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Regulation of the Lactoperoxidase System in the Airway

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REGULATION OF THE LACTOPEROXIDASE SYSTEM IN THE AIRWAY

By

Miryam Araceli Fragoso

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

December 2007
REGULATION OF THE LACTOPEROXIDASE SYSTEM IN THE AIRWAY

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The lactoperoxidase (LPO) antimicrobial system has been shown to play an important role in maintaining sterile conditions in several tissues including the mammary gland, the salivary gland, and the airway. The LPO system in the airway consists of the enzyme LPO and its substrates hydrogen peroxide and an anion. LPO catalyzes the oxidation of a halide or pseudohalide ion for example SCN⁻ or I⁻ by hydrogen peroxide producing a product, OSCN⁻ or OI⁻ which have antibacterial, antifungal, and antiviral properties. In order to have a functional antimicrobial system all the components need to be present at appropriate concentrations. The LPO system has been suggested to be deficient in cystic fibrosis. There are three possible regulatory mechanism of this antimicrobial system and these involve the secretion and availability of the three components of the LPO system in the luminal fluid. The studies presented in this dissertation examine two of the possible regulatory mechanisms of the LPO system in the airway; the availability and transport of SCN⁻ to the luminal surface, and the expression of LPO. The knowledge obtained from these studies could be utilized to develop treatments to control infection in diseases characterized by chronic infections such as cystic fibrosis.
ACKNOWLEDGEMENTS

First and foremost I would like to thank my mentor Dr. Gregory E. Conner for his guidance, encouragement, patience and moral support during my graduate studies. I would also like to thank the members of my committee: Dr. Nevis Fregien, Dr. Theodore Lampidis and Dr. Roger Fenna for their time, effort and helpful advice during the course of my graduate studies. A sincere thanks to the current and former members of the Airway Biology Lab for their helpful suggestions and friendship throughout the years. Finally, I would like to thank my parents and brother for their unconditional love, encouragement and support, for without them this would not have been possible.
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<tbody>
<tr>
<td>ALI</td>
<td>Air liquid interface</td>
</tr>
<tr>
<td>APO</td>
<td>Airway Peroxidase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BL</td>
<td>Basolateral</td>
</tr>
<tr>
<td>bLPO</td>
<td>Bovine lactoperoxidase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>DIDS</td>
<td>4,4’-diisothiocyanatostilbene-2,2’disulphonic acid</td>
</tr>
<tr>
<td>DNDS</td>
<td>Dinitrostilbene-2,2’-disulphonic acid</td>
</tr>
<tr>
<td>DPC</td>
<td>Diphenylamine-2carboxylicacid</td>
</tr>
<tr>
<td>Duox</td>
<td>Dual function NAD(P)H oxidases/peroxidases</td>
</tr>
<tr>
<td>ENac</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>EPO</td>
<td>Eosinophil peroxidase</td>
</tr>
<tr>
<td>Ex4p1</td>
<td>Exon 4 peptide</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HBE</td>
<td>Papilloma virus-immortalized human tracheobronchial epithelial cell line</td>
</tr>
<tr>
<td>HSCN$^-$</td>
<td>Hypothiocyantate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IKB</td>
<td>Nuclear factor kappa B inhibitor</td>
</tr>
<tr>
<td>IKK</td>
<td>IKB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinases</td>
</tr>
<tr>
<td>KHL</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS binding protein</td>
</tr>
<tr>
<td>LC/MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LPO</td>
<td>Lactoperoxidase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>Muc2</td>
<td>Mucin 2</td>
</tr>
<tr>
<td>Muc5AC</td>
<td>Mucin 5AC</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NIS</td>
<td>Sodium Iodide Symporter</td>
</tr>
<tr>
<td>NPPB</td>
<td>5-nitro-2-(3-phenylpropylamino)-benzoate</td>
</tr>
<tr>
<td>NTS</td>
<td>Sodium thiocyanate symporter</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBT-T</td>
<td>0.5% Tween-20 phosphate buffered saline</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase</td>
</tr>
<tr>
<td>rhLPO</td>
<td>Recombinant human lactoperoxidase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCN^-</td>
<td>Thiocyanate</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory leukocyte protease inhibitor</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloro acetic acid</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>T-TBS</td>
<td>Tris buffered saline, 0.5% Tween-20</td>
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<td>V1</td>
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</tr>
<tr>
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CHAPTER 1

Introduction

Host defense mechanisms against infection in the airway

Mammalian have evolved innate and adaptive defense mechanisms due to the continuous threat of invasion by microorganisms. For example the respiratory system is constantly under attack by microorganisms and other substances present in inhaled air. The innate immune system is an important part of respiratory defenses and includes mechanical, humoral, chemical and cellular defense mechanisms. Mechanical defense mechanisms in the airway involve physical barriers such as the presence of a glycocalyx and mucus which protect the epithelium by entrapping microorganisms, and mechanical clearance of microorganisms by coughing or ciliary movement, for review (Ratner & Prince, 2000; Zaas & Schwartz, 2005).

In the airway, the epithelium acts an interface between the inside and outside environment of an organism and plays an important role in the humoral and chemical defense mechanisms. Many studies have shown that the airway mucosa secretes antimicrobial substances such as reactive oxygen species including hydrogen peroxide (Geiszt et al., 2003; Forteza et al., 2005; Harper et al., 2005). The epithelium has also been shown to secrete antimicrobial peptides (Hiemstra, 2001) and macromolecules having antimicrobial properties such as lysozyme (McClelland & van Furth, 1975; Tournier et al., 1990; Akinbi et al., 2000; Singh et al., 2000), defensins (Zhao et al.,
lactoferrin (Gawel et al., 1979; Inoue et al., 1993; Singh et al., 2000), Secretory leukocyte protease inhibitor (SLPI) (Merten et al., 1992; Hiemstra et al., 1996; Singh et al., 2000), collectins (Grubor et al., 2006; Gupta & Surolia, 2007), and lactoperoxidase (Salathe et al., 1997; Gerson et al., 2000). The cellular defense mechanisms of the innate immune system include recruitment of leukocytes such as macrophages, neutrophils, dendritic cells mast cells, eosinophils, basophils, lymphocytes and natural killer cells. These cells are important as part of the adaptive immune system or in activating the adaptive immune system by secreting cytokines and other substances involved in the immune response. (Bals & Hiemstra, 2004). A series of cytokines are released into the airway. Primary inflammatory mediators such as interferon γ (IFN γ) and Tumor Necrosis Factor α (TNF α) up-regulate the production of secondary mediators such as IL-4, IL-5, IL-6, IL-8, IL-10, IL-13 IL-17, and granulocyte macrophage cytokine stimulating factor (GM-CSF), amongst others. (Esche et al., 2005).

The innate immune system is non-specific and recognizes microorganisms in a generic way but is able to discriminate between self and a variety of microorganisms (Akira et al., 2006). Microorganisms are recognized by the innate immune system by means of recognition receptors including Toll-like receptors (TLRs) among others, which recognize microbial components. These components are constitutive, conserved and include lipopolysacharride (LPS), flagellin and peptidoglycans. (Koehler et al., 2004; Akira et al., 2006).
Signaling pathways involved during an innate host defense response

Airway epithelial cells express a variety of TLR as well as cytokine receptors which are involved in the recognition of viral products (TLR3), fungal products (TLR2) (Goldman et al., 1997) and bacterial products such as LPS (TLR4), and flagellin (TLR5). The nuclear factor-kappa B (NF-κB) and the JAK-STAT signaling pathways are activated upon recognition of microbial products and cytokines; NF-κB pathway (LPS, flagellin, TNFα), and JAK-STAT pathway (IFNs) (Esche et al., 2005).

NF-κB signaling pathway

In unstimulated conditions, subunits of the NF-κB transcription factor are bound by the NF-κB inhibitor (IκB) in the cytoplasm. Activation of NF-κB occurs when molecules such as microbial products (LPS, flagellin) or cytokines (TNFα) bind to TLRs or cytokine receptors respectively, thus stimulating IκB phosphorylation by IκB kinase (IKK) the main regulator of the NF-κB among other factors. This causes the ubiquitinylation and degradation of IκB which allows NF-κB to translocate to the nucleus, bind to the DNA and regulate gene expression (Koehler et al., 2004). Following is an example of how this pathway is activated upon recognition of bacterial products such as LPS. LPS recognition involves binding of LPS to the LPS binding protein (LBP), CD-14 and MD-2, all of which form a complex with TLR4 (Dziarski et al., 2001) and activates the NF-κB signaling pathway (Dziarski et al., 2001; Koehler et al., 2004; Esche et al., 2005) causing changes in expression of genes involved in inflammation. If one of the components in the recognition of LPS is missing for example, MD-2, recognition of
LPS will not take place in airway epithelium as observed by Dziarski et al. (Dziarski et al., 2001).

**Jak-STAT signaling pathway**

Other signaling pathways are also activated during a host defense immune response. The JAK-STAT signaling pathway is activated upon binding of interferons, which have been shown to be present in the airway during infection and which are secreted by lymphocytes activated during the immune response. In this pathway, the Janus Kinases (JAKs) are bound to the intracellular domain of a cytokine receptor. When a cytokine binds to its receptor, activation of JAKs takes place, an event, which causes the phosphorylation of tyrosine residues on the receptors. This event enhances the binding of the signal transducers and activators of transcription (STATs), which are also phosphorylated. Dimerization of STATs takes place and this is followed by translocation of the activated dimmers to the nucleus where regulation of gene expression occurs (Hebenstreit et al., 2005).

Recognition of pathogens by airway epithelial cells cause activation of signaling pathways including the ones aforementioned resulting in an innate immune response which includes increased secretion of mucus, peptides, hydrogen peroxide and other molecules which have antimicrobial properties including peroxidases (Kinbara et al., 1992; Li et al., 1997; DiMango et al., 1998; Ratner & Prince, 2000).
Peroxidases in the airway

Peroxidases are important contributors to innate host defense in a variety of tissues. Several members of the mammalian peroxidase family are present in the airway lumen and are either secreted by the airway mucosa (lactoperoxidase) (Salathe et al., 1997; Gerson et al., 2000) or by cells recruited by the innate defense system such as granulocytes (myeloperoxidase) and eosinophils (eosinophil peroxidase). Peroxidases have been shown to be a highly conserved gene family that forms a cluster on the long arm of chromosome 17, suggesting that these genes were duplicated from a single ancestral gene (Daiyasu & Toh, 2000; Sakamaki et al., 2002). The members of this heme peroxidase family share 50-70% identity. The active site-related residues are highly conserved (Fenna et al., 1995; Andersson et al., 1996).

