction to high levels of ozone. This could be by increasing mucin secretion, altering the composition of airway surface liquid, or secreting inflammatory cytokines. The precise mechanism connecting respiratory symptoms and ozone levels is not fully understood.

Calpain

Calpains are a family of 15 calcium-dependent cysteine proteases. Two archetypes of the calpain family are μ-calpain and m-calpain. These were named for the molar concentration of calcium ions required for their activation. Although they share the regulatory subunit Calpain small subunit 1, a product of the CAPNS1 gene, their catalytic subunits are different: μ-calpain contains calpain 1, a product of the gene CAPN1 and m-calpain contains calpain 2, product of the gene CAPN2 (134-136).

Calpains require calcium and a neutral pH for their proteolytic activity. Calpains are heterodimers consisting of an enzymatic 80 kDa subunit and a regulatory 28 kDa subunit. The catalytic subunits contain four domains (DI – DIV) and the regulatory subunits contain two domains (DV and DVI). These subunits are counted from the N- to the C-terminal. When calcium activates calpains, D1 autolyses (although this is not required for activation). DII is the conserved catalytic domain; it actually is two separate subdomains that come together, with a change in conformation, when they bind calcium. This forms the catalytic cleft (137-140). DIII contains a C2 domain, consisting of 8 beta sheets, which bind 2-3 calcium ions and confers a structural change to calpain when it binds calcium (139, 140). DIV binds to the DVI on the regulatory subunit and contains five EF hands, a helix-loop-helix structure that binds calcium in the loop region. The fifth EF hand is a site important for binding the two subunits together (141, 142). DVI contains repeating glycine residues that are thought to interact with the plasma membrane. These are autolysed when calpain is activated. (143). Calpstatin, an endogenous calpain

inhibitor in cells, occludes the catalytic cleft of heterodimeric calpain as a means of regulating calpain activity endogenously.

Calpains lack a specific primary sequence for cleavage; they are thought to have site recognition based on tertiary structure. The first calpain was discovered by Guroff in rat brain in 1964 (144). Interestingly, Calpain has been shown to cleave MARCKS protein, although the location(s) of the cleavage site(s) are still uncertain (145, 146). Reducing the expression of Calpain genes leads to accumulation of MARCKS in cultured myogenic cells (147). Calpain cleavage may increase accessibility of the phosphorylation site domain (PSD) on MARCKS, thereby increasing its ability to bind actin (148). This cleavage site is possibly the same site identified between asparagine 147 and glutamate 148 in the bovine sequence of MARCKS, only three amino acids away from the amino-terminal side of the PSD (146). It also has been speculated that MARCKS may be cleaved between the 6th and 7th amino acid from the N-terminus (145). A convergent point between MARCKS and Calpain activation in these cells could be phospholipase C, a common cellular signaling pathway. Activated phospholipase C will cleave phosphatidylinositol into diacylglycerol (DAG) and inositol triphosphate (IP3). DAG can then go on to activate PKC, which phosphorylates MARCKS, while IP3 binds to specific receptors on endoplasmic reticular membranes, resulting in release of intracellular stores of calcium into the cytoplasm, which may activate Calpains (along with other calcium-sensitive molecules) (149).

Calpains have been shown to have an effect on secretion in other tissues. A calpain inhibitor was shown to decrease insulin secretion from pancreatic cells *in*

vitro (150). The presence of calpain-10 mRNA in pancreatic islets, muscle, and liver, the three most important tissues that control blood glucose levels, suggests that calpain-10 may regulate pathways that affect insulin secretion, insulin action, and hepatic glucose production, each of which is altered in patients with type 2 diabetes (150).

Calpains have been implicated in an increasing number of diseases. Since calpain often function to partially proteolyse substrates, it modifies rather than terminates the function of the substrate, leaving fragments of proteins that may retain function. For example, calpain may alter talin via affecting the structural integrity of adhesive complexes and/or affecting signaling pathways (151). Direct and indirect roles for calpains have been proposed in intracellular processes including cell-cycle regulation (152), cell differentiation (153) and cell motility (154).

Over-expression of calpain has been implicated in tumorigenesis in several types of cancer. Increased expression of μ-calpain is observed in schwannomas and meningiomas (155), increased expression of CAPN1 mRNA is seen in renal cell carcinoma (156), and increased expression of m-calpain in colorectal adenocarcinomas has also been observed (157). However, calpain expression is not altered in all cancers. Basal and squamous carcinomas of the skin have normal calpain levels (158) and contradictory results have been presented for prostate cancer(159, 160). Also, decreased expression of calpastatin has been observed in endometrial cancer (161, 162). There are reports of a calpain-activator protein that is specific for μ-calpain(163-166). Both μ-calpain and m-calpain can be phosphorylated

by protein kinase Cι (PKCι), and this phosphorylation has been associated with increased cell migration and invasion of lung cancer cells (167).

Calpain and Secretion

Calpain has been associated with regulation of secretion in several different cell systems. Benzyloxycarbonyl-L-leucyl-L-leucinal (ZLLal), a calpain inhibitor, reduced growth hormone (GH) secretion, leading to intracellular accumulation, in a GH-secreting rat pituitary tumor cell. Pulse-chase experiments demonstrated that ZLLal retarded the turnover of clathrin and adaptins in these cells, and ZLLal-treatment co-immunoprecipitated increased amounts of GH with clathrin and adaptins compared to control cells, suggesting intracellular accumulation of immature secretory granules. Adaptins can be proteolyzed by m-calpain in vitro, indicating that calpain may be involved partly in the maturation of secretory granules in endocrine cells via the process of clathrin uncoating (168, 169).

Several mechanisms can assist in activating calpain by reducing the concentration of calcium necessary for activation. This is done by causing calpain to autolyse in the DI domain, one of the regulatory domains (170), (171). Another mechanism of reducing calcium concentration required for activation is interacting with plasma membrane phospholipids;phosphatidylinositol, phosphatidylcholine, phosphatidylglycerol, phosphatidylserine, phosphatidyl ethanolamine, phosphatidic acid and sphingomyelin have all been found to be effective at reducing the calcium requirement for m-calpain autolysis (172).

As mentioned above, the endogenout inhibitor of calpain is called Calpstatin. Calpastatin is a multiheaded inhibitor capable of inhibiting more than one calpain molecule. Each inhibitory domain of calpastatin has three subdomains, A, B, and C; The A domain binds to domain IV of calpain and domain C binds to domain VI of calpain. Crystallographic evidence shows that binding of C to domain VI involves hydrophobic interactions at a site near the first EF-hand in domain VI. Sequence homology suggests that binding of A to calpain domain IV also involves hydrophobic interactions near the EF1- hand of domain IV. (173) Calpastatin occludes the catalytic cleft of heterodimeric calpain. (140)

The calpain cleavage site(s) on MARCKS would suggest that calpain cleaves MARCKS and either limits the secretory function. Alternately, by cleaving MARCKS, Calpain could change it into a form that is more conducive to secretion. nhibiting calpain would leave more full length MARCKS to participate in the secretory pathway.

Inflammation

Lipopolysaccharide

Lipopolysaccharide (LPS), an endotoxin produced by Gram-negative bacteria, consists of chains of covalently bound lipids and polysaccharides. Mammals have evolved a defense against endotoxins that involves a significant immune response after exposure. These include Toll-like receptors (TLRs), a group of receptors that serve as sensors for pathogens or tissue damage. As TLRs bind their ligand they will dimerize, forming both heterodimers and homodimers, and, through adapter protein such as TRIF, TRAM and TRAP, this allows MyD88 to bind the complex.

TLRs mostly signal through a MyD88 pathway, with the exception of TLR3 (179). The MyD88 attached to the receptor will activate IRAKs 1&4. IRAK will cause TRAF6 to become unbiquinated and bind to a complex of TAK1, TAB2 and TAB3 (174, 175). When TRAF6 bind to the complex, TAK1 will first phosphorylate IKK, and then IKBα. IKBα will then release NF-κB allowing it to translocate into the nucleus. This ultimately leads to the nuclear translocation of NF-κB to serve as a transcription factor causing the production of inflammatory cytokines such as TNF, IL-1α, IL-1β, IL-6, and IL-8. This signaling cascade causes LPS to increase mucus secretion in the airways. (176).

TNFα

Tumor Necrosis Factor alpha (TNF-α) is an important and very potent cytokine that recruits leukocytes into peripheral tissue, including the lungs.

While it is produced by many cell types, the most important sources of TNFα are activated macrophages and mast cells. TNF-a production is mediated by several chemokines, which act through their receptors on the surface of the plasma membrane of leukocytes (177-179). TNF-a binds to two receptors, TNF-R1 and TNF-R2. Upon binding, the receptors form trimers, which allows for the inhibitory protein SODD to dissociate from the "Death Domain" on the TNF-R. TRODD protein can then bind to the receptor and initiate three signaling cascades: activation of NF-kB pahways, MAPK pathways, and caspase signaling. LPS will cause an increase in TNFα secretion.

Septic Shock

Septic shock is a complex condition starting from an infective stimulus and resulting in an exaggerated immune response. The inflammatory response that was initiated to fight the infection ultimately leads to damage of various organs thorough out the body. During the onset of sepsis, the inflammatory system becomes hyperactive, involving both cellular and humoral defense mechanisms Endothelial and epithelial cells, as well as neutrophils, macrophages and lymphocytes, produce powerful pro-inflammatory mediators, especially TNF-a, IL-1, IL-6, and IL-8. At the same time, acute-phase proteins (such as C-reactive protein) are produced, and humoral defense mechanisms such as the complement system are activated, resulting in production of pro-inflammatory mediators, including C5a, the complement split product, which ultimately enhances cytokine and chemokine production. Furthermore, the coagulation system becomes activated through various mechanisms, often resulting in disseminated intravascular coagulopathy (174, 177). The hallmarks of sepsis are excessive inflammation, excessive coagulation and suppression of fibrinolysis.

When a patient enters septic shock, blood levels of TNFα rise. TNFα can contribute to Multi-Organ Dysfunction Syndrome (MODS), and administration of an anti-TNF-alpha antibody increases survivability after septic shock by reducing the symptoms of MODS (180). Exposure to TNFα or LPS will cause MARCKS to become highly expressed and also become myristoylated. (175). Interestingly, approximately 90% of all new protein synthesis in neutrophils after exposure to

TNFα is MARCKS protein, indicating that MARCKS is an important part of the immune response to TNFα.

MicroRNA

Definition and Post-transcriptional Processing

MicroRNAs or miRNAs are fairly short strands of non-coding RNA. They are typically only 21–25 nucleotides in length. They serve an important regulatory role in proliferation (181), differentiation (182), development, migration (183, 184), angiogenesis (185), apoptosis (186) and carcinogenesis (187). They are created from cleavage of a much larger precursor, which can be up to a few kilobases in length. An important feature of the precursors is a stem loop structure with imperfect base pairing. Typically the final micro RNA is part of one strand of this hairpin. In humans and other mammals, MicroRNAs bind to the 3′ untranslated region of their target gene forming an imperfect complement. This serves to act as a repressor of translation. If they pair imperfectly with their target, they can physically prevent the RNA for entering the ribosome. Alternately, if they pair perfectly with their target, they can form a double stranded RNA complex that can be targeted by the immune system. The double stranded RNA mimics the genetic material of some viruses, such as rotavirus, and the immune system is programmed to recognize the strands and break them down. It is worth noting that a single miRNA will have multiple gene targets and that one mRNA may be targeted by more than one miRNA.

There are two post-transcriptional steps which are necessary to create mature miRNA. This involves being cleaved twice by two different types of RNaseIII,

Drosha and Dicer, which cleave the precursor into the final/active microRNA. Drosha is located primarily in the nucleus. It contains two tandem RNase-III domains, a dsRNA binding domain and an amino-terminal segment of unknown function (188, 189). Regardless of the diverse primary sequences and structures of pri-miRNAs, Drosha cleaves these into ~70-bp pre-miRNAs that consist of an imperfect stem-loop structure. The original RNA (pri-miRNA) is cleaved into ~70 nucleotide precursor (pro-miRNA) by Drosha while the pri-mRNA is still in the nucleus. This pro-miRNA has a stem loop structure where the miRNA is part of one strand of the loop. It will then translocate into the cytoplasm. The second step of processing is cleavage by Dicer, which will create a mature miRNA ~21-25 nucleotides in length (190).

Both Dicer and Drosha contain two RNase III domains. In order for a RNase III to cleave RNA, it must fold to dimerize its two RNase III domains in order to have a functional catalytic site. Crystallographic modeling of RNase III indicates that a dimer is necessary to create an active enzymatic site (191). Drosha was the first human RNase III enzyme identified and cloned. The sequential processing of miRNA maturation requires a precise mechanism of target identification, but at this point the mechanism by which Drosha identifies pri-miRNA remains unknown.

Also, the methods by which pri-miRNAs transcription is regulated remain mostly unknown. A number of pri-miRNA genes are located within introns of genes that code for protein and others are located on non-coding RNA. It is likely that these genes have some level of transcriptional regulation. Those located in an intron would be subject to the same transcriptional regulation as the host gene with

its promoters and might therefore be transcriptionally regulated through their host-gene promoters. Also some miRNAs are clustered into polycistronic transcripts, or one messenger RNA that contains more than one open reading frame indicating that these miRNAs are regulated as a group (186).

After being cleaved to their active size, miRNAs will dissociate from their complementary strand, become linear, and assemble into a ribonucleoprotein complex (miRNP) also called an effector complex. One type that targets mRNA for degradation is the RNA-induced silencing complex, or RISC. RISC is composed of proteins and ribonucleic acids. One strand of micro RNA (miRNA) or small interfering RNA (siRNA) is bound into this complex. The complex facilitates identification of a complementary mRNA. Upon binding, the RNase is activated and the mRNA is cleaved. This is a conserved mechanism also used by cells as a defense against viruses, which use dsRNA as their genetic material (192). The mature miRNA will bind to a heterodimer of Dcr-2 and R2D2 protein forming the initiator complex. The 5'end of the miRNA interacts with complex of Dicer-2 bound to HIV-1 TAR RNA binding protein (TRBP). TRBP is required to recruit Agronaute 2 to the miRNA. Agronaute is the molecule responsible for cleavage of the target mRNA while retaining the miRNA intact. It has two(193) domains: the PIWI, which contains the endonuclease, and PAZ, which contains a groove for substrate binding. (188) Agronaute 2 protein activates the pre-RISC complex, which will recruit additional proteins to make and activate RISC (189).

