ABSTRACT


This dissertation focuses on two critical functions mediated by the airway epithelium: mucus production and inflammatory mediation. Both processes are important for pulmonary defense, but may be deleterious to the surrounding airway if regulated inappropriately. In the first study, a novel monoclonal antibody developed against guinea pig Muc2 mucin and a commercially available anti-MUC5AC mucin antibody were characterized for use in guinea pig studies. Muc2 and Muc5AC mucin production was then measured in guinea pig tracheal epithelial (GPTE) cells stimulated with pro-inflammatory cytokines, TNF-α, IL-1β, and IFN-γ (cytomix). It was demonstrated that Muc2, but not Muc5AC, mucin secretion increased over constitutive production in cytomix-stimulated cells, but intracellular protein and mRNA increased similarly for both mucin subtypes. It was concluded that differential mechanisms for mucin subtype secretion are present in the guinea pig airway epithelium. This differential regulation of mucin subtype expression is an important finding, as it may affect the interactive properties of mucus in both normal and diseased airway.

In the second study, the anti-inflammatory role of inducible nitric oxide synthase (iNOS) was evaluated in normal human bronchial epithelial (NHBE) cells. Using iNOS-specific inhibitor, cGMP-dependent kinase inhibitor, and exogenous nitric oxide and cGMP-analogue application, it was demonstrated that an iNOS/cGMP/PKG-mediated pathway suppresses granulocyte macrophage colon stimulating factor (GM-CSF), but not interleukin-8, expression in cytomix-stimulated NHBE cells.

The third study demonstrated that an anti-inflammatory action of the β2-adrenergic agonist, (R)-albuterol, is dependent on iNOS. Through the use of small interfering RNA targeted against iNOS, it was demonstrated that (R)-albuterol, and not its inert enantiomer, (S)-albuterol, suppressed GM-CSF message and protein release in IL-1β/IFN-γ-stimulated NHBE cells by augmenting iNOS expression. In addition, through the use of various kinase-specific inhibitors and activators, it was demonstrated that (R)-albuterol-mediated iNOS augmentation requires protein kinase Cδ, but not cAMP or cGMP-dependent kinase, activity.
Overall, this study identifies a novel pathway in which β2-adrenergic agonists may exhibit anti-inflammatory effects in the airway epithelium and surrounding milieu.
DIFFERENTIAL MUCIN SUBTYPE REGULATION AND ANTI-INFLAMMATORY EFFECTS OF INDUCIBLE NITRIC OXIDE SYNTHASE IN STIMULATED AIRWAY EPITHELIAL CELLS IN VITRO

by

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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

COMPARATIVE BIOMEDICAL SCIENCES

Raleigh

2005

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Lawrence and Dale
Brian Chorley was born on June 13th, 1976 in Jacksonville, North Carolina. He is the son of Joseph and Bonnie Chorley and the older brother of Joey Chorley. He grew up in Jacksonville where he made several life-long friends. He moved to Raleigh, North Carolina in 1994 to attend college at North Carolina State University. He currently resides in northern Raleigh with his girlfriend of 5 years, Meredith, and their two cats, Tigger and Joey-Cat.

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Duane M. Alvens
‘Life-long Friend’
ACKNOWLEDGEMENTS

I would like to acknowledge my advisor and mentor, Dr. Ken Adler, for his guidance, input, and support during my doctoral studies. He has taught me what it takes to be a Ph.D. and has forever altered my approach to career and to life.

I would additionally like to thank my committee, Drs. Linda Martin, Sarah Gardner, Fred Fuller, and Ida Smoak for their support throughout the years. I would like to particularly thank Linda for her invaluable advice and guidance for which I am eternally grateful.

I would like to thank all past and present members of Drs. Adler and Martin’s lab in which I have been in contact. Every member has helped me in some capacity to achieve my academic goal. I must also personally thank Dr. Yuehua Li who initially took me under his wing and taught me the ways of research. His instruction and support gave me the confidence to initialize an independent approach to my studies.

I would like to thank my friends and family. My long-time buddies - Ally, Cory, Duane, and Justin - and good friends I made during my time at NCSU - Christine, Drew, Eric, John, and Mike - have supported me and provided many opportunities for much needed distractions from my studies. Dad, Mom, and my brother, Joey, have always encouraged me during whatever endeavor I have pursued. Their love and support has kept me going through the tough times that are inherently linked to a doctoral education. Dad, in particular, gave me inspiration by example of his own pursuit for higher education and desires for a better life for himself and his family. Last, but certainly not least, I would like to thank the love of my life, Meredith. Not only did her own aspirations to obtain a DVM inspire and encourage me, her love, support, and companionship came at a time when it was needed most.
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CHAPTER I

INTRODUCTION
BACKGROUND

The studies described herein focus on two important functions of the airway epithelium: mucus expression and inflammatory mediation. The following introductory chapter will summarize the current knowledge base concerning these processes. The first section will focus on regulation of mucin subtypes (the major protein components of mucus) and the animal models used to study mucus regulation in airway research. The second section will give a general overview of how these cells mediate airway inflammatory response, with focus on nitric oxide and the β2-adrenergic receptor. The final section will briefly summarize each of the novel studies presented in this dissertation.
MUCIN EXPRESSION

The Airway Epithelium and Mucus Expression

Traditionally, the airway epithelium was only considered a physical barrier between the outside environment and the internal milieu. Now, however, the airway epithelium is known to mediate many critical functions pertinent to pulmonary homeostasis and environmental insult response. These roles are not limited to a single cell type; rather the burden is shared by at least eight different morphological cell types which compose the human epithelium (1). These cell types fall into three broad categories which superficially characterize this pseudostratified cellular layer: basal, ciliated, and secretory (reviewed in (1)). Basal cells are known progenitors of mucous and ciliated cells, but recent evidence suggests they play additional roles including inflammatory mediation, anti-oxidant release, growth factor production, and fluid modulation (2). The central role of the ciliated cell is movement of viscoelastic, gel-like mucus, from the conductive airways to the throat through highly coordinated, wave-like motions of the cilia. Over 50% of the normal epithelial makeup are ciliated cells (3). The secretion function of the airway epithelium is primarily mediated by the goblet cells, although the serous and Clara cells may also secrete bronchiolar surfactant as well as other secretions (reviewed in (1)). Goblet cells are physiologically characterized by large, membrane-bound granules that store and release mucin, the structural components of mucus, as well as other components of mucus (4).

The airway epithelium is the first line of defense against invading pathogens, allergens, and pollutants that populate the external environment. As a preemptive mechanism, the epithelium secretes the mucosal barrier that lines airway surfaces.

Mucus is composed of a complex mixture of antioxidants, lysozymes, immunoglobulins, defensins, ions, water, and lipids, among others (5). The structural components of mucus are large, heavily glycosylated, multimeric proteins called mucins. Physically, these multi-mega-Dalton glycoproteins serve to trap inhaled particulates and pathogens, thereby exposing these elements to the various anti-proteolytic and anti-microbial substances that compose the fluid-like portion of mucus (6). In addition, the trapped
substances are cleared from the intra- and extra-pulmonary airway by both ciliary and mechanical action (via cough) (7).

A major hallmark of chronic airway diseases (e.g., asthma, cystic fibrosis, chronic obstructive pulmonary disease) is mucin hypersecretion, which subsequently leads to mucociliary clearance impairment, poor gas exchange, bacterial colonization, inflammation, and mechanical injury of the surrounding airway (5). For this reason, major research efforts have been mounted to uncover the mechanisms that regulate mucin production and expression in both normal and diseased airways.

**Major Airway Gel-forming Mucin Types**

The twenty currently known mucin subtypes are grouped into two broad categories based on their characteristics and localization: *membrane-bound* and *secreted*. The *gel-forming* subset of the secreted mucins plays an important role in mucus composition. Four of the gel-forming mucins (MUC2, MUC5AC, MUC5B, and MUC6) are of particular interest due to their oligomerizing nature which is thought to be regulated by their “D-domains” (cysteine-rich motifs, located at the carboxyl- and N-terminal regions) (8-11). These genes cluster on the human chromosome 11p15.5 and span some 400 kB in the order of telomere-MUC6-MUC2-MUC5AC-MUC5B-centromere (12). MUC2, MUC5AC, and MUC5B are known to be expressed in the human airway (13-15).

*MUC2*

MUC2 was first described as an intestinal mucin and has since been isolated from a number of organs, including colon, bronchus, cervix, and gallbladder (16-18). Respiratory diseases, through alteration of many inflammatory mediators, are also known to upregulate MUC2 gene expression. In the human cell line H-292, the TH2-phenotypic cytokine, interleukin-4 (IL-4), was shown to increase MUC2 expression over 3-fold after a 24 hour exposure (19). In guinea pig tracheal epithelial cells, tumor necrosis factor-α exposure over a short-time course augmented Muc2 (rodent homolog of human MUC2) message 3-fold over constitutive expression (20). In a transgenic mouse lung where the inflammatory
secretagogue interleukin-9 was expressed constitutively, Muc2 message was significantly elevated over control mouse levels (21).

Despite reports of enhanced gene expression during inflammation, MUC2 has not been considered a major airway gel-secreting mucin. In human tracheobronchial epithelial culture, MUC2 was barely detected in all stages of culture differentiation (22). This low level detection was also reported in six commonly used respiratory carcinomic cell lines (23). In addition, Muc2 gene expression was not reported in normal mouse and equine airway tissue (5, 24, 25). Furthermore, MUC2 mucin was not detected in normal preparations of human sputum (26) or in the airways of the inbred FVB/N mouse strain (27). While gene expression studies have shown Muc2 message increase during inflammation, there is evidence that MUC2 mucin may be translationally and postranslationally regulated (28, 29). This may explain why studies do not measure MUC2 mucin secretion in normal and diseased airways of many species.

MUC5AC

MUC5AC was originally considered to be two separate mucins (5A and 5C) but was later discovered to be clones of the same gene (30). MUC5AC is expressed in the epithelial goblet cells and the mucous cells of the submucosal glands, but is generally localized in the goblet cells in normal respiratory tissues (31). In contrast to MUC2, MUC5AC has been characterized as a dominant mucin in the airway of many mammalian species. An immunolocalization study of human tracheal goblet cells indicated strong presence of MUC5AC (13). In addition, human respiratory tract cell lines (Calu-3, H292, and A-549) dominantly express MUC5AC transcript in relation to MUC2 and MUC5B (32). In mice, where submucosal glands are notably absent from the airway, Muc5AC message is present in normal lungs, and protein expression can be augmented with ovalbumin challenge (allergic model of asthma) or interleukin-13 exposure (32). The equine MUC5AC transcript was also noted in horse airways with fatal cases of recurrent airway obstruction, where MUC2 was non-existent (24). Muc5AC gene expression was also present in rat airways after exposure to the respiratory tract irritant, acrolein (33). In differentiated guinea pig epithelial cell culture,
Muc5AC protein is present but, interestingly, has only been shown to express only Muc2 transcript at measurable levels (20, 34).

MUC5AC is induced or upregulated by various inflammatory mediators. Table 1 summarizes what is currently known regarding stimulated expression/regulation of gel-forming airway mucins. At quick glance, it is apparent that MUC5AC is commonly modulated with treatment or allergic challenge. Briefly, MUC5AC (message and/or glycoprotein) was found to be upregulated with exposures to interleukin-1β (35, 36), tumor necrosis factor-α (36-38), lipopolysaccharide (37), platelet activating factor (37, 39), prostaglandin-E2 (37), interleukin-4 (19, 34), interleukin-6 (40), interleukin-9 (21), interleukin-17 (40), neutrophil elastase (41), adenosine 5′-triphosphate (42), and uridine 5′-triphosphate (43).

**MUC5B**

MUC5B mucin has recently been demonstrated to be a major mucosal component in the human airway. In contrast to MUC5AC, MUC5B production *in vivo* is primarily limited to the mucous cells of the submucosal glands (31), although some production has been noted in goblet cells (44). Interestingly, in normal human bronchial epithelial cells, MUC5B has been described as the dominant mucin component of mucus (22).

MUC5B is considered to be expressed constitutively and resistant to sudden modulation. Stimulus with epidermal growth factor, thyroid hormone (T₃), or retinoic acid dramatically and rapidly altered MUC5AC mRNA expression, with only a gradual, abbreviated change in MUC5B levels (45). Interestingly, two separate glycoforms of MUC5B (based on charge differences) are found in the human airway (46). Although the reason for the variation is unclear, it has been suggested that the two charged variants are produced in two separate locations, specifically the submucosal glands (low-charge) and the goblet cells (high-charge) of the epithelium (47). Therefore, it is plausible that initiating production from different sources can alter the mucus makeup. This may be of major relevance in disease studies. For example, it has been shown that patients suffering from
status asthmaticus exhibited viscous mucus plugs consisting of the low-charged variant of MUC5B mucin (15).

MUC5B production and expression in other species has not been well established. However, in the mouse, tissue-specific expression analysis of the gel-forming mucins found that Muc5B was the only mucin normally expressed in laryngo-tracheal tissue, where Muc5AC expression was weak and Muc2 was not found (25). Moreover, Muc5B expression was enhanced in the asthmatic model of ovalbumin-sensitized mice (48). Muc5B expression patterns in other commonly used rodent models, including guinea pig and rat, are unknown. As sequence information becomes available for these species, it is certain studies examining Muc5B expression and probable roles in respiratory function will emerge.

Mucin Composition

MUC2, MUC5AC, and MUC5B not only characterize the secreted gel-forming mucins of the mammalian airway, they also belong to a special subclass defined by their ancestral lineage. These mucins are related to the endothelial-derived von Willebrand Factor (vWF), which is a protein essential to platelet-blood vessel wall function. A common characteristic shared by the mucins are cysteine-rich subdomains, located near the carboxyl- and amino- terminal regions of these proteins, whose sequences are evolutionarily conserved (10, 49). It is assumed these domains play vital roles in the functionality of the mature mucin, including packaging and trafficking, as well as dimerization and interaction with other mucins (50, 51), indicative to what is observed with von Willebrand Factor (52). The central and most populated region of each mucin is composed of a collection of repeated domains rich in the hydroxylated amino acids, serine and threonine (53, 54). These domains are responsible for carbohydrate linkage to the peptide backbone (55-57). While the sequences of the cysteine-rich domains are conserved among the three mucin subtypes, and are thought to mediate critical functions, the repeated regions (referred to as variable number-tandem repeats (VNTR) regions) vary for each mucin and may elicit unique functionality for each mucin subtype.

Each initial O-glycosylated linkage of N-galactosamine on the mucin backbone leads to further branching of oligosaccharide chains including fucose, galactose, N-
acetylglucosamine, and N-acetylneuraminic acid (27, 58). Sulfate or neuramic acid may then be linked, which gives mucin its acidic properties (59). Of note, increased incidence of sulfation and sialylation has been observed in patients suffering from cystic fibrosis (60). Therefore, functionality may vary with carbohydrate alterations - which are ultimately dependent on transcriptional, translational, and post-translational mechanisms for each mucin type.

**Mucin Subtype Regulation in Disease**

MUC2, MUC5AC, and MUC5B mucin over- or under-production may exacerbate disease. In the small intestine and the colon, lack of MUC2 production or defective polarization has been linked to incidence of colon cancer or ulcerative colitis, respectively (61, 62). In the airway, allelic MUC2 gene variants were found to be more prominent in atopic patients with chronic asthma as compared to atopic individuals without asthma (63). Although inconclusive, the authors suggested that the longer MUC2 allelic variants could better protect the epithelium and surrounding milieu from asthmatic development.

Differential MUC5AC expression has been shown to be a prognostic indicator in cases of gastric carcinoma. MUC5AC mucin expression has been shown to decrease with tumor incidence, but patients with MUC5AC-positive tumors generally have lower survival rates (64). In airway, MUC5AC is considered to be the inducible mucin and may play an important role in causation and exacerbation of disease. *MUC5AC* gene expression was augmented in mild to moderate cases of inflammatory respiratory disease, specifically asthma (65), as well as in ovalbumin-sensitized mice infected with the respiratory syncytial virus (commonly associated with childhood asthma) (66). Muc5AC induced expression and secretion was also suggested to exacerbate obstructive airway disorders due to cigarette smoke component (acrolein) exposure using rat models (33). Interestingly, differential production of Muc5AC compared to Muc2 expression was shown, suggesting pulmonary impairment may be affected by differential regulation of a specific mucin subtypes.

MUC5B production has been linked to increased morbidity and mortality in pulmonary disease. Thornton et al. investigated the quantitative amounts of MUC2, MUC5AC, and MUC5B in the sputum of normal samples and samples from patients
suffering from respiratory disease (47). MUC5AC and MUC5B were detected in collected samples; however, MUC5B (in particular, the glandular-derived, low-charged form) was dominant in samples collected from patients with cystic fibrosis and chronic obstructive pulmonary disease. It was theorized that inflammatory stimulation leads to increased production of the low-charged variant of MUC5B. This variant may adopt a more compact structure, leading to denser, less mobile mucus (characteristic of obstructive pulmonary disease). In addition, MUC5B upregulation on the surface epithelium has been shown in animal asthmatic models (48).

**Common Rodent Models in Mucin Research**

Because of convenience, cost, accessibility, and available tools, many different animal and cell culture models are used today in pulmonary research. While relevant information can be obtained when using non-human subjects, it is important to situate the findings in an appropriate context. It is therefore important to review current knowledge about an animal and highlight obvious differences that may affect overall assumptions regarding human pulmonary activity and function.

**Mice**

The mouse is the most commonly used mammalian model of pulmonary research, including transgenic, exposure, and behavioral studies, among others. Significant advancements in mouse mucin research were made with a recent study conducted by Escande et al. (25). *Muc2, Muc5AC, Muc5B, and Muc6* gene sequences and organization, as well as expression patterns, were elucidated in mouse. As in human, the deduced coding sequences of the gel-forming mucins showed striking similarity to the von Willebrand Factor. Muc5B presence was strong, but Muc2 was absent in the laryngo-tracheal tract. Muc5AC expression was weak or non-existent. This difference, as compared to human airway, might be due to low goblet cells numbers in normal mouse airways. This is not the only histologically relevant difference, as sub-mucosal glands are completely absent in mouse airways (67). It has been suggested that this characteristic may be advantageous to mucus obstructive studies, as the
mouse model would then be ideal for focusing on the role of goblet cells in mucus overproduction in airway disease (32). Interleukin-4 and interleukin-13 have been shown to initiate mucin overproduction in murine models with differential expression of mucin subtypes (68-71). However, contribution from the mucous cells of the submucosal glands cannot be assessed using this model.

Rats

Rat models are commonly used to study chronic exposure to pollutants and particulates, especially cigarette smoke. As an example, multiple studies have been conducted using sulfur dioxide (SO₂)-induced chronic bronchitis in the large airways of rats (72, 73). Specifically, Muc2 expression was upregulated in virus-infected rats after SO₂ exposure (74). Notably, the rat airway exhibited near-zero constitutive expression (75). This, however, differs with findings in human studies where neutrophil elastase (a common inflammatory mediator in chronically bronchitic airways) elicited an increase in MUC5AC expression and message stability (76). A more recent study, focusing on rat Muc5AC gene expression in response to wood smoke, found that there was increased expression over 7 days after a short-30 minute exposure (77). Theses studies, therefore, show that both Muc2 and Muc5AC are expressed and can be modulated in response to stimuli in rat airways.

Physiologically, the rat airway does process particulate matter differently than human and other rodent models. It has been stated that rat airways demonstrate a faster clearance pattern and seem to be more reactive to dust burden insult in toxicology studies than of human airways (78). In addition, the SO₂-exposed rat model of induced bronchitis, although exhibiting similar patterns of mucus hypersecretion, did not exhibit persistent fibrosis and chronic inflammation usually seen in human bronchitis (79). Physiological and histological differences from human in the cellular makeup of the rat tracheal epithelium are also evident. For example, the rat airway epithelium rarely expressed mucosal cells, which, interestingly, differed from that of rat tracheal cell culture (80). However, the Brown Norway rat, a commonly used pathogen-free model of airway hypersensitivity, exhibited submucosal gland
expression and abundant mucus production (81), but the specific mucin subtype makeup of these secretions has yet to be determined.