All hemoprotein peroxidases utilize H$_2$O$_2$ to catalyze the oxidation reaction of pseudohalide ions whose product has antimicrobial properties (Fig. 1.1). The relative antimicrobial activity of these peroxidases depends on the halide ion. Even though the enzymes are very similar these enzymes differ in amino acid sequence molecular weight, subunit structure, immunological reactivity and substrate specificity (Thomas et al., 1991a). For example all of the peroxidases found in the airway oxidize SCN$^-$, Br$^-$ and I$^-$ but only myeloperoxidase (MPO) is able to oxidize Cl$^-$. (Thomas et al., 1991a).
Lactoperoxidase (LPO) consists of a single polypeptide chain of about 78kDa and has a high isoelectric point (pH 9.2) (Thomas et al., 1991c). LPO contains one heme prosthetic group per molecule and absorbs strongly at 412-423 nm at neutral pH and the major absorbance was shown to be at 438nm for the reduced form of LPO (Carlstrom, 1969; Mansson-Rahemtulla et al., 1988; Thomas et al., 1991a). Comparison of LPO amino acid sequences to the cloned cDNAs and mRNA transcripts predicted from the LPO gene suggests that LPO is proteolytically processed after synthesis to remove both a signal peptide and a propeptide similar to myeloperoxidase (MPO) biosynthesis (Thomas et al., 1991b; Hansson et al., 2006) based on their sequence similarity. Sequencing LPO by Edman degradation shows that much of the protein purified from milk, has a blocked N-terminus (Dull et al., 1990; Shin et al., 2000). Although, the sequences obtained suggest that LPO is proteolytically processed at the N-terminus, it is possible that unprocessed LPO is also present with a blocked N-terminus. Heterologous expression of
LPO following cDNA transfection also results in truncated heterogeneous, N-terminal LPO sequences (Shin et al., 2000). Thus, the function of LPO proteolytic processing is not known, however similar processing of MPO apparently does not play a role in regulating activity of the enzyme (Hansson et al., 2006).

LPO expression appears to be up-regulated in lactating mammary tissue since LPO is found in colostrum and milk. The mechanisms that regulate the expression and activity of LPO in response to the needs of epithelial host defense appear to vary among different tissues and little is known about factors regulating its expression and activity. Peroxidase in rat tracheal glands was reported to be increased by *Mycoplasma pulmonis* exposure of animals maintained in pathogen free conditions (Kinbara et al., 1992). In contrast, LPO appears to be constitutively present in saliva of several species (Ihalin et al., 2006) and in sheep, human (Salathe et al., 1995; Salathe et al., 1997; Gerson et al., 2000; Wijkstrom-Frei et al., 2003), and rat (Novak et al., 2006) airways. To date, no *in vitro* cell culture systems have been described that synthesize and secrete endogenous LPO in the amounts expected from its levels in milk, saliva, or airway secretions.

**LPO system in airway host defense**

The lactoperoxidase (LPO) antibiotic system provides protection against infection in a variety of tissues including salivary gland, lacrimal glands, mammary glands, and airways. (for review, Reiter & Perraudin, 1991; Thomas et al., 1991). The antimicrobial LPO system was characterized by Reiter et al. in 1963 in milk (Reiter et al., 1963). In this work LPO was shown to catalyze the oxidation of an anion, SCN⁻ or I⁻ utilizing H₂O₂ as its substrate producing the antimicrobial products, OSCN⁻ or OI⁻ that are antibacterial
(Reiter & Perraudin, 1991), antifungal (Majerus & Courtois, 1992; Benoy et al., 2000; Bosch et al., 2000), and antiviral (Pourtois et al., 1990; Yamaguchi et al., 1993; Mikola et al., 1995; van Hooijdonk et al., 2000). Christensen et al. reported the expression of a peroxidase in rodent airways (Christensen et al), but this Airway Peroxidase (APO) was not identified as LPO until 1997 in this lab (Salathe et al., 1997). Gerson et al. (Gerson et al., 2000) presented evidence that LPO plays an important role in the antimicrobial defense system in the airway and thus is one of the components of the innate-immunity system present in airway secretions. Components of the LPO system; LPO, hydrogen peroxide, and SCN- have been shown to be present in the airway at appropriate concentrations to have efficient antimicrobial activity (Gerson et al., 2000; Wijkstrom-Frei et al., 2003). The LPO system inhibits the growth, respiration and acid production in bacteria (Thomas et al., 1991b). As proposed by Thomas et al. the LPO system can be both bactericidal and protective when excess H₂O₂ is present (Thomas et al., 1991b) since LPO has been shown to be the major scavenger of hydrogen peroxide (El-Chemaly et al., 2003).

**Innate Immune Response and Cystic fibrosis**

Failure of airway innate-immune defense mechanisms can result in chronic microbial airway infections evident in diseases such as cystic fibrosis (CF). Mutations on the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene lead to a nonfunctional anion channel resulting in the cystic fibrosis disease characterized by chronic infections and neutrophil dominated inflammation (Danel et al., 1996; Sheppard & Welsh, 1999; Wojnarowski et al., 1999; Berger, 2002). CFTR is a member of the ABC transporter
proteins. The CFTR channel is composed of five domains: two membrane-spanning domains, two nucleotide-binding domains, and a regulatory domain. It has a molecular weight of 170 kDa (for review see Sheppard & Welsh, 1999). CFTR is localized primarily on the apical surface of epithelial cells and transports Cl ions among other ions across epithelial cell membranes. The CFTR channel is found in the epithelial cells of many organs including the lung, liver, pancreas, digestive tract, reproductive tract, and skin. However, it is still not clear whether the diseased state is mainly due to the genetic defect or the host’s immune response itself, although there is evidence that inflammation is present in CF airways even in the absence of infection (Khan, 1995). Various reports have reported IFNγ and other cytokines including IL-1, IL-4, IL-6, IL-8, IL10 and TNFα, to be present only in CF airways when compared to normal healthy control airways (Wojnarowski et al., 1999; Kelley & Elmer, 2000; Coyne et al., 2002).

Since the LPO system is antimicrobial and is highly abundant in bronchial lavage (1% of total protein) as reported by Salathe et al. (Salathe et al., 1997), we hypothesized that a deficit in the LPO system could be involved in the pathogenesis of diseases characterized by chronic airway infection such as cystic fibrosis. To study this we first needed to learn about how the availability for each of the LPO system’s components; LPO, SCN- and H2O2, is regulated since each of these are required to be present at appropriate concentrations to have a functional LPO system. In order to do so the regulation of the production and transport of these components to the airway lumen needed to be studied.
Hydrogen peroxide production

Various studies have shown that reactive oxygen species (ROS) have specific functions in host defense and signaling (Martin et al., 1997). Since ROS have also the capability of damaging the epithelium it is important that there is equilibrium between the presence of ROS and antioxidants. Airway lactoperoxidase for example is one of several natural ROS scavengers in the airway (Salathe et al., 1997; Conner et al., 2002; El-Chemaly et al., 2003). According to El-Chemaly et al. LPO is one of the major scavengers for hydrogen peroxide in airway secretions. The airway mucosa is known to produce ROS (Adler et al., 1992; Wright et al., 1996; Geiszt et al., 2003; Forteza et al., 2005; Harper et al., 2005).

There are various sources of H$_2$O$_2$ in the airway. Dual function NADPH oxidase/heme peroxidase (Duox) protein is one of the sources of H$_2$O$_2$ in the airway (Geiszt et al., 2003; Forteza et al., 2005; Harper et al., 2005; Harper et al., 2006). Duox is a transmembrane protein localized in the apical domain of airway epithelial cells. Duox has been shown to be up-regulated upon after challenge with cytokines such as IFN$\gamma$, IL-13, and IL-4, IFN $\gamma$ showing the highest effect on Duox expression (Harper et al., 2005). This work suggested that inflammatory mediators can regulate H$_2$O$_2$ production and that the production of H$_2$O$_2$ is part of the innate host defense response in the airway.

SCN$^-$ transport in the airway

To maintain adequate LPO enzyme activity, the epithelia must provide sufficient SCN$^-$ substrate concentrations (Wijkstrom-Frei et al., 2003). The primary source of SCN$^-$ is food, the minor sources being cyanide detoxification and amino acid degradation.
SCN$^-$ is present in saliva (Dacre & Tabershaw, 1970; Schultz et al., 1996), milk (Reiter, 1978), and airway secretions (Wijkstrom-Frei et al., 2003). In the case of saliva, SCN$^-$ is normally present at 0.5 - 3 mM (Dacre & Tabershaw, 1970; Schultz et al., 1996). Airway secretions have recently been shown to contain 0.4 mM SCN$^-$ (Wijkstrom-Frei et al., 2003). These values are more than 10 fold higher than serum values (0.05 mM). This suggests that active transport seems to be responsible for SCN$^-$ movement from the blood compartment to the epithelial surface. The regulation of SCN$^-$ secretion by epithelia provides a possible mechanism to manage peroxidase-mediated host defense activity on the luminal surface.