Another ribonucleoprotein complex that affects gene expression is the RNA-induced Transcriptional Silencing (RITS) complex. The RITS complex works co-

transcriptionally. It comes into contact with a specific sequence of RNA that has begun to be transcribed. The complex methylates the proximal histones, causing condensation of DNA by heterochromatin. This causes the gene to be downregulated pre-transciptionally (194, 195).

Mir-21

In humans, the pri-miR-21 is encoded by the *MIR21* gene. After processing, the mature RNA is called miR-21. A high level of evolutionary conservation is typical with miRNAs, and this gene is conserved exactly between different groups of mammals. Mir 21 is mapped at chromosome 17q23.2, and is present within the protein-coding gene VMP1 (or TMEM49). miR-21 and VMP1 are independently regulated (196) (201). The nuclear factor Activation Protein 1 (AP1) has been shown to cause the pri-miR-21 gene to be transcribed. AP-1 is continually activated through a double-negative feedback mechanism. Nuclear Factor I (NFI) functions as a negative regulator of the mir-21 promoter, miPPR-21. CCAAT-enhancer-binding proteins (C/EBP) have also been shown to function as a negative regulator of miPPR-21. (196) (201). miR-21 is one of the most extensively studied micro RNAs, being associated with cancer and inflammation, described in detail below.

Mir-21 in Cancer

Mir-21 has been shown to target many tumor suppressor genes. Targets include PTEN (197), PDCD4 (198), Tropomyosin (199), TGFBRI (200), RhoB (194), Bcl2 (201), IL-12 (202) and CDK2AP1 (203) as well as many others. MiR-21

downregulates the tumor suppressor Programmed cell death protein 4 (Pdcd4). It has also been shown to stimulate invasion, intravasation (invasion through the basal membrane) and metastasis in colorectal tumors (204).

VMP1 is the homologue in humans to the rat vacuole membrane protein (196). In non-small cell lung cancer, VMP1 is only observed in cancerous cells and it has a positive correlation with the clinical stage of disease (205). Upregulation of mir-21 has been shown to lead to cell proliferation, while downregulation causes less proliferation and an increase in rates of apoptosis (206). Additionally, the 17q chromosomal region is amplified in many cancers including prostate (191) and breast (207). It also occurs in about half of medulloblastoma patients (195) and frequently in Hodgkin's lymphoma (204). However, the genomic locus encoding miR-21 is not amplified in many cancers, which still express very high levels of miR-21, such as Glioblastoma multiforme (GBM) and B-cell chronic lymphocytic leukemia (B-CLL) (208). There is no definitive correlation between the increased expression of mir-21 and the amplification of the genomic locus of mir-21. Deregulation in the expression of mir-21 may occur at either the transcriptional or the post-transcriptional level or possibly both. Mir 21 is also located in the fragile site FRA17B, which is one of the sites of integration for Himan Papiloma Virus HPV16 (219). Infection with HPV 16 or 18 is one of the major risk factors for cervical cancer. HPV integrates into the host cell genome, causing both genetic and epigenetic changes. This suggests that HPV may be having an effect on the expression of mir-21, which is upregulated in cervical carcinoma (209).

miR-21 also has been shown to modulate apoptosis of endothelial cells and

vascular constriction (204). Some of the predicted targets of mir-21 are in the TGF-beta pathway such as TGFBR2, TGFBR3 and DAXX. TGF-ß signaling has been associated with inducing apoptosis in cancer cells. TFGBR2 can activate the SMAD3/4 pathway leading to apoptosis and TGFBR3 activates the Death-associated protein 6 (DAXX) pathway, activating the JNK pathway, and activating the transcription factor AP-1. This pathway also leads to apoptosis (210). When activated, TGF-ß receptors bind to ß2-adaptin, which activates AP-2 (157), and AP-2 activity has been shown to inhibit the growth of cancer cells and lead to activation of p21, leading in turn to apoptosis. MiR-21 expression has been associated with increased mortality in patients with colon adenocarcinoma (197), and Mir-21 expression is elevated in leukemias, lymphomas and solid tumors (196). MiR-21 has been shown to target genes related to the TGF-ß pathway, such as TGFBR2, TGFBR3, and DAXX, in glioblastoma cells (211). Mir-21 also decreases apoptosis and increases eNOS and NO production in endothelial cells (212).

Mir-21 in Inflammation

Many types of lung cancer bear resemblance to pulmonary fibrosis since they have a highly fibrotic phenotype. Mir -21 plays an important role in T cells, as it is expressed highly in both effector and memory T cells. It is further upregulated in activated CD4 T cells (198). Micro RNAs are thought to play a role in asthma, in which miRNAs could be involved in the changes in gene and protein expression within the lungs which occur in this disease (213). In the asthma sensitivity gene HLA-G, a single nucleotide polymorphism at the 3′ untranslated region affects the

binding of three miRNAs (214). MiR-21 inhibits the TGF-beta signaling pathway, which is known to inhibit adipogenesis and stimulate inflammation (199). Mir-21 specifically targets TGF-beta 1 and 2 and TGFß receptors (211). Upregulation of mir-21 causes cell proliferation; downregulation allows cell to stop dividing and or undergo apoptosis. Mir-21 is a trigger for fibroblast dysfunction and fibrosis and is upregulated in cardiac infarctions (200).

Mir-21 is also upregulated in individuals with idiopathic pulmonary fibrosis (193). Mir-21 is upregulated in the lungs of mice with bleomycin fibrosis, and pretreatment of these mice with antisense peptides to mir-21 diminished the severity of fibrosis. The possible targets for mir-21 in fibrosis are Smad7, an inhibitory Smad, which is an important regulator of TGF-beta (215). During fibrosis there is an increase in fibroblasts and collagen. Some of these fibroblasts derive from other fibroblasts, but others are thought to derive from epithelial cells in a process called Epithelial Mesenchymal Transition (EMT). During this process, epithelial cells become undifferentiated and lose their polarity due to disappearance of differentiated junctions, the rearrangement of organelles and the reorganization of the cytoskeleton (201). EMT causes cells to regain some of the properties of stem cells such as increased CD44 and decreased CD24 (216). Later these cells can divide and differentiate into mesenchymal cells and then fibroblasts. EMT also occurs in cancers, as EMT is inducible by collagen I, and TGF-beta signaling in non–small cell lung cancer cell lines (196). Mir-21 prevents Smad7 from being made, which stops TGFß from being inhibited. This allows Smad 3 to become activated, increasing collagenase activity and ultimately leading to increased deposition of collagen (212).

Mir-21 has been shown to directly target MARCKS and increases resistance to apoptosis and promotes invasion in prostate cancer cell lines. Mir-21 binds MARCKS in the 3' untranslated region from the nt713-734, which is highly conserved between species. Treatment with siRNA analogous to mir-21 caused a decrease in cell motility in pancreatic cancer cells (202). Currently there is little known about direct interactions between mir-21 and MARCKS, however, significant work has been done using siRNA to the MARCKS gene. Treatment with siRNA to the MARCKS gene has been shown to inhibit the secretion of nuerotensin, decrease cell adhesion, spreading, and invasion (203).

The previous research indicates that MARCKS is directly targeted by mir-21. In airway epithelial cells, modulating the level of mir-21 should regulate the amount MARCKS transcribed thereby lowering the amount of MARCKS protein present in the cells. Reducing the amount MARCKS protein in these cells should lead to decreased mucin secretion.

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CHAPTER 2

CALPAIN AND MARCKS PROTEIN REGULATION OF AIRWAY MUCIN SECRETION

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Abstract

Hypersecretion of mucin plays an important role in the pathophysiology of many inflammatory airway diseases, including asthma, chronic bronchitis, and cystic fibrosis. Myristoylated alanine-rich C-kinase substrate (MARCKS) protein has been shown to play an important role in regulation of airway mucin secretion, as peptides analogous to the amino (N)- terminus of MARCKS attenuate mucin secretion by airway epithelium *in vitro* and *in vivo*. Here, we investigated a potential role for the protease *Calpain*, a calcium-dependent cysteine protease that can cleave MARCKS, in the MARCKS-related secretory mechanism. We theorized that Calpain might cleave MARCKS near the N-terminus, thereby attenuating the ability of MARCKS to bind to membranes and/or creating a small N-terminal peptide that could act as a competitive intracellular inhibitor to remaining endogenous full-length MARCKS molecules. Primary normal human bronchial epithelial (NHBE) cells and the virally-transformed human bronchial epithelial HBE1 cell line were exposed to phorbol-12-myristate-13-acetate (PMA) to stimulate the Protein Kinase C (PKC) pathway, leading to enhanced mucin secretion, and Calpain activity within the cells was measured with a fluorescent cleavage assay. Calpain activity was increased by PMA, and pretreatment of the cells with Calpain inhibitors reduced both Calpain activity and mucin secretion in a concentration-dependent manner. Thus, as opposed to the original hypothesis, inactivating Calpain caused a decrease rather than an increase in secretion. HBE1 cells transfected with DNA constructs encoding a MARCKS-YFP fusion protein showed cleavage at a putative site near the N-terminus in response to PMA. Cleavage of MARCKS by Calpain may have an

important role in regulation of the PKC/MARCKS pathway regulating airway mucin secretion.

KEY WORDS: Airway, Mucin, MARCKS, Calpain, Secretion

1. Introduction

Hypersecretion of mucus plays an important role in the pathophysiology of many inflammatory airway diseases, as excessive mucus can obstruct airways, leading to inhibited respiration, enhanced susceptibility to infection, and even mortality. Mucus is a gel made up of water and mucins, which are complex glycoproteins 20 to 200kDa in size. They can be extensively post-translationally modified by myristoylation, glycosylation and/or phosphorylation, and their carbohydrate content may account for between 50-90% of their total mass. It is mucins that give mucus its viscosity and elasticity [1]. At least 20 different human mucins have been identified, and they are present in the respiratory, gastrointestinal, ocular and reproductive systems as either membrane-tethered or secreted. In the respiratory system, 11 different mucin genes have been identified at the protein or mRNA level, and the mucins MUC5AC, MUC5B, MUC19 and MUC2 are secreted by both goblet cells and submucosal glands, with MUC5AC and MUC5B predominating [2].

Myristoylated alanine-rich C-kinase substrate (MARCKS) protein, specifically the evolutionarily-conserved N-terminal region, has been shown to have an important role in airway mucin secretion, as peptides analogous to the N - terminus of MARCKS attenuate mucin secretion by airway epithelial cells both *in vitro* (72)] and *in vivo* (87)]. MARCKS can be activated by PKC, especially the delta isoform, in airway epithelium (147, 210)]

Calpain is a calcium-dependent cysteine protease lacking a specific primary sequence for cleavage; it is thought to have site recognition based on tertiary

structure. Interestingly, Calpain has been shown to cleave MARCKS protein, although the location(s) of the cleavage site(s) are still uncertain [12,13]. Reducing the expression of Calpain genes leads to accumulation of MARCKS in cultured myogenic cells [14]. (211)Calpain cleavage may increase accessibility of the phosphorylation site domain (PSD) on MARCKS, thereby increasing its ability to bind actin [15]. This cleavage site is possibly the same site identified between asparagine 147 and glutamate 148 in the bovine sequence of MARCKS, only three amino acids away from the amino-terminal side of the PSD (212)]. It also has been speculated that MARCKS may be cleaved between the 6th and 7th amino acid from the N-terminus (212)].

A convergent point between MARCKS and Calpain activation in these cells could be phospholipase C, a common cellular signaling pathway. Activated phospholipase C will cleave phosphatidylinositol into diacylglycerol (DAG) and inositol triphosphate (IP3). DAG can then go on to activate PKC, which phosphorylates MARCKS, while IP3 binds to specific receptors on endoplasmic reticular membranes, resulting in release of intracellular stores of calcium into the cytoplasm, which may activate Calpains (along with other calcium-sensitive molecules) [16].

In the studies described here, a possible role for Calpain in modulating the MARCKS-related mechanism of airway mucin secretion was investigated, utilizing cultured primary human airway epithelial cells and a virally – transformed human airway epithelial cell line. The results suggest that inhibition of Calpain in these cells decreases mucin secretion in response to PKC activation, and that MARCKS

might be cleaved near its N-terminus by Calpain during the secretory event. The exact role of Calpain in the MARCKS-related secretion mechanism, however, remains speculative.

2. Materials & Methods

2.1. Cell Culture

Primary normal human bronchial epithelial (NHBE) cells, purchased from Lonza (Walkersville, MD), and human papilloma virus-transformed bronchial epithelial cells (HBE1 cells; a generous gift from Dr. Reen Wu, University of California, Davis, CA) [17] were seeded on Corning® Transwell® collagen-coated membrane inserts and maintained in a humidified air/5% CO2 incubator for 14 days in air-liquid interface culture, as described previously [3,18]. Two different cell types were used in these studies:HBE1 cells were used when experiments called for molecular manipulations (eg transfection) as the transfection efficiency is low (~ 5%) for primary NHBE cells and over 50% for HBE1 cells. When studies were complementary to these molecular manipulations, HBE1 cells were used for consistency. Studies on mucin secretion were performed using primary NHBE cells because these cells produce more mucin than the HBE1 cell line.

2.2. Treatments

NHBE or HBE1 cells were exposed to 250nM PMA (EMD Biosciences, La Jolla, CA) for 3 min to provoke mucin secretion. Two separate inhibitors of Calpain, Z-LLY-FMK (MBL International Corporation, Woburn, MA) or Z-LLY-CHO (Enzo Life Sciences, Farmingdale, NY) were added at 20 μM to cells for 15 min prior to addition of PMA (or medium control) and Calpain activity or mucin secretion after exposure

to PMA for the indicated time periods measured as described below. All reagents were applied both apically and basolaterally.