**Guinea pigs**

Historically, guinea pigs have been a popular choice for respiratory study because of striking pathophysiological similarities to the human airway. The antigen-sensitized and -challenged guinea pig mimics asthmatic human airway hyperresponsiveness and eosinophilic infiltration (82-85). In addition, the guinea pig trachea, unlike mice, expresses submucosal glands (86), but the complement has been suggested to be smaller than that of the human condition (87). Also, unlike normal mice and rat airways, mucus-producing goblet cells are constitutively present in the airway epithelium (39). Many studies focusing on mucus secretion in guinea pig, however, have shown that these animals do indeed exhibit patterns of goblet cell hyperplasia and hypersecretion (39, 88, 89). Guinea pig tracheal epithelial cells also exhibit a mucus phenotype in air-liquid culture, which modulates mucus expression with stimulus (see Table 1 for examples), thereby expanding the versatility of the guinea pig model (90). Unfortunately, few studies have attempted to identify specific mucin subtypes in the guinea pig mucus makeup, primarily due to the lack of available molecular tools. However, a recent study conducted by our laboratory has identified Muc2 and Muc5AC transcript in the guinea pig lung, both of which were upregulated in ovalbumin-sensitized airways (20). Interestingly, in epithelium tracheal cell culture, only Muc2 (and not Muc5AC) was present, suggesting that the guinea pig surface mucous cell production may differ from what is commonly seen in human and other mammalian airways. It has been shown, however, that Muc5AC protein can be induced in guinea pig tracheal epithelial cells with interleukin-13 exposure (34), although its contribution to the final mucosal makeup was not reported.
### Table 1. Airway Epithelial Mucin Upregulation by Treatment and Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Type</th>
<th>Message?</th>
<th>Protein?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Interleukin-1β</td>
<td>5AC</td>
<td>Y</td>
<td>Y</td>
<td>(35, 37)</td>
</tr>
<tr>
<td></td>
<td>Interleukin-4</td>
<td>2</td>
<td>Y</td>
<td>?</td>
<td>(19)</td>
</tr>
<tr>
<td></td>
<td>Interleukin-6</td>
<td>5AC, 5B</td>
<td>Y</td>
<td>?</td>
<td>(40)</td>
</tr>
<tr>
<td></td>
<td>Interleukin-8</td>
<td>5AC</td>
<td>Y</td>
<td>?</td>
<td>(91)</td>
</tr>
<tr>
<td></td>
<td>Interleukin-9</td>
<td>5AC, 2</td>
<td>Y</td>
<td>?</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>Interleukin-17</td>
<td>5AC, 5B</td>
<td>Y</td>
<td>?</td>
<td>(40)</td>
</tr>
<tr>
<td></td>
<td>Tumor necrosis factor-α</td>
<td>5AC, 2</td>
<td>Y</td>
<td>Y</td>
<td>(36-38, 92)</td>
</tr>
<tr>
<td></td>
<td>Prostaglandin-E₂</td>
<td>5AC</td>
<td>?</td>
<td>Y</td>
<td>(37)</td>
</tr>
<tr>
<td></td>
<td>Lipopolysaccharide</td>
<td>5AC</td>
<td>Y</td>
<td>Y</td>
<td>(37)</td>
</tr>
<tr>
<td></td>
<td>Platelet activating factor</td>
<td>5AC</td>
<td>Y</td>
<td>Y</td>
<td>(37)</td>
</tr>
<tr>
<td></td>
<td>Adenosine 5'-triphosphate</td>
<td>5AC</td>
<td>?</td>
<td>Y</td>
<td>(42)</td>
</tr>
<tr>
<td></td>
<td>Uridine 5'-triphosphate</td>
<td>5AC, 5B</td>
<td>Y</td>
<td>?</td>
<td>(43)</td>
</tr>
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<td></td>
<td>Neutrophil elastase</td>
<td>5AC</td>
<td>Y</td>
<td>Y</td>
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</tr>
<tr>
<td></td>
<td>Retinoids</td>
<td>5AC, 5B, 2</td>
<td>Y</td>
<td>Y</td>
<td>(45, 93)</td>
</tr>
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<td>Epidermal growth factor</td>
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<td>Y</td>
<td>?</td>
<td>(94)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Interleukin-1β</td>
<td>5AC</td>
<td>?</td>
<td>Y</td>
<td>(37)</td>
</tr>
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<td>Interleukin-4</td>
<td>5AC</td>
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<td>(19)</td>
</tr>
<tr>
<td></td>
<td>Interleukin-9</td>
<td>5AC, 2</td>
<td>Y</td>
<td>?</td>
<td>(21)</td>
</tr>
<tr>
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<td>Interleukin-13</td>
<td>5AC</td>
<td>Y</td>
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<td>(32)</td>
</tr>
<tr>
<td></td>
<td>Prostaglandin-E₂</td>
<td>5AC</td>
<td>?</td>
<td>Y</td>
<td>(37)</td>
</tr>
<tr>
<td></td>
<td>Ovalbumin challenge</td>
<td>5AC</td>
<td>Y</td>
<td>?</td>
<td>(32)</td>
</tr>
<tr>
<td></td>
<td>Acrolein</td>
<td>5AC</td>
<td>Y</td>
<td>?</td>
<td>(33)</td>
</tr>
<tr>
<td>Rat</td>
<td>Platelet activating factor</td>
<td>5AC</td>
<td>Y</td>
<td>?</td>
<td>(39)</td>
</tr>
<tr>
<td></td>
<td>Acrolein</td>
<td>5AC</td>
<td>Y</td>
<td>Y</td>
<td>(33)</td>
</tr>
<tr>
<td></td>
<td>Epidermal growth factor</td>
<td>5AC</td>
<td>Y</td>
<td>?</td>
<td>(95)</td>
</tr>
<tr>
<td></td>
<td>Retinoids</td>
<td>5AC</td>
<td>Y</td>
<td>?</td>
<td>(96)</td>
</tr>
<tr>
<td>Guinea</td>
<td>Interleukin-13</td>
<td>5AC</td>
<td>?</td>
<td>Y</td>
<td>(34)</td>
</tr>
<tr>
<td>Pig</td>
<td>Tumor necrosis factor-α</td>
<td>2</td>
<td>Y</td>
<td>?</td>
<td>(20)</td>
</tr>
<tr>
<td></td>
<td>Ovalbumin challenge</td>
<td>5AC, 2</td>
<td>Y</td>
<td>?</td>
<td>(20, 85)</td>
</tr>
<tr>
<td></td>
<td>Residual oil fly ash</td>
<td>2</td>
<td>Y</td>
<td>?</td>
<td>(97)</td>
</tr>
</tbody>
</table>
INFLAMMATION

The Airway Epithelium and Inflammation

The respiratory airways are lined both extrapulmonarily and intrapulmonarily by epithelial cells. The epithelium, therefore, is the first area of contact to potentially hazardous external environmental agents, including pathogens, particulates, allergens, cytotoxic agents, and irritants. Inflammatory response to these agents is generally assumed to be coordinated by the classic inflammatory cells, such as neutrophils, eosinophils, leukocytes, and macrophages. However, there is overwhelming evidence that the airway epithelium is a major mediator of pulmonary inflammation. The airway epithelium utilizes paracrine/autocrine, exocrine, and endocrine signaling mechanisms in mediating inflammatory response.

Paracrine/autocrine inflammatory regulation in epithelial cells

Many agents expressed by airway epithelial cells act to stimulate secondary or feedback mechanisms in the epithelium itself. This type of mediation helps to either augment or suppress an initial or established response. One way a cell may achieve this is by expressing a chemical inflammatory mediator, also known as a cytokine.

A major cytokine that regulates many epithelial cellular processes via auto/paracrine mechanisms is tumor necrosis factor-α (TNF-α). TNF-α has been shown to stimulate production of other epithelial-derived cytokines, such as interleukins-5, 6, and 8, and granulocyte colony stimulating factor (GM-CSF), as well as adhesion molecules vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), E-cadherin, and beta- and gamma-catenin (98-102). Conversely, TNF-α promotes production of the anti-inflammatory molecule Clara cell secretory protein (CCSP) through a post-transcriptional mechanism (103). Tumor necrosis factor-α has also been shown to modulate mucin secretion, which has already been reviewed in Part I of this introduction. TNF-α exerts its functions by binding to specific cell surface receptors. To date, two receptors have been identified, TNF-R1 and the less abundant TNF-RII. Both receptors exist in lung tissue (104),
as well as rat epithelium (105), but only TNF-R1 transcript is expressed in NHBE cell culture (106). While not much is known about receptor activation/regulation in the airway epithelium, these two receptors may play differential roles. For example, increased shedding of TNF-RII, but not TNF-RI, in the lung tissue of a mild inflammatory pneumonia model of mice, suggests that the TNF-RI to TNF-RII ratio may play an important role in development or exacerbation of inflammatory disease (107).

Another major epithelium-derived cytokine that has been shown to modulate many secondary processes through autocrine and paracrine action is the interleukin-1 family of cytokines, in particular interleukin-1β (IL-1β). In addition to mucin modulation, IL-1β has been shown to alter tight junction ion selectivity (108), augment nerve growth factor (NGF) production (109), induce ICAM-1 and VCAM-1 expression (110), enhance β2-adrenergic receptor expression (111), induce sTNF-RII shedding (112), and augment production of secondary inflammatory mediators including GM-CSF, cyclooxygenase-2 and prostaglandin E2 (113, 114). Recently, a study utilizing the respiratory carcinomic cell line NCI-H292 focused on gene expression response to 6 and 24-hour exposures of 10 ng/ml IL-1β (115). Expression of 413 genes that mediated metabolism, cell communication, cell cycle, transcription, and translation were modulated by IL-1β.

Interferon-γ (IFN-γ) is a very potent Th1-response cytokine, whose global effects counteract allergic-type inflammatory response. In the airway epithelium, IFN-γ has been shown to attenuate transforming growth factor-β, eotaxin-3, and 15-lipoxygenase, whereas these inflammatory mediators are augmented by Th2-type signaling pathways (116-118). However, IFN-γ also can upregulate inflammatory mediators, including the inducible synthase of nitric oxide (iNOS, see review below) through a STAT 1 (signal transducer and activator of transcription) mediated pathway (119). In addition, IFN-γ exposure to human bronchial epithelial cells infected with rhinovirus (common respiratory infection associated with virus-induced asthma) enhanced the monocyte chemoattractant RANTES (Regulated on Activation, Normal T cells Expressed and Secreted) (120). Similarly, IFN-γ initiated fractalkine expression, which also works as a potent chemoattractant of monocytes and T lymphocytes (121).
Secondary mediators of epithelial-derived inflammation – focus on GM-CSF

Once initiated by signals from inflammatory cells and surrounding milieu, or triggered by environmental antigens, the airway epithelium responds by secreting a number of chemical mediators that directly and indirectly combat invasion. Unfortunately, in chronic, pre-existing conditions, such as asthma and chronic bronchitis, increased amounts of inflammation may be deleterious to the surrounding airway, causing irreparable damage and further exacerbation of disease. These mechanisms may include upregulation of viral-promoting adhesion molecules, release of reactive oxygen/nitrogen species, and unnecessary longevity of inflammatory cell life in an already overpopulated environment.

The airway epithelium produces a wide assortment of cytokines that promote recruitment, activation, and longevity of inflammatory cell types. These inflammatory cells, with their subsequent epithelial-derived mediators in parentheses, include: T lymphocytes (RANTES, IL-6, IL-16), natural killer cells (IFN-α/β, MIP-1α), macrophages (IL-1β, MIP-1α, MCP-1, TNF-α), eosinophils (GM-CSF, eotaxin 1/2, RANTES, MIP-1α), and neutrophils (IL-8, Groα, ENA78) (reviewed in (122)). In addition, the airway epithelium can produce lipid mediators, including prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids (HETES), and platelet-activating factor (PAF), as well as numerous reactive oxygen/nitrogen species.

Granulocyte macrophage colony stimulating factor (GM-CSF) was first described as a generator of granulocytes and macrophages from an inflammatory precursor cell population (123), however, its role has been expanded to include regulation of the functionality of these inflammatory cells (124). In addition, GM-CSF is a powerful activator of dendritic cells, an antigen presenting subtype that regulates the activation and differentiation of naïve T-cells (reviewed in (125)). Bronchial epithelial cells are known to produce GM-CSF (113). Strikingly, GM-CSF levels, as measured in bronchoalveolar lavage fluid (BALF) of asthmatics, correlate with severity and eosinophilic infiltration (126, 127). Additionally, bronchial epithelial cells isolated from asthmatic patients and cultured in vitro expressed more GM-CSF than non-asthmatic cultures after ozone exposure (128). This increased production has also been observed in nitrogen dioxide and diesel exhaust-exposed epithelial
cell cultures (128, 129), and in biopsied bronchial mucosal tissue after 2-hour ozone exposure (130).

GM-CSF may play a significant role in the initiation and exacerbation of asthma. Asthma sufferers are marked with a T-cell helper type-2 dominated profile (humoral-dominated immunity) (131). Development of this T-cell subtype is dependent on the differentiation of the naïve T-cell, which relies on communication with antigen-presenting cells (APC). The most prominent APC is the dendritic cell (DC). Interestingly, GM-CSF has been shown to differentiate and induce proliferation of dendritic cells of the myeloid lineage (132). Adoptive transfer of GM-CSF-stimulated myeloid DCs into mice elicited an immune response characterized by the presence of Th2-associated cytokines and immunoglobulins (133). Furthermore, allergen challenge considerably lowered blood-circulating levels of myeloid DCs in asthmatic patients (134), presumably due to migration toward the airway mucosa (135). Consequently, GM-CSF has been shown to play a vital role in the inflammatory state of the airway as GM-CSF neutralizing antibody significantly reduced airway hyperresponsiveness and inflammation in experimentally-induced asthmatic mice (136).

Nitric Oxide is a Key Mediator of Airway Inflammation

One of the most potent and pervasive biomessengers is the free radical nitric oxide (NO). This gaseous molecule, lipophilic in nature, can freely diffuse through cellular barriers and react with proteins through an unpaired electron in its outer atomic orbit. Since the pioneering cardiovascular work by Nobel Prize winners Robert Furchgott, Ferid Murad, and Louis Ignarro, NO has since been universally recognized to play varied biological roles throughout all human tissues and cell types. Nitric oxide was first described as endothelial-derived relaxant factor (EDRF) (137) but has since been identified to act as a neurotransmitter in the nervous system (138, 139), regulator of gastrointestinal peristalsis (140), important mediator in penile erectile dysfunction (141), and progenitor and repressor of tumorogenesis (142), among others (see reviews (143-145)).

Nitric oxide mediates many processes in the lung and conducting airways. Initially, NO was shown to have profound effects on airway smooth muscle (146). This effect was
mediated by an intracellular rise in cyclic-GMP levels, caused by direct activation of guanylyl cyclase (147, 148). This increase in cyclic-GMP levels activate its dependent kinase (PKG), which activate Ca^{2+}-dependent K^{+} channels (which relax smooth muscle), initially described by Tamakage et. al. in porcine airway smooth muscle cells (149). In addition to the traditional cGMP-mediated pathway, direct nitrothiosylation of K^{+} channels or sequestration of “Ca^{2+} sparks” into the plasmalemma via the oxidative form of nitric oxide (NO^+) also mediate airway smooth muscle relaxation (150, 151). NO also regulates the ciliary movement of ciliated cells of the airway epithelium, which significantly contributes to mucosal clearance in the conducting airway. β-adrenergic agonists, cytokines, and methacholine have all been described to influence ciliary beat via nitric oxide formation (152-155).

Of focus are the varied, and sometimes contradicting, effects on airway inflammation. For example, contrasting regulation of the pro-inflammatory transcription factor NF-κB by NO has been described. Nam et al. found that particulate matter could activate NF-κB, and hence promote pro-inflammatory gene transcription through a pathway involving NO synthase in alveolar type II epithelial cells (156). However, NO has also been shown to stabilize inhibitory κB (IκB), a protein that sequesters NF-κB and prevents nuclear translocation (157). Reyanaert et al. demonstrated that this may be accomplished through NO-mediated inhibition of IκB kinase, the enzyme responsible for IκB degradation (158). A number of factors can influence how NO mediates it actions, including the location, amount, and time of NO production, redox status and bioavailability of anti-oxidant and reactive-oxidant species, as well as the inflammatory state of the surrounding milieu. Given the multiple routes that NO can elicit its effects, it was no surprise that researchers later demonstrated NO could suppress NF-κB activity while the reactive NO-metabolite, peroxynitrite (ONOO^-), could sustain NF-κB activity (159).

**Inducible Nitric Oxide Synthase and its Regulation**

Nitric oxide production is achieved in mammalian cells by an enzymatic process mediated by three distinct subtypes of nitric oxide synthase (NOS). Neuronal NOS and endothelial NOS
(nNOS and eNOS) are characterized by low NO output and are dependent on Ca\(^{2+}\) for their activity. These subtypes are considered to be constitutively expressed in many cell types, including neurons, endothelial cells, and epithelial cells. Their NO output can be measured at a picomolar level (160). The third subtype, inducible nitric oxide synthase (iNOS), is regulated by inflammatory mediators, such as cytokines and microbial products, and is not dependent on Ca\(^{2+}\) for its activity (160, 161). iNOS is known for its high NO output, and is usually measured in nanomolar levels.

iNOS, while only present when induced in many cell types, is constitutively expressed in the normal airway epithelium (162). Constitutive iNOS expression is uncommon in mammalian cell types and therefore elicits the theory that iNOS plays a unique and essential role in the airway epithelium. Indeed, iNOS is considered critical to inflammatory response, as it can upregulated by pro-inflammatory cytokines such as TNF-\(\alpha\), IL-1\(\beta\), or IFN-\(\gamma\) (163-165). Interestingly, constitutive iNOS expression is lost in human airway epithelial cells placed in culture (162). This is presumably due to the loss of exogenous signaling from other cell types in vivo or as a result of an undifferentiated cell culture, however, iNOS expression can be recovered with exogenous stimulation (166).

iNOS is considered to be transcriptionally regulated. Numerous binding sites for transcription factors have been located on the iNOS promoter in various species, including AP-1, ARE, C/EBP, c-ETS-1, CREB, GATA, HIF, IRF-1, NF-1, NFAT, NF-\(\kappa\)B, NF-IL6, Oct-1, PEA3, p53, Sp1, SRF, and STAT-1\(\alpha\) (reviewed in (167)). In the human alveolar epithelial cell line A549, TNF-\(\alpha\), IL-1\(\beta\), and IFN-\(\gamma\) synergistically induced maximal iNOS transcription, with removal of one cytokine reducing iNOS gene induction 38%, 41%, and 83%, respectively (168). Kwon et al. studied the synergistic effects of these three cytokines on iNOS monomer, co-factors, and nitric oxide production in the human alveolar epithelial cell line A549 (169). The authors suggested that the synergy between TNF-\(\alpha\) and IL-1\(\beta\) might be due to increased production of iNOS monomer and tetrahydrobiopterin (BH4), an essential factor for iNOS dimer formation. Production of these proteins is mediated through NF-\(\kappa\)B, where it was additionally shown that IFN-\(\gamma\) augmented NF-\(\kappa\)B translocation by degrading I\(\kappa\)B in the presence of IL-1\(\beta\). IFN-\(\gamma\), presumably through a STAT-1 mediated
pathway, induced IRF-1 production and nuclear translocation, which can also promote iNOS expression (164, 170).

Once induced, many different agents may actively augment or attenuate iNOS expression. NO itself has been described, to both induce and suppress expression of its own synthase. For example, chemical inhibition of iNOS enhanced its own transcription, and exogenous NO application attenuated iNOS expression in a mouse macrophage cell line (171-173). NO, conversely, augmented LPS/cytokine-induced iNOS expression in rat mesangial cells (174). Possible mechanisms by which NO regulates iNOS synthesis include pathways involving cGMP, p53, hypoxia-induced factor-1, NF-κB, and STAT-1 (reviewed in (167)). cAMP can also modulate iNOS expression in a stimulated environment. In rat aortic smooth muscle cells, Durante et al. demonstrated that cAMP could augment iNOS expression synergistically with IL-1β in rat aortic smooth muscle cells (175).

iNOS may also be regulated by message stabilization. iNOS message has been shown to be the target of rapid degradation because of a highly susceptible 3’-gene sequence (176). Consequently, Carpenter et al. demonstrated that PKCδ activity was required for IL-1β augmentation of iNOS message in rat insulinoma cell line INS-1 through a mechanism that stabilized iNOS message (177). The authors speculated that PKCδ stabilized iNOS message through the same mechanism that PKC stabilized lactate dehydrogenase, which shares the same degradation-prone region of the 3’ transcript sequence (178).

Nitric Oxide’s Influence on Inflammation May Depend on Reactive Metabolites

NO has an odd number of electrons, which is the foundation of its highly reactive and radical nature. NO can either accept an electron and form nitoxyl anion (NO⁻) or lose an electron to form nitrosonium cation (NO⁺). NO, NO⁻, or NO⁺ may then react with reactive oxygen species, other free radicals, transition metals, thiols, and molecular oxygen to form other reactive species (179). NO reacts with superoxide (O₂⁻) to form peroxynitrite (ONOO⁻), one of the best-understood and most studied reactive nitrogen species. Peroxynitrite may be protonated to yield peroxynitrous acid (ONOÖOH), an unstable, highly reactive species, capable of both oxidizing and nitrating reactions. ONOOH can, for example, irreversibly
nitrate aromatic amino acids (in particular, tyrosine), lipids, and DNA bases (180-182). Other NO-derived metabolites include nitrite (NO\textsubscript{2\textsuperscript{-}}), nitrate (NO\textsubscript{3\textsuperscript{-}}), nitrogen dioxide (NO\textsubscript{2}), nitrosoamines, and S-nitrosothiols (183). Depending on surrounding environmental conditions and localized concentration, a vast array of reactive metabolites may form, and, therefore, it is not surprising that nitric oxide can mediate simultaneously opposing events.