For these reasons, we tested the hypothesis that airway epithelia actively transport SCN$^-$ and characterized the properties of SCN$^-$ transport with regard to possible molecular components at both the basolateral and apical membranes. In this study we also tested the hypothesis that LPO expression was regulated by substances found in infected airways such as cytokines and bacterial products. The data obtained in this study will provide an insight on how the expression and availability of the components of the LPO system are regulated in the airway. The knowledge obtained from these studies could be utilized to develop treatments to compensate for a deficient LPO system in diseases characterized by chronic infections such as cystic fibrosis.
CHAPTER 2

Transcellular Thiocyanate Transport by Human Airway Epithelia

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Introduction

The lactoperoxidase (LPO) antibiotic system is made by epithelia of the mammary, salivary, lacrimal and airway glands and provides protection against infection. To function in epithelial host defense, LPO uses $\text{H}_2\text{O}_2$ to oxidize an anion, for example, $\text{SCN}^-$ or $\Gamma$, forming $\text{OSCN}^-$ or $\text{OI}^-$ that are antibacterial, antifungal and antiviral (for review, Reiter & Perraudin, 1991; Thomas et al., 1991). To maintain adequate enzyme activity, the epithelia must provide sufficient substrate concentrations. Since plasma levels of the used anions are low, diffusion rates are most likely insufficient in secretions of these glands to maintain significant LPO activity. Thus, epithelia must concentrate the anion from the blood compartment to secretions to provide sufficient substrate for the enzyme. In the case of saliva, $\text{SCN}^-$ is normally present at 0.5 – 3 mM (Dacre & Tabershaw, 1970; Schultz et al., 1996). Airway secretions have recently been shown to contain 0.4 mM $\text{SCN}^-$ (Wijkstrom-Frei et al., 2003). These values are more than 10 fold higher than serum values (0.05 mM, Lundquist et al., 1995). As $\text{SCN}^-$ is not made by epithelial cells in sufficient amount to account for these secreted amounts of $\text{SCN}^-$, active

transport seems to be responsible for SCN\(^{-}\) movement from the blood compartment to the epithelial surface. However, studies of the mechanism responsible for transport and concentration of SCN\(^{-}\) have not been reported for salivary, lacrimal and airway glands, the major sites of active LPO.

Most secretions that contain SCN\(^{-}\) also contain I\(^{-}\). Iodide is present at 10-20 fold higher concentration in these secretions compared to serum (Brown-Grant, 1961) and iodide is known to be transported across the thyroid epithelium in a Na\(^{+}\)/I\(^{-}\) symporter (NIS)-dependent fashion (De La Vieja \textit{et al.}, 2000). Iodide is used by thyroid peroxidase for the synthesis of thyroid hormones but can also be utilized by other heme-peroxidases such as lactoperoxidase. Mammary, salivary and gastric glands express NIS, and iodide transport has been demonstrated in these tissues as well as in the kidney. NIS transcripts and protein have been shown to be present in the lungs (Wapnir \textit{et al.}, 2003) but no reports of functional NIS in airway have been made. SCN\(^{-}\) is well known to be a competitive inhibitor of I\(^{-}\) transport by NIS and NIS is known to transport SCN\(^{-}\) in the thyroid gland (for review, De La Vieja \textit{et al.}, 2000). However, the function of NIS has not been experimentally linked with SCN\(^{-}\) transport in these extrathyroidal tissues.

LPO enzymatic activity has been shown to be a component of host defense in airways (Wijkstrom-Frei \textit{et al.}, 2003) and thus transport of SCN\(^{-}\) across the airway epithelium might play a role in maintaining airway sterility. The regulation of SCN\(^{-}\) secretion by epithelia provides a possible mechanism to manage peroxidase-mediated host defense activity on the luminal surface. For these reasons, we studied whether airway epithelia actively transport SCN\(^{-}\) and characterized the properties of SCN\(^{-}\)
transport with regard to possible molecular components at both the basolateral (BL) and apical membranes.

**Materials and Methods**

**Materials**

Unless otherwise noted all materials were obtained from Sigma Chemical Company (St. Louis, MO).

**Cell Culture**

Human airways were obtained from organ donors whose lungs were not to be used for transplant from the Life Alliance Organ Recovery Agency of the University of Miami. All tissues were obtained following appropriate consent and local IRB approved protocols. Airway epithelial cells were isolated, grown and re-differentiated at an air-liquid interface (ALI) on either 6.5 mm or 24 mm T-clear filters (Costar) having a porosity of either 0.4 µm or 3 µm (only for permeabilization experiments) and coated with human placental collagen Type IV as previously described (Bernacki et al., 1999; Nlend et al., 2002).

**Transport Experiments**

All cultures had a resistivity ≥ 300 Ωcm. All experiments were performed under open circuit conditions.

*Time Course of Thiocyanate Accumulation in the Apical Compartment.* To determine the time course of SCN⁻ accumulation from initial addition of ¹⁴C-SCN⁻ to the BL media
to the point where \([^{14}C-\text{SCN}^-]\) is near steady state, \(^{14}C-\text{SCN}^-\) (85 \(\mu M\), 50 mCi mMole\(^{-1}\), Amersham Pharmacia, Piscataway, NJ) was added to the BL culture media of fully redifferentiated ALI cultures and Dulbecco’s Phosphate Buffered Saline (PBS pH 7.4, Gibco, NY) was added to the apical surface of the culture. No unlabeled SCN\(^-\) was added to any of the media, thus the specific activity of \(^{14}C-\text{SCN}^-\) was that supplied by the manufacturer. Samples of the apical PBS were collected at different times and transported SCN\(^-\) determined by liquid scintillation counting.

In experiments using cultures that were permeabilized basolaterally with \(\alpha\)-toxin from *Staphylococcus aureus*, ALI cultures were differentiated on 24 mm inserts with 3 \(\mu m\) pores. BL and apical compartments were washed with PBS and then 140 mM potassium gluconate, 0.33 mM CaCl\(_2\), 10 mM NaCl, 20 mM HEPES, pH of 7.2 containing *Staphylococcus aureus* \(\alpha\)-toxin (10,000 U ml\(^{-1}\)) was added to the BL compartment for 30 min at 37°C in the absence of CO\(_2\). Permeabilization was apparent by the near absence of ciliary beating. The BL compartment was briefly rinsed with same buffer lacking \(\alpha\)-toxin but containing 5 mM MgATP, creatine phosphokinase (50 U ml\(^{-1}\)), 10 mM creatine phosphate, and \(^{14}C-\text{SCN}^-\). Addition of ATP and the ATP regeneration system to the BL compartment restored ciliary beating suggesting the BL membrane was permeabilized as reported previously by others (Ostedgaard et al., 1992; Illek et al., 1999). After addition of \(^{14}C-\text{SCN}^-\) to the BL compartment, appearance of \(^{14}C-\text{SCN}^-\) at the apical surface was then measured by sampling small amounts of apical PBS followed by scintillation counting. Similar treatment of the apical surface did not decrease ciliary beating supporting the idea that \(\alpha\)-toxin was not able to permeabilize the apical membrane (Ostedgaard et al., 1992).
Unidirectional transport. To measure unidirectional transport of SCN$^-$ from the BL to apical compartments in the absence of accumulated apical SCN$^-$, non-permeabilized cultures were washed with PBS and incubated in BL media containing different concentrations of $^{14}$C-SCN$^-$ (50 mCi mMole$^{-1}$) until the system approached steady state with regard to apical [$^{14}$C-SCN]$^-$ as determined from time course experiments described above. To measure iodide (I$^-$) transport unlabeled I$^-$ was used. Transport of these anions was then determined by rapidly washing the apical surfaces of the cultures three times with PBS to remove anion accumulated on the apical surface. Following the third wash, additional aliquots (e.g. 500 µl/ 24 mm filter) were placed on the apical surface for sequential 2 min incubations at 37°C in humidified 5% CO$_2$ until a steady flow of anions to the apical surface was obtained. Removal of washes after 2 min and replacement with fresh PBS prevent accumulation of anion in the apical compartment. BL media were sampled after the last wash. $^{14}$C-SCN$^-$ in BL media and apical washes was determined by liquid scintillation counting. All of the collected $^{14}$C-SCN$^-$ was soluble following 10% trichloroacetic acid (TCA) precipitation showing that radiolabel was not covalently attached to protein.

A colorimetric iodide assay (O’Kennedy et al., 1989) based on the Sandell-Kolthoff reaction was utilized to measure I$^-$ transport. A standard curve ranging from 0.01 - 0.6 µM potassium iodide was utilized. Reagent addition was precisely timed and transmittance at 414 nm was measured in a microplate reader (SpectraMax, Molecular Devices) at 10 sec intervals for 15 minutes after addition of ceric ammonium sulfate. The time points giving a linear range of transmittance with respect to standards (usually 5 min) were used to determine iodide concentrations in samples.
To determine the transport of SCN\(^{-}\) from the apical to BL compartment (back transport), apical surfaces of cultures were incubated with PBS containing \(^{14}\)C-SCN\(^{-}\) and BL media were removed at 2 min intervals for liquid scintillation counting.

Apparent Km’s were estimated by non-linear regression fitting to the Michaelis-Menten equation: \(V = \frac{V_{\text{max}}}{S/(S+K_m)}\). Inhibition data were fit to \(V = \frac{V_{\text{max}}S}{[(1+i/K_i)S]+K_m}\)-L, using the \(K_m,_{\text{app}}\) determined from the experiments described. L corrects for small amounts of non-inhibitable SCN\(^{-}\) transport.

**RT-PCR**

RNA was extracted from normal human bronchial epithelial (NHBE)-ALI cultures using Trisolv (Invitrogen, NY) and cDNA was obtained using SuperScript First Strand Synthesis System for RT/PCR kit (Invitrogen, NY). NIS specific oligonucleotide primers were designed according to Ajjan et al. (sense, 5’CTCCCTGTAACGACTCCAG3’; antisense 5’CTATCTCTATTACGGTGC3’). NIS cDNA was amplified by 35 cycles of 1 minute at 94°C, 55°C and 72°C, which was followed by a final 3 min elongation at 72°C. PCR product was cloned using pGEM-T Easy Vector system (Promega, Madison, WI) and sequenced using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, CA).