2.3. Calpain Activity Assay

After exposure of cells to PMA (or control) for 30 min, cells were assayed for Calpain activity using a Calpain Activity Assay Kit (Abcam, Cambridge, MA) according to the manufacturer's suggestions. Cell lysates were exposed to substrate bound to AFC fluorophore (7-Amino-4-trifluoromethylcoumarin). Calpain cleaves the substrate, releasing the AFC fluorophore and allowing it to fluoresce. Relative activity of Calpain vs. a standard was then determined using a fluorescent plate reader.

2.4. Measurement of Mucin Secretion

Mucin was collected and assayed as described previously [3]. Briefly, after "baseline" mucin samples were collected, cells were rested overnight and exposed to test reagents the next day for indicated times. After each treatment period, secreted mucin was collected as the treatment sample and quantified by sandwich enzyme-linked immunosorbent assay using the 17Q2 antibody (Covance Research Products, Berkeley, CA), a monoclonal antibody that reacts specifically with a carbohydrate epitope on human airway mucins [19]. The 17Q2 antibody was purified using an ImmunoPure(G) IgG purification kit (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol and then conjugated with alkaline phosphatase (EMD

Biosciences). To account for variability between cultures and experiments, levels of mucin secretion were reported relative to non-treated controls, as described previously [3].

2.5. Transfections

HBE1 cells were grown to approximately 50% confluence and transfected with plasmids containing yellow fluorescent protein (YFP) fused to the N-terminus of full length human MARCKS. FuGENE® 6 (Roche, Indianapolis, IN) was used as the transfection reagent according to the manufacturer's protocol. Forty-eight hrs later, cells were exposed to either 100 uM ATP (a potent activator of Calpain), or control media, for 3 min. Cells were lysed and protein was extracted in buffer containing Complete Mini Protease inhibitor (Roche).

2.6. Western Blot Analysis

Briefly, cells were washed with cold PBS and scraped into lysis buffer [50 mmol/L Tris-Cl (pH 7.6), 1mmol/L ethylenediamine tetraacetic acid, 100 mmol/L NaCl, 100 mmol/L MgCl,1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 1% (v/v) protease inhibitor cocktail, and phosphatase inhibitor cocktail (Sigma, St. Louis, MO). Western blots used a primary antibody against GFP (which cross reacts with YFP in the constructs) followed by biotinylated goat anti-mouse secondary (both from Abcam) and then Streptavidin HRP (Santa Cruz, Santa Cruz, CA) After these blots were performed, they were normalized to actin using an antibody from Santa Cruz,

2.7. Statistical Analysis

Graphpad Prism® version 5 was used for the statistical analysis. A one way analysis of variation (ANOVA) was used in the time course study of PMA treated HBE1 cells. Student's T-test was used for the remainder of the experiments. A p value < 0.05 for the two-tailed t test was considered statistically significant.

3. Results

3.1. Effects of PMA on Calpain Activity

An approximate twofold increase in Calpain activity compared to untreated cells was observed in HBE1 cells exposed to 250nM PMA within a two min period post exposure. This activity appeared to plateau and remained elevated for at least 10 min (Figure 1).

3.2. Effects of Calpain Inhibitors on Calpain Activity

Pretreatment with 20 μM of the commercially available Calpain inhibitors Z-LLY-FMK or Z-LLY-CHO for 15 minutes significantly decreased the PMA-induced enhancement of Calpain activity in HBE1 cells (Fig 2).

3.3. Effects of Calpain Inhibitors on Mucin Secretion

As illustrated in Figure 3, pretreatment of NHBE cells for 15 min with 20 μM of either Z-LLY-FMK (FMK) or Z-LLY-CHO (CHO) significantly attenuated mucin secretion in response to exposure of the cells to 250nM PMA. When NHBE cells were pretreated for 15 min with CHO over a range of concentrations (0.2 - 20 μM), there was a concentration-dependent attenuation of mucin secretion in response to PMA measured 3 min after PMA exposure (Figure 4).

3.4. Western Blot Analysis of Cells Transfected with MARCKS Constructs

HBE 1 cells transfected with the YFP-labeled MARCKS showed, upon Western blot of lysed cells, enhanced induction of potential cleavage products after ATP exposure

and thus presumptive Calpain activity. As shown in Figure 5, these cleavage products were observed at a molecular weight of approximately 27-32 kDa, suggesting that Calpain cleaves MARCKS near the YFP-tagged amino terminus.

Discussion

In this study, Calpain was shown to be activated in response to PMA in human airway epithelial cells in vitro. In addition to its previously-described secretagogue effect on airway mucin secretion [3,5], PMA also activates a number of PKC (typical and atypical) channels, which could then open calcium channels in the plasma membrane, with the resultant increase in intracellular calcium potentially activating Calpain proteases. The increased activity of Calpains in response to PMA in airway epithelial cells was effectively blocked by pretreatment of cells with Calpain inhibitors, and, interestingly, pretreatment with these Calpain inhibitors caused a decrease in PMA-provoked secretion of mucin. It is interesting to note that mucin secretion in cells treated with FMK (see Figure 3) was attenuated to a level below that seen in control cells. This is a phenomenon that occurs often in cells exposed to strong inhibitors of various molecules involved in the secretory process, as was seen with MARCKS – inhibitory peptides both in vitro and in vivo in previous studies [3,6]. This would indicate that Calpain could be involved in both stimulated and constitutive mucin secretion by human airway epithelial cells. Of the two commercially – available inhibitors, FMK appears to be the more potent in attenuating both Calpain activity mucin secretion, as equimolar concentrations appeared to have a greater inhibitory effect on both parameters (see Figures 3 and 4). In extracts of HBE1 cells, which were transfected with a fusion protein of MARCKS with YFP joined to the N-terminus, a band possibly corresponding to a protein fragment where MARCKS has been cleaved near the N-terminus was

present in Western Blots probed for GFP/YFP. This fragment could be the result of Calpain cleaving MARCKS near the N-terminus, possibly at a putative cleavage site between Lysine 6 and Threonine 7 [12].

MARCKS is a highly conserved and ubiquitously expressed protein. It has been shown to have major roles in, for example: secretion, including airway mucin secretion [3-11], cellular migration [20-24] brain development [25], cell adhesion [14], phagocytosis [26], and cell cycle regulation [27]. While precise mechanisms for these diverse functions remain unkown, MARCKS is known to interact with cytoskeletal components and can bind and crosslink actin filaments within cells [28]. Structurally, MARCKS contains three evolutionarily – conserved sites: a multiple homology (MH2) region of unknown function, an effector or phosphorylation site domain (PSD) that serves as the site for PKC phosphorylation as well as binding of actin and calmodulin, and a myristoylated, hydrophobic N-terminal domain associated with binding of MARCKS to the plasmalemma and other intracellular membranes.

In elucidating the function of MARCKS, the N-terminus recently has received much interest. This highly conserved domain has been shown to have an important role in airway mucin secretion based on inhibition of secretion, both in vitro and in vivo, by a peptide, the MANS peptide, which corresponds to the conserved myristic acid + 24 amino acid - containing N-termina l region of MARCKS [3]. Although the exact nature of the inhibition of MARCKS function in the secretory process by the MANS peptide is uncertain, it may act as a competitive inhibitor for endogenous,

full length MARCKS, preventing MARCKS from interacting with membranes of secretory granules as part of the mucin secretion process [6].

Calpains are calcium-activated cysteine proteases. Calpains 1 and 2 (known as μ- and m-calpain, respectively, from the relative molarity of calcium needed to activate them) are ubiquitously expressed. Calpains do not cleave based upon primary sequence of their substrates. Rather, they are thought to recognize their cleavage sites based upon the higher order structure of the substrate. Calpain 1 has been demonstrated to cleave MARCKS on the N-terminal side of the PSD in a manner dependent on the activity of PKC phosphorylation of MARCKS. MARCKS cleaved at this site appears only in cytoplasm, as MARCKS bound to the cell membrane does not appear susceptible to Calpain cleavage (213)]. MARCKS and Calpain 2 have been shown to co-localize in caveolae, and Calpain can indirectly affect MARCKS phosphorylation by modulating PKC activity and thereby affecting MARCKS binding to membranes and translocation to the cytosol after it is phosphorylated (210)].

The original hypothesis behind these studies was that Calpain-induced cleavage of MARCKS protein would inactivate MARCKS, therefore increased Calpain activity would increase secretion of mucin in airway epithelial cells. However, the results appeared to be opposite of what was hypothesized; inactivating Calpain caused a decrease in secretion. If, as it seems, cleavage of MARCKS by Calpain occurs at the predicted site between the 6th and 7th amino acid of the N-terminus, as has been suggested [2], this cleavage would separate the bulk of the molecule from its intensely hydrophobic end. Alternately, if the relevant site of MARCKS cleavage is

the other known site, close to the PSD, this could allow for more favorable access of the site to allow MARCKS to bind to actin, leading to increased actin crosslinking. How these factors affect the MARCKS-related mechanism of mucin secretion is not known, and clearly additional studies to determine the mechanism of Calpain-induced modulation of mucin secretion in these cells clearly are needed. For example, the exact site of Calpain cleavage of MARCKS needs to be determined, as does the role of cellular compartmentalization and intracellular localization of Calpain where it can affect MARCKS function in secretion. Other important information may relate to, for example, determining the specific Calpain gene(s) that are important in this function and/or determining other potential cellular targets of Calpain whose cleavage may be relevant to the secretory process. Clearly, there is a connection between Calpain, MARCKS and mucin secretion, but at this point this connection has not been fully elucidated.

It also should be pointed out that these studies were done with both a cell line (HBE1 cells) and primary human bronchial epithelial cells (NHBE) maintained in air/liquid interface culture to maintain their differentiated characteristics. In order to elucidate more about the role of Calpain in airway mucin secretion, it is clear that *in vivo* studies using physiologically – relevant stimuli for mucin secretion in animal models would be an appropriate next step. In fact, most of our work in the literature related to mechanisms of MARCKS function in secretion were first reported using these *in vitro* approaches [3-5, 10, 11] and later expanded to animal studies [6- 9].

In conclusion, the results of this study show that Calpain is activated in human airway epithelial cells by exposure to PMA, suggesting that PKC increases

Calpain activity by raising intracellular Ca^{++}; treatment of airway epithelial cells with commercially available Calpain inhibitors reduces Calpain activity in these cells; inhibiting Calpain activity appears to attenuate mucin secretion, and a MARCKS YFP fusion protein transfected into airway epithelial cells appears to be cleaved at a site near the N-terminus, possibly at a suspected Calpain cleavage site.

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Figure Legends

Figure 1. PMA exposure increases Calpain activity in HBE1 cells. Relative levels of Calpain activity in HBE1 cells after stimulation with 250nM PMA are shown. Label on X axis (time) refers to time post PMA addition. Calpain activity is significantly increased within 2 min of exposure to PMA, and this increase plateaus for at least 10 min. (* $p<0.05$ relative to 0 time control, all values expressed as mean ± SEM, n=3).

Figure 2. Calpain inhibitors decrease Calpain activity in airway epithelium. HBE1 cells were exposed to 250nM PMA for 3 min after 15 min pretreatment with 20 μM of the Calpain inhibitors Z-LLY-FMK or Z-LL-CHO (* $p<0.05$ † $p<0.005$ relative to PMA – stimulated, all values expressed as mean ± SEM, n=3).

Figure 3. Pretreatment of NHBE cells with Calpain inhibitors attenuates mucin secretion. Relative levels of mucin secretion in NHBE cells after treatment with 250nMPMA with or without pre-treatment with Calpain inhibitors: 20μM Z-LLY-CHO (CHO) or 20μM Z-LLY-FMK (FMK) for 15 min. Secretion was measured 3 min after PMA addition. († $p<0.01$, †† $p<0.001$ relative to PMA- stimulated, all values expressed as mean ± SEM, n=4).

Figure 4. Pretreatment of NHBE cells for 15 min with the Calpain inhibitor Z-LL-CHO (CHO) attenuates mucin secretion in response to 250nMPMA in a concentration-dependant manner. Secretion was measured 3 minutes after PMA

addition and is normalized to media control. (* $P < 0.05$ relative to PMA-stimulated, all values expressed as mean ± SEM, n=3).

Figure 5. Western Blot probing for YFP (using antibody against GFP) in HBE1 cells transfected with YFP fused to the N-terminus of MARCKS. A) the entire blot; B) the area of the entire blot underneath the dotted line in "A"; this region was heavily exposed to bring out blot details; C) actin used for normalization. Lanes 1,2: MARCKS constructs; Lanes 3,4: Empty Vector. Treatment of cells for 3 min with 100 μM ATP (B; lane 2) shows apparent induction of MARCKS cleavage products of molecular weight 27 – 32 kDa (indicated by arrows) presumably near the YFP-tagged amino terminus of the transfected MARCKS construct.