Reactive nitrogen species may support the progression of many respiratory diseases. For example, asthmatic patients have increased levels of 3-nitrotyrosine in the lung, a major by-product of peroxidase activity (184). Increased peroxynitrite exposure in the lung can significantly contribute to airway inflammation in mice (185). Indeed, the possibility of deleterious reactive nitrogen species formation is of concern, since available substrate (e.g. superoxide) production in airway can increase after antigen exposure in individuals with allergic disease (186). Epithelial injury as a result of peroxynitrite and nitrogen dioxide exposure can also result from induced cell death via transcriptionally and translationally-dependent mechanisms (187). In contrast, many studies have shown NO, its metabolites, and its inducible synthase are beneficial and sometimes necessary in suppressing an overzealous inflammatory response. In mouse knockout studies, it was shown that iNOS -/- mice exhibited increased susceptibility to ovalbumin challenge, which included augmented fibrosis and inflammation (188). NO-depletion in pulmonary cell culture led to cell cycle perturbations, decreased DNA synthesis, and enhanced oxidative stress (189). iNOS activity also served to balance oxidative radical production, as demonstrated in mice models of acute hyperoxic lung injury (190). In addition, Sanders et al. demonstrated that GM-CSF production in bronchial epithelial cells, induced by rhinovirus exposure, was attenuated with nitric oxide exposure in a dose- and time-dependent manner (191).

\textbf{β\textsubscript{2}}-adrenergic Receptor Activation Suppress Airway Inflammation

The \(β\)-adrenergic receptor is a post-ganglionic neuroeffector of sympathetic signaling specific for autonomic response of the heart and lung. The \(β\)-adrenoceptor family is divided into three groups: \(β\textsubscript{1}, β\textsubscript{2}, \) and \(β\textsubscript{3}\). While augmented alveolar fluid clearance regulation has been reported with \(β\textsubscript{1}\) activation in the rat lung, such (192), \(β\textsubscript{2}\)-adrenergic receptors are the most widely distributed throughout the airway. They are associated with airway smooth
muscle, epithelial, endothelial, type II alveoli, and mast cells (193). β2-adrenergic agonism activates adenylate cyclase via a trimeric Gs protein signal transduction event, which mediates smooth muscle relaxation (194).

Other signaling mechanisms can also be linked to the β2-adrenergic receptor. In the airway epithelium, agonists can augment ciliary beat frequency (195, 196), ion transport (197), repair (198), and attenuation of inflammatory response (199-201). In addition, monocytic production of interleukin-8 by lipopolysaccharride exposure was attenuated by β2-adrenergic agonists via NF-κB-mediated pathway (202). Similar suppressive activities of interleukin-8 were demonstrated in neutrophils using long-acting agonists (203). β2-adrenergic stimulation in airway smooth muscle cells responded by downregulating inflammatory cytokines GM-CSF, RANTES, and eotaxin induced by TNF-α and IL-1β stimulation (204).

In the airway epithelium, the β2-agonist fenoterol attenuated IFN-γ-induced ICAM-1 expression in a dose-dependent manner (199). In addition, GM-CSF was suppressed with β2-adrenergic activity in stimulated bronchial culture (200, 201), while, interestingly, interleukin-8 was augmented (200). It is unclear how β2-adrenergic receptor mediates inflammation in airway epithelial cells, but cAMP-dependent kinase (PKA) may be involved. A recent study found that salbutamol-mediated GM-CSF inhibition was blocked by a PKA inhibitor (201). It is also known that nitric oxide synthase activity increased in airway epithelial cells with long-acting β2-agonist administration in rats (205). β2-agonist induced NO might well influence the inflammatory response in airway epithelial cells.
SUMMARY AND HYPOTHESES

The studies outlined in this dissertation focus on two critical processes of the airway epithelium: mucus expression and inflammatory mediacion. While these two processes are unique and may involve separate mechanistic pathways, they both are vital to normal pulmonary function. In addition, inappropriate, untimely, or unabated mucus expression or inflammation can contribute to morbidity and/or mortality of respiratory diseases. It is therefore important to delineate the signaling mechanisms that coordinate these processes so that succinct, targeted therapies can be developed.

The hypothesis of the first study states that pro-inflammatory stimulation of guinea pig airway epithelial cells results in differential expression of specific mucin subtypes. It has been shown in other models that mucin subtypes are differentially expressed, depending on the stimuli, and that this differential production may alter the normal mucus makeup, thereby altering its interactive and clearance properties. The guinea pig model has historically been used in pulmonary research because of pathophysiologic similarities to human airway, but mechanistic studies have been limited due to a lack of appropriate molecular tools. Therefore to address the hypothesis, two monoclonal antibodies to Muc2 and Muc5AC mucin were developed and characterized, respectively. The two newly characterized monoclonal antibodies were then used to analyze Muc2 and Muc5AC message expression, protein production, and secretion in guinea pig tracheal epithelial (GPTE) cell culture maintained in a biphasic air/liquid interface system. The results provide the first evidence that the guinea pig epithelium increase secretion of Muc2, but not Muc5AC, in response to a pro-inflammatory cytokine mixture in guinea pig tracheal epithelial cell culture.

Interestingly, this is the first evidence that these two mucin subtypes can be differentially secreted, when both intracellular protein production and message expression increase similarly upon pro-inflammatory stimulation. In addition, the finding that Muc2 mucin is preferentially secreted over Muc5AC contradicts many findings in the literature that state Muc5AC mucin is primarily modulated upon pro-inflammatory stimulation in other mammalian species besides guinea pig, including human.
The second study demonstrated that regulation of the inflammatory cytokine, granulocyte macrophage colony stimulating factor (GM-CSF), is influenced by inducible nitric oxide synthase via a cGMP-PKG-mediated pathway in airway epithelial cells. While other studies (see prior review) have shown that nitric oxide application attenuates GM-CSF production in stimulated airway epithelial cells, my study defines a mechanistic pathway involving iNOS may mediate this anti-inflammatory response. The findings of this study served as a foundation for the final study of this dissertation.

The hypothesis of the third study states that anti-inflammatory effects attributed to β2-adrenergic agonists are mediated through a signaling pathway involving inducible nitric oxide synthase (iNOS). Indeed, it is known that catecholamines, such as β2-adrenergic agonists, attenuate inflammatory mediators, but the signaling mechanisms involved are largely unknown. To address this hypothesis, normal human bronchial epithelial (NHBE) cells were grown in a submerged culture and stimulated with pro-inflammatory cytokines interleukin-1β and interferon-γ to induce iNOS transcription and augment GM-CSF. It was demonstrated that co-incubation with β2-adrenergic agonist (R)-albuterol attenuates GM-CSF expression and protein production. In addition, (R)-albuterol dose-dependently augmented iNOS message, a novel finding. Also, inhibition of iNOS, through RNA silencing, blocked β2-adrenergic-mediated attenuation of GM-CSF message. The results of this study define a novel mechanistic pathway in which β2-adrenergic activation mediates its anti-inflammatory effects, thereby expanding the knowledge base concerning current and future drugs used to treat respiratory disease.
CHAPTER II

DIFFERENTIAL MUC2 AND MUC5AC MUCIN SECRETION BY STIMULATED
GUINEA PIG TRACHEAL EPITHELIAL CELLS IN VITRO

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Submitted to: Respiratory Research

Key Words: guinea pig, airway epithelium, Muc2, Muc5AC, air-liquid interface
ABSTRACT

Mucus overproduction is a characteristic of inflammatory pulmonary diseases including asthma, chronic bronchitis, and cystic fibrosis. Expression of two mucin genes, MUC2 and MUC5AC, and their products, is modulated in certain disease states. Understanding the signaling mechanisms that regulate the production and secretion of these major mucus components may contribute significantly to development of effective therapies to modify their expression in inflamed airways. To study the differential expression of Muc2 and Muc5AC mucins, a novel monoclonal antibody recognizing guinea pig Muc2 mucin and a commercially-available antibody against human MUC5AC mucin were optimized for recognition of guinea pig Muc2 or Muc5AC mucin by ELISA, Western blot, and immunohistochemistry. These antibodies were then used to analyze Muc2 and Muc5AC mucin expression in guinea pig tracheal epithelial (GPTE) cells stimulated with a mixture of pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ). The anti-Muc2 (C4) and anti-MUC5AC (45M1) monoclonal antibodies specifically recognized proteins located in Muc2-dominant small intestinal and Muc5AC-dominant stomach mucosae, respectively, in all experimental protocols. In the resting state, Muc2 mucin was found to be the dominant intracellular mucin in GPTE cells. Following cytokine exposure, secretion of Muc2, but not Muc5AC, mucin from the GPTE cells was increased, with a concomitant increase in intracellular expression of both mucins. Given the tissue specificity in IHC and hybridization to appropriately-sized, high molecular weight proteins by Western blot, we conclude that the antibodies used in this study can specifically recognize their respective mucin subtypes in guinea pig epithelial tissues and in proteins from GPTE cells. In addition, Muc2, rather than Muc5AC, is a dominant mucin gene product expressed constitutively, modulated by inflammation, and secreted differentially in guinea pig tracheal epithelial cells. This finding is in contrast to expression patterns in the airway epithelium of a variety of mammalian species in which Muc5AC mucin predominates.
INTRODUCTION

In the mammalian airway, mucus secreted by the epithelium and submucosal glands provides a defensive barrier between the outside environment and the airways. Mucus traps, neutralizes, and eliminates inhaled irritants, pollutants, and pathogens. Unfortunately, conditions that provoke overexpression of gel-forming mucin glycoproteins (the major structural components of mucus) can clog the conducting airways, and, ultimately, impair effective gas exchange. Many airway diseases, including asthma, chronic bronchitis, and cystic fibrosis, exhibit mucus overexpression (206-208). Thus, understanding the mechanisms of expression and secretion of airway mucins has obvious pathophysiological significance and may assist in designing novel therapeutics for asthma and other airway diseases.

Airway mucins are derived from either epithelial goblet cells or epithelial cells of the submucosal gland (209). At least twenty mucin genes have been reported, with expression of eight detectable in the human airway (59, 210-213). Four of these mucin genes encode gel-forming mucins: MUC2, MUC5AC, MUC5B, and MUC6. MUC2 and MUC5AC expression are altered in inflamed airways (63, 76, 214, 215) and, therefore, may contribute to the pathogenesis of several respiratory diseases. These mucins also exhibit cell- and tissue-specific expression in mammals, where (other than airway) MUC2 is expressed primarily in gastrointestinal epithelium and MUC5AC in gastric epithelium (25, 31). Differential mucin subtype regulation may affect mucus composition in disease states, although little is known currently regarding mechanisms that might regulate such differential functions (216-220).

The antigen-sensitized and -challenged guinea pig is an excellent model of allergic asthma, exhibiting major hallmarks of human asthma, including airway hyperresponsiveness and eosinophilic inflammation (82-84, 221). However, research using the guinea pig model has been hampered by the lack of available molecular tools, especially for studying mucin subtypes. Recently, Muc2 and Muc5AC-specific probes were synthesized based on gene sequence information available from related mammalian species (20). It was found that Muc2 gene expression increased with tumor necrosis factor-α (TNF-α) stimulation in guinea pig tracheal epithelial cells, whereas little, if any, Muc5AC expression was measured. Muc2
expression in airway epithelium is not commonly reported in other mammalian species, whereas Muc5AC mucin is described frequently as the major gel-forming mucin in the airway epithelium of humans, horses and rodents (22, 24, 32, 33, 45).

The purpose of this study was to determine whether or not Muc2 and Muc5AC mucin subtypes were regulated differentially in the guinea pig tracheal epithelium. To this end, a monoclonal antibody against Muc2 apomucin was developed and a commercially available monoclonal antibody against human MUC5AC mucin was optimized for detection of guinea pig Muc5AC mucin. Guinea pig tracheal epithelial (GPTE) cells were exposed to a pro-inflammatory cytokine mix of TNF-α, interleukin-1β (IL-1β), and interferon-γ (IFN-γ), and subsequently tested for differential expression of Muc2 and Muc5AC mucin. While intracellular Muc2 and Muc5AC mucin production and message expression increased similarly, only Muc2 mucin secretion increased significantly over constitutive levels following inflammatory stimulation. These results demonstrate, for the first time, that mucin subtypes are regulated differentially in guinea pig epithelial cells, suggesting that different mechanisms may exist for mucin subtype storage and/or secretion.
MATERIALS AND METHODS

Cell Culture
Primary cultures of differentiated guinea pig tracheal epithelial (GPTE) cells were established using the air-liquid interface procedure (90). Briefly, guinea pig tracheas were excised from euthanized animals. Epithelial cells were dissociated proteolytically, washed, and seeded in Transwell inserts (Corning Costar, Cambridge, MA) coated with rat tail collagen type I (BD Biosciences, Franklin Lake, NJ) at a density of 5 X 10^4 cells/cm^2 in Dulbecco’s modified Eagle’s medium (DMEM)/F12 supplemented with 5% fetal bovine serum (FBS), 4 mM L-glutamine (Invitrogen, Carlsbad, California), 1% HL-1, 25 ng/ml recombinant human epidermal growth factor (Serologicals, Norcross, GA), 50 nM retinal acetate, 100 µg/ml gentamicin, 40 U/ml nystatin (Sigma, St. Louis, MO), and 0.5 µg/ml amphotericin-B. Cells were cultured at 37°C in an atmosphere of 3% CO₂. Medium was renewed every other day until the cells were 70-80% confluent (4-5 days), at which time medium was removed from the apical surfaces and cultures were fed basally with serum-free medium for an additional 7 days to allow mucous cell differentiation. Reagents noted above were purchased from Cambrex Corporation (East Rutherford, New Jersey), unless otherwise noted.

The Institutional Animal Care and Use Committee of North Carolina State University approved all protocols for the use of animals that pertain to this study.

Production of Anti-Mucin 2 Monoclonal Antibody
A 522 base pair fragment of guinea pig Muc2 cDNA, determined previously to be a coding region of the carboxy-terminal cysteine-rich region of guinea pig Muc2 mucin (20, 222), was cloned into a bacterial protein expression vector (pCAL-n; Stratagene, La Jolla, CA). The inserted sequence was verified by sequencing (DNA Sequencing Facility, University of North Carolina at Chapel Hill). The bacterial host strain BL21(DE3)pLysS (Stratagene, La Jolla, CA) was then transformed with the ligated vector. An overnight, ampicillin-selected BL21(DE3)pLysS culture was expanded in Luria-Bertani broth at 37°C for 3-5 hours with shaking. Vector expression was then induced by 1 mM isopropyl-1-thio-β-D-
galactopyranoside (IPTG; Sigma, St. Louis, MO) during logarithmic growth (culture density yielding 0.6 at OD₆₀₀ via UV spectrometry). Culture lysates of induced (+IPTG) and uninduced (-IPTG) cells were resolved on a 10% SDS-PAGE gel. Staining of total protein in the gel indicated high-level induction of a ~29 kDa protein, which formed an insoluble inclusion body. The Muc2 protein fragment was purified by elution from an electrophoresis gel, using high concentrations of detergent to keep the inclusion body soluble. After elution, the Muc2 protein fragment was verified by SDS-PAGE and dialyzed into phosphate buffered saline, pH 7.4 (PBS).

A monoclonal antibody was raised against this purified Muc2 protein fragment by the North Carolina State University Hybridoma facility. Specifically, a BALB/c mouse was inoculated 3 times with 100 µg of purified Muc2 mucin protein fragment mixed with Freund’s Incomplete Adjuvant over a 3 month time period. Serum was collected two weeks after the final inoculation.

Antibody against the protein fragment was detected in a 1:100 dilution of mouse serum collected from the antigen-challenged mouse via an enzyme-linked immunosorbent assay (see ELISA protocol below). Results indicated the presence of Muc2 mucin antibody in the serum as a reaction was observed in test wells coated with 50 ng of purified Muc2 protein fragment. Three additional booster injections were administered before antibody-producing spleen cells were collected and hybridized with murine myeloma cells (SP2/0-Ag14). Fused cells were diluted and plated to ~1 cell per well in eight 96-well plates and grown for two weeks in modified RPMI 1640 medium with 10% fetal bovine serum, 2 mM L-glutamine, 1X hypoxanthine-thymidine, 5 U/ml penicillin, and 5 µg/ml streptomycin at 37°C, 5% CO₂. All culture reagents were purchased from Cambrex, East Rutherford, New Jersey. 37 of 768 cultures were positive for Muc2 mucin antibody when assayed by ELISA. These cultures were expanded and retested. One clone (clone 4.68a) was selected for best overall performance based on immunohistochemistry, Western blot, and ELISA testing and was used for monoclonal selection and expansion.

Two additional rounds of small-scale expansion and selection were performed. Media containing secreted antibody from each clone was screened against the purified protein fragment by ELISA. The hybridoma clone that secreted the antibody with the
strongest immunoreactivity was selected for final large-scale expansion (clone C4). Culture expansion was carried out in 225 cm$^2$ flasks (Corning Costar, Cambridge, MA) using the same media as above, with the exception that only 5 % FBS was added in addition to 5% P388D1-derived growth supplement (American Type Culture Collection, Manassas, VA). The monoclonal antibody was purified using a Protein L column (Pierce, Rockford, IL) according to manufacturer’s instructions. Column fractions containing antibody, were verified by ELISA, desalted and resuspended in 50% glycerol. The purified monoclonal antibody was isotyped as mouse IgM using a commercially available isotyping kit (Roche, Basel, Switzerland).

**Collection of Guinea Pig Tissues**
Trachea, stomach, liver, and small intestine were dissected from euthanized adult, male Hartley guinea pigs (Charles River, Stone Ridge, NY). For frozen sections, small portions of tissue were submerged in ice-cold PBS and then frozen in liquid nitrogen before sectioning. For paraffin-embedded sections, tissue was placed in 2.0% formaldehyde, rested overnight, and then paraffin-embedded and sectioned.

Mucus secretions were also collected from small intestine, stomach and trachea tissue samples. The internal epithelial surface of each tissue was exposed, rinsed repeatedly with PBS, and then scraped with a rubber policeman to remove mucus. Mucus was diluted (1:1) into PBS containing Complete Mini protease inhibitor cocktail (Roche, Basel, Switzerland). Isolated secretions were stored at -80°C.

**Western Blot**
Samples of isolated mucus secretions were solubilized in sample buffer (23), spun at 10,000 rpm for 5 minutes, and loaded in a 1.0% agarose gel using a horizontal gel apparatus (BioRad, Hercules, CA). Electrophoresis of samples was carried out at 15 V (1.9V/cm gel) for 18 hours. After the gel was equilibrated in tris/glycine (BioRad, Hercules, CA) buffer for 30 minutes, proteins were transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Transfer occurred after approximately 2 hours at 12V. The nitrocellulose membrane was washed in
PBS for 5 minutes, and then blocked in 3% milk in PBS for 1 hour with slight agitation at room temperature. The membrane was hybridized with primary antibody (newly developed C4 or 45M1, Cat# MS145-PABX from NeoMarkers, Fremont, CA), at stated dilutions, in 1% milk at 4°C overnight. After washing the nitrocellulose membrane three times in PBS at room temperature (10 minutes each wash), the membrane was incubated with HRP-conjugated goat anti-mouse antibody (MP Biomedicals, Irvine, CA) diluted 1:2000 in 1% dry milk for 2 hours on a rocker at room temperature. The membrane was then washed three times in PBS, washed additionally in PBS + 0.05% Tween-20, and finally rinsed five times with double-distilled H₂O. ECL chemiluminescent detection (Amersham Biosciences, Piscataway, NJ) was used for final visualization. Molecular masses of the resultant bands were predicted using a computer software program (Vector NTI, Invitrogen, Carlsbad, California).