**Immunolocalization**

*Tracheal tissue.* Immunolocalization in tissue was performed according to Castro et al. (1999b) with slight modifications. Normal human tracheas were fixed in 4% paraformaldehyde and prepared for embedding in paraffin in a Microwave Tissue Processor (Microwave Materials Technologies Inc., Knoxville, TN) according to the
manufacturer’s protocol using a non-xylene method. Embedding and sectioning were performed by the Histology Laboratory at the University of Miami Hospital & Clinics-Sylvester Comprehensive Cancer Center. Auto-fluorescence of de-paraffinized sections was reduced by incubating sections in sodium borohydride in PBS (5 mg ml$^{-1}$). This was followed by quenching of endogenous peroxidase activity for 2 hours in 3% H$_2$O$_2$ in methanol. Sections were subjected to antigen retrieval by incubating in 10 mM citrate buffer (pH 6) for 15 min at 80°C followed by blocking for 1 h at room temperature in 5% normal goat serum (NGS, Chemicon, Tamecula, CA) in PBS/0.05% Tween-20 (PBS-T). Biotin autofluorescence was reduced utilizing Endogenous Biotin Blocking kit according to manufacturer’s instructions (Molecular Probes, Eugene, OR). Sections were incubated overnight at 4°C with either anti-human NIS monoclonal antibody against amino acid residues 469-643 (Castro et al., 1999a) or nonimmune mouse IgG (both from Chemicon, Tamecula, CA) as a negative control. Both antibodies were used at 10 µg ml$^{-1}$ in 1% NGS-PBS-T. Sections were incubated with affinity purified goat-anti-mouse IgG conjugated with horseradish peroxidase at a concentration of 0.02 µg ml$^{-1}$ (KPL, Gaithersburg MD) for 45 min at room temperature followed by labeling utilizing Tyramide Signal Amplification Kit (TSA, Molecular Probes, Eugene, OR) according to manufacturer’s protocol.

Cultures. ALI cultures were fixed in 4% paraformaldehyde and permeabilized with methanol for 10 min. Auto-fluorescence of fixed ALI cultures was reduced by incubating filters in sodium borohydride in PBS (5 mg ml$^{-1}$). Cultures were subjected to antigen retrieval by incubating in 10 mM citrate buffer (pH 6) for 15 min at 80°C followed by blocking for 1 h at room temperature in 100% NGS. Cultures were incubated overnight at
4°C with either anti-human NIS monoclonal antibody or nonimmune mouse IgG as a negative control both at 10 µg ml⁻¹ in 100% NGS. ALI cultures were incubated with purified goat-anti-mouse IgG conjugated with AlexaFluor 555 (Molecular Probes, Eugene, OR) for 1 h at a concentration of 2 µg ml⁻¹ in 100% NGS. Fluorescent images were obtained using a Zeiss LSM-510 Confocal Laser Scanning Microscope in the University of Miami Analytical Imaging Core Facility.

Results

Time Course of Thiocyanate Accumulation

To assess the ability of human airway epithelial cells to accumulate SCN⁻ in the apical compartment, de-differentiated human tracheobronchial epithelial cells were cultured in a two-chamber system that allowed re-differentiation at the air-liquid interface. Differentiation was evident by the presence of cells with beating cilia and goblet cells that secrete mucus. These cultures maintain a small volume of liquid on the apical surface (Matsui et al., 1998) and do not normally secrete lactoperoxidase (as measured by enzyme activity and Northern blots, data not shown) that might metabolize transported SCN⁻, thus providing an ideal system to study the transport of this anion.

The accumulation of SCN⁻ from the BL compartment to the apical compartment was first examined by adding ¹⁴C-SCN⁻ to the BL media and then following the time course of ¹⁴C-SCN⁻ appearance at the apical surface. PBS, not containing SCN⁻ was added to the apical surface and then sampled at different times after addition of isotope and ¹⁴C [SCN⁻] determined by scintillation counting. After a brief lag, ¹⁴C-SCN⁻ began accumulating at the apical side and to a 10.0 ± 1.1 fold higher concentration (mean ±
s.e.m., n = 6) than that found in the BL media. An example experiment is shown in Figure 2.1A. Accumulation of SCN\(^-\) on the apical side also occurred using culture media in both the apical (instead of PBS) and BL compartments. Addition of \(^{14}\)C SCN\(^-\) to the apical compartment did not result in accumulation in the BL compartment and the appearance of apically applied \(^{14}\)C-SCN\(^-\) in the BL compartment occurred at a much slower rate (see below).

The transepithelial potential of the re-differentiated cultures before initiation of experiments was \(-13.7 \pm 1.8\) mV (mean ± s.e.m., n = 15), i.e. the BL compartment was positive with respect to the apical compartment. Within 10 min after adding PBS to the apical surface, this potential dropped to \(-7.6 \pm 0.7\) mV (mean ± s.e.m, n = 15) and then increased back to baseline over the following 2 h. Thus, the electrical potential was unfavorable for movement of SCN\(^-\) from the BL to apical compartment and the observed 10-fold accumulation of SCN\(^-\) could not be attributed to electrical potential differences.

In identical experiments using separate cultures permeabilized with \(\alpha\)-toxin in the BL compartment, apical [SCN\(^-\)] rapidly approached the BL [SCN\(^-\)] but did not increase above that level (Fig. 2.1B). Permeabilization of the BL membrane was apparent by a dramatic decrease in ciliary beating that was restored upon addition of ATP and an ATP regeneration system in the BL compartment. Apically applied \(\alpha\)-toxin did not alter ciliary beating consistent with other reports that \(\alpha\)-toxin does not permeabilize the apical surface. Thus, an intact BL membrane was apparently needed for concentration of SCN\(^-\) at the apical surface of the ALI cultures.
The difference in accumulation of SCN\(^-\) by the intact and permeabilized cultures is reflected in the rate at which SCN\(^-\) appeared in the apical compartments (Fig. 2.1C). The rate of SCN\(^-\) appearance in intact cells increased, presumably as the cellular compartment filled, and then decreased over several hours as the apical \(^{14}\text{C-SCN}^-\) increased and the system approached the steady state. In contrast, the culture permeabilized with \(\alpha\)-toxin in the BL compartment, the rate of SCN\(^-\) appearance reached a maximum after about 30 min and then declined to near zero for the remainder of the experiment. The \(^{14}\text{C}\) collected from the apical surface was soluble in 10% TCA indicating that the radiolabeled SCN\(^-\) had not been bound to protein.

**Thiocyanate Transport Rates**

To quantify transport rates, cultures were instead preincubated with \(^{14}\text{C-SCN}^-\) in the BL media until apical \(^{14}\text{C-SCN}^-\) approached a steady value. The rate of appearance was then followed by adding and removing aliquots of PBS at the apical surface at 2 min intervals and determining the amount of \(^{14}\text{C}\) in the collected PBS as a measure of apical [SCN\(^-\)]. These short washes insured that apical [SCN\(^-\)] remained very low (< 1 \(\mu\)M) during transport measurements, thus transport was not inhibited by accumulation of SCN\(^-\) in the apical compartment as suggested by Figure 2.1.

The first wash showed a high level of isotope suggesting that SCN\(^-\) had accumulated in the small volume of apical surface liquid present during the preincubation period. Later washes contained a lower constant amount of isotope suggesting a constant rate of \(^{14}\text{C-SCN}^-\) appearance (Fig. 2.2A). This constant rate obtained in the later washes increased with increasing BL \(^{14}\text{C-SCN}^-\). Experiments were done on triplicate filters at each
different BL [SCN$^-$] used. The experiments used cultures from two different donors and four different days ($n = 52$ cultures). The rate was concentration dependent and appeared to be saturable at higher BL concentrations (Fig. 2.2B). Fitting the data to the Michaelis-Menten equation predicted an apparent $K_m$ of $69 \pm 25 \, \mu M$ and an apparent $V_{\text{max}}$ of $24 \pm 3$ nMoles $h^{-1} \, cm^{-2}$ (fit coefficient $\pm$ S.D.) for transepithelial transport of SCN$^-$. These experiments were conducted under open circuit conditions, thus these constants are not corrected for any effects of electrical potentials. In addition, these values do not distinguish the different contributions of different subcellular compartments and surface membranes and thus are \textit{apparent} values for the entire transepithelial transport process under the experimental conditions.

Back transport was measured by an identical procedure except cultures were preincubated with $^{14}$C-SCN$^-$ in the apical PBS and the BL surface was washed with culture media at 2 min intervals. The SCN$^-$ transport rate from the apical to BL compartment was less than 0.05 that of the BL to apical transport rate at the same [SCN$^-$].

\textbf{Na$^+/I^-$ Symporter}

Since SCN$^-$ can be actively transported by the Na$^+/I^-$ symporter (NIS) in thyroid gland and since NIS has also been detected in salivary gland, mammary gland and stomach (Ajjan \textit{et al.}, 1998; Spitzweg \textit{et al.}, 1998; Spitzweg \textit{et al.}, 1999), all organs that transport SCN$^-$, airway epithelia were probed for the presence of NIS mRNA and protein. RT PCR using total RNA isolated from re-differentiated airway epithelial cultures showed a band of the expected size (Fig. 2.3). Sequence analysis of this band showed that
it was 100% identical to NIS nucleotide sequence (GenBank U66088) suggesting that NIS is expressed in airway epithelia.

Immunolocalization (Fig. 2.4) with a monoclonal antibody against recombinant human NIS (Castro et al., 1999a), confirmed the protein’s expression in human trachea. Immunoreactivity was mainly associated with submucosal gland cells (Fig. 2.4, A and B) at the basal portion of the acinar cells (Fig. 2.4, C and D). In contrast to NIS localization in salivary gland (Jhiang et al., 1998), NIS in airway submucosal glands was not restricted to ducts. A smaller amount of product was also specifically associated with superficial epithelial cells (data not shown). Since cultures studied here were derived mainly from surface epithelia but also contained cells with glandular phenotype (Gray et al., 1996; Yoon et al., 1997), we examined the cultures directly for the expression of NIS. Confocal images of immunostained epithelial cell cultures re-differentiated on filters showed that approximately half of the cells expressed NIS (Fig. 2.4 G and H) apparently in the lateral membrane of the cells (circular profiles, Fig. 2.4 H). Phase contrast images suggested that NIS expressing cells were not ciliated (data not shown). Together these data strongly suggest that NIS is expressed in large human airways and that it may be the molecule responsible for SCN\(^-\) transport at the basolateral membrane.

Biochemical and pharmacological properties of NIS activity in thyroid gland are well characterized (De La Vieja et al., 2000) and thus we assessed the airway epithelial SCN\(^-\) transport with respect to the known properties of NIS. To measure the requirement of Na\(^+\) for SCN\(^-\) transport, cultures were incubated with \(^{14}\)C-SCN\(^-\) containing normal BL culture media. After establishing a baseline transport rate, the media was replaced with \(^{14}\)C-SCN\(^-\) containing HBSS in which choline was substituted for Na\(^+\) and SCN\(^-\) transport was
monitored for changes in transport (Fig. 2.5). Replacement of Na\(^+\) by choline in the BL media blocked \(^{14}\)C-SCN\(^-\) transport. Transport could be restored by transferring the cultures to Na\(^+\)-containing medium.