Figure 1

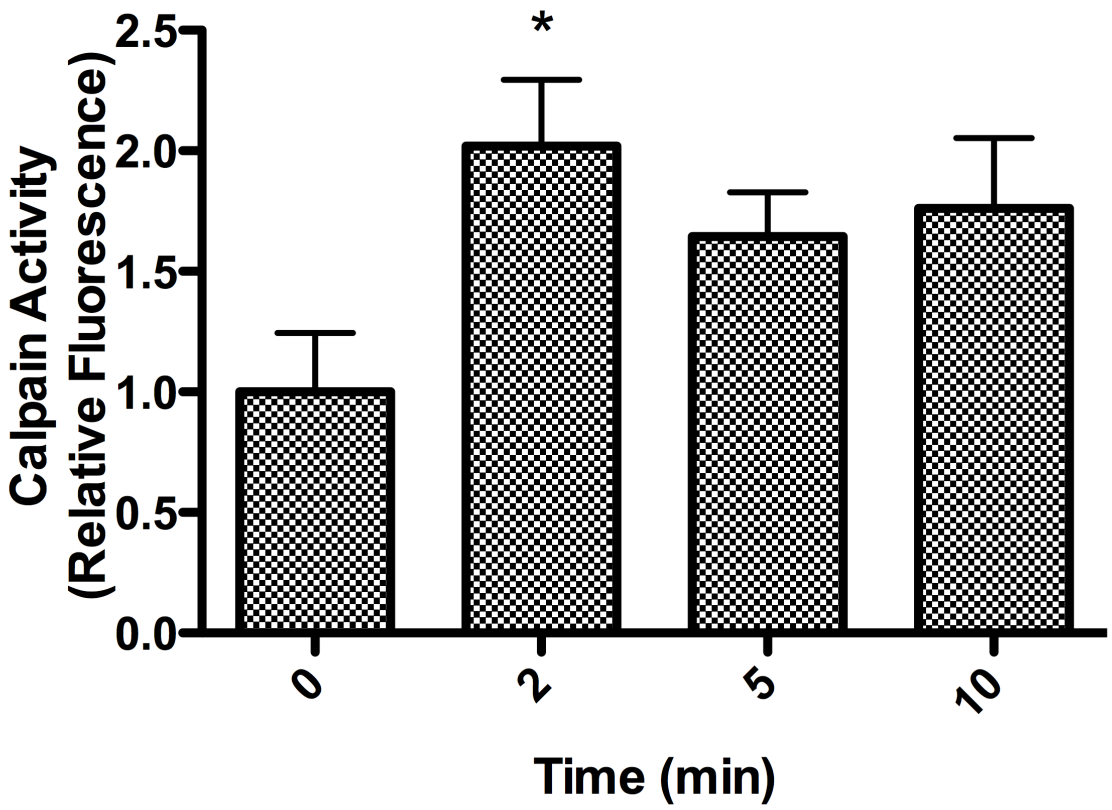

Calpain Activity
(Relative Fluorescence)
2.5
2.0
1.5
1.0
0.5
0.0
*
0
2
5
10
Time (min)

Figure 2

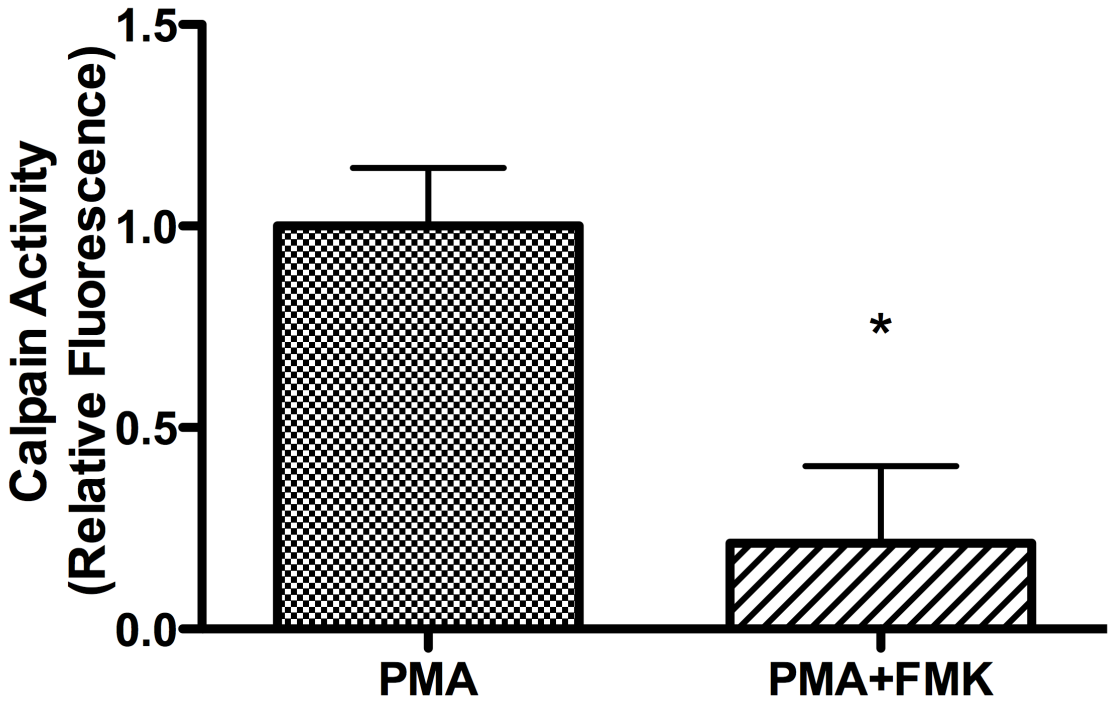

Calpain Activity
(Relative Fluorescence)
1.5
1.0
0.5
0.0
*
PMA
PMA+FMK

Figure 3

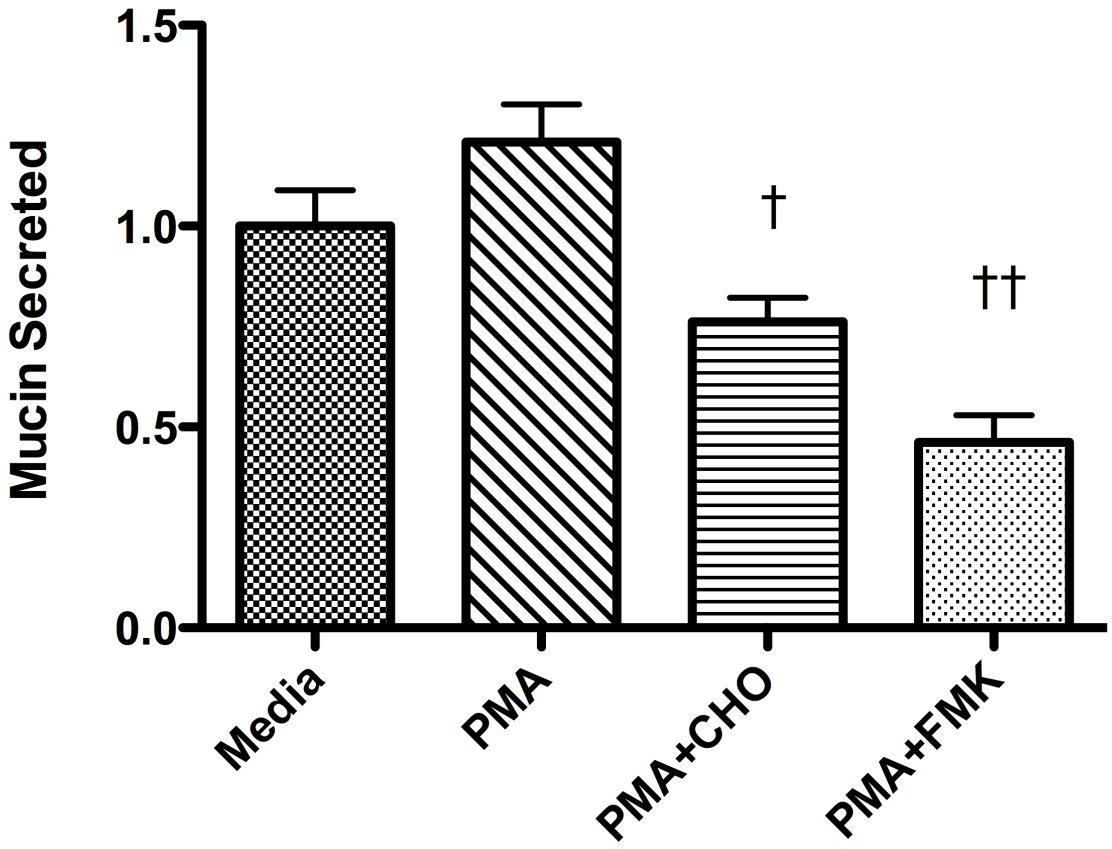


Figure 4

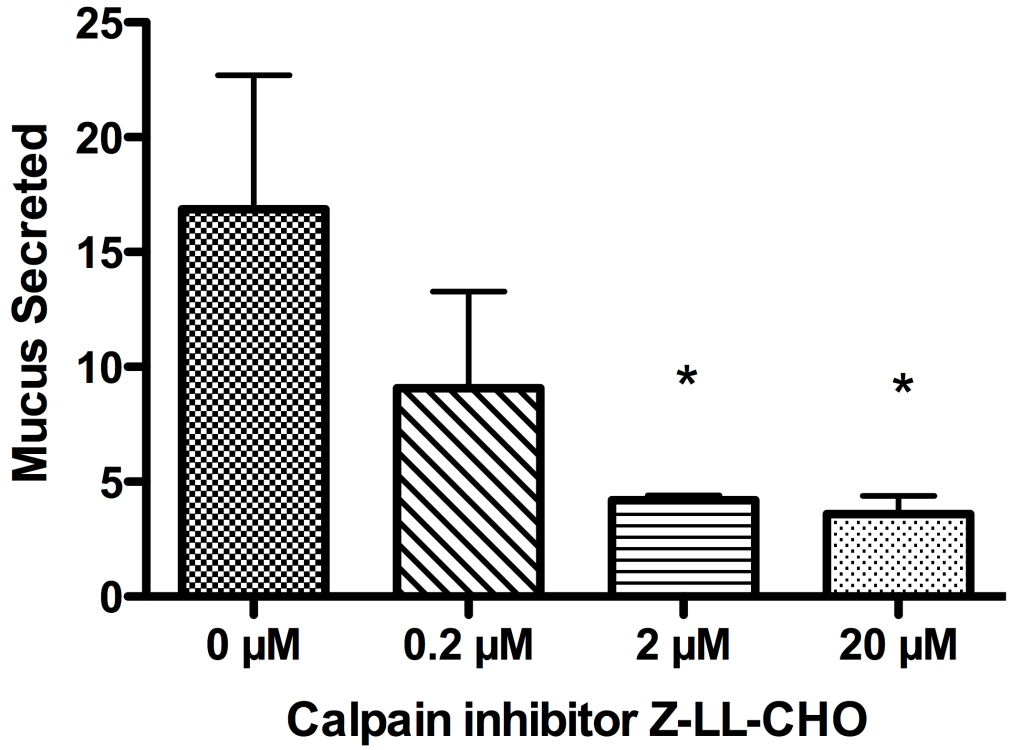
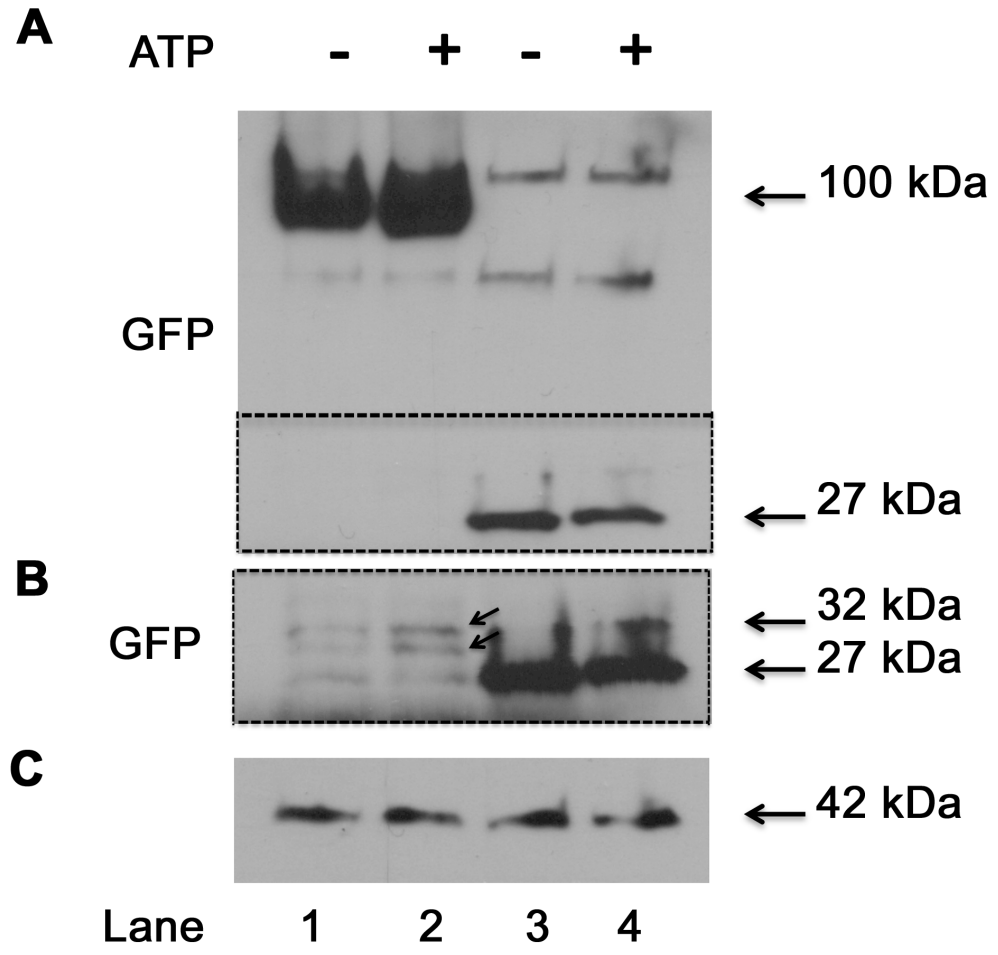

25
20
15
10
5
0
Mucus Secreted
*
*
0 µM
0.2 µM
2 µM
20 µM
Calpain inhibitor Z-LL-CHO

Figure 5



CHAPTER 3

MiR-21 REGULATION OF MARCKS PROTEIN AND MUCIN SECRETION IN AIRWAY EPITHELIAL CELLS

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Abstract

Hypersecretion of mucus characterizes many inflammatory airway diseases, including asthma, chronic bronchitis, and cystic fibrosis. Excess mucus causes airway obstruction, reduces pulmonary function, and can lead to increased morbidity and mortality. MicroRNAs are small non-coding pieces of RNA which regulate other genes by binding to a complementary sequence in the target mRNA. The microRNA miR-21 is upregulated in many inflammatory conditions and, interestingly, miR-21 has been shown to target the mRNA of Myristoylated Alanine-Rich C Kinase Substrate (MARCKS), a protein that is an important regulator of airway mucin (the solid component of mucus) secretion. In these studies, we determined that exposure of primary, well-differentiated, normal human bronchial epithelial (NHBE) cells to the pro-inflammatory stimulus lipopolysaccharide (LPS) increased expression of both miR-21 and MARCKS in a time-dependent manner. To investigate whether miR-21 regulation of MARCKS played a role in mucin secretion, two separate airway epithelial cell lines, HBE1 (papilloma virus transformed) and NCI-H292 (mucodepidermoid derived) were utilized, since manipulation of miR-21 is performed via transfection of commercially-available miR-21 inhibitors and mimics/activators. Treatment of HBE1 cells with LPS caused concentration-dependent increases in expression of both miR-21 and MARCKS mRNA and protein. The miR-21 inhibitor effectively reduced levels of miR-21 in the cells, coincident with an *increase* in MARCKS mRNA expression over time as well as enhanced mucin secretion, while the miR-21 mimic/activator increased levels of miR-21, which coincided with a *decrease* in expression of MARCKS and a decrease in

mucin secretion. These results suggest that miR-21 is increased in airway epithelial cells following exposure to LPS, and that miR-21 downregulates expression of MARCKS, which may decrease mucin secretion by the cells. Thus, miR-21 may act as a negative feedback regulator of mucin secretion in airway epithelial cells, and may do so, at least in part, by downregulating expression of MARCKS.