**Enzyme Linked Immunosorbent Assay (ELISA)**
A 96-well high-binding ELISA plate (Corning Costar, Cambridge, MA) was coated with experimental samples at 4°C overnight. The plate was then washed twice with PBS. Wells were blocked with a solution of 3% cold-water fish gelatin (Sigma, St. Louis, MO) in PBS for two hours at room temperature. Following two washes with PBS, samples were exposed to the primary antibody (newly developed C4 or 45M1, Cat# MS145-PABX) diluted as indicated in 0.3% cold-water fish gelatin for 1 hour at room temperature. The plate was washed three times, labeled with a horseradish peroxidase-conjugated goat anti-mouse IgG (MP Biomedicals, Irvine, CA) diluted 1:2000 in 0.3% cold-water fish gelatin, and incubated for 1 hour at room temperature. After secondary antibody incubation, the plate was washed five times with PBS, and the color developed for 5 minutes to 3 hours, depending on primary antibody concentration and immunoreactivity. A 1M H₂SO₄ solution was then added to halt color development and absorbance read at 450 nm.
**Immunohistochemistry**

Frozen or paraffin-embedded sections of guinea pig stomach, small intestine, liver or trachea were immunostained by the Histopathology Laboratory at the North Carolina State University, College of Veterinary Medicine. Briefly, sections were rinsed with deionized water before a 10 minute immersion in 3% H$_2$O$_2$ in methanol. After rinsing with ice-cold deionized water and then PBS, sections were blocked with normal goat serum for 20 minutes. Immediately after, primary antibody (C4 to detect Muc2 and 45M1 to detect Muc5AC mucin) was applied for 30 minutes. A 1:20 to 1:100 dilution of C4, or 45M1, supplied diluted by the manufacturer (Cat # MS-145-R7 from NeoMarkers, Fremont, CA), was used. Sections were rinsed with PBS and incubated with biotin-labeled goat anti-mouse antibody (Vector Labs, Burlingame, CA) for 20 minutes. Sections were rinsed again with PBS, and incubated with streptavidin peroxidase complex for 20 minutes. Sections were then rinsed with PBS, and developed with 0.05% 3,3’-diaminobenzidinetetrahydrochloride (DAB) for 30 seconds to 2 minutes. Sections were rinsed in tap water and counterstained with hematoxylin for 20 to 40 seconds. Sections were rinsed for 5 minutes under tap water, then dehydrated with xylene and mounted. As positive controls, normal mouse stomach and intestine were prepared and immunostained similarly. Negative controls were performed by substituting normal mouse serum or PBS for primary antibody. Both paraffin-embedded and frozen tissue sections were used in IHC. Both the C4 and 45M1 antibodies were capable of staining both types of sections, however higher background was evident in paraffinized sections.

**Real-time Reverse Transcriptase Polymerase Chain Reaction**

Total RNA was isolated from lysates of GPTE cells using the RNeasy Mini Kit according to manufacturer’s instructions (Qiagen, Valencia, CA). Each sample was treated with RNase-free DNase for 15 minutes to reduce DNA contamination. RNA with $A_{260}/A_{280}$ ratio of 1.95 or greater was used for reverse transcription. 1 µg of RNA from each sample was reverse transcribed using iScript cDNA synthesis kit according to recommended conditions (BioRad, Hercules, CA). 0.5 µl of each reverse-transcribed sample was added to 1X iQ SYBR Green Supermix (BioRad, Hercules, CA), 200 nM of forward and reverse primers, and nuclease-free water to a 25 µl final reaction volume. Primer sequences and cycling conditions for
Muc2, Muc5AC, and γ-actin were as described previously (20). Amplifications were performed on an iCycler iQ Real-Time PCR Detection System. Starting amount of cDNA template was extrapolated from amplification curves by the method of Peirson (223). Values reported are normalized to γ-actin levels and expressed as percentage of control. Melt-curve analysis was performed to verify that only a single product was amplified in each reaction.

**Exposure of Guinea Pig Trachea Epithelial Cells to Cytomix**

On the 7th day after GPTE cell cultures were exposed to air, secreted mucus was removed from the apical surface with two washes (1X PBS). The basal media was changed and the cultures incubated at 37°C, 3% CO₂ for 12 hours at which time the secreted apical mucus was collected with a single PBS wash (0.5ml). Mucins in these collections were measured by ELISA (see above) to determine BASELINE values for each culture. Basal media was again changed to begin the EXPERIMENTAL protocol. The EXPERIMENTAL protocol spanned an additional 12 hours, during which time a pro-inflammatory cytokine mixture (10 ng/ml each of TNF-α, IL-1β, and IFN-γ or “cytomix”) was added to the culture media at 8, 4, and 0 hours after EXPERIMENTAL time zero. This approach resulted in EXPERIMENTAL cultures exposed to cytomix for 4, 8, or 12 hours. In addition, control cultures were exposed only to media for the 12-hr EXPERIMENTAL period to allow measurement of CONSTITUTIVE mucus secretion. After the EXPERIMENTAL exposure period, mucus secretions were collected with 0.5 ml PBS wash. BASELINE and EXPERIMENTAL or CONSTITUTIVE mucin samples for each culture well were assayed in duplicate on the same 96-well ELISA plate to minimize interplate variability. Undiluted mucus samples were always used in the ELISA (unless otherwise noted) as these corresponded to OD values for Muc2 or Muc5AC that appeared to be in the linear range (Fig. 4), while more dilute mucus corresponded to lower OD values prone to greater instrumental error. Normalized values were calculated by dividing the OD reading corresponding to mucin secreted for the EXPERIMENTAL period by the OD reading corresponding to mucin secreted during the BASELINE period, allowing each well to serve as its own control. Final EXPERIMENTAL/BASELINE results were expressed as a percentage of normalized CONSTITUTIVE mucin production for the 12-hr EXPERIMENTAL period. All mucin samples collected from GPTE cell cultures, unless otherwise noted, were treated with
neuraminidase enzyme originating from *Arthrobacter ureafaciens* (Calbiochem, La Jolla, CA) by a method described previously (224).

**Statistical Analysis**

Experimental data were analyzed for significance using paired Student’s *t* test or ANOVA with Tukey’s post-test comparisons, where appropriate. Differences between treatments were considered significant at *p* < 0.05. Data are represented as means ± standard error of the mean (SEM).
C4 Monoclonal Antibody Recognizes Muc2 Mucin

To verify that the novel monoclonal antibody (C4) developed in this study would recognize guinea pig Muc2 apomucin, a dilution series of purified antigen was examined by ELISA. C4 bound as little as 50 ng per test well, and absorbance values increased in a concentration-dependent manner (Fig. 1).

With the knowledge that C4 readily recognizes a Muc2 apomucin epitope, the ability of this antibody to recognize Muc2 mucin was further tested by probing the mucin-rich mucosae of the small intestine and stomach using IHC. In mice, the small intestine is known to express a large amount of Muc2 mucin while the stomach expresses Muc5AC mucin preferentially (25, 225). Using mouse sections, C4 stained the epithelial lining of the small intestine (Fig. 2a), but comparatively less staining was observed in stomach (Fig. 2b). Similar staining patterns were found using guinea pig small intestine and stomach (Figs. 2c and 2d). Negative controls (guinea pig liver and IHC substituting mouse sera for primary antibody) showed no staining (data not shown). Thus, the C4 antibody appears to selectively recognize protein within tissues known to express large amounts of Muc2 mucin, such as the murine small intestine. Similarly, the antibody selectively recognizes protein in the guinea pig small intestine, suggesting this tissue, like its murine counterpart, expresses abundant Muc2 mucin.

Western blot analysis was then used to verify specificity of the C4 antibody. C4 hybridized to mucus from the guinea pig small intestine but not from the stomach (Fig. 2e). Specifically, the C4 antibody labeled two discernable proteins; a smaller band estimated to be 2.4 MDa and a larger, more intense band or “smear” spanning the 2.6 to 4.8 MDa size range. These sizes are similar to those described by Axelsson et al. (226) following hybridization of a MUC2 antibody to reduced colonic mucus in a Western blot. In that study, two bands were evident; the smaller band (2.7 MDa) was described as the MUC2 monomer, whereas the larger, more intense band was thought to consist of MUC2 oligomers resistant to reduction treatment, a finding confirmed elsewhere (227). Although our small intestine mucosal samples were processed differently than those used in these previous studies, a similar
banding pattern and mass estimation is observed in our Western blot analysis, lending confidence to the ability of the C4 antibody to recognize Muc2 mucin isolated from guinea pig epithelial tissue.

To expand the utility of the C4 antibody for relative quantitation of specific mucins, we established conditions for its use in a more sensitive ELISA. Low concentrations of antibody bound mucus from both guinea pig small intestine and stomach by ELISA (Fig. 2f). However, a 1:500 dilution of C4 antibody (0.8 µg/ml) selectively recognized mucus isolated from the guinea pig small intestine. Thus, we chose to use this dilution for all subsequent ELISAs, as it appeared to allow greater differential recognition of Muc2 mucin.

45M1 Monoclonal Antibody Recognizes Muc5AC Mucin in Guinea Pig Tissue

To examine expression of Muc2 versus Muc5AC mucin in guinea pig tissues, we developed guinea pig-specific assays using a commercially-available antibody known to recognize Muc5AC mucin in tissues from monkey, rat, rabbit, pig, and chicken (228, 229). Muc2-rich small intestine and Muc5AC-rich stomach sections were utilized to examine differential staining by the 45M1 antibody. As seen in Fig. 3a, this antibody (45M1) clearly labeled the epithelial surface of sectioned guinea pig stomach. 45M1 did not label the small intestine from guinea pig (Fig. 3b). Negligible, seemingly non-specific, labeling occurred when the antibody was reacted to sections of liver or when tissue sections were reacted with control mouse sera in place of the primary antibody (data not shown).

To further verify that the 45M1 monoclonal antibody could recognize Muc5AC mucin from guinea pig with specificity, we used the antibody to hybridize Western blots containing mucus from guinea pig stomach and small intestine (Fig. 3c). As anticipated, 45M1 recognized only the stomach sample. Specifically, a 1:5000 dilution of stock 45M1 antibody labeled a smeared, high molecular weight band. This band was similar to those observed in Western blots containing rat mucosal samples (230) or samples of mucus secreted from cell lines of human mucosal origin (23).

Using the 45M1 in an ELISA, a 1:1000 or 1:100 dilution of the antibody recognized 5.8 µg of total stomach mucus per well, yielding high absorbance values (Fig. 3d). The same dilutions of 45M1, however, did not bind to wells coated with an equal amount of total protein isolated from the small intestinal mucosa.
Neuraminidase Treatment Improves 45M1 Performance in ELISA

Both the anti-Muc2 antibody (C4) created in this study as well as the 45M1 antibody are thought to preferentially recognize core mucin proteins. Since mucin populations isolated from lung are known to be heavily O-glycosylated (231), it is sometimes possible to enhance the specific performance of mucin-recognizing antibodies by removing carbohydrate moieties that obscure the protein backbones (224). The enzyme neuraminidase (*Arthrobacter ureafaciens*), which cleaves neuraminic acid from mucin glycoproteins (224), was used to determine whether the binding of C4 or 45M1 antibodies could be enhanced similarly in our ELISA method. Serially-diluted collections of secreted mucus from GPTE cells, with and without neuraminidase treatment, were subjected to ELISAs using both mucin subtype monoclonal antibodies (Fig. 4). C4 performance in ELISA appeared to improve minimally with neuraminidase treatment in some assays; however, this increase in absorbance values was not consistent from assay to assay (Fig. 4a). Both treated and non-treated curves always had similar slope and $R^2$-fit, leading us to conclude that C4 performance was not enhanced following neuraminidase treatment of isolated mucus samples. Conversely, neuraminidase treatment considerably improved 45M1 ELISA performance (Fig. 4b). Without treatment, the 45M1 antibody poorly recognized mucin when total mucus concentrations were greater than 35 ng/well. This paradoxical trend was reversed with neuraminidase treatment wherein consistent increases in absorbance corresponding to increased mucus concentration were observed over the range of 0.07 to 280 ng/well. Based on these findings, all mucin collected from GPTE cells was subjected to neuraminidase treatment prior to ELISA to avoid potential underestimation of the amount of Muc5AC mucin.

Muc2 and Muc5AC Mucins Secreted Differentially from GPTE Cells

Finally, we sought to determine whether specific mucin subtypes were expressed differentially in GPTE cells. To examine constitutive Muc2 and Muc5AC mucin secretion versus intracellular production, equal amounts of total protein derived from extracellular secretions or intracellular lysates were quantified for both mucin subtypes by ELISA (Fig. 5a). While direct quantitative comparisons between the two subtypes cannot be made, the higher ratio of intracellular to secreted Muc2 mucin compared to the ratio for Muc5AC
mucin suggests that Muc2 is secreted at a slower rate than Muc5AC under normal circumstances, leaving Muc2 mucin stores within the cells.

To determine if expression of the mucin subtypes can be modulated differentially in response to inflammation, GPTE cells were exposed to a mixture of pro-inflammatory cytokines (TNF-α, IL-1β, and IFN-γ or “cytomix”) for 4 hours. This exposure was carried out after a baseline mucus collection and a subsequent 8-hour rest period. Secretion of Muc2 mucin increased 100% over constitutive levels following cytokine stimulation, with no significant change in Muc5AC mucin secretion observed (Fig. 5b). In contrast, intracellular production of both Muc2 and Muc5AC mucins increased similarly, showing a 50% elevation over constitutive levels following cytokine stimulation (Fig. 5c). These findings suggest inflammation may induce a differential release of pre-formed Muc2 mucin from GPTE cells, while at the same time stimulating the cells to increase intracellular mucin stores of a variety of mucins.

**No Significant Change in Muc2 and Muc5AC Gene Expression with Cytokine Exposure**

To determine if Muc2 and Muc5AC gene expression patterns correlate with mucin protein expression following exposure to pro-inflammatory cytokines, GPTE cells were exposed to cytomix over a time course of 0 to 12 hours and total RNA was extracted from the cells. There appeared to be an overall trend toward an increase in the steady-state levels of both Muc2 and Muc5AC mRNAs (Fig. 5d); however, this increase was not statistically significant (p>0.05 by ANOVA) due to high variability between cultures. Even with this, the small increase in both mRNAs was ultimately similar, although induction of Muc5AC gene expression seemed to lag behind that of Muc2. It may be that the early increase in Muc2 mRNA is induced by the preferential secretion of stored Muc2 mucin, rather than by direct induction due to cytomix exposure. These results suggest a trend toward an increase in Muc2 and Muc5AC gene expression in GPTE cells can be induced by pro-inflammatory cytokines. Such an increase would likely impact the cumulative increase in intracellular Muc2 and Muc5AC mucin also observed in these cells (Fig. 5c).
While mucus overproduction is a hallmark of inflammatory airways disease (206-208), relatively little is known about its mechanistic regulation. For a number of years, the guinea pig has been used as an animal model of human allergic asthma. The similarities in airway physiology and hypersensitivity upon allergen challenge between humans and the guinea pig make this model particularly effective (20, 82-84, 221). Unfortunately, detailed mechanistic studies focusing on mucous cell hyperplasia and mucus overproduction in the guinea pig model have been limited by the lack of appropriate molecular tools.

Recently, we developed DNA probes specific for guinea pig Muc2 and Muc5AC, which are useful for delineating regulated expression of these mucins at the level of their mRNAs (20). In the present study, we continue to develop molecular tools applicable to the study of mucus production in the guinea pig model, now targeting mucin protein expression. Specifically, we report the successful creation of a monoclonal antibody to guinea pig Muc2 mucin (C4). In addition, we have optimized use of a commercially available anti-human MUC5AC monoclonal antibody (45M1) for detection of Muc5AC mucin from guinea pig. These newly employed antibodies have also been used successfully to examine the expression of guinea pig Muc2 and Muc5AC mucins during constitutive growth of airway epithelial cells and following exposure of these cells to inflammatory cytokines. The discovery that the differential expression of these two mucins can be readily measured opens avenues to molecular mechanistic studies which could not be done using previously reported anti-guinea pig mucin antibodies which most likely target the extensive, non-specific carbohydrate moieties on multiple mucin types (222).

Mucin specificity of each monoclonal antibody was demonstrated using ELISA, Western blot, and immunohistochemistry (IHC). While previous experimentation with 45M1 has led to its acceptance as MUC5AC-specific (225, 232, 233), further proof was needed to establish the novel C4 monoclonal antibody as specific for Muc2 mucin. We therefore used C4 to immunostain tissue sections of small intestine and stomach from mouse because it has been established that secretions from these tissues are Muc2 and Muc5AC-dominant, respectively (25). The C4 staining patterns were generally tissue specific regardless of
whether tissues from mouse or guinea pig were used. Similarly, by Western blot, C4 hybridized only to mucus from small intestine. The C4 antibody did, however, react minimally with guinea pig stomach mucus by ELISA, which is considered a more sensitive assay. Therefore, the possibility exists that some Muc2 mucin is expressed in guinea pig stomach. The 45M1 antibody selectively reacted with gastric samples by ELISA, Western blot, and IHC, with little to no labeling of small intestinal mucosa observed.

Experimentally, use of the C4 and 45M1 antibodies have now provided the first complete picture of Muc2 and Muc5AC mucin production and secretion in the guinea pig airway epithelium in vitro, with these mucins present at the extracellular, intracellular, and mRNA levels. These findings expand previous work, such as that by Nagai et al. demonstrating intracellular MUC5AC protein production in differentiated GPTE cells in vitro (34). Additionally, we have previously examined expression of the mRNAs corresponding to the Muc2 and Muc5AC mucins in guinea pig (20), determining that the Muc2 message predominates over the Muc5AC message in RNA isolated from GPTE cells. Although it is difficult, even with the current study, to ascertain relative amounts of Muc2 and Muc5AC glycoproteins due to differences in assay kinetics and lack of coordinated standards, examination of the ratio of intracellular to extracellular mucin for each of the two mucin subtypes indicates that Muc2, rather than Muc5AC, mucin is more abundant as an intracellular reserve in cells growing constitutively. Studies examining mucin expression in bronchial or tracheal tissue, or in epithelial cells from these tissues, in rat, mouse, horse or humans generally portray MUC5AC and/or MUC5B as the dominantly expressed mucins both in healthy and in altered airways. In these studies, however, MUC2 is often detected minimally, or not at all (24, 27, 45, 231, 234). Thus, the finding that Muc2 mRNA and intracellular Muc2 mucin appear to predominate over Muc5AC mucin in guinea pig tracheal epithelial cells, both constitutively and following exposure to pro-inflammatory cytokines, is novel. This result suggests that mechanistic regulation of mucin subtypes in guinea pig may vary from that observed in other mammalian species, including humans. More generally, it may be that different mucins respond to airway inflammation differently depending on the mammalian species. Such a possibility suggests the need to fully characterize which mucins
respond maximally to inflammatory stimuli when a new animal model of mucus hypersecretion is being developed.

Regulatory mechanisms governing differential expression of specific mucin subtypes can exist at transcriptional and/or translational levels. It has been shown, for example, that inflammatory stimulation can augment the MUC5AC message while MUC5B levels remain unchanged in the human airway-derived cell lines A549 and H-292 (235). One mechanism by which MUC5AC expression can be upregulated involves modulation of mRNA stability. Such regulation has been demonstrated in A549 and NHBE cell cultures, where the inflammation-associated protease, neutrophil elastase, induced an increase in MUC5AC message stability (41), via a signaling mechanism involving secondary production of reactive oxygen species (ROS) (76). Transcriptional regulation of MUC5AC has also been demonstrated in epithelial cell lines, where ROS-dependent activation of an AP-1 response element upstream of the MUC5AC gene occurs after cigarette smoke exposure (236). In addition, MUC2 transcripts have been observed in both mucin-expressing and non-expressing cells, suggesting regulation of MUC2 mucin expression can occur at the level of protein translation (29). In our study, we observe yet another level of differential mucin regulation when an increase in Muc2 mucin secretion is induced by cytokine stimulation, while the level of secreted Muc5AC mucin remains unchanged. This differential secretion occurs even though cytokine-induced effects on the message and intracellular protein production of the two mucin subtypes do not appear to be regulated differentially. This finding supports the theory that mucin subtypes may be secreted differentially as a post-translational regulation of mucin expression. Evidence from previous studies has demonstrated that mucin secretion occurs through coordinated cycles of phosphorylation/dephosphorylation of the PKC-substrate, MARCKS, in a mechanism which induces mucin granule movement toward the plasma membrane (237). Assuming this pathway is non-selective for a particular mucin subtype, the mucin granules themselves may be packaged differentially with regard to mucin subtypes. While differential secretion of specific mucin subtypes in the absence of differential transcriptional or translational control has not been reported previously, it has been suggested that different mucin granules within a single goblet cell may contain structurally distinct mucin subpopulations (238). The
differential regulation of mucin secretion from such mucin-specific granules in response to pro-inflammatory stimuli might be an important mechanism whereby the properties of airway mucus can be altered. This can have the effect of providing greater protection to the underlying epithelial cells, but also may result in a deleterious effect on lung function.