To rule out the possibility that the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransporter was responsible for SCN\(^-\) transport in ALI cultures, transport was measured in the presence of 100 µM furosemide (Fig. 2.6A). No alteration in SCN\(^-\) transport was observed, however, transport was inhibited by inclusion of 100 µM perchlorate a known inhibitor of the NIS (Fig. 2.6A). Perchlorate inhibition was reversible (Fig. 2.6B). Perchlorate inhibition was concentration dependent (Fig. 2.7). The \(K_{\text{app}}\) was estimated by non-linear regression fitting of the data to the Michaelis-Menten equation to be 0.6 ± 0.05 µM (fit coefficient ± S.D.), a value near the previously reported \(K_i\)'s (1-2 µM) in other tissues (Dohan et al., 2003).

If NIS was responsible for uptake of SCN\(^-\) into airway epithelia, I\(^-\) should compete with SCN\(^-\) and should be transported. Addition of I\(^-\) to the BL media reversibly inhibited SCN\(^-\) transport (Fig 2.6B) and competed with SCN\(^-\) for transport across the airway epithelial cell cultures in a concentration dependent manner (Fig. 2.8B) with a \(K_{\text{app}} = 9 \pm 8\) µM. To measure I\(^-\) transport, varying concentrations of nonradioactive I\(^-\) were included in the BL media, and the appearance of I\(^-\) at the apical surface was determined by a chemical assay (O'Kennedy et al., 1989). I\(^-\) was effectively transported across airway epithelial cultures with a \(K_{\text{m,app}}\) of 111 ± 69 µM (Fig. 2.8A), close to that observed in thyroid (20-36 µM, De La Vieja et al., 2000).
Thus, NIS is expressed in airway mucosa and NIS activity is similar to the one in thyroid gland. Therefore, the observed properties of SCN\(^{-}\) and \(\Gamma\) transport in ALI cultures suggest that NIS is likely responsible for BL transport of these anions, at least in this experimental system.

NIS in thyroid gland concentrates \(\Gamma\) in epithelial cells and \(\Gamma\) then exits via a channel in the apical membrane. To characterize the apical pathway by which SCN\(^{-}\) exits airway epithelial cells, various inhibitors of anion channels were added to apical PBS washes to assess their ability to inhibit transport of SCN\(^{-}\). 4,4\-'-dinitrostilbene-2,2\-'-disulfonic acid (DNDS, 100 µM), an inhibitor of anion exchangers and Cl\(^{-}\) channels, had no effect on SCN\(^{-}\) transport (Fig. 2.9) nor did 4,4\-'-diisothiocyanatostilbene-2,2\-'-disulfonic acid (DIDS, 200 µM) (data not shown). In contrast, the sulfonyleurea glibenclamide (500 µM), an inhibitor of CFTR, blocked SCN\(^{-}\) transport, as did the arylaminobenzoate, diphenylamine-2-carboxylic acid (DPC, 1 mM) (Fig. 2.9). Inhibition by glibenclamide and DPC was reversible on removal of the compounds indicating that the cells remained viable during treatment (Fig. 2.9). 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) also reversibly inhibited SCN\(^{-}\) transport (data not shown).

These data suggested that CFTR might be the anion channel responsible for SCN\(^{-}\) transport and if so, predicted that increased intracellular cAMP would stimulate transport. Inclusion of forskolin (10 µM) and dibutyryl cAMP (500 µM) in the apical washes was associated with an immediate increase in SCN\(^{-}\) transport that was blocked by glibenclamide (500 µM) (Fig. 2.10A), consistent with a role for CFTR. Glibenclamide inhibited both basal and cAMP stimulated SCN\(^{-}\) transport and the inhibition by glibenclamide was again reversible (Fig. 2.10A).
Back transport from the apical to BL compartment was low in comparison to BL to apical transport (Fig. 2.10B). Inclusion of perchlorate in the BL media of these cultures had no effect on back transport unless forskolin and dibutryl cAMP were used to stimulate protein kinase (PKA). When forskolin and dibutryl cAMP were added to the apical PBS, back transport was stimulated (Fig. 2.10B). This suggested that back transport, in the absence of PKA stimulation was primarily not through BL NIS, and may occur via a paracellular route.

Since CFTR mediated Cl\(^-\) current is increased in the presence of the ENaC blocker amiloride (Knowles et al., 1983), the effect of amiloride (100 µM) was also tested for its effect on SCN\(^-\) transport. Inclusion of amiloride in apical washes was not associated with a significant increase in SCN\(^-\) transport (Fig. 2.11), perhaps due to the smaller contribution of SCN\(^-\) to the overall anion flux through CFTR. The pharmacological profile of SCN\(^-\) transport suggested that CFTR might be involved in transepithelial transport.

**Discussion**

The data presented here support the hypothesis that NIS may play an important role in moving and concentrating SCN\(^-\) into airway epithelial cells and that CFTR may regulate transport into the airway lumen. In a similar fashion, NIS in salivary, mammary, and gastric glands also may be responsible for developing the significant transepithelial [SCN\(^-\)] gradient seen in those tissues, although the apical channel releasing SCN\(^-\) into the lumen of these tissues remains unidentified.
The data suggested that SCN\textsuperscript{−} transport occurred primarily via a transcellular route in ALI cultures and not paracellular since SCN\textsuperscript{−} was concentrated in the apical compartment in opposition to an electrical potential, transport was reversibly blocked by competitive inhibitors of NIS in the BL compartment and inhibitors of CFTR in the apical compartment, and required Na\textsuperscript{+} in the BL media and was stimulated by treatments to increase intracellular cAMP. Thiocyanate accumulated to values similar to those reported previously for airway secretions (Wijkstrom-Frei et al., 2003) suggesting that the ALI cultures resemble \textit{in vivo} epithelial SCN\textsuperscript{−} transport. Importantly, these cultures do not make LPO under the conditions used for growth and differentiation. Finally, transported SCN\textsuperscript{−} was not bound to protein by organification reactions.

Apparent kinetic constants estimated for SCN\textsuperscript{−} and I\textsuperscript{−} transport were within the range of published values for NIS in other tissues (Dohan \textit{et al.}, 2003) despite the fact that calculations of airway epithelial kinetic constants were performed under open circuit conditions and did not take into account effects on transport by the apical membrane of the cells. Measurement of transport across intact epithelial layers under open circuit conditions more closely resembles the physiological circumstances in airways but in turn prevents accurate measurement of kinetic constants of the NIS in the BL membrane. Despite this, the measured $K_m^{\text{app}}$ for SCN\textsuperscript{−} transport by airway epithelia (70 \textmu M) was between that reported for SCN\textsuperscript{−} transport by thyroid slices (30 \textmu M, Wolff, 1964) and oocytes expressing recombinant NIS (96 \textmu M, Eskandari \textit{et al.}, 1997). In thyroid samples, the $K_m$ of NIS for SCN\textsuperscript{−} and I\textsuperscript{−} are identical although in oocytes expressing NIS, $K_m$’s for these anions are 3 fold different (36 \textmu M vs. 96 \textmu M). The $K_m^{\text{app}}$ and $K_i^{\text{app}}$ for I\textsuperscript{−} were dissimilar (111 \textmu M vs. 9 \textmu M). Although the reasons for this are unclear, a similar
difference was reported for recombinant NIS expressed in oocytes (Dohan et al., 2003). In this regard, both SCN\(^-\) and \(\Gamma^-\) have higher lipid bilayer permeability than other anions of similar size that could contribute. Our experiments were conducted under open circuit conditions and measured transport across the intact epithelium and thus are influenced by movement across the apical membrane as well.

The perchlorate \(K_{i,app}\), using SCN\(^-\) as a substrate, was comparable to that reported for NIS using \(\Gamma^-\) as a substrate (Wolff, 1964; Eskandari et al., 1997) and thus the selectivity resembled that of NIS, perchlorate > \(\Gamma^-\) \(\approx\) SCN\(^-\). These apparent kinetic constants together with the \(Na^+\) requirement of transport support the hypothesis that NIS may be the BL transporter responsible for uptake of SCN\(^-\). The absence of furosemide inhibition and the inhibition by perchlorate rule out a major contribution by the \(Na^+/K^+/2Cl^-\) cotransporter.

Back transport studies showed low levels of apical to BL SCN\(^-\) movement despite a transepithelial potential that favored transport in this direction. Since apical to BL movement was not inhibited by perchlorate unless cells were first stimulated with forskolin and db cAMP, this low level of back transport suggested that the paracellular route probably contributed a small amount to the overall movement of the anion in the BL to apical direction. Back transport was increased by stimulation of PKA and this increase was blocked by perchlorate in the BL media consistent with the idea that CFTR was involved in regulating movement of SCN\(^-\) across the apical membrane.

In addition to the kinetic data, RT-PCR and immunolocalization support the presence of NIS in airway mucosa. Interestingly, immunolocalization suggested that NIS
expression is heaviest in airway submucosal glands. Both RT-PCR and immunocytochemistry showed that ALI cultures used for these transport studies contained cells that expressed NIS basolaterally.