Introduction

Hypersecretion of mucus characterizes many inflammatory airway diseases including asthma, chronic bronchitis, and cystic fibrosis. Excessive mucus can obstruct airways, inhibit respiration, increase susceptibility to infection, and lead to increased morbidity and mortality. Mucus is a gel made up of water and mucins, large complex glycoproteins) that are post-translationally modified by myristoylation, glycosylation and/or phosphorylation. Mucins provide mucus with its viscosity and elasticity (23). At least 20 different mucins have been discovered in humans, 11 of which have been identified in the lungs. MUC5AC and MUC5B are the most prominent types in the airways (214).

Myristoylated alanine-rich C-kinase substrate (MARCKS) protein is a ubiquitous Protein Kinase C (PKC) substrate that has been shown to play an important role in regulation of mucin secretion by airway epithelium *in vitro* (72, 86, 210) and *in vivo* (87, 88). The evolutionarily-conserved N-terminal region of MARCKS (87) is clearly involved in this action, as peptides analogous to the MARCKS N-terminus attenuate mucin secretion in airway epithelial cells both *in vitro* (72) and *in vivo* (87).

MicroRNAs are small non-coding pieces of RNA typically about 22 bases long. They regulate other genes binding to a complementary sequence in the 3′-untranslated region of the target mRNA. microRNAs serve an important regulatory role in proliferation (181), differentiation (182), development, migration (183) angiogenesis (185), apoptosis (100, 215) and carcinogenesis (182). The micro RNA

miR-21 has been shown to target many tumor suppressors and it is upregulated in many types of cancers and in various inflammatory conditions (186, 192, 201, 202, 205, 207). Interestingly, miR-21 has been shown to specifically target the mRNA of MARCKS (208).

Given these associations, we investigated whether or not miR-21 could be involved in mucin secretion by airway epithelial cells in response to the proinflammatory stimulus, LPS, and, if so, whether miR-21 regulation of MARCKS could be part of the mechanism. The results indicate that: a) treatment of well-differentiated primary normal human bronchial epithelial (NHBE) cells with LPS derived from *E. coli* provoked time-dependent increases in expression of both miR-21 and MARCKS; b) LPS treatment caused a similar increase in expression of both miR-21 and MARCKS in the virally-transformed HBE1 human airway epithelial cell line; c) Inhibition of miR-21 via transfection of a miR-21 inhibitor after LPS treatment increased expression of MARCKS coincident with an increase in mucin secretion in another human airway epithelial cell line, NCI-H292 cells (derived from a mucoepidermoid carcinoma); d) Activation of miR-21 via transfection of a mimic/inhibitor decreased expression of MARCKS and decreased LPS-provoked mucin secretion in these cells; and e) Inhibition of MARCKS protein with a peptide identical to the MARCKS N-terminus inhibited mucin secretion in these cells regardless of treatment. Thus, it appears that miR-21 may play an important role as a negative feedback regulator of MARCKS expression and mucin secretion following inflammatory stimulation in airway epithelial cells.

Materials and Methods

Cell Culture

Well-differentiated NHBE cells from two separate donors were utilized for the initial studies. NHBE cells were purchased from Lonza corporation (Walkersville, MD), and grown and maintained in air/liquid interface as described previously (216) until, after approximately 18 days in culture, a well-differentiated epithelium was formed. After initial experiments indicated that expression of both miR-21 and MARCKS were enhanced by exposure of cells to lipopolysaccharide (LPS) from E. coli (**Figures 1 and 2**), studies to determine if there was a connection between miR-21 and MARCKS expression were performed using both a commercially-available inhibitor and an activator/mimic of miR-21 (described below) that required use of cells with a high transfection efficiency, so a human bronchial epithelial cell line, papilloma virus-transformed HBE1 cells (217); a generous gift from Dr. Reen Wu, University of California, Davis, CA) were used. HBE1 cells were cultured as previously described (218). In additional studies examining the effects of these reagents on airway mucin secretion, a second cell line, NCI-H292 cells (derived from a human pulmonary mucoepidemoid carcinoma; purchased from the American Type Culture Collection [ATCC, Manassas, VA) were chosen, as these cells have been used previously to study mucin production (219). RPMI 1640 + 10% FBS with penicillin/streptomycin and amphotericin added was the medium used and cells were maintained in a humidified air/5% CO2 environment until they reached ~70% confluence before they were transfected with the miR-21 inhibitor or mimic. Forty-

eight hrs post transfection, cells were exposed to a range of concentrations of LPS and responses related to miR-21 and MARCKS expression and function monitored, as described below.

MiR-21 Inhibitor and MiR-21 mimic

To alter miR-21 levels in these cells, we utilized both an anti-miR-21 inhibitor and a pre-miR-21 activator, both purchased from Ambion (Forster City, CA). MicroRNA inhibitors are small, chemically modified single-stranded RNA molecules designed to specifically bind to and inhibit endogenous miRNA molecules and enable miRNA functional analysis by down-regulation of miRNA activity and endogenous miRNA function after transfection into cells. For these studies, we utilized the *mirVana*® miRNA inhibitor containing the hsa-miR-21-5p sequence: UAGCUUAUCAGACUGAUGUUGA. In contrast, miRNA mimics are small, chemically modified double-stranded RNAs that mimic endogenous miRNAs and enable miRNA functional analysis by up-regulation of miRNA activity. Here, we utilized the *mirVana*® miRNA mimic (also from Ambion) containing the stem loop sequence: GUCGGGUACAUCGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAG UCGAUGGGCUGUCUGACA. Effective use of these reagents in other cell types has been described previously [25].

Transfections

Here, we utilized the *mirVana*® miRNA mimic (also from Ambion) containing the stem loop HBE1 and NCI-H292 cells were grown, submerged in medium, in plastic wells to approximately 70% confluence, and at that point transfected with either the miR-21 inhibitor or activator. Transfections were performed using Qiagen (Roche, Indianapolis, IN) "HiPerFect" transfection reagent, a unique blend of cationic and neutral lipids suited to both low- and high-throughput transfection of miRNA mimics or inhibitors, according to the manufacturer's protocol. Forty-eight hours later, cells were exposed to either a range of concentrations of LPS or control media, and appropriate experiments performed.

Analysis of mRNA Expression via RT-PCR

NHBE cells were treated with 100ng/ml of LPS for various time periods. Cells were harvested and RNA was extracted with an RNeasy kit (Qiagen). For miRNA analysis, real-time qPCR was carried out on a iQ5Detection System (Bio-Rad) using 5 ng RNA input, 2× iQ SYBR Green Supermix (Bio-Rad). For the detection 50 ng RNA input, 2× iQ SYBR Green Supermix, and 5 pmol gene-specific primer pairs were used. Thermal cycling conditions were 95°C for 3 minutes, 40 cycles at 95°C for 10 seconds, and 55°C for 30 seconds, followed by melting curve analyses. RNA input was normalized to endogenous controls:ß-actin or 36B4. The

$2^{-\Delta\Delta ct}$ method was used to calculate the fold relationships in miRNA expression among the tested samples.

Analysis of Protein Expression via Western Blot

Westerns blots were used to evaluate levels of MARCKS within the cells after exposure to LPS or control media. After the predetermined time interval, cells were lysed and protein was extracted in buffer containing Complete Mini Protease inhibitor (Roche), washed with cold PBS and scraped into lysis buffer [50 mmol/L Tris-Cl (pH 7.6), 1mmol/L ethylenediamine tetraacetic acid, 100 mmol/L NaCl, 100 mmol/L MgCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 1% (v/v) protease inhibitor cocktail, and phosphatase inhibitor cocktail (Sigma, St. Louis, MO)]. Western blots used a primary antibody against MARCKS (Upstate,Lake Placid, NY) followed by biotinylated goat anti-mouse secondary (both from Abcam, Cambridge, MA) and then Streptavidin HRP (Santa Cruz, Santa Cruz, CA). Blots were developed with Supersignal (Thermo, Waltham Massachusetts) and normalized to β-actin (Santa Cruz) using Adobe® Photoshop® CS3.

Measurement of Mucin Secretion via ELISA

Mucin was collected and assayed as described previously (72). Briefly, after the treatment period, medium was collected and the content of secreted mucin (measured as the major respiratory mucin, MUC5AC) quantified via a sandwich enzyme-linked immunosorbent assay using an antibody to MUC5AC (Neomarkers, Freemont, CA) as the capture antibody with the reporter antibody being a 17Q2 pan-

mucin antibody (220)].The 17Q2 antibody was purified from murine acites fluid (Covance, Gaithersburg MD) and further purified using an ImmunoPure(G) IgG purification kit (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol and then conjugated with alkaline phosphatase (EMD Biosciences). The ELISA Substrate was 4-nitrohenyl phosphate (Sigma, Saint Louis, MO).

Statistical Analysis

Graphpad Prism® software was used to perform unpaired two-tailed Student's T-test with unequal variance as indicated in the figure legends.

Results

Exposure to LPS enhances expression of both miR-21 and MARCKS in human airway epithelium.

As illustrated in **Figure 1A**, exposure of NHBE cells to LPS (100ng/ml) results in increased expression of miR-21 in a time – dependent manner. Both mRNA and protein expression of MARCKS also was enhanced by exposure to LPS at the six-hour time point, but plateaued or decreased after this time, coinciding with an increase in miR-21 expression (**Figure 1B,C**). Both miR-21 and MARCKS expression were enhanced similarly in HBE1 cells after exposure to LPS for 6 hrs (**Figure 2**).

Transfection of the *mir*Vana® miR-21 inhibitor reduces expression of miR-21 in HBE1 cells; this correlates with enhanced expression of MARCKS

Transfection of the *mir*Vana® miR-21 (200nM) inhibitor into HBE1 cells for 48 hrs effectively reduced expression levels of miR-21. This correlated with an increase in expression of MARCKS RNA (**Figure 4**).

Transfection of the mirVana pre-miR-21act activator into HBE1 cells increases expression of miR-21; this correlates with decreased expression of MARCKS

Transfecting HBE1 with 200nM of the pre-miR-21 activator increases levels of miR-21 in the cells, correlating with decreased expression of MARCKS mRNA (**Figure 4**).

Mucin secretion by NCI-H292 cells is affected by the miR-21 inhibitor and mimic, and further by peptides targeting MARCKS protein

As illustrated in **Figure 5**, LPS (100ng/ml) increased mucin secretion by NCI-H292 cells after 30 min exposure. Pretreatment of the cells with the mirVana® miR-21 inhibitor resulted in higher levels of secreted mucin in response to LPS than cells exposed to the HiPerfect transfection reagent used as a control, while pretreatment with the mirVana® pre-miR activator decreased mucin secretion in response to LPS compared to cells without the activator. Additional pretreatment of the cells for 30 min with 50 μM of the MANS peptide, a reagent that inhibits function of MARCKS in airway epithelial cells (72, 87), further attenuated the mucin secretory response, implicating MARCKS in the secretory pathway, as described previously (221)

Discussion

MicroRNAs are fairly short (21-25 nucleotides in length) strands of non-coding RNA. They serve an important regulatory role in proliferation (181), differentiation (182), development and migration (183), angiogenesis(185), apoptosis [12] and carcinogenesis (186). In humans and other mammals, miRNAs bind to the 3' untranslated region of their target gene, forming an imperfect complement. This serves to act as a repressor of translation.

Human miR-21, mapped at chromosome 17q23.2, and present within the protein-coding gene VMP1 (or TMEM49) is one of the most extensively studied microRNAs, as it has been associated with both cancer and inflammation. MiR-21 targets many tumor suppressor genes, such as PTEN, PDCD4, Tropomyosin,

TGFBRII, RhoB, Bcl2, IL-12 and CDK2AP1 (200, 204), and has been shown to stimulate invasion, intravasation, and metastasis [14].

MiR-21 also has been associated with inflammation. It inhibits the TGF-β signaling pathway, which is known to inhibit adipogenesis and stimulate inflammation (204, 222). It has been shown to specifically target TGF-beta 1 and 2 and TGFß receptors [15]. Upregulation of miR-21 causes cell proliferation, while downregulation allows cell to stop dividing and/or undergo apoptosis. MiR-21 is a trigger for fibroblast dysfunction and fibrosis and is upregulated in cardiac infarctions (205). It is also upregulated in individuals with idiopathic pulmonary fibrosis, and in lungs of mice with bleomycin-induced fibrosis. A possible target for miR-21 in fibrosis is Smad7, an inhibitory Smad, which is an important regulator of TGF-β. MiR-21 prevents Smad7 from being made, which stops TGF- β from being inhibited. This allows Smad3 to become activated, increasing collagenase activity and ultimately leading to increased deposition of collagen in the lung parenchyma (222).

Interestingly, miR-21 recently has been shown to also directly target MARCKS protein, binding to MARCKS in the 3' untranslated region from the nt713-734, a region of MARCKS highly conserved among species. Since in previous studies from this laboratory, MARCKS has been shown to be an important regulatory molecule in the process of airway mucin secretion as well as inflammation (72, 85, 87), we looked here at a possible connection between airway inflammation, mucin secretion, MARCKS and miR-21.