In summary, we have used newly developed molecular tools to examine expression of Muc2 and Muc5AC mucins in mucus collected from guinea pig tissues and GPTE cells. Specifically, a novel mouse monoclonal antibody was raised against guinea pig Muc2 mucin, and it, along with a commercially-available antibody to Muc5AC mucin, were optimized for specific recognition of mucin from guinea pig by ELISA, Western blot, and IHC. Using GPTE cells, it was found that pro-inflammatory stimulation with a cytokine mixture of TNF-\(\alpha\), IL-1\(\beta\), and IFN-\(\gamma\), stimulated increased secretion of Muc2, and not Muc5AC, mucin, while intracellular protein and mRNA expression increased similarly for both mucin subtypes. These findings highlight differences in regulation of specific mucins within the same organism, as well as suggest comparative differences with regard to which mucins are most highly expressed among a variety of mammalian species. This differential regulation associated with inflamed airways may well influence the mucosal makeup, and, ultimately, the physical properties of airway mucus in a manner that could deleteriously affect clearance and gas exchange.
Figure 1. Anti-Muc2 monoclonal antibody (C4) binds to Muc2 apomucin in ELISA.
Guinea pig Muc2 apomucin fragment (10, 50, 100, and 200 ng/well) purified from transformed bacterial culture lysate was measured by ELISA using purified C4 monoclonal antibody (400 µg/ml), diluted 1:100 in 0.3% cold-water fish gelatin. Results indicate the C4 monoclonal antibody binds to the Muc2 apomucin fragment in a concentration-dependent manner. Absorbance values read at 450 nm are shown corrected for background. Each sample was measured in triplicate, and data are shown as mean ± SEM. This assay was repeated three times with similar results.
Figure 2. C4 antibody recognizes small intestinal mucus preferentially by immunohistochemistry, Western blot, and ELISA. **Immunohistochemistry.**
(Magnification for panels a-d is 100X.) a) The C4 antibody (diluted 1:50) recognizes the epithelial lining of mouse small intestine by immunohistochemistry. (Note dark brown staining.) b) Using the same dilution of the C4 antibody, slight staining of the murine stomach mucosal surface is observable. The amount and intensity of staining, however, is less than that observed with murine intestinal tissue (panel a). c) Immunohistochemistry using the C4 antibody (diluted 1:20) shows this antibody reacts with sections of small intestine from normal adult guinea pig. Strong staining is present along the epithelial-lumen barrier. d) Using a similar dilution of the C4 antibody, little staining is observed in the guinea pig stomach section, a finding similar to that observed in the murine stomach (panel b). e) **Western blot hybridized with the C4 antibody.** 23.4 µg of mucus collected from either the guinea pig small intestine (I) or the stomach (S) was subjected to electrophoresis through a 1.0% agarose gel and then transferred to nitrocellulose membrane. Hybridization with the C4 antibody (1:100) labeled a high molecular weight smear and a second high molecular weight band in the intestinal mucus, but did not recognize the stomach mucus. Molecular weight estimates were made using a gel prediction program (included in Vector NTI Suite 9, Informax). Western shown is representative of three separate blots. f) **ELISA plate was coated with equal amounts of stomach or small intestinal mucus (11.7 µg per well).** The C4 antibody (1:500) preferentially recognizes the small intestinal mucus, although less differentiation is observed at lower working concentrations of the antibody. Each sample was assayed in triplicate, and data are shown as mean ± SEM. The assay was done twice using two independent sample isolations, with similar results obtained each time.
Figure 3. 45M1 antibody recognizes stomach mucus preferentially by immunohistochemistry, Western blot, and ELISA. Immunohistochemistry (Panels a,b; magnification 100X.): a) 45M1, pre-diluted by the manufacturer, recognizes the epithelial lining and neck cells (brown color) in a section of guinea pig stomach. b) 45M1, used at the same dilution as in panel a, did not label a section of guinea pig small intestine. c) Western blot hybridized with the C4 antibody. 23.4 µg of mucus collected from either small intestine (I) or stomach (S) was subjected to electrophoresis through a 1.0% agarose gel and then transferred to nitrocellulose membrane. Hybridization with the 45M1 antibody labeled a high molecular weight protein in stomach mucus, with no labeling evident for small intestine mucus. Molecular weight estimates were made using a gel prediction program (Vector NTI Suite 9, Informax). Western blot shown is representative of three separate blots. d) ELISA wells were coated with 5.8 µg of stomach or small intestinal mucus. 45M1 (1:100 and 1:1000) recognized stomach mucus, but not small intestinal mucus. Each sample was assayed in triplicate and data are shown as mean ± SEM. The assay was done twice using two independent sample isolations, with similar results obtained each time.
Figure 4. **Neuraminidase treatment enhances 45M1 performance in ELISA.** Mucin secretions were collected from GPTE cell cultures on day 9 after exposure to air. Samples were treated with neuraminidase (0.625 mU/µg total protein) from *Arthrobacter ureafaciens* for 2 hours at 37°C [35]. Both neuraminidase-treated and untreated samples were diluted 2-fold in series, then coated on ELISA plates in duplicate.  

**a)** While neuraminidase treatment seemingly increased absorbance values using C4 antibody (diluted 1:500) in some experiments, such as the one shown here, this shift was not consistent in repeated experimentation. Logarithmic slope and trend line fit of both treated and non-treated samples were similar.  

**b)** Detection by the 45M1 monoclonal antibody (diluted 1:1000) improved with neuraminidase treatment, as indicated by better $R^2$ value fit (calculated for ranges of increasing absorbance with increasing concentration) of logarithmic trend line. In addition, treatment extended the upper detection range above 35 ng/well, a concentration at which non-treated samples showed reduced antigenicity.
Figure 5. Muc2, but not Muc5AC, mucin secretion increases with pro-inflammatory stimulation in GPTE cells.  

a) Total protein concentration of pooled samples of mucin collected from the apical surface or as intracellular lysates of non-stimulated cultures was determined using the Bradford assay. Equal amounts of protein were used to coat ELISA wells (76 ng/well), and then the presence of Muc2 or Muc5AC mucin was examined. Results indicate there is a higher ratio of intracellular to secreted Muc2 compared to Muc5AC mucin in unstimulated GPTE cells. Samples from six cultures were pooled. Error bars represent assay variance of triplicate measurements. 

b) GPTE cells were exposed to air for 7 days during which time goblet cell differentiation occurred and mucus secretion began. Apical surfaces were washed, media changed, and then collected for secreted mucus baseline after 12 hours incubation at 37°C, 5% CO₂. Media was then changed and cultures were rested for 8 hours and exposed to cytomix for 4 additional hours. Relative amounts of Muc2 or Muc5AC mucin in experimental and baseline collections were determined by ELISA. Experimental absorbance values were then corrected with baseline values, allowing each well to serve as its own control. These normalized values are expressed as a percentage of constitutive secretion observed during the 12-hr rest/exposure period. Results indicate Muc2 mucin secretion is augmented with cytomix exposure, where Muc5AC mucin secretion remains unchanged. n=5 in each group. * = Significant change from constitutive mucin secretion with Student’s t-test (p<0.05). 

c) GPTE cells were exposed to cytomix for 4, 8, or 12 hours, then cells were lysed and assayed for intracellular mucin. After 4 hours, both Muc2 and Muc5AC production increased 50% over constitutive mucin production. Subsequent time points show a return to constitutive intracellular levels (data not shown). n=6 in each group. ** = Significant change from constitutive mucin production with Student’s t-test (p<0.01).

d) GPTE cultures were exposed to 4, 8, and 12 hours of cytomix. Total RNA was collected and Muc2 and Muc5AC transcript levels were determined using real-time RT-PCR. Results show a trend toward increasing Muc2 and Muc5AC mRNA levels following cytomix exposure when compared to constitutive expression. This increase, however, is not significant when measured by ANOVA due to a high degree of expression variability between cultures. n=4-6 in each group. All data points are presented as mean ± SEM.
CHAPTER III

INDUCIBLE NITRIC OXIDE SYNTHASE MEDIATES ANTI-INFLAMMATORY EFFECTS IN NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS IN VITRO

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Key Words: iNOS, GMCSF, IL-8, cGMP, PKG

Citation: Chorley, B. N., and K. B. Adler. Inducible Nitric Oxide Synthase/Protein Kinase G Signaling Pathway Suppresses Granulocyte Macrophage Colony Stimulating Factor Transcription in Normal Human Bronchial Epithelial Cells [abstract]. Am J Respir Crit Care Med 2004; 169:A421.
ABSTRACT

During inflammation, bronchial epithelial cells increase production of the transcriptionally-regulated inducible nitric oxide synthase (iNOS), which significantly increases nitric oxide above normal levels produced by constitutive species of NOS. Nitric oxide activates the soluble enzyme guanylate cyclase, which causes an increase of intracellular cGMP and activation of its dependent kinase, protein kinase G (PKG). We theorize that iNOS, and therefore nitric oxide, plays an anti-inflammatory role in bronchial epithelial cells by attenuating transcription of pro-inflammatory cytokines through this pathway. Normal human bronchial epithelial (NHBE) cells in primary culture were grown until confluent and pre-treated with a cytokine mixture (10 ng/ml each of tumor necrosis factor-α, interferon-γ and interleukin-1β), to induce iNOS production, or directly treated with a potent NO donor, S-nitroso-N-acetylpenicillamine (SNAP). Cytokines granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-8 (Il-8) messenger RNA levels were measured using TaqMan real-time reverse transcriptase polymerase chain reaction, normalized to β-actin message, in a multiplex reaction. We demonstrate that GM-CSF message is increased significantly over constitutive expression by blocking iNOS activity using specific inhibitor N6-(1-iminoethyl)-L-lysine hydrochloride (L-NIL). In addition, SNAP-induced activation of the cGMP-PKG pathway (shown by augmented cGMP production) significantly increased GM-CSF expression with the addition of a PKG-inhibitor, (Rp)-8-pCPTS-cGMPS. Il-8 message levels were not altered with same treatments. We conclude that inhibition of this signaling pathway leads to increased GM-CSF transcription when NHBE cells are stimulated with NO-producing agents, suggesting that iNOS plays a role in inflammatory suppression.
INTRODUCTION

Nitric oxide is a key mediator of many functions throughout all major tissues of mammalian organisms. In the lung, nitric oxide has been shown to modulate airway tone, regulate pulmonary vascular tone, stimulate mucin secretion, modify ciliary beat frequency, and impose tumoricidal and bactericidal effects (see review (239)). Endogenous nitric oxide (NO) is produced by three different synthases: two low-output synthases, neuronal and endothelial (eNOS and nNOS), and a high-output inducible synthase (iNOS). Airway epithelial cells in vivo uniquely express iNOS constitutively (162) and may play a homeostatic role.

iNOS is involved in inflammatory response, as it can be upregulated in the lung in response to injury (240) or by pro-inflammatory cytokines such as TNF-α, IL-1β, or IFN-γ (163-165). iNOS expression changes in many inflammatory-mediated diseases of the lung, such as asthma, where increased iNOS immunostaining has been demonstrated in the human airway epithelium (241). However, there has been little consensus on the definitive role that iNOS and nitric oxide plays in pulmonary disease. iNOS can be both pro- and anti-inflammatory. iNOS-deficient mice exhibit enhanced susceptibility to ovalbumin-challenge, marked with increased fibrosis and inflammation compared to normal ovalbumin-challenged mice (188). In contrast, selective inhibition of iNOS prevented neutrophil-mediated acute lung injury in a rat model of ruptured abdominal aortic aneurysm (242). Differential effects of nitric oxide on inflammation can be attributed to its ability to form numerous highly reactive metabolites, as well as location, amount, and longevity of production.

Nitric oxide mediates many of its effects by activating soluble guanylyl cyclase (sGC) (243). Activated sGC metabolizes GTP into guanosine 3’, 5’-cyclic monophosphate (cGMP), which activates its dependent protein kinase, PKG. PKG influences ciliary beat frequency, mucin production, and ion transport in the airway epithelium (237, 244, 245), but mediation of inflammatory response via the iNOS/NO/cGMP/PKG signaling pathway is not well established in airway epithelial cells. Therefore, the goal of this study is to elucidate the role of this pathway in modulating two major chemical inflammatory mediators known to be
influenced by nitric oxide (191, 246): granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-8 (IL-8).
MATERIALS AND METHODS

Primary Culture of Normal Human Bronchial Epithelial Cells

One aliquot of normal human bronchial epithelial (NHBE) cells (Cambrex, San Diego, CA) was thawed at 37°C for 2 minutes and seeded into vented T-75 tissue culture flasks (500 cells/cm²) until 75-80% well coverage. Cells were maintained at 37°C in an atmosphere of 5% CO₂ and air. The expansion medium used was bronchial epithelial base medium (BEBM; Cambrex, San Diego, CA) containing 130 µg/ml bovine pituitary extract (BPE) prepared by methods of Bertolero et al. (247), 25 ng/ml human recombinant epidermal growth factor (huEGF; Intergen, Purchase, NY), 5 X 10⁻⁸M all-trans retinoic acid (Sigma, St. Louis, MO), 1.5 mg/ml bovine serum albumin (Intergen, Purchase, NY), 40 U/ml nystatin (Sigma, St. Louis, MO), 500 µg/ml hydrocortisone, 500 µg/ml epinephrine, 10 mg/ml transferrin, 5 mg/ml insulin, 6.5 µg/ml triiodothyronine, 50 µg/ml gentamicin, and 50 µg/ml amphotericin-B (Cambrex, Dan Diego, CA). Once confluent, cultures were dissociated with trypsin/EDTA and frozen in liquid nitrogen as passage-2 according to methods provided by Cambrex Corp.

Following the expansion, passage-2 NHBE cells were seeded on plastic 6- or 12- well culture plates at a density of 1.0 X 10⁴ cells/cm². Supplemented BEBM growth media was changed every other day, using incubator conditions described above, until cultures were 80-90% confluent. Cultures were then incubated in BEBM growth media without BPE and huEGF for 24 hrs to force cells into a non-proliferative state. Confluent cultures were then used for experimentation.

Treatments including 10 or 100 ng/ml each of tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, MN), interleukin-1β (IL-1β), and/or interferon-γ (IFN-γ) were prepared in warmed (37°C) serum-free media before application to culture surface. This “cytomix” treatment was used to induce iNOS transcription and augments GM-CSF and IL-8 expression. In other experiments, the potent NO donor S-nitroso-N-acetylpenicillamine (SNAP) at stated concentrations was used to increase nitric oxide levels in culture. Some studies utilized the cGMP-analogue, 8-bromo-cGMP, to simulate increased intracellular cGMP levels, at concentration previously used in NHBE cells (237). For inhibitor studies,
chemical inhibitors were added 15-30 minutes prior to cytokine administration. L-Lysine ω-acetamindine hydrochloride (L-NIL), a specific iNOS inhibitor, and 8-(4-Chlorophenylthio)-guanosine 3’-5’-cyclic monophosphorothioate, Rp Isomer triethylammonium salt ((Rp)-8-pCPTS-cGMPS), a PKG I and II inhibitor, were used at previously tested concentrations (156, 237) and stated time periods. All chemicals, unless otherwise stated, were obtained from Sigma Corporation, St. Louis, MO.

**TaqMan Reverse Transcriptase Polymerase Chain Reaction**

Total RNA was isolated from culture lysate using RNeasy Mini Kit, according to manufacturer’s instructions (Qiagen, Valencia, CA). Each sample was treated with RNase-free DNase for 15 minutes to reduce DNA contamination. Total RNA was then quantified and checked for purity by measuring A$_{260}$/A$_{280}$ ratio on UV spectrophotometer. Only those with ratios above 1.95 were used for amplification.

Messenger RNA levels were assayed using One-Step TaqMan Real Time RT-PCR Master Mix (Applied Biosystems, Foster City, CA). 0.5 µg of each RNA sample was added to a 25 µl reaction volume containing 200 nM forward and reverse primers, 50 nM dual-labeled nonextendable oligonucleotide hydrolysis (TaqMan) probe, 1X One-Step TaqMan Real Time RT-PCR Master Mix, and nuclease-free water in a thin-walled 96-well reaction plate (BioRad, Hercules, CA). Amplifications for each sample were performed in triplicate on an iCycler iQ Real-Time PCR Detection System (BioRad) with the following cycling conditions: 30 minutes 48°C reverse transcription, 10 minutes 95°C polymerase activation and 45 cycles of amplification [15 seconds 95°C denaturing cycle, 1 minute 60°C annealing/extending]. Both target and β-actin amplifications were performed in the same well to minimize well-to-well variability (multiplex).

IL-8 primer and probe sequences were designed using Beacon Designer software (Premier Biosoft, Palo Alto, CA) based on published mRNA obtained from the NCBI sequence online database ([http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov); GI accession #28610153). All other TaqMan primer and probe sets were previously designed and sequences have been published (248-250).
Relative quantification of targets, normalized to β-actin message, was achieved according to a method described by Peirson et. al. (223), which estimates initial fluorescence (R₀) value of each individual amplification based on estimated amplification efficiency and measured fluorescence at the amplification threshold cycle (R₉Cₐ). The resulting target R₀ value for the target amplification of each treated sample is normalized to the R₀ value of β-actin for the same sample. This normalized value is then expressed as a ratio of the normalized R₀ value of a control sample.

**cGMP Assay**

cGMP was assayed by a commercially available EIA kit (Amersham Biosciences, Piscataway, NJ). 10 µM zaprinast, a phosphodiesterase-V inhibitor, was added 15 minutes before culture stimulation to prevent cGMP breakdown before assay. Values were compared to a standard curve and expressed as fmol/well. These values were normalized to total protein values determined by Bradford assay (BioRad).

**Statistical Analysis**

Data were analyzed for significance using Student’s t-test or one-way analysis of variance (ANOVA) with Tukey’s post-test correction for multiple comparisons. Data were considered significant at $p < 0.05$. 
RESULTS

Cytomix Augments iNOS, GM-CSF, and IL-8 Message
It is known that normal human bronchial epithelial (NHBE) cells lose their constitutive expression of inducible nitric oxide synthase in vitro (162), so it is necessary to initiate iNOS expression with a cytokine mixture of TNF-α, IL-1β, and IFN-γ or “cytomix” (166). It is also known that these cytokines can increase expression of GM-CSF and IL-8 in NHBE cells (113, 251). Fig. 1 illustrates that expression of each target is augmented with cytomix exposure; however, the GM-CSF expression over the time course differs from that of iNOS and IL-8 suggesting that GM-CSF may have different transcriptional regulation. Since it has been shown that nitric oxide can attenuate GM-CSF message in airway epithelial cells (191), increased iNOS expression may suppress GM-CSF expression. Additionally, both iNOS and IL-8 message expression levels decrease after NHBE cells are exposed to 12 hrs of cytomix. Although the reason is uncertain, it is most likely due to autoregulatory mechanisms.

iNOS Inhibition Augments GM-CSF Message
To test the theory that iNOS expression/activity may suppress GM-CSF message in cytomix-stimulated NHBE cells, an iNOS-specific chemical inhibitor, L-NIL, was used at concentrations previously reported (156). Fig. 2 shows that L-NIL augments GM-CSF message in cytomix-stimulated NHBE cells in a dose-dependent manner, whereas L-NIL treatment does not affect IL-8 message. This evidence supports the theory that iNOS activity suppresses cytomix-induced GM-CSF, but not IL-8, expression.

Nitric Oxide Suppresses GM-CSF Through cGMP/PKG Pathway
Fig. 3 illustrates that cGMP production is increased both with cytomix exposure and exogenous nitric oxide application. Nitric oxide can mediate its actions through a cGMP/PKG pathway in airway epithelial cells (252-254). Fig. 4 shows that exogenous nitric oxide application, via potent NO donor, SNAP, or cGMP addition, via analogue 8-Br-cGMP, can suppress GM-CSF transcription in cytomix-stimulated cells. To elucidate cGMP-dependent kinase (PKG) involvement, a PKG-specific chemical inhibitor was added to
selected cells before SNAP application. Fig. 5 shows that SNAP will augment GM-CSF message when PKG is inhibited, where SNAP or PKG inhibitor alone has no effect, suggesting that NO may be pro-inflammatory if PKG is inhibited.
DISCUSSION

Nitric oxide is modulated in many types of pulmonary disease; however, there has been no consensus on whether or not nitric oxide plays an overall beneficial or detrimental role in the airway epithelium. Nitric oxide can interact with anti-oxidants, protein moieties, oxygen reactive species, other radicals, and nucleotides, which vary from location to location. These various factors ultimately determine how nitric oxide affects the surrounding airway. It is difficult to estimate or control specific variables \textit{in vivo}; therefore it is helpful to reduce influence from other cell types by using an isolated cell culture system. This allows for elucidation of mechanistic pathways influenced by nitric oxide specifically in the airway epithelium.

Constitutive expression of iNOS is lost in airway epithelium culture, but can be induced with a cytokine mixture of TNF-\(\alpha\), IL-1\(\beta\), and IFN-\(\gamma\) (cytomix) (162, 168). These cytokines act synergistically to induce iNOS expression by activating both NF-\(\kappa\)B and IRF-1 promoters (169). In addition, NF-\(\kappa\)B is also associated with GM-CSF and IL-8 transcriptional regulation (255, 256). In our study, iNOS, GM-CSF, and IL-8 expression all increased with cytomix exposure in NHBE cells; however GM-CSF expression over the time course differed from that of iNOS and IL-8, suggesting differential regulatory mechanisms. Curiously, GM-CSF levels decreased when iNOS expression was at its highest. Sanders et. al. have previously demonstrated that exogenous application of nitric oxide can reduce rhinovirus-induced production of GM-CSF in airway epithelial cells (191). Similarly, we attenuated GM-CSF message with SNAP (potent NO donor) in cytomix-stimulated NHBE cells. To implicate iNOS involvement in GM-CSF message suppression, we co-incubated cytomix-stimulated culture with a specific iNOS inhibitor, L-NIL, which augmented GM-CSF message in a dose-dependent manner. This evidence supports the claim that both exogenous and endogenous NO production suppresses GM-CSF expression. This differs from nitric oxide mediated suppression of TNF-\(\alpha\)-induced matrix metalloproteinase-9 (MMP-9) (257). Okamoto et. al. distinguished the effects of S-nitrosothiols (e.g. SNAP) and nitric oxide by demonstrating that SNAP, but not NO or iNOS, suppressed MMP-9 expression. They hypothesized that nitrosothiols may act by inhibiting NF-\(\kappa\)B signaling.
pathway through a thiol-to-thiol NO\(^+\) redox state transfer, which cannot occur with nitric oxide in its native redox state (NO\(^-\)). In our study, both SNAP and iNOS are capable of suppressing GM-CSF message in cytomix-stimulated NHBE cells. Since we show that both SNAP and cytomix can induce cGMP production, most likely through activation of soluble guanylyl cyclase, we focused on this signaling pathway in which nitric oxide, regardless of redox state, may mediate its actions.