The observation that SCN\(^{-}\) transport responded to apically applied compounds with an inhibition profile similar to CFTR, suggested that CFTR might regulate or possibly be the channel that passes SCN\(^{-}\) to the apical surface. Like other chloride channels, CFTR has been shown to carry SCN\(^{-}\) although these studies primarily used SCN\(^{-}\) as probe for channel properties (Tabcharani et al., 1993; Linsdell et al., 1997; Linsdell, 2001). The possibility that CFTR regulates SCN\(^{-}\) transport in airway epithelia cells raises important questions regarding the effect of impaired CFTR activity on LPO-mediated airway host defense and other potential effects of a loss of LPO activity on airway homeostasis in cystic fibrosis.
Figures

Figure 2.1. Time course of SCN$^-$ transport by airway epithelial cells in open circuit conditions. Panel A: PBS was added to the surfaces of two cultures followed by the addition of 85 µM $^{14}$C-SCN$^-$ to the BL media. The [SCN$^-$] in the apical compartment (●) was measured as a function of time by sampling small aliquots of the apical PBS for liquid scintillation counting (LSC). The decrease in [SCN$^-$] of the BL compartment is also plotted (■). Mean values of the two filters are shown. In these intact cultures, after 240 min, the apical [SCN$^-$] was 10 fold higher than the BL [SCN$^-$] despite an unfavorable electrical potential. Panel B: The BL membranes of another culture were permeabilized with α-toxin as described in Methods and 100 µM $^{14}$C-SCN$^-$ was added to the BL compartment. The apical compartment [SCN$^-$] (●) increased to, but did not exceed, the level of the BL compartment [SCN$^-$] (■). Panel C: The rates of appearance of SCN$^-$ in the apical compartments of the experiments in panels A (●) and B (■) are compared.
Figure 2.2. Concentration dependence of thiocyanate transport by airway epithelial cultures. Cultures, re-differentiated at the ALI, were incubated with $^{14}$C-SCN$^-$ in the BL media under open circuit conditions. After incubation, the apical surfaces were washed at 2 min intervals with PBS. $^{14}$C-SCN$^-$ in washes was determined by LSC of triplicate samples at each time point. Panel A shows the basal transport rates of single cultures incubated with 31 (●), 92 (■), 153 (▲), and 306 (▼) µM SCN$^-$ in the BL media (concentrations determined from the specific radioactivity of BL media). Panel B shows mean (± S.E.M.) basal transport rates from triplicate cultures at each BL [SCN$^-$]. Cultures in panel B were obtained from two different lungs and assayed on three separate days, n = 51 cultures. Fitting the data to the Michaelis-Menten equation by non-linear regression analysis gave apparent Km and Vmax for the transport process of 69 ± 25 µM and 24 ± 3 nMoles h$^{-1}$ cm$^{-2}$, respectively (fit coefficient ± S.D.) under open circuit conditions.
Figure 2.3. RT-PCR detects NIS message in airway epithelial cells. Total RNA from re-differentiated airway epithelial cells was isolated and reverse transcribed. PCR was performed using human NIS specific oligonucleotide primers (lanes 1 and 3) and GAPDH primers (lane 2 and 4). Bands of the expected size (346 bp) for hNIS (lane 1) and GAPDH (lane 2) were seen in RNA prepared from ALI cultures. Control PCR reactions without reverse transcriptase (lanes 3 and 4) and without primers (lanes 5 and 6) showed no amplified products. The NIS band was sequenced and was identical to the expected portion of human NIS (GenBank U66088). Markers (lane M) were Hae III digested ΦX174.
Figure 2.4. Immunolocalization of NIS in human airway mucosa and cultured airway epithelia. Sections of human trachea (panels A–F), and ALI cultures of human airway epithelia (panels G–J) were incubated with monoclonal antibody against human NIS (panels A, B, C, D, G and H) or nonimmune mouse IgG (panels E, F, I and J), followed by anti-mouse IgG coupled to horseradish peroxidase (A-F) or AlexaFluor 555 (G-J). Tyramide was used to localize HRP-coupled antibody (A-F). NIS in human trachea was primarily seen in submucosal glands (panel B) and localized to the basal surface of gland acinar cells (panel D, corresponding to region outlined by a square in panel A). Arrowheads point toward lateral cell borders and the asterisks mark the small lumen in these acini. NIS was also present in about half of the cells in ALI cultures (panel H). Panels A, B, E, F, bar = 100 µm. Panels C-D, bar = 20 µm. Panels G-J, bar = 10 µm.
**Figure 2.5. Thiocyanate transport is sodium dependent.** ALI cultures, incubated with 70 µM $^{14}$C-SCN$^{-}$ in the BL media, were washed normally to determine baseline transport rates (0-20 min, not shown). Two cultures with similar transport rates were transferred to BL HBSS containing 70 µM $^{14}$C-SCN$^{-}$ and baseline transport re-determined with 10 additional apical washes (20-40 min). BL HBSS was then exchanged for HBSS (●) or Na-free HBSS (■), both containing 70 µM $^{14}$C-SCN$^{-}$, and washing was continued (40-80 min). Cultures were then transferred back to BL culture media containing 70 µM $^{14}$C-SCN$^{-}$ and washed at the predetermined intervals (80-95 min). Transport rates were determined by LSC of triplicate samples from the apical washes. Standard errors typically fell within the plotted point. Replacement of Na$^{+}$ by choline reversibly blocked the transport of SCN$^{-}$.
Figure 2.6. Thiocyanate transport is resistant to furosemide but sensitive to perchlorate and iodide. Panel A. Three ALI cultures, incubated with 10 µM $^{14}$C-SCN$^-$ in the BL media, were apically washed with PBS at 2 min intervals. After 5 washes, cultures were transferred to BL media containing $^{14}$C-SCN$^-$ alone (●) or BL media with 100 µM furosemide (■) or 100 µM perchlorate (▲) and washing with PBS continued (12-48 min). Furosemide had no effect while perchlorate inhibited transport. Panel B. Three ALI cultures, incubated with 10 µM $^{14}$C-SCN$^-$ in the BL media, were apically washed with PBS at 2 min intervals. After 8 washes, cultures were transferred to BL media containing $^{14}$C-SCN$^-$ alone (●) or BL media with 100 µM potassium iodide (■) or 10 µM perchlorate (▲) and washing with PBS continued (18-38 min). Cultures were then transferred to normal BL media without inhibitors containing $^{14}$C-SCN$^-$ for additional washes (40-70 min). Transport rates were determined by LSC of triplicate samples from the apical washes. Standard errors typically fell within the plotted point. Furosemide had no effect while perchlorate and iodide reversibly inhibited transport.
Figure 2.7. Perchlorate is an inhibitor of thiocyanate transport. Airway epithelial cell cultures were incubated with $^{14}$C-SCN$^{-}$ containing BL media and then various concentrations of perchlorate were added to the BL media. Transport at each perchlorate concentration was determined with triplicate cultures and values are means ± S.E.M. Perchlorate inhibited SCN$^{-}$ transport in a concentration dependent manner. Transport rates were fit by nonlinear regression to Michaelis-Menten kinetics for a competitive inhibitor and predicted an apparent $K_i = 0.6 \pm 0.05 \mu$M (fit coefficient ± S.D.).
Figure 2.8. Iodide transport by airway epithelial cultures. Panel A: airway epithelial cells were incubated with BL media containing various concentrations of $\Gamma^{-}$. Transport of $\Gamma^{-}$ was measured by colorimetric assay of apical washes from three individual cultures at each BL $[\Gamma^{-}]$ in two separate experiments and plotted values are means ± S.E.M. Transport rates were fit by nonlinear regression to the Michaelis-Menten equation and predicted an apparent $K_m = 111 \pm 69 \mu M$ and apparent $V_{max} = 37 \pm 16$ nMoles h$^{-1}$ cm$^{-2}$ (fit coefficient ± S.D.). Panel B: airway epithelial cell cultures were incubated with 70 $\mu M$ $^{14}$C-SCN$^{-}$ containing BL media and then various concentrations of $\Gamma^{-}$ were added to the BL media. Transport at each $[\Gamma^{-}]$ was determined with triplicate cultures and means ± S.E.M. were plotted. Iodide inhibited SCN$^{-}$ transport in a concentration dependent manner. Transport rates were fit by nonlinear regression to Michaelis-Menten kinetics for a competitive inhibitor and predicted an apparent $K_i = 9 \pm 8 \mu M$ (fit coefficient ± S.D.).
Figure 2.9. Thiocyanate transport is sensitive to apically applied inhibitors of CFTR. Three ALI cultures, incubated with 90 μM $^{14}$C-SCN$^{-}$ in the BL media, were rapidly and apically washed 3 times with PBS and then at 2 min intervals. After 10 washes (20 min) to establish baseline transport rate, PBS washing was continued in the presence of either glibenclamide (500 μM) (●), DPC (1mM) (■) or DNDS (100 μM) (▲) (22-42 min). Compounds were then removed and washing continued in PBS to show reversibility (44-58 min). SCN$^{-}$ transport was determined by LSC of triplicate samples from each time point. Standard errors typically fell within the plotted point. DPC and glibenclamide reversibly inhibited SCN$^{-}$ transport while DNDS had no effect.
Figure 2.10. Thiocyanate transport is stimulated by increased intracellular cAMP. Panel A. Two ALI cultures, incubated with 70 µM $^{14}$C-SCN$^-$ in the BL media, were rapidly and apically washed 3 times with PBS and then at 2 min intervals. After 10 washes (20 min), dibutyryl cAMP and forskolin were added to the next 6 PBS washes (22 –32 min) causing an abrupt increase in transport. Inclusion of glibenclamide, in addition to the dibutyryl 500 µM cAMP and 10 µM forskolin, caused an immediate block of both the stimulated and baseline SCN$^-$ transport (34-46 min). Removal of compounds from washes (48-56 min) restored SCN$^-$ transport to the pre-cAMP level demonstrating the reversibility of glibenclamide inhibition. Panel B. Two ALI cultures were incubated with 100 µM $^{14}$C-SCN$^-$ in the apical PBS, and either normal BL media (●) or BL media containing 100 µM perchlorate (■). The BL compartment was then washed at 2 min intervals with culture media to measure back transport from the apical to BL compartment. After 16 washes (32 min), dibutyryl cAMP and forskolin were added to the apical PBS (34 –60 min) to 500 µM and 10 µM respectively. Stimulation of PKA caused an increase in transport in control but not in the culture containing perchlorate in the BL media. Transport rates were determined by LSC of triplicate samples from each wash. Standard errors typically fell within the plotted point.
Figure 2.11. Thiocyanate transport is insensitive to apical amiloride. Two ALI cultures, incubated with 70 μM $^{14}$C-SCN$^-$ in the BL media, were washed rapidly 3 times and then at 2 min intervals with PBS (●) or with PBS containing amiloride (■). After 9 washes (18 min), dibutyryl cAMP and forskolin was added to the next 5 PBS washes (20 –28 min) and then, glibenclamide, in addition to the dibutyryl cAMP and forskolin, was added to the final 6 washes (caused an immediate block of SCN$^-$ transport (30-40 min). Transport rates were determined by LSC of triplicate samples of each wash. Standard errors fell within the plotted point. Inclusion of amiloride did not significantly affect either basal or stimulated SCN$^-$ transport.
Chapter 3

Regulation of Lactoperoxidase mRNA Suggests a Role in Inflammation

In revision*

Introduction

Lactoperoxidase, a member of the mammalian heme peroxidase family, uses hydrogen peroxide ($H_2O_2$) to catalyze the oxidation of thiocyanate ($SCN^-$) and produce hypothiocyanite ($OSCN^-$), a biocidal compound. LPO acts against bacteria (Reiter & Perraudin, 1991), viruses (Pourtois et al., 1990; Yamaguchi et al., 1993; Mikola et al., 1995; van Hooijdonk et al., 2000; Wang et al., 2000) and fungi (Majerus & Courtois, 1992; Benoy et al., 2000; Bosch et al., 2000) and is important in the prevention of mucosal infection. The LPO system has been identified in a variety of mucosal glandular tissues including salivary, lacrimal, and mammary glands, as well as in tracheal and bronchial submucosal glands. Little is known about how the LPO system is regulated.