In studies using primary well-differentiated normal human bronchial epithelial cells cultured at an air/liquid interface, and using exposure to LPS as a model of inflammation, we found that, indeed, LPS exposure increased of miR-21. Coincident with that increase, expression of MARCKS protein also was enhanced by LPS, but MARCKS expression plateaued after approximately 6 hours of exposure, while miR-21 expression continued to increase. This suggested that miR-21 might be downregulating expression of MARCKS in these cells, which could have a downstream effect of attenuating mucin secretion since MARCKS is integral to the mucin secretory pathway. Thus, we performed additional studies utilizing a commercially-available miR-21 inhibitor as well as a miR-21 activator. Since these studies required efficient transfection of these reagents into cells, we switched the model system from primary NHBE cells to the papilloma virus-transformed HBE1 cell line, as described previously [23].

LPS exposure had the same effect on HBE1 cells, increasing expression of both miR-21 and MARCKS. Treatment with the miR-21 inhibitor decreased significantly levels of miR-21 in the cells with or without exposure to LPS, and this coincided with an increase in expression of MARCKS at the mRNA and protein levels. In contrast, treatment of HBE1 cells with the miR-21 mimic resulted in downregulation of expression of MARCKS under baseline conditions and after exposure of the cells to LPS. Thus, it appears from these findings that miR-21 may act as a negative regulator of MARCKS expression in airway epithelial cells, similar to its role as a negative regulator of MARCKS in prostate cancer cells [20]. One

could speculate that miR-21 functions as part of a negative-feedback mechanism that buffers cellular responses to inflammatory stimuli.

Since MARCKS has been shown to be integral to the mucin secretory process, we then examined how expression of miR-21 and subsequent regulation of MARCKS expression could affect mucin secretion. We turned to a second cell line for these studies, the NCI-H292 cell line, derived from a human epidermoid tumor, since these cells are excellent models for airway mucin secretion, especially of MUC5AC, the predominant human airway mucin [1, 2, 23]. The results of the secretion studies supported the potential anti-inflammatory role of miR-21 in airway epithelium, as treatment with the miR-21 inhibitor, which increases MARCKS expression, also provoked secretion of mucin by cells treated with LPS, while treatment with the miR-21 mimic, which downregulates MARCKS expression, resulted in decreased mucin secretion. To ascertain that indeed MARCKS was functionally associated with the secretory responses, pretreatment of the cells with the MANS peptide, a reagent that is identical to the evolutionarily-conserved N-terminus of MARCKS and which has been shown to inhibit mucin secretion and other functions of MARCKS [3,6,(73, 85, 89, 91), reduced secretion in cells treated with the either the control HiPerFect transfection reagent, cells transfected with the miR-21 inhibitor, or cells treated with the miR-21 mimic/activator.

In summary, it appears that inflammatory stimulation of airway epithelial cells, in this case by exposure to LPS, provokes enhanced expression of the microRNA, miR-21. MiR-21 then appears to target MARCKS mRNA, decreasing levels of MARCKS protein in these cells, and via this mechanism apparently also

decreases the mucin secretory response to LPS. These results, while limited to *in vitro* studies, suggest that miR-2, as well as MARCKS, might be therapeutic targets for treatment of respiratory diseases characterized by mucus hypersecretion.

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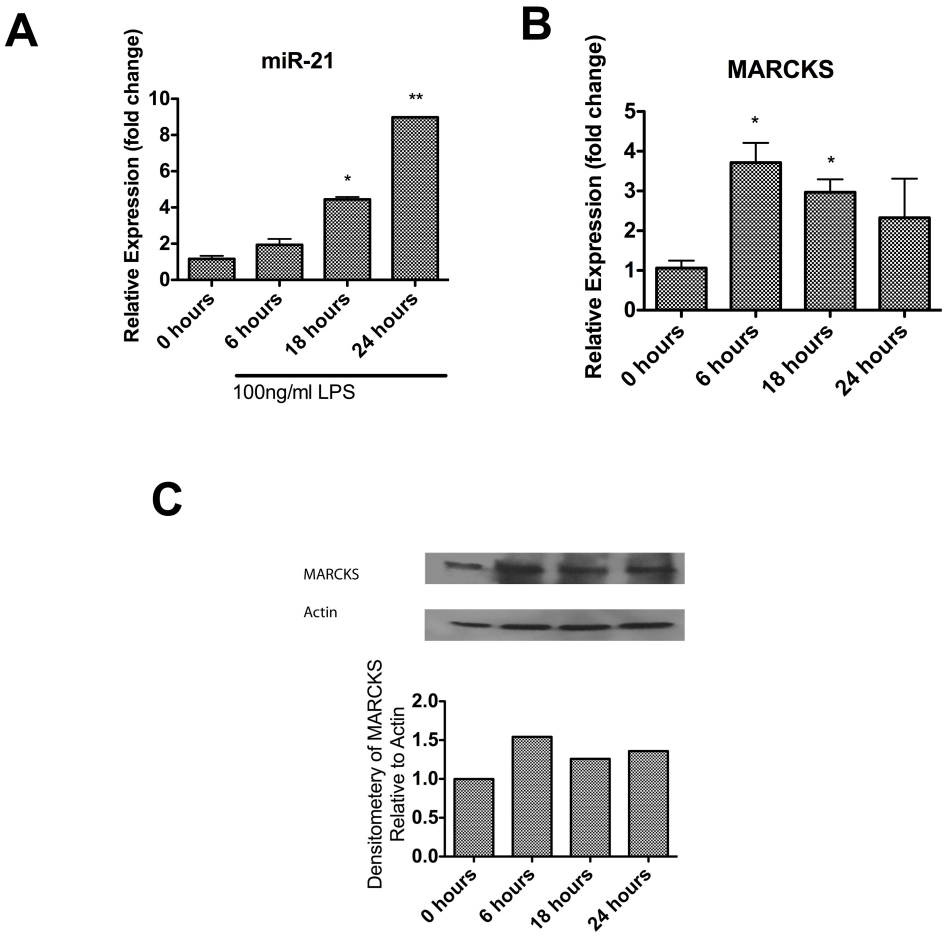

Figure 1. Exposure of well-differentiated NHBE cells to LPS (100ng/ml) increases expression of: A) miR-21 expression over time, from 0 – 24 hrs; (* = $p<0.005$ ** = $p<0.0005$ using Student's T-test, n=4) B) mRNA levels of MARCKS, which peak at 6 hrs and gradually decline over the next 18 hrs; (* = $p<0.005$, n=4) and C) Protein expression of MARCKS, which mimics the mRNA response by peaking at 6 hrs and then plateauing or decreasing slightly from 6-24 hours.

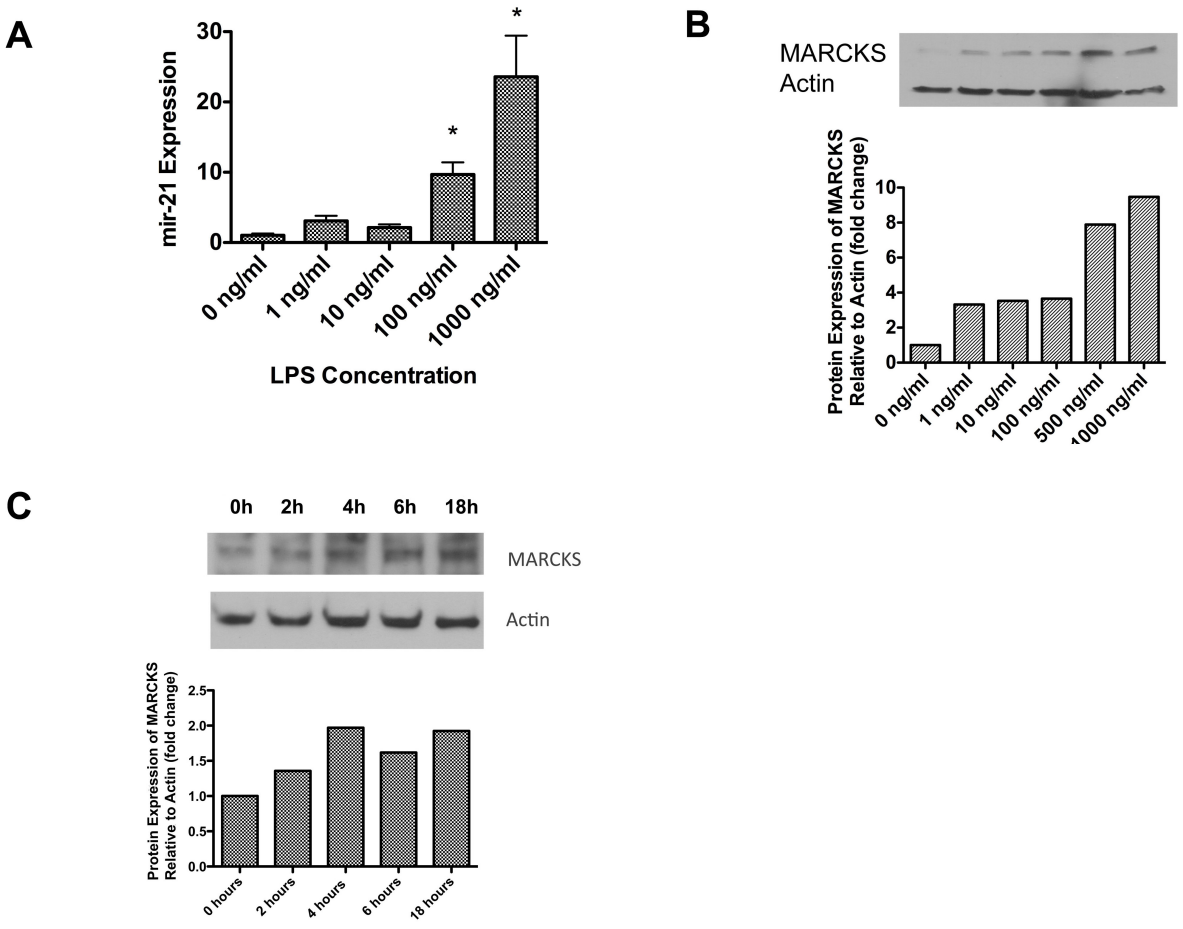

Figure 2. **A)** Expression of miR-21 in HBE1 cells is increased in a concentration – dependent manner after exposure to LPS for 6 hrs, with significant increases between 100 and 1000 ng/ml. (*= $p<0.05$, using Student's t-test, n=3). **B)** Protein expression of MARCKS also is increased at 500 and 1000 ng/ml LPS. C) Protein expression of MARCKS increases at 4 hrs post LPS (500 ng/ml) exposure and plateaus thereafter, similar to what is observed in NHBE cells illustrated in Figure 1.

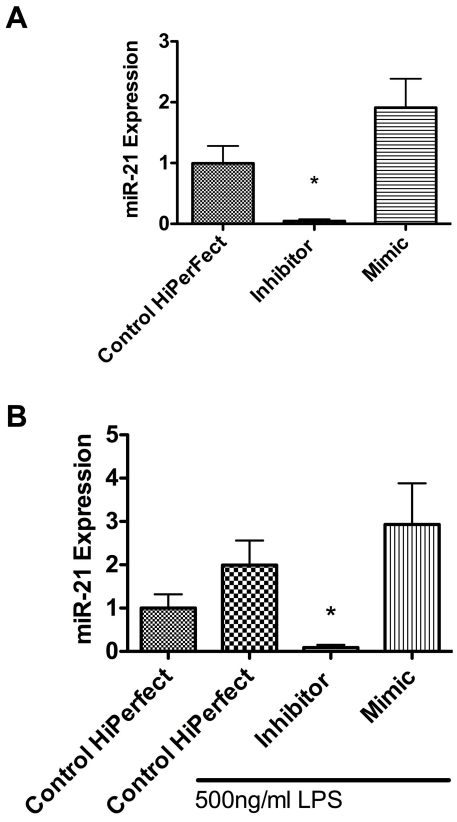

Figure 3. Transfection of the *mir*Vana® miR-21 inhibitor (200 nM) or the *mir*Vana® pre-miR-21 mimic (200nM) into HBE1 cells for 48 hrs shows a decrease in expression of miR-21 with the inhibitor and an increase in expression of miR-21 with the mimic **(3A)**. When these cells were treated with 500 ng/ml of LPS for 6 hrs, **(B)**, miR-21 expression increased in control cells treated with the HiPerFect transfection reagent, while expression was inhibited with the *mir*Vana® miR-21 inhibitor (200 nM) and enhanced with the *mir*Vana® pre-miR-21 mimic (200nM). (* = $p<0.05$ using Student's T-test, n=3)

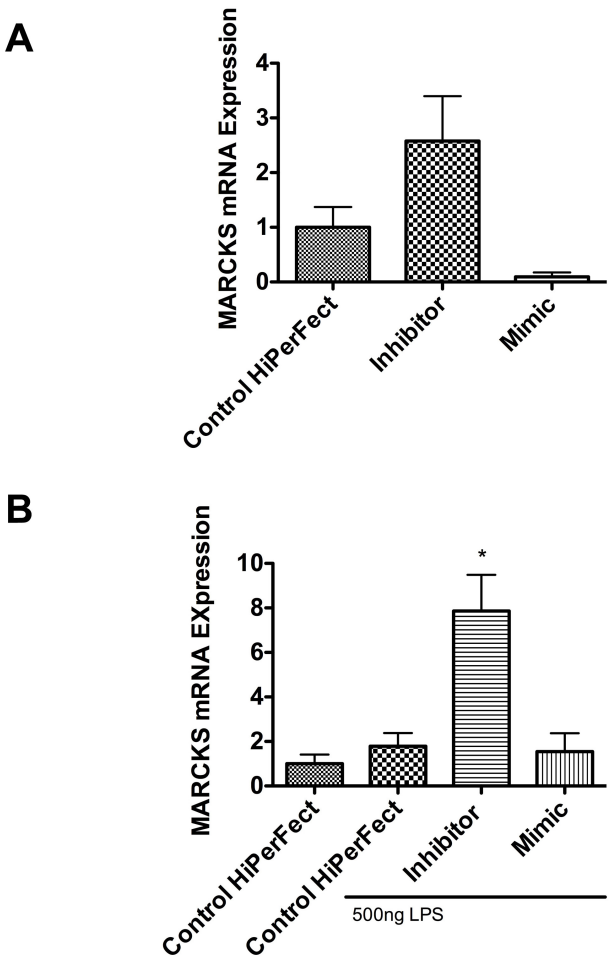

Figure 4. **A)** Transfection of the *mir*Vana® miR-21 inhibitor (200 nM) or the *mir*Vana® pre-miR-21 mimic (200nM) into HBE1 cells for 48 hrs, followed by examination via RT-PCR of MARCKS mRNA expression in these cells, shows that treatment with the inhibitor increases mRNA expression of MARCKS, while treatment with the mimic decreases it. **B)** When these cells were treated with 500 ng/ml of LPS for 6 hrs, mRNA expression of MARCKS was enhanced in cells transfected with the inhibitor and slightly decreased in cells treated with the mimic. (* = $p<0.05$ using Student's T-test, n=3)