Critical roles of the airway epithelium, including ciliary movement and mucin secretion, are influenced by nitric oxide, specifically through a cGMP/PKG-mediated pathway (244, 252). In this study, we demonstrate, for the first time, that the cGMP/PKG signaling pathway is anti-inflammatory in airway epithelial cells. We show that cytomix-induced GM-CSF can be suppressed by addition of the cGMP analogue, 8-Br-cGMP. In addition, chemical inhibition of PKG increased GM-CSF transcription when co-incubated with SNAP. The data suggest that nitric oxide may induce GM-CSF through alternate pathways, however, is suppressed through NO-induced PKG. A possible mechanistic explanation for this is through PKG-mediated suppression of the ERK signaling pathway via interference of Ras/Raf interaction, which has been demonstrated in vascular smooth muscle cells (258). This suppression may influence GM-CSF expression, which is regulated by the Raf1-MEK-ERK pathway in NHBE cells (259). However, further study will be required to clarify PKG’s definitive role in GM-CSF expression.

The data suggest that IL-8 expression is not modulated by iNOS, NO, or PKG activity in NHBE cells in culture. While both GM-CSF and IL-8 are regulated by NF-\(\kappa\)B transcriptional elements, there are clearly other separate regulatory mechanisms involved, in particular mechanisms influenced by nitric oxide. This conclusion contrasts findings presented by Sparkman and Boggaram utilizing the human lung adenocarcinoma bronchial cell line, NCI-H441 (246). They demonstrated that nitric oxide can increase IL-8 expression and protein production through a cGMP-independent pathway. However, greater concentrations and longer exposure times of SNAP, in a cancer-derived cell line, were used in their study. In addition, endogenous NO production through inducible nitric oxide synthase was not considered, which, given our results, has no influence on IL-8 expression.
Therefore the Sparkman study may not accurately represent what may occur in primary airway epithelial cells.

In conclusion, nitric oxide, in particular production from its inducible synthase, can suppress GM-CSF in a cGMP/PKG-dependent signaling pathway. This study sheds new light on the anti-inflammatory effects mediated by iNOS in airway epithelium cells, and may lead to a better understanding of its influence in pulmonary disease.
Figure 1. Exposure to 10 ng/ml each of TNF-α, IL-1β, and IFN-γ (cytomix) upregulates iNOS, GM-CSF, and IL-8 expression in NHBE cells. Message levels were measured after total RNA isolation using TaqMan real-time RT-PCR and normalized to β-actin message. All transcripts levels rise with cytomix exposure. GM-CSF exhibits different expression pattern from iNOS and IL-8 over 24 h time course. Dotted lines represent constitutive message expression (no constitutive iNOS expression was noted in NHBE cells). Symbols represent without cytomix (-) and with 12 h exposure (+). Data represented as mean ± SEM (n=6). Statistical analysis performed with ANOVA using Tukey’s post-test comparison. Symbols represent significant change from constitutive mRNA production (* = p<0.01), (**) = p<0.001). Significantly more message expression compared to 1 h cytomix exposure (‡ = p<0.001). Significantly less message expression compared to 6 h cytomix exposure († = p<0.05), (†† = p<0.001). Significantly more message expression compared to 12 h cytomix exposure (§ = p<0.001).
Figure 2. iNOS inhibition augments GM-CSF, but not IL-8, message expression.
NHBE cells were exposed to 10 ng/ml cytomix for 12 h, with or without specific iNOS inhibitor, L-NIL. Results indicate that iNOS inhibition increases GM-CSF mRNA expression in a concentration-dependent manner, whereas IL-8 expression did not change. Dotted lines represent message expression after 12 h cytomix stimulation. Data represented as mean ± SEM (n=3). Statistical analysis performed using Student’s t-test. All bars represented exhibited significantly higher mRNA expression than constitutive state (p<0.05; data not shown) * = significantly more mRNA expression than with cytomix stimulation only (p<0.05).
Figure 3. Cytomix exposure or nitric oxide application augments cGMP production in NHBE cells. NHBE cells were exposed to 100 ng/ml TNF-α, IL-1β, and IFN-γ for 3 or 6 h, or potent NO donor, SNAP for 0.5 or 1 h. Zaprinast, a phosphodiesterase-V inhibitor, was added to prevent cGMP breakdown before assay. cGMP protein of cell lysates was measured by ELISA and normalized to total protein levels of cell lysates. Cytokine mixture and nitric oxide increase cGMP production in NHBE cells. Data represented as mean of cGMP (pg/ml lysate) divided by total protein (mg/ml lysate) for each treatment group ± SEM (n=3). Statistical analysis performed using Student’s t-test. * = significant change from constitutive cGMP production (p<0.05).
Figure 4. Nitric oxide and cGMP analogue attenuates GM-CSF expression in cytomix-stimulated NHBE cells. NHBE cells stimulated to 12 h of 10 ng/ml cytomix were co-exposed with NO donor, SNAP, or cGMP analogue, 8-Br-cGMP, over a time course ranging the final 0 to 2 h. Both SNAP and 8-Br-cGMP significantly reduce cytomix-induced GM-CSF mRNA expression at 60 or 30 min exposure. — signifies no cytomix stimulation. Dotted lines represent constitutive message expression. Data represented as mean ± SEM (n=4). Statistical analysis performed using Student’s t-test. † = significant change from cytomix-induced GM-CSF mRNA expression (p<0.05).
Figure 5. PKG inhibition augments GM-CSF, but not IL-8, mRNA expression in SNAP-exposed NHBE cells. Potent NO donor, SNAP, was added to NHBE cell medium for 0.5 h, with or without PKG inhibitor, (Rp)-8-pCPTS-cGMPS (denoted as Rp). SNAP augments GM-CSF, but not IL-8, message when PKG is inhibited. Dotted lines represent constitutive message expression. Data represented as mean ± SEM (n=6). Statistical analysis performed with ANOVA using Tukey’s post-test correction. * = significant change from constitutive mRNA expression (p<0.05).
CHAPTER IV

(R)-ALBUTEROL ELICITS ANTI-INFLAMMATORY EFFECTS IN NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS IN VITRO VIA A PATHWAY INVOLVING INDUCIBLE NITRIC OXIDE SYNTHASE

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Submitted to: American Journal of Respiratory and Critical Care Medicine

Key words: inducible nitric oxide synthase, human bronchial epithelial cells, inflammatory cytokines, albuterol, asthma
ABSTRACT

It is widely recognized that catecholamines suppress production of inflammatory mediators in multiple cell types, including airway epithelium. However, downstream signaling mechanisms involved in regulation of these anti-inflammatory effects are largely unknown. We theorized that acute β₂-adrenergic stimulation of airway epithelial cells with albuterol could suppress production and release of inflammatory mediators, specifically granulocyte macrophage colony stimulating factor (GM-CSF), via a pathway involving inducible nitric oxide synthase (iNOS). Normal human bronchial epithelial (NHBE) cells in primary culture were exposed to a cytokine mixture (10 ng/ml each interferon-γ and interleukin-1β) to induce iNOS expression. (R)- and (S)-enantiomers of albuterol, as well as racemic mixtures, were added with these cytokines and effects on GM-CSF expression and production were assessed. Specific inhibitors and activators of protein kinases, β₂-adrenergic receptor antagonists, and small interfering RNA against iNOS were used to delineate signaling pathways involved. iNOS message was significantly upregulated in a concentration-dependent manner by the active (R)-enantiomer of albuterol. (R)-albuterol also attenuated cytokine-induced increases in GM-CSF steady state mRNA expression and protein release. The (S)-enantiomer of albuterol had no effect on these parameters. Protein kinase C (PKC), specifically the δ isoform, was required for iNOS message increase, but protein kinases A and G were not involved in the pathway. Overall, this study identifies a novel pathway by which β₂-adrenergic agonists may exhibit anti-inflammatory effects in airway epithelium and surrounding milieu.
INTRODUCTION

Clinically, β2-adrenergic agonists are a widely used treatment in asthmatic patients, where they act as potent bronchodilators. The β2-adrenergic receptor mediates smooth muscle relaxation by activating adenylate cyclase via a trimeric Gs protein signaling pathway (194). In addition, β2-adrenergic agonists in the airway can regulate ciliary’s beat frequency (195, 196), ion transport (197), and repair of injury (198). β2-adrenergic agonists also may suppress pro-inflammatory responses in several airway cell types, including attenuation of monocytic and neutrophilic production of interleukin-8 (202, 203), downregulation of production of granulocyte-macrophage colony stimulating factor (GM-CSF), and eotaxin in airway smooth muscle cells (204), and suppression of production of ICAM-1 and GM-CSF production by airway epithelial cells (199, 200).

As the first line of defense to pathogens, irritants, and environmental particulates, the airway epithelium plays a critical role in coordinating inflammatory responses. A prominent mechanism by which the airway epithelium regulates inflammatory response is through the pluripotent radical, nitric oxide (NO). Airway epithelium cells in vivo uniquely expresses the inducible isoform of nitric oxide synthase (iNOS) constitutively (162). iNOS appears to be involved intimately in local inflammatory response, as it is upregulated in the lung in response to injury (240) or by pro-inflammatory cytokines such as TNF-α, IL-1β, or IFN-γ (163-165). There is some controversy as to whether iNOS activity is beneficial or detrimental in the inflamed airway. Mice that have been genetically altered so as to not express the iNOS gene exhibit enhanced susceptibility to ovalbumin-challenge, with increased fibrosis and inflammation compared to normal ovalbumin-challenged mice (188). Conversely, asthmatic patients have increased levels of peroxynitrite (ONOO⁻) activity, an active and potentially toxic reactive nitrogen species which forms in the presence of NO and superoxide (184). Increased peroxynitrite in the lung can significantly enhance airway inflammation in mice (185). Nitric oxide can therefore elicit a multitude of effects depending on production, location, metabolite formation, and antioxidant contribution.

Interestingly, nitric oxide synthase expression and activity have been shown to increase in the bronchial epithelium in response to β-adrenergic agonists in both rats and
dogs (205, 260). As exposure of human bronchial epithelial cells to NO can attenuate the production of the pro-inflammatory cytokine GM-CSF (191), the possibility exists that β2-adrenergic receptor agonists may regulate their anti-inflammatory properties through an iNOS-mediated pathway. The present study examined the effects of the short-acting β2-adrenergic agonist, (R)-albuterol, on expression of iNOS in normal human bronchial epithelial (NHBE) cells in vitro. The results demonstrate for the first time that cytokine-induced expression of iNOS expression is enhanced by (R)-albuterol in these cells. In addition, we show that knocking-down the expression of iNOS inhibits the suppressive effects elicited by (R)-albuterol on IL-1β + IFN-γ-induced augmentation of GM-CSF message and protein release. Additional studies demonstrated that iNOS expression requires PKC activity, but does not involve activation of either PKA or PKG.
MATERIALS AND METHODS

Primary Culture of NHBE Cells
Expansion and cryopreservation of normal human bronchial epithelial cells were performed as described previously (261). Following expansion, passage-2 NHBE cells were seeded on plastic 6- or 12- well culture plates at a density of 1.0 X 10^4 cells/cm^2. Supplemented bronchial epithelial cell basal media (BEBM) was changed every other day until cultures were 80-90% confluent, at which time the cell were incubated in supplemented BEBM from which bovine pituitary extract and human recombinant epidermal growth factor for were removed for 24 h to force cells into a quiescent, non-proliferative state. Confluent cultures were then used for the experiments described below. Unless otherwise described, agents added to cells were prepared in warmed (37°C) serum-free media before application to the culture surface.

Treatments
Treatments including 10 or 50 ng/ml each of tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, MN), interleukin-1β (IL-1β), and/or interferon-γ (IFN-γ) were prepared in warmed (37°C) serum-free media before application to culture surfaces. (R)-, (S)-, or racemic albuterol (provided by Sepracor Inc., Marlborough, MA) were added in selected studies. For inhibitor studies, pharmacological inhibitors were added 0-30 min prior to cytokine/albuterol administration. Inhibitors for cAMP-dependent kinase (H-89; IC_{50} < 50nM) (262), protein kinase C (calphostin C; IC_{50} = 50nM) (263), protein kinase C δ (rottlerin; IC_{50} = 3-6µM) (264), protein kinase C ε (translocation inhibitor peptide; Calbiochem, La Jolla, CA), cGMP-dependent kinase (8-[4-cholorophenylthio]-guanosine 3’,5’-cyclic monophosphorothioate, Rp Isomer triethylammonium salt, also known as (Rp)-8-pCPT-cGMPS; IC_{50} = 0.5µM) (265), and β2-adrenergic receptor (erythro-D,L-1[7-lethylindan-4-yloxy]-3-isopropylamino-+++butan-2-ol, also known as ICI 118,551; IC_{50} = 15nM) (204) were used. To test for PKC-mediated mediation of iNOS expression, the general PKC-activator phorbol 12-myristate 13-acetate (PMA), and PKCδ/ε isozyme activator, bryostatin 1, were used at concentrations previously published (237, 266). An inert
PMA analogue, 4-α-PMA, was used as a negative control. All reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO).

For some time course studies, reagents of interest were co-incubated with cytokines IL-1β and IFN-γ for 6 or 8 h, as noted. For some studies requiring shorter exposures (e.g., 30 min, 1 h, etc.) agents were added to the cytokine-exposed culture medium so that experimentation stopped simultaneously with the appropriate IL-1β and IFN-γ exposure time.

Some cells were lysed and collected for total protein determination or assayed for PKC activity. Briefly, medium was vacuum-aspirated from cultures and then washed once with phosphate-buffered solution (PBS). Cells were then scraped into lysis buffer (~50 µl volume/well) containing 50 mM Tris-HCl [pH7.5], 1mM EDTA, 100 mM NaCl, 5mM MgCl₂, 10 mM dithithreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), with 10% (v/v) protease inhibitors cocktails (P-8340, P-2850; Sigma, St. Louis, MO).

**TaqMan Real-time Reverse Transcriptase Polymerase Chain Reaction**

Total RNA was isolated from cell culture lysate using RNeasy Mini Kit, according to manufacturer’s instructions (Qiagen, Valencia, CA). Each sample was treated with RNase-free DNase for 15 min to reduce DNA contamination. Total RNA was then quantified and checked for purity by measuring A₂₆₀/A₂₈₀ ratio on UV spectrophotometer, and only those with ratios above 1.95 were used for reverse transcription. 1 µg of RNA from each sample was reverse transcribed using iScript cDNA synthesis kit according to recommended thermal conditions (Bio-Rad, Hercules, CA).

Real-time PCR was performed using a hydrolysis (TaqMan) probe. 0.5 µl of each reverse-transcribed sample was added to a 25 µl reaction volume containing 200 nM forward and reverse primers, 50 nM dual-labeled nonextendable oligonucleotide hydrolysis (TaqMan) probe, 1X Absolute QPCR Mix containing DNA polymerase (ABGene, Epsom, Surrey, UK), and nuclease-free water in a thin-walled 96-well reaction plate (BioRad, Hercules, CA). Amplifications for each sample were performed in triplicate on an iCycler iQ Real-Time PCR Detection System (BioRad) with the following cycling conditions: 15 minutes 95°C DNA polymerase activation and 45 cycles of amplification [15 s 95°C denaturing cycle, 1
min 58°C annealing/extending]. Each sample was run in triplicate and amplifications without template were run as negative controls.

Primer and probe sequences were designed using Beacon Designer software (Premier Biosoft, Palo Alto, CA) based on published mRNA sequences for human iNOS, GM-CSF, and β-actin obtained from the NCBI sequence online database (http://www.ncbi.nlm.nih.gov). Forward and reverse primer sequences for each target were located on separate exons to reduce possibility of amplifying contaminating DNA in samples. Table 1 lists the target sequence GI accession number, primer and probe sequences, and amplicon length and location. Single amplification products void of primer-dimer formation for each target were verified using SYBR Green (BioRad) melt-curve analysis. Hydrolysis (TaqMan) probes were dual-labeled with 6-carboxy-fluorescein (FAM) as a fluorescent reporter dye, and Black Hole Quencher-1 (BHQ-1; Integrated DNA Technologies, Coralville, IA) as a quencher.

Relative quantification of targets, normalized to β-actin message, was achieved according to a method described by Peirson, et. al. (223), which estimates initial fluorescence (R₀) value of each individual amplification based on estimated amplification efficiency and measured fluorescence at the amplification threshold cycle (Rᶜₒ). The resulting target R₀ value for the target amplification of each treated sample is normalized to the R₀ value of β-actin for the same sample. This normalized value is then expressed as a ratio of the normalized R₀ value of a control sample.
Table 1. Primer and Probe Sequence Information

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<th>Human β-actin (GI# 5426604)</th>
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<td>5’/FAM/CCGACCTGC CTACAGACCCGCTG/BHQ-1/3’</td>
<td>5’/FAM/CCTGGCTGC CTCCACCCACTCCC/ BHQ-1/3’</td>
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<tr>
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<td>94 [212-305]</td>
<td>130 [1642-1772]</td>
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GM-CSF Enzyme-Linked Immunosorbent Assay
GM-CSF protein released from NHBE cells was assayed with a commercially available enzyme-linked immunosorbent assay (BD-Biosciences, San Diego, CA) according to manufacturer’s instructions. GM-CSF values were normalized to total protein (Bradford method; BioRad, Hercules, CA).

NHBE Transfection and RNA Silencing
NHBE cells were grown in 12-well plates in growth medium void of antibiotics until -60-75% confluent. Medium was then replaced with 450 µl (volume) of growth medium without antibiotics, bovine pituitary extract and human recombinant epidermal growth factor, and the cells incubated at 37°C/5% CO₂ until transfected. To prepare transfection mixture for an individual cell culture well, 2 µl of Fugene 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN) was added directly to 48 µl of serum- and antibiotic-free media without touching reagent to the plastic walls of the tube. After brief vortexing incubation at room temperature for 5 min, 0.1 nmole annealed small interfering RNA (siRNA) for human iNOS (catalog #8678; Ambion, Austin, Texas) was added to the transfection preparation, vortexed briefly, and incubated at room temperature for 15 min. After incubation, 50 µl transfection mixture was then added to each culture well to a final concentration of 0.45% Fugene and
225 nM iNOS siRNA and returned to 37°C/5% CO₂ for 24 h. These concentrations were experimentally optimized to elicit the greatest iNOS message silencing percentage in NHBE cell cultures (~60%) without adverse cellular effects. As a control, some cultures were transfected with scrambled siRNA (catalog #4611, Ambion) or Fugene 6 reagent alone. At 0.45% final concentration, Fugene 6 transfection reagent did not alter iNOS, GMCSF, or β-actin message levels from non-treated cultures. Scrambled siRNA did elicit some knockdown of iNOS message (~30%, see Appendix II), however, iNOS siRNA consistently elicited a greater knockdown. After 24 h transfection incubation, cultures were treated after serum- and antibiotic-free media change for no longer than 24 hrs post-transfection.

**PKC Activity Assay**
Protein kinase C activity was assessed using a PepTag® assay for non-radioactive detection of PKC following the manufacturer’s protocol. Reaction mixtures were separated on 0.8% agarose gels and phosphorylated peptides were quantified by Labworks image acquisition and analysis software (Ultra Violet Products, Ltd., Upland, CA). Values were extrapolated from a linear standard curve based on a series dilution of purified PKC supplied with kit (0-20 ng).

**Cytotoxicity Assay**
All reagents were tested for cytotoxicity to NHBE cells by measuring lactate dehydrogenase (LDH) release/retention. The assay was performed using a commercially available kit (Promega Madison, WI) according to manufacturer’s instructions. All treatments shown in this study elicited LDH release comparable to non-treated cultures (between 3-7%) and were therefore not considered cytotoxic (see Appendix I).

**Statistical Analysis**
Data were analyzed for significance using one-way analysis of variance (ANOVA) with Tukey’s post-test for multiple comparisons or Student’s t-test. Data were considered significant at $p < 0.05$. 