Comparison of LPO amino acid sequences to cloned cDNAs and to mRNA transcripts predicted from the LPO gene suggests that LPO is proteolytically processed after synthesis to remove both a signal peptide and a propeptide similar to myeloperoxidase (MPO) biosynthesis (Hansson et al., 2006). Sequencing LPO by Edman

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degradation shows that much of the protein purified from milk, saliva and tracheal secretions has a blocked N-terminus (Dull et al., 1990; Gerson et al., 2000; Shin et al., 2000b). Although, the sequences obtained suggest that LPO is proteolytically processed at the N-terminus, it is possible that unprocessed LPO is also present with a blocked N-terminus. Heterologous expression of LPO following cDNA transfection also results in truncated heterogeneous, N-terminal LPO sequences (Shin et al., 2000b). The function of LPO proteolytic processing is not known, however similar processing of MPO apparently does not play a role in regulating activity of the enzyme (for review, Hansson et al., 2006).

LPO expression appears to be upregulated in lactating mammary tissue since LPO is found in colostrum and milk. Peroxidase in rat tracheal glands was reported to be increased by *Mycoplasma pulmonis* exposure of animals maintained in pathogen free conditions (Kinbara et al., 1992). In contrast, LPO appears to be constitutively present in saliva of several species (Ihalin et al., 2006) and in sheep and human airways (Salathe et al., 1997; Gerson et al., 2000; Wijkstrom-Frei et al., 2003). Computer modeling of LPO system activity in human airway secretions suggests that enzyme activity and biocidal function are regulated by availability of H$_2$O$_2$ rather than levels of LPO protein (Conner et al., 2007). H$_2$O$_2$ in airway secretions is produced by the NADPH oxidases, Duox 1 and 2 (Geiszt et al., 2003; Forteza et al., 2005) and Duox 2 is upregulated by IFN$\gamma$ (Harper et al., 2005) that is increased after infection although it is no known whether LPO is similarly regulated.

The mechanisms that regulate the expression and activity of LPO in response to the needs of epithelial host defense appear to vary among different tissues and little is known
about factors regulating its expression and activity. To date, no \textit{in vitro} cell culture systems have been described that synthesize and secrete endogenous LPO in the amounts expected from its levels in milk, saliva, or airway secretions. In this study we used primary airway epithelial cell cultures that expressed LPO mRNA, but very little protein, and assessed the forms and levels of LPO mRNA in response to challenge of the cultures by bacterial products and cytokines. The data showed the existence of at least three alternatively spliced LPO mRNA variants and suggest that LPO expression is only slightly upregulated in response to bacterial products consistent with predictions that \( \text{H}_2\text{O}_2 \) levels regulate acute changes in enzyme activity. LPO mRNA was highly upregulated in response to pro-inflammatory cytokines suggesting a possible role for LPO during inflammation.

\textbf{Materials and Methods}

\textbf{Materials}

Unless otherwise noted all materials were obtained from Sigma Chemical Company (St. Louis, MO).

\textbf{Cell Culture}

As a model system to study LPO’s role in mucosal defense, human airways were obtained from organ donors whose lungs were not to be used for transplant from the Life Alliance Organ Recovery Agency of the University of Miami. All tissues were obtained following appropriate consent and local IRB approved protocols. Airway epithelial cells were isolated, grown and differentiated at an air-liquid interface (ALI) on 24 mm T-clear
filters (0.4µm pores Costar Corning, Corning, NY) and coated with human placental collagen Type IV as previously described (Bernacki et al., 1999; Nlend et al., 2002; Fulcher et al., 2005).

**Amplification and Cloning of LPO Sequences**

Oligonucleotide primers described by Shin et al. (Shin et al., 2000a) were utilized to amplify the entire LPO coding sequence from a human tracheal cDNA library (Wijkstrom-Frei et al., 2003). The amplified products were cloned utilizing the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) and sequenced. To amplify all splice variants, specific oligonucleotide primers were designed flanking the exons 3 and 4 (sense, 5’ TTCCCTCATCTTGCTTCAGG; antisense 5’ GCAGTCTCCCGTAATGGTG’). RNA was extracted from airway epithelial ALI cultures using TRIzol (Invitrogen, Carlsbad, CA) and cDNA was made using SuperScript First Strand Synthesis System for RT/PCR (Invitrogen Carlsbad, CA). RNA integrity was confirmed using RNA 6000 Labchips and a bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) by the University of Miami DNA Microarray Facility. LPO cDNA was amplified by 35 cycles of 30 sec at 94°C, 30 sec at 60° C. and 45 sec at 72°C and followed by a final 5 min elongation at 72°C. PCR products were cloned using pGEM-T Easy Vector system (Promega, Madison, WI) and sequenced using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems) at the Cardiovascular Genetics Lab Sequencing Facility at the University of Miami.

**Production of Antibodies to Human LPO**

Full length LPO cDNA (i.e. containing propeptide exons) was expressed in a baculovirus-insect cell system as described by Shin et al (Shin et al., 2000a). Hi-Five
insect cells were infected with recombinant baculovirus and cultured in suspension in the presence of δ-aminolevulinic acid for 2 days at 28° C with stirring at 100 RPM. Culture medium was collected and centrifuged at 1000 RPM for 5 min at 4° C. Recombinant human LPO (rhLPO) was purified from culture supernatant as described by Shin et al. (Shin et al., 2000a). rhLPO was excised from SDS gels and utilized for the immunization of rabbits by Covance Laboratories (Hazelton, PA). The identity of purified rhLPO was confirmed following trypsinization and LC/MS/MS at the Protein Chemistry Core Facility at the University of Florida (Gainesville, FL). Antibodies were affinity purified using either native rhLPO or SDS denatured rhLPO coupled to agarose.

The LPO amino acid sequence MSSETPTSRQLSEYLK from exon 4 was synthesized, coupled to KLH and utilized to immunize rabbits by Covance Laboratories. Antibodies (anti-Ex4p1) were affinity purified using peptide coupled to beads with the SulfoLink Kit (Pierce Biotechnology, Rockford, IL) following the manufacturer’s instructions.

Immunoblotting

Human tracheal aspirate samples were obtained using a protocol approved by the University of Miami Institutional Review Board. The samples were collected from patients undergoing general anesthesia for elective surgery indicated for non-pulmonary reasons, as previously described (Campos et al., 2004). Saliva samples were collected from normal healthy individuals 2 hours after food intake. Prior to collection, mouth was rinsed with water and approximately 3ml of saliva was collected. Samples were precipitated with TCA. Protein concentrations were determined using the Micro BCA
Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). Extracts were electrophoresed in Ready Gel SDS 7.5% polyacrylamide gels (Biorad, Hercules, CA), transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) and blocked overnight with 1% gelatin in 0.05% Tween 20 in Tris pH 7.6 buffered saline (T-TBS). Primary antibodies were used at 1 µg/ml in 10% heat inactivated human serum in T-TBS for 1 hour. Second antibody was alkaline phosphatase-conjugated affinity purified goat anti-rabbit IgG Fc fragment specific (Jackson Immunoresearch Laboratories, West Grove, PA) used at 0.02 µg/ml in 10% heat inactivated human serum in T-TBS. The blot was developed utilizing 5-bromo-4chloro-3-indoyl phosphate and nitroblue tetrazolium.

Immunocytochemistry

Normal human tracheas were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned by the Histology Laboratory at the University of Miami Hospital and Clinics, Sylvester Comprehensive Cancer Center. Autofluorescence of de-paraffinized sections was reduced by treatment with 5 mg/ml sodium borohydride in PBS. Sections were subjected to antigen retrieval by incubating in 10 mM citrate buffer (pH 6) for 15 min at 80°C followed by blocking for 1 hr at room temperature in 1% BSA in PBS. Sections were incubated overnight with 5 µg/ml anti-rhLPO or anti-Ex4p1 in blocking solution. Either non-immune rabbit IgG or peptide competed affinity purified anti-Ex4p1 were utilized as negative controls. Visualization was achieved using Alexa 555 labeled anti-rabbit IgG (Molecular Probes-Invitrogen, Carlsbad, CA). Sections were then labeled with either mouse anti- secretory leukocyte protease inhibitor (SLPI) (HyCult Biotechnology (Uden, The Netherlands) or mouse anti-Muc5AC (Chemicon, Temecula,
CA) and visualized with Alexa 488 labeled anti-mouse-IgG (Molecular Probes-Invitrogen, Carlsbad, CA). Fluorescent images were obtained at the University of Miami Analytical Imaging Core Facility.

**Cytokine, Flagellin and LPS Stimulation**

For cytokines, replicate air liquid interface cultures (2-5) of airway epithelial cells from different lung donors were treated for 48 h basolaterally with TNFα (50 ng/ml), or IFNγ (100 ng/ml). Total RNA was isolated using QIAshredder and the Qiagen RNeasy Plus Mini kit (Qiagen, Valencia, CA). RNA was quantified and cDNA was prepared utilizing the iScript cDNA synthesis Kit (Biorad, Hercules, CA).

For LPS (*E. coli*), replicate cultures were stimulated at the apical or basolateral surfaces (50 or 100 ng/ml) for 24h or 48h. Total RNA was isolated, quantified and cDNA was prepared as described above.