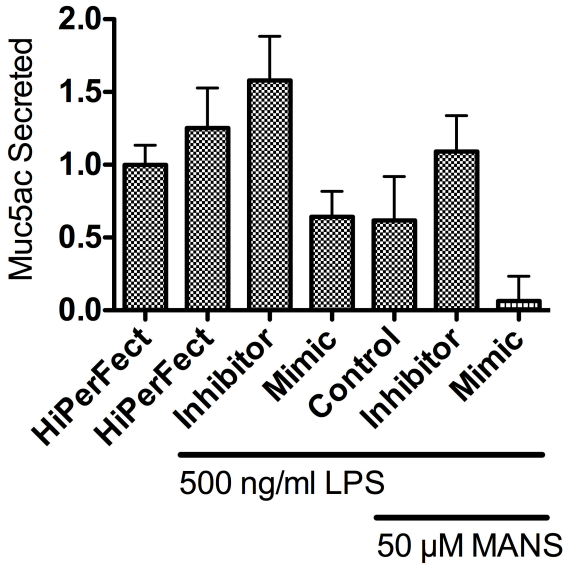

Figure 5. Effects of miR-21 inhibitor and mimic, and of a MARCKS-inhibitory peptide (MANS) on secretion of mucin (MUC5AC) by NCI-H292 cells exposed to 500 ng/ml LPS. NCI-H292 cells were transfected with the *mir*Vana® miR-21 inhibitor or with the *mir*Vana® pre-miR-21 mimic, both at 200nM, for 48 hrs, then treated with LPS for 30 min and mucin secretion measured by ELISA as described. Transfection with the inhibitor increased secretion, while transfection with the mimic decreased secretion. Preincubation of cells for 30 min with 50 μM of the MANS peptide, which inhibits function of MARCKS protein, attenuated secretion in cells whether they were exposed to the HiPerfect® transfection reagent only, to the miR-21 inhibitor, or to the miR-21 mimic/activator, implicating MARCKS in the secretory response.. Cells transfected with the miR-21 mimic secrete significantly less mucus when treated with MANS peptide. (* = $p<0.001$ using Student's T-test) Values are means ± SE, n=6 at each point.

APPENDICES

APPENDIX A

Abstract

Ozone is created by the interaction of oxides of nitrogen and volatile organic compounds in the presence of sunlight. It has been declared a priority pollutant by the EPA and has been shown to exacerbate asthma, bronchitis and emphysema. Although extensive research has linked ozone exposure to inflammation and airway constriction, it remains unknown how ozone exposure affects the airway mucous layer. Hypersecretion of mucus can exacerbate the effects of inflammation and constriction by further blocking air flow. The role of mucus secretion in the lungs' response to ozone will be examined using air-liquid interface cultures of human bronchial epithelial cells and the HBE1 cells, an immortalized human bronchial epithelial cell line. Ozone has been shown to cause the activation of a diverse group of cell signaling molecules. Included in this group are Prostaglandin E2, Thromboxane B2, Interleukin 6, Complement 3a fibronectin, plasminogen, IL-8, and cysteinyl leukotrienes. These cytokines could activate Phospholipase C which will produce diacylglycerol, which activates the protein kinase C. As PKC is activated, it translocates from the cytosol to the plasma membrane. PKC alpha, delta and epsilon have been shown to phosphorylate MARCKS causing it to translocate from the plasma membrane to the cytoplasm and participate in mucus secretion. Cytosolic and membrane protein extracts will be examined for the translocation of Protein kinase C via Western Blots. Mucus secretion is measured by ELISA

detecting all mucin proteins.

Introduction

High levels of tropospheric ozone casue a higher incidence of cough, phlegm and breathing difficulty. Ozone has been shown to impair pulmonary function during heavy exercise (223) and lower peak expiratory flow in children with mild asthma2 Asthma is characterized by three major hallmarks which include airway obstruction by mucus, airway inflammation, and exaggerated airway constriction. These factors all contribute to airway hyper-responsiveness. Asthma affects 15 million Americans and continues to increase in both incidence and severity despite vast improvements in both therapeutic options and understanding of the disease. Protein kinase C (PKC) is a serine/threonine kinase involved in exocytosis in various cell types and plays a role in the secretion of mucus, insulin, neurotransmitters, and platelet dense granules. Mucus secretion in airway epithelial cells involves PKC. Upon activation PKCδ translocates from the cytosol to the plasma membrane and phosphorylates myristoylated alanine-rich C kinase substrate (MARCKS), which will then detach from the membrane and move into the cytosol participating in mucus secretion (72, 87, 210) Three classes of PKC exist, conventional, novel and atypical. PKCα is a conventional isozyme of PKC while PKCδ and PKCε are novel isozymes. Both of these classes PKC are activated by diacylglycerol within the cell. In these experiments we use Phorbol-12-myristate-13-acetate (PMA), which is a stable analogue of diacylglycerol as a positive control for activation of PKC.

Materials and Methods

Culture of Bronchial Epithelial Cells Primary NHBE cells purchased from Lonza. Human papilloma virus-transformed human bronchial epithelial cells (HBE1)5 were seeded on Corning® Transwell® collagen-coated membrane inserts and maintained in a humidified air/5% CO2 incubator for 14 days in air-liquid interface culture.

Exposure of Cells to Ozone and PMA Ozone was generated by electric discharge in pure oxygen and levels were measured by sensors calibrated to known standards. Well-differentiated NHBE and HBE1 cells were exposed to 0.4ppm and 0.8ppm ozone for up to 240 minutes in uncovered culture plates. Control cells were placed in identical chambers in the absence of ozone. Microscopic observation post-treatment revealed no evidence of cytotoxicity. Well-differentiated NHBE and HBE1 cells were exposed to 500nm PMA (EMD Biosciences, La Jolla, CA) in media applied to the apical side only.

Measurement of Mucin Secretion Mucin was collected both at baseline and after treatments in 300μL of phosphate buffered saline. Baseline mucin secretion was used to normalize variation between different culture wells. After baseline mucin samples were collected, cells were incubated overnight and exposed to test reagents the next day for indicated periods of time. After each treatment period, secreted mucin was collected as the baseline sample and quantified by sandwich enzyme-linked immunosorbent assay using the 17Q2 antibody (Covance, Gaithersburg MD),

a monoclonal antibody that reacts specifically with a carbohydrate epitope on human airway mucins. (220) The 17Q2 antibody was purified using an ImmunoPure(G) IgG purification kit (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol and then conjugated with alkaline phosphatase (EMD Biosciences). To account for variability between cultures and experiments, levels of mucin secretion were reported as percentage of the same wells, non-treated, a day earlier.

Subcellular Localization of PKC Isoforms Activation of PKC was assessed by subcellular fractionation following the protocol described by Kajstura et al (224) and subsequent Western blot analysis using a PKC- specific antibody (Cell Signaling Technology, Inc., Danvers, MA). Briefly, cells were washed with cold PBS and scraped into lysis buffer [20 mmol/L Tris-Cl (pH 7.5), 1mmol/L ethylenediamine tetraacetic acid, 100 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 1% (v/v) protease inhibitor cocktail, and phosphatase inhibitor cocktail (Sigma, St. Louis, MO)]. The lysates were then sonicated and pelleted at 16,000 g for 30 minutes. The supernatant was collected and kept as the cytosolic fraction at -80°C until used. The remaining pellet was resuspended in lysis buffer containing 1% Triton X-100, sonicated, and centrifuged at 16,000 g for 30 minutes at 4ºC. The supernatant fraction was stored at -80°C until analyzed.

Results

Protein kinase C delta resides primarily in the cytosolic fraction in ozone treated and air control cells. PMA serves as a positive control for PKCδ (Figure 1).

The intracellular location of PKC isotypes in NHBE cells was examined by Western Blot in response to 0.8ppm ozone exposure. 500nM PMA caused translocation of PKC alpha, delta, and epsilon to the plasma membrane while 0.8 ppm ozone did not increase translocation. (Figure 2)

This indicates that these airway epithelial cells do respond to the activation of PKC but the effect of ozone on these three PKC isoforms is minimal. When exposed to 0.8 ppm for 1 hour, there is a trend towards increased mucus secretion, but is not quite statistically significant. Even when exposed to PMA, a potent activator of PKC and secretion, there is only approximately a 50% increase in mucus secretion in these NHBE cells. (Figure 3)

After 20, 40 and 60 minutes of exposure to 0.8 ppm ozone, there was some movement of MARCKS protein away from the membrane, indicating the phosphorylation and activation of MARCKS protein. When exposed to PMA for 60 minutes there is a similar move away from the membrane. (Figure 4)

Discussion

Ozone has been shown to cause the activation of a diverse group of cell signaling molecules. Included in this group are Thromboxane B2, Prostaglandin E2, Interleukin 6, Complement 3a (225) fibronectin, plasminogen, IL-8, and cysteinyl leukotrienes (226).

Thromboxane B2 is an inactive product created by the hydrolysis of the very short lived Thromboxane A2, which activates TBXA2R, a Gq-protein coupled receptor. In the presence of Thromboxan A2, TBXA2R activates Phospholipase C, which will cleave phosholipids to produce diacylglycerol molecules. Diacylglycerol activates the protein kinase C family. PKCα, PKCδ and PKCε have been shown to phosphorylate MARCKS causing it to translocate from the plasma membrane to the cytoplasm and participate in mucus secretion.

Other possible ozone induced activators could participate in this pathway by activating Phospholipase C. Any of a number or Gq-protein coupled receptors could stimulate the same response resulting in mucus secretion. Other likely candidates include the cysteinyl leukotirenes LTC4, LTD4, and LTE4. These activate the CysLT1 and CysLT2 receptors, which most commonly are coupled with Gq-protein.

Another possibility would be Interleukin-8, which have been shown to activate IL8R, which is coupled to either Gi or Gq protein receptors. It should be noted that using this method for isolating the outer membrane from the cytosol is likely to discard the intercellular membranes of organells and/or vesicles. MARCKS is thought to bind to the secretory vesicles found in goblet cells.(227) This could

explain the absense of MARCKS protein in the cytosolic samples even after MARCKS has been activated.

Further studies of the response of airway epithelial cells to ozone exposure should be studied further. Since ozone is a very transient molecule, it is not known how much ozone can actually diffuse into the cell. It is possible that the primary effects of ozone upon epithelial cells are due to the peroxidation of the phospholipids in the plasma membrane. The level of peroxidation of the membrane by exposure to ozone should be measured. Future work could focus on the location and sites of action of ozone molecules to better understand the pathways by which the worsen symptoms in those with airway disease.

To expand upon this study, repeating the exposure to ozone and its effect on mucus secretion in primary airway epithelial cells from more individuals, especially those who had suffered from airway disease. Also subcellular fractions should be prepared to determine if acute ozone exposure has a noticeable affect upon the location and therefore activity of different Protein Kinase C isoforms notably delta epsilon and alpha, which have been shown to phosphorylate MARCKS (77, 96)

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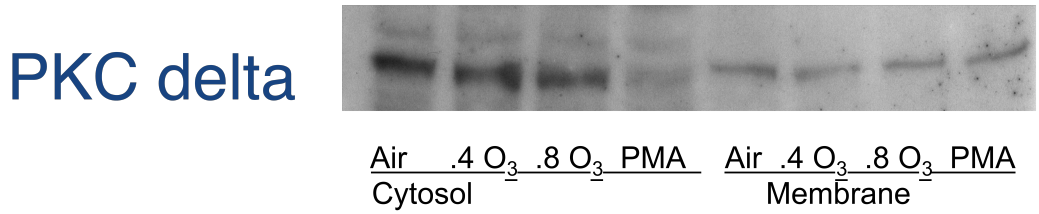

Figure 1. The localization of Protein Kinase C delta (PKCδ) in HBE1 cells was examined by Western Blot. 500nM PMA caused translocation to the membrane while .4 and 0.8 ppm ozone for 60 minutes had no effect.