RESULTS

IL-1β + IFN-γ Increases iNOS Message in NHBE cells
To mimic pro-inflammatory conditions, NHBE cells were exposed to a cytokine mixture (“cytomix”: 10 ng/ml each of TNF-α, IL-1β, and IFN-γ) over a time course of 0 to 48 h. iNOS message was induced after 6 h of cytomix exposure, peaked at approximately 12 h, and then fell to near-undetectable levels by 48 h (Fig. 1a). Cytomix exposure also increased GM-CSF message after 1 h, but then seemed to decrease it slightly over the next 12 h period. At 24 hrs, GM-CSF began to rise again, and at 48 h GM-CSF message levels were approximately 15-fold higher than 6 h levels (Fig. 1b). To determine the optimal cytokine mixture that would synergistically induce iNOS message in NHBE cells, different combinations of cytokines were tested. Interestingly, the addition of TNF-α to IL-1β and IFN-γ did not have an additive or synergistic effect on iNOS expression as shown in other studies (see iNOS section of Chapter 1). The reason is not entirely clear, however, it may be due to a donor-specific response or a differential response to media reagents used to grow our NHBE cell cultures. Regardless, the combination of IL-1β and IFN-γ induced the highest levels of iNOS expression (Fig. 2) so the combination of 10 ng/ml IL-1β and 10 ng/ml IFN-γ (IL-1β/IFN-γ) was used for the remainder of the experiments below.

(R)-albuterol Suppresses Cytokine-Induced GM-CSF Augmentation
As illustrated in Fig. 3, (R)-albuterol attenuated the increase in GM-CSF message in NHBE cells exposed to IL-1β/IFN-γ. The increase in GM-CSF message was significantly (p<0.01) attenuated in cytokine-exposed NHBE cells when co-incubated with 10⁻⁶ M (R)- or racemic albuterol after 12 h. (S)-albuterol had no significant effect on GM-CSF message at any time point. Cytokine-stimulated GM-CSF protein release was also attenuated by (R)-albuterol at all time points tested, with statistically significant suppression (p<0.01) at 18 and 24 h. Racemic albuterol treatment had the same effect as (R)-albuterol, suggesting that (S)-albuterol does not affect the GM-CSF suppression mediated by (R)-albuterol.
(R)-albuterol Augments Cytokine-Induced iNOS Transcription via β2-adrenergic Receptor Activation

NHBE cells were exposed to IL-1β/IFN-γ in the presence of (R)- or (S)-albuterol over a range of concentration (10⁻⁸ to 10⁻⁵M) for 12 h. As illustrated in Fig. 4, (R)-albuterol augmented cytokine-induced iNOS message in a concentration-dependent manner, reaching statistical significance (p<0.001) at 10⁻⁵M. (S)-albuterol, in contrast, did not augment iNOS message, even at the highest concentration tested (10⁻⁵M). To ensure that (R)-albuterol was mediating its suppressive effects through the β2-adrenergic receptor, selected cultures were incubated with the specific β2-adrenergic antagonist, ICI 118,551. As illustrated in Fig. 5, 1 µM ICI 118,551 negated (R)-albuterol-mediated augmentation of iNOS message, suggesting that β2-adrenergic receptor activation is required for this action.

GM-CSF Suppression by (R)-albuterol is Mediated Through iNOS

To link (R)-albuterol mediated iNOS augmentation and GM-CSF suppression, RNA silencing of iNOS message was used. Although we have observed that chemical inhibition of iNOS can augment GM-CSF message (267), RNA silencing is a more exact method to specifically target a particular transcript. NHBE cells were transfected using Fugene 6 reagent (Roche) and a 21-base annealed small interfering RNA (siRNA) designed using human iNOS mRNA sequence and verified to be effective in silencing experiments (Ambion; Austin, TX). Fugene 6 reagent alone did not affect iNOS transcription in cytokine-stimulated NHBE cells, but the addition of iNOS siRNA inhibited transcription by about 60% (see Appendix II). As illustrated in Fig. 6a, silencing iNOS message negated the suppressive effect of (R)-albuterol and racemic albuterol on GM-CSF message levels. (S)-albuterol had no effect on GM-CSF message, with or without silencing. As illustrated in Fig. 6b, iNOS silencing only partially negated (R)-albuterol-mediated suppression of GM-CSF protein release, suggesting that (R)-albuterol may suppress GM-CSF post-transcriptionally though additional pathway(s) not involving iNOS.
**PKCδ Mediates iNOS Expression**

To determine protein kinase involvement in (R)-albuterol upregulation of iNOS message, chemical inhibitors of PKA, PKC, PKCδ, and PKG (H-89, calphostin C, rottlerin, (Rp)-8-CPTS-cGMPS, respectively) were used [PKA, PKC, and PKG activity have been shown to induce iNOS transcription (268-270)]. The results indicated that PKA and PKG inhibition does not block (R)-albuterol mediated augmentation of iNOS message in cytokine-stimulated NHBE cells (Fig. 7a and 7b). The PKC inhibitor calphostin C (500nM), however, did attenuate the (R)-albuterol mediated increase of iNOS expression. In addition, PKCδ inhibition with rottlerin (3µM) not only inhibited (R)-albuterol-mediated augmentation of iNOS message; but also partially inhibited IL-1β/IFN-γ induction of iNOS message (Fig. 7c).

To further implicate PKC involvement in this signaling pathway, PKC activity was assessed. As shown in Fig. 8, 10⁻⁵ M (R)-albuterol augmented PKC activity in NHBE cells. Maximal PKC activity was obtained when cells were incubated with IL-1β/IFN-γ for 3 h, and then 10⁻⁵ M (R)-albuterol added for another 3 h. (Of note, longer exposures to (R)-albuterol resulted in decreased PKC activity, potentially due to proteolytic regulatory mechanisms).

To demonstrate that PKC activation can increase iNOS message, specific chemical activators were added to cytokine-stimulated NHBE cells over a time course. As illustrated in Fig. 9a, activation of PKC with the phorbol ester, phorbol 12-myristate 13-acetate (PMA), or with specific PKC δ/ε activator, bryostatin 1, augmented iNOS message over cytokine-induced levels. Further evidence of PKCδ involvement in mediation of iNOS message is illustrated in Fig. 9b. PMA-augmented iNOS expression was completely blocked with PKCδ inhibition (rottlerin), while inhibition with PKCε inhibition with a translocation inhibitor peptide had no effect.
DISCUSSION

Nebulized administration of $\beta_2$-agonists is the most commonly used medication for treatment of bronchoconstriction associated with pulmonary diseases such as asthma. Activation of these receptors by both long- and short-acting agonists has been linked to anti-inflammatory actions in various pulmonary cells (see review (271)). $\beta_2$-adrenergic receptor agonists suppress GM-CSF production in macrophage, smooth muscle cell, and epithelial cell lines (200, 201, 204, 271, 272). Modulation of GM-CSF is particularly interesting because the airway epithelium has been suggested to be a major source of GM-CSF production, especially in asthmatic airways (127), and GM-CSF levels in bronchoalveolar lavage fluid (BALF) of asthmatics, correlate with severity and eosinophilic infiltration (126). The results described here suggest the existence of a novel signaling pathway involving iNOS and its product, NO, in this mechanism, and suggest possible target(s) by which $\beta_2$-agonists may elicit their suppressive effects on GM-CSF production and release in airway epithelium.

The short-acting $\beta_2$-adrenergic agonist (R)-albuterol is commonly administered in racemic form, containing equal parts of its stereoisomers, (R)- and (S)-albuterol. In vitro binding experimentation have demonstrated that (R)-albuterol is a potent ligand for $\beta_2$-adrenergic receptor, exhibiting over 100-fold greater affinity than that of (S)-albuterol (273). Based on this, much of the beneficial effects of racemic albuterol have been attributed to the ‘active’ stereoisomer, (R)-albuterol, discounting the (S)-enantiomer as largely inert. However, several recent reports suggest that (S)-albuterol may have deleterious effects, counteracting many of the therapeutic effects of (R)-albuterol when the racemic mixture is administered to patients (274-276). A recent study in cultured airway smooth muscle cells by Ameredes et al. (277) showed (S)-albuterol could partially counteract the suppression of GM-CSF production mediated by the corticosteroid, dexamethasone, whereas (R)-albuterol elicited additional suppression. In our studies using bronchial epithelial cells, however, (S)-albuterol did not significantly affect the increase in GM-CSF message provoked by exposure of the cells to cytokines. In addition, the racemic albuterol-mediated suppression was similar to the effect of (R)-albuterol treatment alone. It seems, therefore, that (S)-albuterol does not have a long-term effect on GM-CSF production in bronchial epithelial cells in culture. At
shorter time points, however, we have observed that (S)-albuterol downregulates iNOS message and subsequent cGMP production in NHBE cells (278), suggesting that (S)-albuterol is not inert in airway epithelial cells.

In vivo, iNOS is expressed in the human bronchial epithelium of normal adults, but constitutive iNOS expression appears to be lost when these cells are placed in tissue culture (162). However, iNOS expression can be recovered with exogenous application of a cytomix (TNF-α, IL-1β, and IFN-γ (166)). In the present study, the combination of 10 ng/ml IL-1β and IFN-γ exposure elicited comparable (or even larger) levels of iNOS expression in NHBE cells after 12 h exposure when compared to the total cytomix, which includes TNF-α. The data agree somewhat with findings reported by Kwon et. al. where dramatic synergy between IL-1β and IFN-γ in iNOS monomer production was observed in the alveolar epithelial cell line, A549 (169). The root of this synergy may stem from co-activation by IL-1β and IFN-γ of two separate transcription factors that promote iNOS expression, namely NF-κB and IRF-1, respectively (170, 279). However, in contrast to the Kwon study, these specific cytokines did not elicit significant iNOS message expression in NHBE cells when administered singly, nor was there any synergistic effect with addition of TNF-α. This may simply be due to the differences between immortalized, transformed or cancer-derived cell lines and the primary cell cultures used in our study. Additionally, the cytokine concentrations used in our study were about 10-fold less than those used with A549 cells, which may “dilute” a potential effect of TNF-α on iNOS expression in these cells.

Nitric oxide and its inducible synthase have been reported previously to play a role in GM-CSF expression in human bronchial epithelial cells. Sanders et. al. elegantly demonstrated that exogenous NO (using the chemical NO donor, NONOate) reduced GM-CSF message and protein levels that were enhanced in human rhinovirus-infected BEAS-2B epithelial lines via a transcriptionally regulated event (191). In addition, we have previously demonstrated that GM-CSF expression was increased in NHBE cells in which iNOS was chemically inhibited, suggesting that iNOS plays a role in suppressing GM-CSF-induced expression (267).

In light of findings of previous studies demonstrating that β-adrenergic agonists increase iNOS activity and NO production in airway epithelium (153, 205, 260), it was
hypothesized that β2-adrenergics may elicit suppressive effects on GM-CSF expression through an iNOS-dependent pathway. We found that (R)-albuterol increased iNOS message levels in IL-1β/IFN-γ-stimulated NHBE cells. We further explored this regulation by investigating the potential roles of specific protein kinases shown in other studies to regulate iNOS. First, cAMP-dependent kinase (PKA) is known to modulate many effects mediated by the β2-adrenergic receptor. PKA is activated by adenylyl cyclase-dependent intracellular increases in cAMP levels in most cell systems, a process mediated by activation of β-adrenergic receptors (194). Surprisingly, PKA did not appear to be involved in GM-CSF modulation by albuterol, as treatment with the PKA inhibitor isoquinolinesulfoamide, H-89, did not prevent (R)-albuterol-mediated upregulation of iNOS. However, it should be noted that isoquinolinesulfonamides has recently been questioned, and these compounds may possibly influence actions of other enzymes (201, 280). Although a low concentration of H-89 was used in this study compared to what is commonly used, we cannot entirely rule out the possibility that PKA may still be involved in β2-adrenergic receptor-mediated augmentation of iNOS expression in NHBE cells.

Secondly, the results suggest that PKC, in particular the δ isoform, seems to be involved in (R)-albuterol-mediated iNOS expression. Rottlerin is a compound that specifically inhibits PKCδ and θ, but, since NHBE cells do not contain the PKCθ isoform (281), it serves as a specific inhibitor of PKCδ in NHBE cells. In our studies, rottlerin not only prevented (R)-albuterol (and PMA) upregulation of iNOS, it also attenuated full induction of iNOS by IL-1β/IFN-γ. This finding is supported by results of a study by Carpenter et. al. which demonstrated that IL-1β activation of PKCδ stabilized iNOS message in pancreatic β-cells (177). In our studies, (R)-albuterol augmented PKC activity initially, but the activity begins to decrease after 6 hours exposure. This was not surprising, as prolonged PKC activation can lead to down-regulation through proteolytic cleavage in particular isozymes that bind biologically active phorbol esters (for review see reference (282)). Increased PKC activity with exposure to PMA did increase iNOS expression, implicating involvement of these PKC isozymes. The PKCδ activator, bryostatin 1, also increases iNOS expression in a similar manner. The data overall identifies that PKCδ plays a critical role in this signaling pathway.
iNOS message silencing, involving transfection of double-stranded iNOS siRNA into primary NHBE cells, was used to prove involvement of iNOS in GM-CSF suppression. While we show that iNOS silencing completely negated (R)-albuterol-induced suppression of GM-CSF message, alteration of GM-CSF protein release, although still statistically significant, was not as dramatic. There are several explanations for this discrepancy. First, siRNA transfection in primary culture is exceedingly difficult, which is why most silencing studies involve established cell lines which better tolerate the invasive reagents used to transfect siRNA or plasmid constructs. Primary cultures are much more sensitive to their environmental surroundings, so a fine balance with the amount of transfection reagent that does not deleteriously alter normal cell function and largest achievable message knockdown must be established. It was found that only an average of ~60% knockdown of induced-iNOS message could be realized without transfection procedures being cytotoxic to the cell or without altering baseline gene expression of our measured targets. Thus, there still existed a significant level of iNOS activity in “silenced” NHBE cells, which could affect iNOS-mediated alteration of our measured endpoints. Given this, alteration of (R)-albuterol induced suppression of GM-CSF protein release with iNOS silencing could have been somewhat underestimated. Secondly, there are likely multiple signaling pathways involved in β2-adrenergic-mediated suppression of GM-CSF including both transcriptional and post-transcriptional mechanisms. There is indeed evidence for post-transcriptional regulation of GM-CSF protein release in A549 cells in response to IL-1β stimulation (283), so this is a distinct possibility in NHBE cells.

In summary, the results of this study indicate that β2-adrenergic receptor activation by the short-acting agonist, (R)-albuterol, can effectively suppress GM-CSF expression and protein release in cytokine-stimulated NHBE cells in primary culture. In addition, we show, for the first time, that this anti-inflammatory activity is mediated through iNOS, where its expression relies on the activity of the PKCδ isozyme. (R)-albuterol-mediated augmentation of iNOS expression also appears to be independent of PKA. Interestingly, GM-CSF production has been positively correlated with eosinophilic influx and function in vivo (284), and asthmatic patients who take β-agonists do have reduced numbers of activated eosinophils in serum and bronchial mucosa (285, 286). A possible beneficial anti-inflammatory effect,
then, of $\beta_2$-adrenergic receptor agonists could be to downregulate, via an iNOS-dependent, epithelial production of GM-CSF.
Figure 1. Exposure to 10 ng/ml each of TNF-α, IL-1β, and IFN-γ (cytomix) upregulates iNOS and GM-CSF message in NHBE cells. iNOS and GM-CSF message were measured after total RNA isolation using TaqMan real-time RT-PCR and normalized to β-actin message. a) iNOS message levels peak after approximately 12 h of cytomix exposure, then fall to barely detectable levels by 48 h. Significantly more iNOS expression after 0, 0.5, 1, and 3 hrs cytomix exposure (** = p<0.001). Significantly less iNOS expression after 6 and 12 hrs cytomix exposure († = p<0.01), (†† = p<0.001). Significantly less iNOS expression after 24 hrs cytomix exposure (§ = p<0.001). b) GM-CSF message levels rise slightly after initial cytomix exposure, then rise by nearly 15-fold after 48 h. Significantly more than constitutive iNOS expression (* = p<0.05). Significantly more iNOS expression than any other time points (** = p<0.001). All data are presented as mean ± SEM (n=6).
Figure 2. Combination of IL-1β and IFN-γ exposure elicits greatest synergistic upregulation in iNOS transcription in NHBE cells primary culture. NHBE cells were exposed to combinations of TNF-α, IL-1β, and IFN-γ, all at 10 ng/ml, for 12 h. iNOS message was normalized to β-actin message. The combination of the cytokines IL-1β and IFN-γ induced the highest levels of iNOS transcription. Data are presented as mean ± SEM (n=3). * = significantly different expression compared to all other treatments (p<0.001), † = significantly more than cytomix-induced expression (p<0.01).
Figure 3. (R)-albuterol significantly suppresses IL-1β+IFN-γ-mediated upregulation of GM-CSF message and protein release. a) NHBE cells were stimulated with 10 ng/ml each of IL-1β and IFN-γ for 12 h in the presence of either control medium, or 10^{-6}M (R)-albuterol or (S)-albuterol, or both (R)- and (S)- (racemic). GM-CSF message was normalized to β-actin message. Both (R)- and racemic albuterol significantly suppressed GM-CSF upregulation by the cytokines, whereas (S)-albuterol had no significant effect. Data represented as mean ± SEM (n=3-7). ∗ = significantly more than constitutive GM-CSF mRNA expression (p<0.05), † = significantly less than cytokine-induced GM-CSF mRNA expression (p<0.01), ‡ = significantly less GM-CSF mRNA expression after cytokine+(S)-albuterol treatment (p<0.01). b) NHBE cells were stimulated with 10 ng/ml each of IL-1β and IFN-γ over a range of times in presence of control medium, 10^{-7}M (R)-albuterol, or racemic (R+S) albuterol, and GM-CSF protein in culture media was measured using ELISA and normalized to total protein of cell lysates. Both (R)- and racemic albuterol significantly attenuated the increase GM-CSF protein release elicited by IL-1β and IFN-γ after 12 h. Data are presented as mean ± SEM (n=4-6). Significantly more than constitutive GM-CSF protein release (∗ = p<0.01), (**) = p<0.001. Significantly less than cytokine-induced GM-CSF protein release (∗† = p<0.01), (∗∗† = p<0.001).
a) GM-CSF mRNA Level

<table>
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<th>2</th>
<th>3</th>
<th>4</th>
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</thead>
<tbody>
<tr>
<td>Media only</td>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β &amp; IFN-γ</td>
<td></td>
<td></td>
<td>†, ‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ (R)-albuterol</td>
<td></td>
<td></td>
<td></td>
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</tr>
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<td>+ (S)-albuterol</td>
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</tr>
<tr>
<td>+ rac. albuterol</td>
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</tbody>
</table>

b) GM-CSF Protein Release

12 Hours 18 Hours 24 Hours

- Media only
- IL-1β + IFN-γ
- + (R)-albuterol
- + (S)-albuterol
- + Racemic albuterol

Exposure Time
Figure 4. (R)-albuterol augments iNOS message in NHBE cells exposed to IL-1β+IFN-γ in a concentration-dependent manner. NHBE cells were exposed to 10 ng/ml each of IL-1β and IFN-γ for 12 h together with either (R)- or (S)-albuterol over a range of concentrations. (R)-albuterol significantly augmented iNOS message at 10^{-5}M, whereas (S)-albuterol did not have a significant effect at this or lower concentrations. Dashed line represents IL-1β/IFN-γ-induced iNOS expression. Data are presented as mean ± SEM (n=6). * = significantly more than cytokine-induced iNOS expression (p<0.001).
Figure 5. β2-adrenergic receptor activation is required for (R)-albuterol-mediated increase in iNOS expression. NHBE cells were stimulated with 10 ng/ml each of IL-1β and IFN-γ with or without 10^{-5} M (R)-albuterol for 18 h. In some wells, β2-adrenergic antagonist, ICI 118,551, also was added. iNOS message, normalized to β-actin message, was assayed by TaqMan real-time RT-PCR. β2-adrenergic receptor antagonism alone did not affect IL-1β/IFN-γ-induced iNOS transcription, but (R)-albuterol augmentation of iNOS message was blocked by ICI 118,551. NHBE cells incubated in media alone exhibited no measurable iNOS message. Dashed line represents IL-1β/IFN-γ-induced iNOS expression. Data are presented as mean ± SEM (n=4). * = significantly more than cytokine-induced iNOS expression (p<0.01). † = significantly less iNOS message expression after cytokine+(R)-albuterol treatment (p<0.01). N.S. = No significant difference (p>0.05).
Figure 6. Silencing iNOS transcription decreases attenuation of GM-CSF message and protein release elicited by (R)-albuterol.  

a) NHBE cells were transfected with iNOS silencing RNA using 0.45% final concentration of Fugene reagent and 225 nM iNOS siRNA (Ambion) for 24 h in growth media void of antibiotics. After transfection period, cells were stimulated with 10 ng/ml each of IL-1β+IFN-γ for 18 h to augment GM-CSF message levels. iNOS silencing had little effect on IL-1β+IFN-γ augmentation of GM-CSF message but, it did negate (R)-albuterol-mediated suppression of GM-CSF message. (S)-albuterol, with or without silencing, had no effect of GM-CSF message expression. These results suggest that iNOS is necessary for (R)-albuterol-mediated GM-CSF message suppression in NHBE cells exposed to cytokines. Dashed line represents constitutive GM-CSF expression. Data represented as mean ± SEM (n=3-4). Significantly more than constitutive GM-CSF expression (* = p<0.05), (**) = p<0.001), † = significantly less GM-CSF expression after treatment with iNOS silencing (p<0.01). § = significantly less than cytokine-stimulated GM-CSF expression (p<0.05). N.S. = No significant difference (p>0.05).