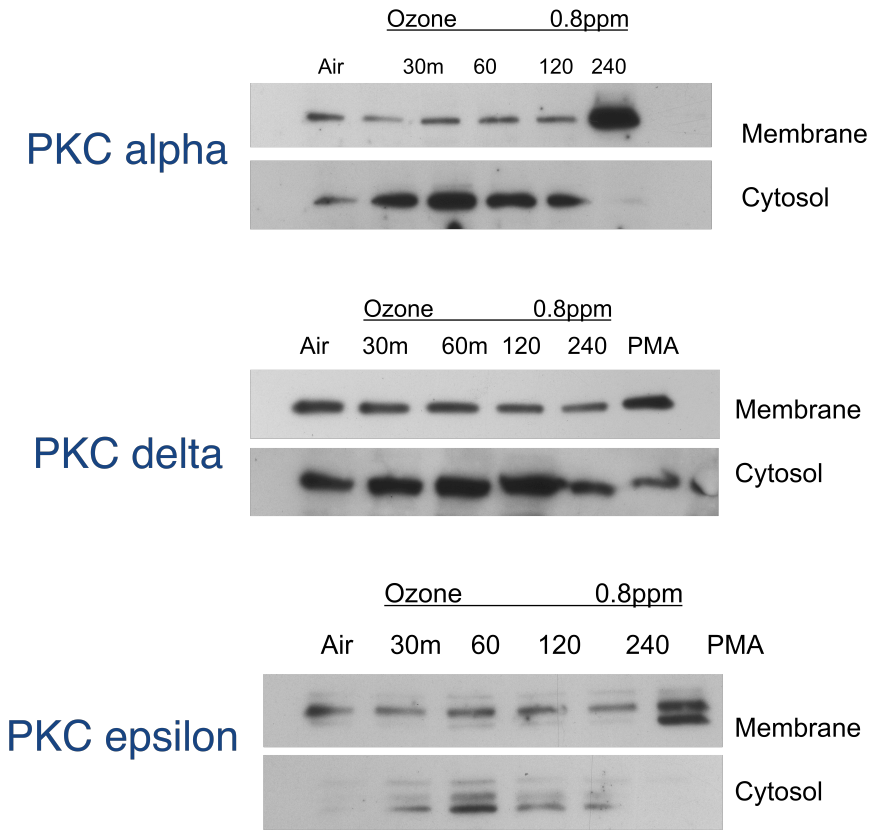

Figure 2. The localization of the PKC isotypes in NHBE cells was examined by Western Blot in response to 0.8ppm ozone exposure. Exposing cells to 500nM PMA caused translocation of all PKC isotypes to the membrane while 0.8 ppm ozone did not increase translocation.

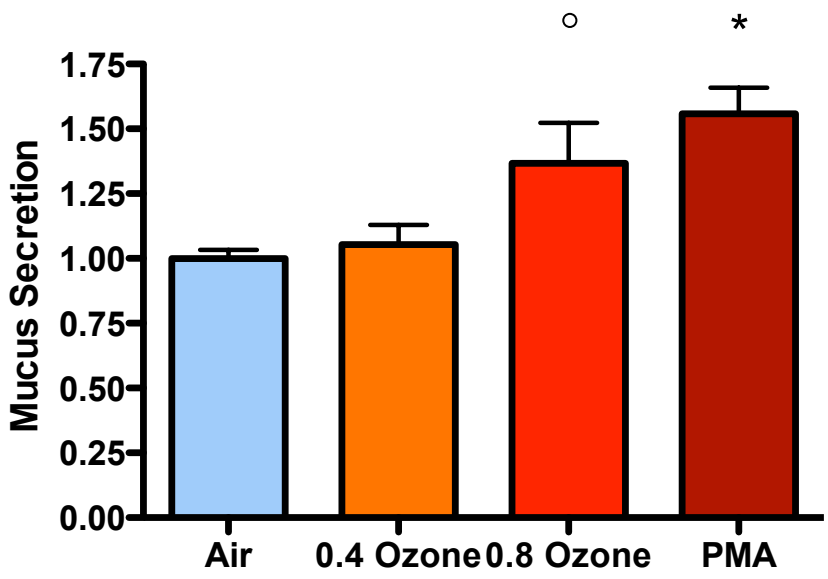

Figure 3. Mucus Secretion of NHBE cells exposed to 0.4 or 0.8 ppm ozone for 60 minutes or 500nM PMA measured by ELISA. Significance vs. air exposed cells (^{o} p=0.067); (*=p<0.05).

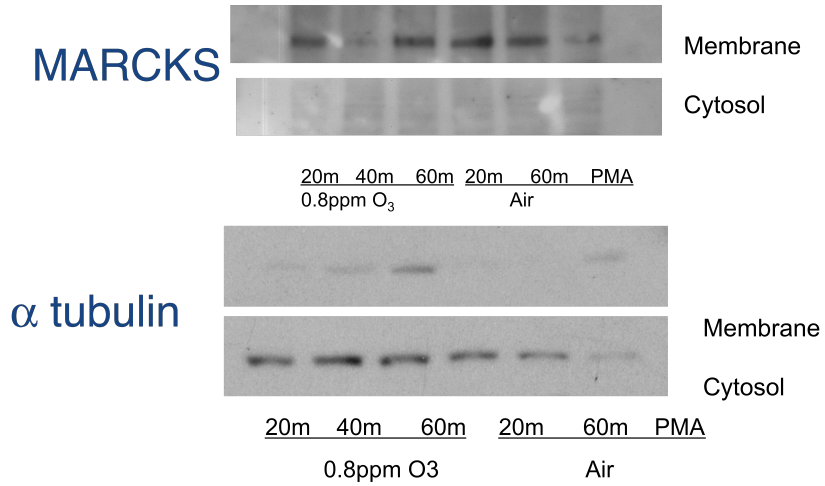

Figure 4. The localization of MARCKS in response to 0.8ppm ozone exposure in NHBE was examined using Western Blots. 500nM PMA caused translocation of MARCKS away from the membrane, while 0.8 ppm ozone caused some movement away from the membrane at 40 minutes.

Appendix B

EXPRESSION OF MARCKS PROTEIN IN AIRWAY DISEASE
AND REACTIVITY TO TNF-ALPHA

Background

TNFα is a cytokine produced by many cell types, which has been shown to have importance in many inflammatory diseases. Bronchioloalveolar lavage fluid samples from asthmatic patients have significantly higher levels of TNFα than samples from non-diseased patients. Predominantly TNFα is secreted by activated macrophages and mast cells. TNFα serves to recruit more leukocytes into peripheral tissue, including the airways. TNF-a production is mediated by several cytokines, which bind to their receptors on plasma membrane leukocytes (175-177). Upon binding to receptor on the plasma membrane, TNFα causes the activation of NF-kß, MAP kinase and caspace pathways. TNFα has been shown to cause an increase in mucin secretion in tracheal epithelial cells (228, 229). Additionally, TNFα increased secretion of both muc5ac and muc5b in a goblet cell line of the middle ear (230).

Hypothesis

• Airway epithelial cells derived from donor with respiratory disease (e.g. asthma, COPD) will have more MARCKS protein than cells from non-diseased patients.

• Exposure to TNFα will cause increases in the amount of MARCKS protein in both cells from diseased patients and normal donors.

Results

The individual with COPD had a higher amount of MARCKS protein than the non-diseased smoker, the asthmatic, and the healthy donor. When cells from diseased and non-diseased individuals were exposed to 100nM TNFα, the cells from the asthmatic patient showed a slight increase in MARCKS protein after three hours. The heightened levels of MARCKS in the COPD cells remained virtually unchanged.

Conclusion

Since MARCKS has been shown to serve as a negative regulator of LPS signaling (231). The increase in MARCKS protein may have an anti-inflammatory affect so that diseased patients will be less sensitive to inflammatory signals such as TNFα or LPS. The increased amount of MARCKS protein may also be part of the cause for increase mucus in inflammatory airway diseases. If heightened protein levels of MARCKS contributed to the morbidity of airway disease, it could be a potential to target in drug therapy or knocking down with siRNA or a relavent microRNA such as miR-21. It would be of interest to learn if the few patients examined are representative of typical patients.

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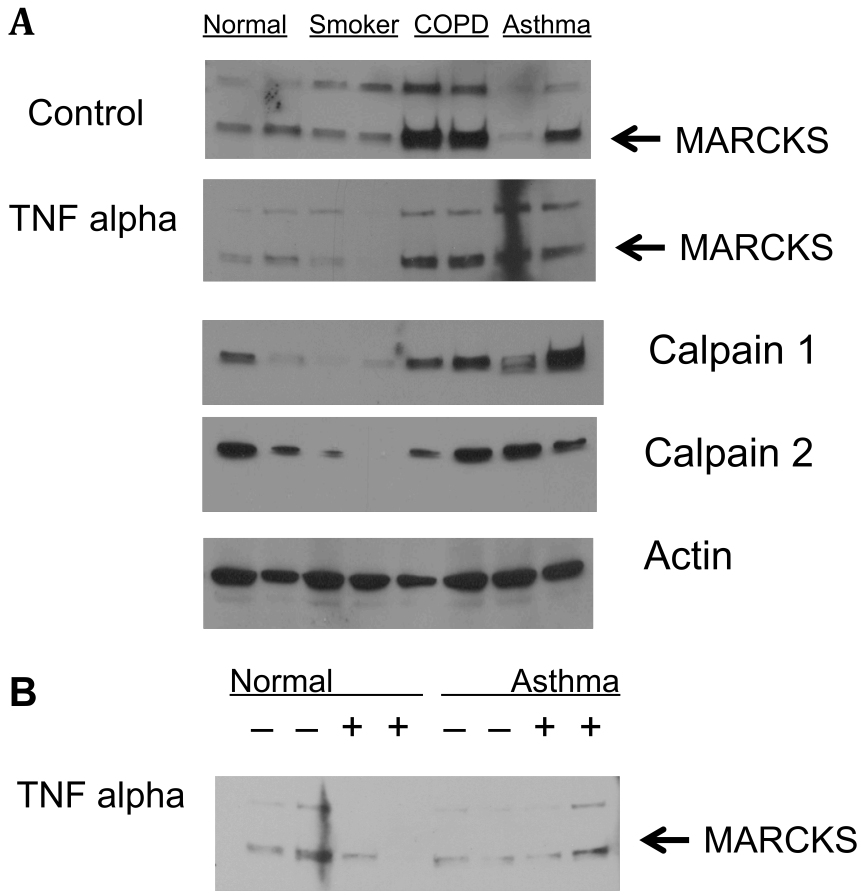

Figure 1. A) An increased amount of MARCKS protein is present in primary epithelial cells from a patient with COPD. The normal donor, non-symptomatic smoker, and asthmatic had comparable levels of MARCKS. B) After 3 hours, there is a slight trend toward 100ng/ml TNF alpha increasing the expression of MARCKS in airway epithelial cells from an asthmatic patient.

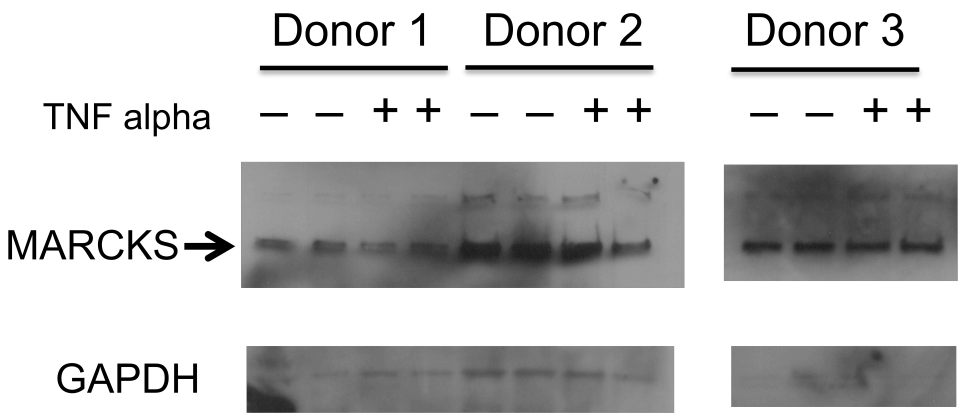

Figure 2. Primary airway epithelial cells from three healthy donors were either exposed to 100ng/ml TNF alpha for 3 hours. Western Blots were used to assess the relative quantity of MARCKS protein. GAPDH serves verifies a comparable amount of protein was loaded in each lane.

Overall Conclusions

1. **Calpain Studies**

   A. Exposing HBE1 cells to 250nM PMA for 2 – 10 min caused an approximate twofold increase in calpain activity.

   B. When cells were pretreated with calpain inhibitors Z-LLY-FMK or Z-LLY-CH, the activity increase in response to PMA was significantly decreased. This indicates that these commercially available calpain inhibitors are effective at reducing calpain activity.

   C. Pretreatment of NHBE cells with either calpain inhibitor significantly attenuated mucin secretion in response to PMA. Z-LLY-CHO showed a concentration - dependent reduction in mucin secretion in response to 250nM PMA.

   D. HBE1 cells transfected with YFP-labeled MARCKS revealed a cleavage product consistent in size with the expected product resulting from the cleavage of MARCKS at a putative calpain cleavage site 6 amino acids from the N-terminus. The fragment was 27-32kDa, which is slightly larger than the size YFP of protein alone.

   E. These results differ from the original hypothesis in that inhibition of calpain resulted in decreased, rather than increased, mucin secretion. The mechanism(s) behind this response are the subject of future studies.

2. miR-21 Studies

A. Exposure of NHBE cells to 100 ng/ml LPS resulted in increased expression of both miR-21 and MARCKS mRNA. MiR-21 continued to increase up to 24 hours whereas MARCKS peaked at 6 hours and then slowly diminished until 24 hours. The difference in time points would suggest that MARCKS levels rise in response to LPS and that further increases in MARCKS expression could be down-regulated as miR-21 levels increase. Thus, mir-21 may down-regulate MARCKS expression.

B. Levels of miR-21 are decreased and increased after pretreatment (transfection) with a commercially – available mir-21 inhibitor or mimic, respectively.

C. Levels of MARCKS mRNA are increased when the cells are pretreated with the miR-21 inhibitor, while the amount of MARCKS mRNA decreases when the cells are treated with the mimic of miR-21.

D. Transfection with the mir-21 inhibitor increased mucin secretion in NCI-H292 cells, while transfection with the mimic decreased secretion. Preincubation of cells for 30 min with 50 μM of the MANS peptide, which inhibits function of MARCKS protein, attenuated secretion in cells whether they were exposed to the HiPerfect® transfection reagent only, to the miR-21 inhibitor, or to the miR-21 mimic/activator, implicating MARCKS in the mir-21 associated secretory response.

3. Ozone Studies

A. Acute exposure to ozone did not cause a significant difference in the cellular location of PKC α, δ or ε from either primary airway epithelial cells or immortalized airway epithelial cell line.

B. PMA (positive control) caused ε translocation of PKC α, δ or ε from the cytosol to the plasma membrane.

C. When primary airway epithelial cells were exposed to ozone, there was a trend towards increased mucin secretion.