b) NHBE cells were transfected and treated as above, and GM-CSF protein in culture medium was measured by ELISA. iNOS siRNA did not significantly affect cytokine-mediated GM-CSF protein release. However, (R)-albuterol (10⁻⁵ M) significantly suppressed cytokine-mediated GM-CSF protein release, and iNOS silencing partially suppressed this effect. Dashed line represents constitutive GM-CSF protein release. Data represented as mean ± SEM (n=12). ** = significantly more than constitutive GM-CSF protein release (p<0.001). †† = significantly less than cytokine-induced GM-CSF protein release (p<0.001). § = significantly more GM-CSF protein release than cytokine+Fugene 6+(R)-albuterol treated cell culture (p<0.05 with Student’s t-test).
**a**

IL-1β + IFN-γ (10 ng/ml)  
Fugene (0.45%)  
iNOS siRNA (225 nM)  
(R)-albuterol (10^{-5} M)  
(S)-albuterol (10^{-5} M)

**b**

GM-CSF Protein Release

GM-CSF mRNA Level

Media only  
IL-1β + IFN-γ  
Fugene Reagent  
iNOS siRNA  
(R)-albuterol  
(S)-albuterol
Figure 7: (R)-albuterol mediated augmentation of iNOS message involves PKC, but not PKA or PKG. NHBE cells were exposed to 10 ng/ml each of IL-1β and IFN-γ for 12 h, together with either the cGMP-dependent kinase inhibitor (Rp)-8-pCPT-cGMPS (1µM), cAMP-dependent kinase inhibitor H-89 (2µM), protein kinase C inhibitor calphostin C (500 nM), or the protein kinase Cδ inhibitor rottlerin (3µM) with and without the addition of 10^{-5}M (R)-albuterol. iNOS message, normalized to β-actin message, was assayed by TaqMan real-time RT-PCR after total RNA isolation. Inhibition of PKG (a) or PKA (b) did not affect (R)-albuterol-mediated iNOS message augmentation in cytokine-stimulated NHBE cells. c) The PKC inhibitor calphostin C attenuated (R)-albuterol-mediated augmentation of iNOS message, suggesting that PKC activity involvement. PKCδ inhibition with rottlerin negated both (R)-albuterol-mediated increase in iNOS transcription and also IL-1β+IFN-γ induced iNOS transcription. There was no detectable constitutive iNOS expression in these cells. Dashed line represents IL-1β/IFN-γ-induced iNOS expression. Data represented as mean ± SEM (n=3-7). Significantly more than cytokine-induced iNOS expression (* = p<0.01), (**) = p<0.001). Significantly more than inhibitor treated control (‡ = p<0.01), (‡‡ = p<0.001). Significantly less than cytokine+(R)-albuterol treated iNOS expression († = p<0.05). N.S. = No significant difference (p>0.05).
a

b

c

IL-1β+IFN-γ
(R)-albuterol
(Rp)-8-CPT-S-cGMPS

IL-1β+IFN-γ
(R)-albuterol
H-89

IL-1β+IFN-γ
(R)-albuterol
Calphostin C
Rottlerin
Figure 8. PKC activity in NHBE cells is increased by (R)-albuterol. NHBE cells were stimulated with 10 ng/ml each of IL-1β and IFN-γ for 6 h. 10⁻⁵ M (R)-albuterol was added to cytokine-stimulated culture 0.5, 1, 3, or 6 h, at which time cells were lysed and immediately assayed for PKC activity. (R)-albuterol augments PKC activity maximally of 3 h of exposure, followed by a diminution of the effect. Band intensity reflects phosphorylation activity by PKC, which was quantified by imaging software (UVP, Ltd., Upland, CA). Data are representative of 3 individual experiments.
Figure 9. iNOS gene expression is reversibly enhanced by PKC activators. a) NHBE cells were stimulated with 10 ng/ml each of IL-1β and IFN-γ for 8 h. The general PKC activator PMA (10 nM) or specific PKC δ/ε activator bryostatin 1 (10 nM) were added for 1, 4, and 8 h iNOS message levels were measured. Both PMA and bryostatin 1 augmented iNOS message at 8 h. Data represented as mean ± SEM (n=6). Significantly more than cytokine-induced iNOS expression (* = p<0.01) (** = p<0.001). b) PMA (10 nM) was applied to NHBE cells for 8 h in the presence of control medium, calphostin C (500 nM), rottlerin (3 μM), or PKCε translocation inhibitor peptide (300 μM), and iNOS message was assessed. In selected wells, PMA negative control, 4-αPMA (10 nM), was added in place of PMA. The increase in iNOS message in response to PMA was attenuated by both calphostin C and rottlerin, suggesting that PKCδ was involved. PKCε translocation inhibitor and PMA-4α had any effect on iNOS expression. PKC activation alone, without IL-1β/IFN-γ exposure, had no effect on iNOS expression (data not shown). Data represented as mean ± SEM (n=4-8). * = Significantly more than cytokine-induced iNOS expression (p<0.001). † = Significantly less than cytokine+PMA-induced iNOS expression (p<0.001). N.S. = No significant difference (p>0.05).
a

IL-1β + IFN-γ (8 hrs)

PMA (10nM)

Bryostatin 1 (10nM)

b

IL-1β + IFN-γ

PMA

4-α-PMA

Calphostin C

Rottlerin

PKCe inhibitor peptide

N.S.

p<0.001

* * *

† † † † † †
CHAPTER V

SUMMARY AND FUTURE DIRECTIONS
SUMMARY AND FUTURE DIRECTIONS

The studies herein focus on two vital functions of the airway epithelium: mucin production/secretion and inflammatory mediation. These studies focused on modulation of these two separate physiologic aspects in response to specific inflammatory cytokines. The primary cell culture system utilized in this dissertation does not allow for other cell types to influence the outcome, such as cellular signaling from neighboring fibroblasts, inflammatory cells, endothelial cells, etc. In addition, environmental elements, temperature, pH, and atmosphere are tightly controlled. This allows for reproducible findings and specific delineation of signaling mechanisms regulated by only the airway epithelial cells. As mucin production and inflammatory regulation are critical to homeostasis and environmental response of the airway, and the airway epithelium plays a large role in these processes, discovery of pertinent signaling mechanisms through these studies may lead to improved strategies to combat debilitating respiratory disease.

The studies have uncovered four novel findings that have not been previously reported. These findings are:

1) Muc2 mucin, but not Muc5AC mucin, secretion increases in guinea pig tracheal epithelial cells with cytokine exposure.
2) iNOS-mediated signaling pathway suppresses GM-CSF message in stimulated NHBE cells.
3) β2-adrenergic agonist, (R)-albuterol, augments iNOS message expression in NHBE cells.
4) (R)-albuterol-mediated increase of iNOS message in NHBE cell culture is dependent on PKCδ activity.

A novel monoclonal for Muc2 mucin was developed and characterized for use in guinea pig. Tracheal epithelial cells isolated from guinea pig airway were grown in air/liquid interface culture as to allow full differentiation and mucous phenotype expression. I demonstrated that Muc2 mucin secretion increased 100% with short-term cytokine exposure,
whereas Muc5AC mucin secretion (as measured with 45M1 antibody from NeoMarkers) did not change compared to constitutive secretion. This differential effect was not due to stimulated transcriptional or translational change, as both Muc2 and Muc5AC mucin intracellular production and message expression increased similarly with cytokine stimulation. However, examination of constitutive mucin production revealed that there was a greater amount of intracellular Muc2 mucin when compared to secreted amounts in equal total protein collections (approximately 4:1). This ratio was near 1:1 when measuring Muc5AC mucin. This data supports the theory that GPTE cells prepare in advance for Muc2 mucin release upon stimulation.

Differential mucin subtype expression may affect mucosal makeup, thereby altering viscosity, polarity, mobility, and other factors. Change in these factors may play important roles in disease. In the small intestine and the colon, lack of MUC2 production or defective polarization has been linked to incidence of colon cancer or ulcerative colitis, respectively (62, 115). In addition, polymorphisms of mucin subtypes, which differ in expression patterns from individual-to-individual, may also influence disease development. Vinall et al. demonstrated that atopic, asthmatic individuals exhibited differences in MUC2 allele distribution as compared to atopic, non-asthmatic individuals (63). Despite some evidence that differential expression may be important, the specific roles of each mucin subtype are not clear. Therefore future studies need to elucidate these individual roles, through modern methods of small interfering RNA, mutational studies, and mouse knockout models. In addition, signaling pathways involved in differential mucin subtype expression should be defined by studying transcriptional, post-transcriptional, translational, or post-translational mechanisms.

Unlike MUC5AC and MUC5B, MUC2 mucin secretion is not commonly reported in human airway epithelial studies. The few studies that have measured MUC2 expression generally do not detect MUC2 or report that expression does not change (see Chapter I). My findings suggest this is not the case for normal guinea pig airway epithelial cell culture. From a general pathophysiologic standpoint, allergic response of guinea pig airway behaves similarly to human; however, these studies suggest that specific mechanisms regarding mucin expression are not the same. This established and easily maintained guinea pig model should
continue to be used to study mucin subtype expression, however, specific assumptions regarding human mucin subtype expression should be made with caution.

The second novel finding is that endogenous production of nitric oxide in the human airway epithelium, through activity of inducible nitric oxide synthase (iNOS), suppresses inflammatory response. While previous studies have shown that exogenously applied nitric oxide can suppress cytokine production, this is the first study to demonstrate that the airway epithelium regulates this process in an endogenous manner. iNOS activity constitutes the major release of nitric oxide during inflammatory conditions and is considered to be a double-edged sword, whereas over abundant NO production can lead to deleterious formation of various reactive oxygen and nitrogen species that damage the interstitial airway. The physiologic purposes of formation of these reactive species is anti-bacterial in nature, however, many pulmonary diseases such as asthma, bronchitis, and lung cancer, are characterized by over-expression of iNOS and increased amounts of exhaled nitric oxide. There are currently many drugs in development that target iNOS to reduce inflammatory-related damage to the airway. My findings using the iNOS-specific inhibitor, L-NIL, clearly show that iNOS has suppressive effects on the inflammatory promoter GM-CSF. Therefore employment of these therapeutic strategies may have contradictive effects in the airway epithelium and perhaps focus on alternative strategies, such as anti-oxidant therapy, should be considered.

The third novel finding is that the anti-inflammatory mediation of the β2-agonist, albuterol involves iNOS. It has been demonstrated that catecholamines, in particular β2-agonists, can inhibit inflammatory cell activation, inflammatory cell recruitment, and inflammatory mediator release (see Chapter I). It also has been demonstrated that these effects on mediator release are achievable at dosages that induce smooth muscle relaxation, therefore relevant for normal drug usage (272). In airway epithelial cells, inflammatory mediator expression of GM-CSF and intracellular cell adhesion molecule-1 are suppressed with β2-agonists (199, 200). While it has been demonstrated that β-agonist induced anti-inflammatory effects can be mediated through a cAMP/PKA signaling pathway in some
inflammatory cells (201), little else is known regarding this mechanistic pathway. Since iNOS can suppress GM-CSF expression in NHBE cells, and β2-agonism can increase nitric oxide synthase activity in the airway epithelium of a bronchial asthma rat model (205), I theorized that the short-acting β2-agonist, (R)-albuterol, suppresses GM-CSF through an iNOS-mediated pathway in NHBE cells. I demonstrated that (R)-albuterol significantly increased iNOS message expression in IL-1β/IFN-γ stimulated NHBE cells after 12 h exposure. With iNOS silenced, I further demonstrated that (R)-albuterol-mediated suppression of GM-CSF message was blocked. These findings are important because it uncovers a novel signaling mechanism for albuterol-mediated anti-inflammatory effects and, in addition, validates the critical importance of iNOS in anti-inflammatory function.

Protein kinase inhibitors were used to delineate the signaling mechanism involved in β2-adrenergic-mediated increase in iNOS expression. β-adrenergic receptors signal primarily by activating the α subunit of adenylyl cyclase stimulatory G-protein (Gs) to stimulate adenylyl cyclase, which produces cAMP (194). Therefore, transient increase of intracellular cAMP levels activates PKA and its downstream targets. Surprisingly, PKA inhibition with the isoquinolinesulfoamide, H-89, did not prevent (R)-albuterol-mediated upregulation of iNOS. In addition, cGMP protein kinase inhibition, with (Rp)-8-pCPT-cGMPS, had no effect. However, use of a PKC inhibitor, calphostin c, blocked (R)-albuterol mediated augmentation of iNOS message. Furthermore, specific inhibition of PKCδ, with rotterlin, also blocked (R)-albuterol mediated iNOS expression increase. iNOS message increase was mimicked with phorbol ester, PMA, and macrolactone (PKCδ/ε specific activator), bryostatin 1. PMA-augmented iNOS expression was also blocked with calphostin c and rotterlin, but not with PKCε translocation inhibitor peptide. PKC-mediated enhancement of iNOS expression is not entirely surprising, as Carpenter et. al. demonstrated that IL-1β activation of PKCδ stabilized iNOS message in pancreatic β-cells (177). PKC has also been implicated in post-translational modification of iNOS and its cofactors (287). My findings demonstrate that PKCδ activity is required for (R)-albuterol (or PMA) mediated increase in iNOS expression. Additionally, PKC activity is increased with (R)-albuterol exposure, demonstrating for the first time that activation of the β2-adrenergic receptor may mediate some of its actions through PKC.
Future studies regarding this mechanism should concentrate on three major areas. First, the mechanism by which $\beta_2$-agonists augment PKC activity should be elucidated. In murine macrophage cell lines, crosstalk between the cAMP signaling system and PKC has been demonstrated in the literature. Specifically, PKC activation with PMA, UTP, and lysophosphatidic acid in RAW 264.7 and J774 cell lines leads to type II adenylyl cyclase activity (288-290). Conversely, several cAMP elevating elements initiate PKC activation in RAW 264.7 and J774 cell lines (290, 291). Given the latency of (R)-albuterol-mediated PKC activation (approximately 3-6 hours of exposure), there may be several key upstream elements in place, however further studies need to done to clarify this.

Secondly, it should be demonstrated how PKC mediates iNOS expression in the airway epithelium. One possibility is that PKC stabilizes iNOS message. It has been shown that the 3’-untranslated region of iNOS mRNA severely destabilizes the RNA structure (176, 292). PKC may therefore regulate iNOS expression by somehow stabilizing this region. Another possibility is that PKC may alter NF-κB translocation or expression, thereby limiting a major contributing factor to iNOS transcription. With the use of various inhibitors, Kuo et al. have demonstrated this in the RAW 264.7 cell line (293). In addition, other transcription factors known to be involved in airway epithelial iNOS expression, such as IRF-1, AP-1, and C/EBP, could also be modulated by PKC activity.

Thirdly, other inflammatory mediators influenced by this mechanism should be identified. In inflammatory cells, release of histamine, leukotriene $C_4$ and $D_4$, prostaglandin, eosinophil peroxidase, TNF-α, IL-3, IL-4, and IL-5 are suppressed with $\beta_2$-agonists (see Chapter 1). Therefore, a number of inflammatory endpoints could be identified in the airway epithelium with silencing strategies and microarray or RNA-dot blot analysis. In addition, in vivo models should be employed to validate in vitro findings. It is conceivable that defects or compromises in this iNOS-mediated signaling pathway might contribute to pulmonary disease by affecting appropriate moderation of inflammatory response and/or limiting drug effectiveness.

In summary, this study has established one signaling pathway in which (R)-albuterol has an anti-inflammatory effect via suppression of the GM-CSF expression through an iNOS-mediated pathway in normal human bronchial epithelial cells. This pathway could possibly
be exploited in future therapeutics to suppress inflammation. Indeed, there are drugs currently in development that have NO-releasing groups linked to enhance therapeutic activity and/or reduce side effects. Of interest is the albuterol-linked drug, NCX-950, which has shown to have anti-inflammatory properties greater than albuterol alone in LPS-stimulated mice (294). Therefore, additional nitric oxide may be of medicinal benefit in pulmonary inflammation.
CHAPTER VI

BIBLIOGRAPHY
REFERENCES


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APPENDICES
APPENDIX I

Cytotoxicity assay. NHBE cells were exposed to all treatments used in the albuterol study and assayed for lactate dehydrogenase release using a commercially available assay (Promega). No treatments caused greater than 7.39% LDH release, which was comparable to non-treated (media only) control, therefore treatments were not considered cytotoxic to the cells.

Table A1. Lactate dehydrogenase release data (cytotoxicity test)

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<th>Treatment</th>
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<tbody>
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<tr>
<td>10ng/ml TNF-α + II-1β + INF-γ (cytomix)</td>
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<td>5.64 +/- 0.69</td>
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<td>Cyto + Fugene 6 + iNOS siRNA+(R)-albuterol 10⁻⁷M</td>
<td>24 + 18 hours</td>
<td>4.88 +/- 0.62</td>
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<tr>
<td>Cyto + Fugene 6 + iNOS siRNA+(S)-albuterol 10⁻⁷M</td>
<td>24 + 18 hours</td>
<td>6.36 +/- 0.56</td>
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</table>
**APPENDIX II**

**Small interfering RNA optimization.** Three different transfection agents were tested in NHBE cell culture for effective attenuation of iNOS transcript using a pre-designed annealed siRNA (catalog# 8678; Ambion, Austin, TX). Lipofectamine 2000 (Invitrogen, Carlsbad, CA), siLentFect (BioRad, Hercules, CA), and Fugene 6 (Roche, Indianapolis, IN) were tested for silencing effectiveness and cytotoxicity to NHBE cells. Initially, a concentration range (50 to 300 nM) of iNOS siRNA was tested for iNOS knockdown percentage following manufacturer’s protocol for Lipofectamine. 200 nM of iNOS siRNA exhibited the greatest knockdown, although all concentrations used significantly attenuated cytomix+Lipofectamine-stimulated induction of iNOS message (p<0.05 with ANOVA; n=3). Unfortunately, while Lipofectamine (0.25% final concentration in incubated media) exhibited an approximate 75% knockdown of iNOS transcript with 24-hour transfection period using 200 nM siRNA, reagent alone caused a 3-fold increase in iNOS transcription (after 12-hour cytokine stimulation). In addition, there was noticeable cell loss, compared to non-transfected wells, and 13.8% LDH release. Similar, but not as pronounced, cell loss was observed with 0.4% siLentFect transfection reagent with an elevated 9.0% LDH release. Lower concentrations of siLentFect lowered these observable cytotoxic effects, however the siRNA was ineffective at these concentrations. Fugene 6, however, did not demonstrate cytotoxic effects at effective concentrations. In fact, 0.45% Fugene 6-treated NHBE cells had less observable cell loss than non-treated wells and only a 5.0% LDH release. Fugene 6 reagent plus 225 nM siRNA silenced iNOS transcript by 60% compared to NHBE cells treated with IL-1β/IFN-γ alone (Fig. A1). A concentration range of Fugene 6 was tested to optimize knockdown percentage, however, 0.23% final concentration Fugene 6 was not effective, and 0.68% and 0.91% Fugene 6 lowered iNOS transcriptional rates 50% and 60%, respectively, without siRNA addition. Consistently, NHBE cell plate coverage between 50-75% was reached before Fugene transfection reagent was applied. Any lower percent plate coverage and cell culture would not reach confluence before silencing was ineffective (approximately 48 hrs after initial transfection). Transfection at higher plate coverage
 (>75%) would reduce silencing effectiveness. Therefore, it was concluded that 24-hours transfection (maximum period of exposure to allow for 12-24 h treatment regimen without reducing siRNA effectiveness) with 0.45% final concentration of Fugene 6 and 225 nM of siRNA would elicit the greatest iNOS knockdown without cytotoxic effects to NHBE cells.

**Figure A1. siRNA effectively attenuates iNOS mRNA expression.** NHBE cell cultures were transfected with iNOS silencing RNA using Fugene 6 (Roche) reagent. Cells were transfected with a 0.45% final concentration of Fugene reagent and 225 nM iNOS small interfering RNA or scrambled siRNA control (Ambion, cat. # 4611) for 24 hrs in growth media devoid of antibiotics. After this transfection period, cells were stimulated with 10 ng/ml each of IL-1β+IFN-γ for 18 hrs to induce iNOS transcription. iNOS message was assayed by TaqMan real-time RT-PCR after total RNA isolation. Addition of the iNOS siRNA reduced cytokine-induced iNOS transcription by ~60%. Some attenuation of message was noted (~30%) with Fugene alone or Fugene+scrambled siRNA, however knockdown was consistently greater with iNOS-specific siRNA. Repeated experiments with Fugene+iNOS siRNA (when compared to Fugene treatment alone) in IL-1β/IFN-γ stimulated NHBE cells demonstrated significant reduction of iNOS message (p<0.01 with Student’s t-test; n=6) (data not shown).