**Real Time PCR**

Quantitative PCR was performed utilizing a pair of primers designed to amplify all forms of LPO; (sense, 5’ GGATGCCAGCTTTGTGTAC 3’; antisense, 5’ TAGGGCAGGTAGGGTAGTC 3’) or only variant form 3 (sense, 5’ ATCTACCACAAGAGATCCAGCCT; anti-sense 5’ AGTCTCCCGTAAATGGTGCGGTAA). Samples were normalized to GAPDH (sense, 5’ TGGTCTCCTCCTCTGACTTCACAG; antisense, 5’ TGCTGTAGCCAATTCGTTGTC). cDNA was assayed in triplicate using the iQ SYBR Green Super Mix (Biorad, Hercules, CA). Thermal cycling was carried out using iCycler ILQ apparatus (Biorad, Hercules, CA). The comparative Ct method was
used for relative quantification. In addition, variant form 1 mRNA was assessed using a pre-made TaqMan assay specific for variant 1 only (Lactoperoxidase Assay ID:Hs00413414_m1 (Applied Biosystems, Foster City, CA). Samples were normalized to GAPDH (GAPDH Assay ID: 4333764T (Applied Biosystems, Foster City, CA). Muc2 was utilized as a positive control to assess adequate stimulation of cultures (Muc2 Assay ID: Hs00159374_m1 Applied Biosystems, Foster City, CA). The quantitative PCR was performed using pre-developed TaqMan Universal PCR Master Mix. Thermal cycling was carried out using ICycler ILQ apparatus (Biorad, Hercules, CA). The comparative Ct method was used for relative quantification.

Results

RT-PCR analysis of LPO mRNA from human tracheal cDNA library (Wijkstrom-Frei et al., 2003) (Fig. 3.1) using primers that flank the previously identified coding sequence (GenBank reference sequence NM_006151.1) showed multiple products of different sizes. Cloning and sequencing of amplimers showed that the largest (2.4 kb) and most abundant amplimer comprised the full-length coding region of LPO corresponding to expected full-length sequence and is referred to as variant 1 (V1). The two smaller, less abundant amplimers were alternatively spliced mRNAs, missing coding exon 3 (V2) or both exons 3 and 4, (based on GenBank reference sequence NM_006151.1) immediately following the predicted signal peptide (Fig. 3.2). To confirm the presence of these and facilitate the detection of other potential variants, specific oligonucleotide primers were designed flanking exons 3 and 4 (Fig. 3.2). Again, RT-PCR analysis with these primers (Fig. 3.3) identified the three splice forms of LPO mRNA: V1 representing full length LPO, V2 lacking only exon 3 that immediately follows the predicted signal
peptide and V3 missing both exons 3 and 4 that encode the propeptide (GenBank accession, EF579964). V2 was previously identified through EST screening (GenBank: BX486530, DB208370, CD722179) but the alternative splice shifts the reading frame relative to the LPO coding region. This new reading frame leads to early termination or perhaps uses alternative translation start sites downstream from the splice site. Sequence analysis of additional bands (arrow heads, Fig. 3.3) showed these likely correspond to heteroduplexes of V1, 2 or 3. Although the relative intensities of all forms varied among cultures from different individuals, V1 was always the predominant form.

To characterize the expression of these variant LPO mRNAs in other tissues, we examined mRNA isolated from salivary and mammary glands, testes, liver and airway epithelial cells redifferentiated at the ALI (Fig. 3.4). All three variants were also present in these glandular tissues and in testes. Liver did not contain LPO mRNA as expected. Expression of the variants appeared to differ in relative quantity suggesting that regulation of LPO splice variant expression might also be important in controlling LPO enzyme activity.

LPO, purified from milk, saliva and tracheal secretions, is proteolytically processed with the new N-terminus encoded near the 3’ end of exon 4 (Shin et al., 2000b; Watanabe et al., 2000), thus effectively removing a polypeptide sequence encoded by the same exons not present in the splice variant V3 and suggesting that these N-terminal coding exons may serve a specific function. To examine the presence of this propeptide region in LPO, an antiserum directed against a peptide in exon 4 (anti-Ex4p1) was raised as well as antiserum against recombinant human LPO (anti-rhLPO) expressed in insect cells.
Western blot analysis was performed to characterize both anti-rhLPO and anti-Ex4p1 antisera. Purified rhLPO, human saliva, human MPO and bovine LPO were examined (Fig. 3.5). As expected, anti-rhLPO detected the purified recombinant antigen (Fig. 3.5, lane 3), as well as bovine LPO (lane 4). Anti-rhLPO did not recognize purified human MPO (generous gift of Dr. Roger Fenna, University of Miami) even when three times more MPO was loaded compared to LPO indicating the antibody’s specificity. A single immunoreactive band was recognized in human tracheal aspirates as reported previously with anti-sheep LPO antibodies (El-Chemaly et al., 2003). Anti-rhLPO identified a major band (~80 kDa) and a slightly faster migrating faint band in human saliva. These results were similar to previous findings in which more than one band were observed in saliva utilizing antibodies against LPO (Carlstrom, 1969; Mansson-Rahemtulla et al., 1988; Shin et al., 2001; Ihalin et al., 2006). Anti-Ex4p1 identified a broad immunoreactive band in human tracheal aspirates and several immunoreactive bands in human saliva (Fig. 3.5). This showed that a portion of immunoreactive LPO in secretions contains the exon 4-peptide and thus was partially unprocessed, in contrast to the reported N-termini for human LPO from milk (Shin et al., 2000b). This inconsistency with the reported N-termini of human (Shin et al., 2000b) and bovine (Watanabe et al., 2000) LPO, may reflect the fraction of LPO with blocked N-termini (Dull et al., 1990; Shin et al., 2000b) in purified fractions that prevents Edman degradation of the longer form. Thus, the anti-Ex4p1 antibodies suggest that in saliva a significant portion of LPO may not be fully processed. Several bands of high MWapp are also detected with anti-Ex4p1 and can be competed with antigenic peptide (data not shown) suggesting they are related to LPO in sequence (Fig. 3.5). Crossreactivity was not observed with purified rhLPO when anti-
Ex4p1 was utilized (Fig. 3.5) although minor amounts of propeptide containing protein was seen when an excessive amount of protein was loaded. Blots performed on saliva samples as well as human tracheal aspirates with either antisera show that the band patterns differ from one individual to another in agreement with other reports (El-Chemaly et al., 2003; Wijkstrom-Frei et al., 2003). Neither anti-rhLPO nor anti-Ex4p1 detected LPO in secretions of airway epithelial cell cultures (data not shown).

Immunofluorescence analysis was done utilizing affinity purified antibody against rhLPO in order to determine the localization of LPO in human airways. LPO was observed primarily in submucosal glands and localized to the basal region of gland acinar cells (Fig. 3.6, B). Anti-rhLPO was used simultaneously with mouse anti-SLPI antibody as a marker for serous cells (Kramps et al., 1981). LPO was co-expressed with SLPI, suggesting that LPO is expressed in serous submucosal cells (Fig. 3.6 D).

Human tracheal sections were also double labeled with anti-rhLPO and anti-Mucin5AC, a marker for mucous secreting cells. In the majority of the lungs studied, LPO was not co-expressed in acini with Muc5AC suggesting that LPO is not normally expressed in mucous acini (Fig. 3.7). Interestingly both anti-rhLPO and anti-Ex4p1 antibodies showed some specific (blocked by antigen competition) cross-reactivity with smooth muscle cells and chondrocytes that suggest an unknown but related peroxidase may exist in these cells (Fig. 3.7).

To determine the relative localization of LPO forms that contained the propeptide region compared to the bulk of LPO, immunohistochemistry was performed using anti-Ex4p1 and anti-rhLPO. Each antisera was used simultaneously with anti-SLPI. The vast
majority of the anti-rhLPO antibody signal (Fig. 3.8 B and N) was in the basolateral aspect of the cells whereas anti-Ex4p1 showed that proLPO was homogeneously distributed throughout the cells (Fig. 3.8F and Q). Thus, LPO retaining exon 4 appears to be differently localized. LPO was also detected in airway surface epithelial cells (Fig. 3.9).

Since LPO’s enzymatic activity provides antibacterial host defense to epithelial surfaces, LPO mRNA levels were examined after challenge at the apical surface of differentiated airway epithelial cells cultures with flagellin purified from Pseudomonas aeruginosa. The primers utilized amplified a portion of exon 6 and thus measured levels of all identified LPO splice variants. Control reactions without reverse transcriptase demonstrated the lack of DNA contamination. Challenge with flagellin slightly increased LPO mRNA levels compared to control (2.8 ± 1.3 fold, n = 5 lungs) but the difference (ΔCt relative to GAPDH) did not reach statistical significance. Muc2, previously reported to be induced by flagellin in an intestinal epithelia cell line (McNamara & Basbaum, 2001), served as a positive control. Flagellin treatment resulted in a statistically significant induction of Muc2 mRNA levels (4.9 ± 1.3 fold, n = 4 lungs). LPS treatment at either the apical or basolateral surface did not increase LPO mRNA. This is consistent with previous reports (Becker et al., 2000; Jia et al., 2004; Talreja et al., 2005), which demonstrated that LPS responsiveness by cultured epithelial cells requires supplementation with components that interact with TLR-4.

In contrast, pro-inflammatory mediators, which increase after infection, caused a relatively large increase in LPO mRNA levels 48 h after treatment. IFNγ increased total LPO mRNA 9.3 ± 2.2 fold (n = 10 lungs). TNFα increased total LPO mRNA 9.6 ± 3.8
fold, (n = 6 lungs). Time course experiments suggested that when cultures (n = 2 lungs) were stimulated with TNFα, LPO mRNA levels increased through the 72h time point whereas IFNγ treatment caused a transient increase in mRNA levels, the highest value obtained at 48h and decreased afterwards (Fig. 3.10). Comparison of V1 and V3 mRNA using variant specific primers showed no differential regulation of splice variant mRNAs in response to any of the used treatments. Comparison of cultures from normal lung donors with asthmatic lung donors showed that LPO mRNA was not elevated in asthmatic cultures compared to normal and these were similarly stimulated by IFNγ (9.9 ± 2.2 fold, n = 3 lungs).

Discussion

The LPO system is a first line innate immune defense mechanism present on many epithelial surfaces and the data presented here represent the only study of LPO mRNA regulation. The data showed that transcripts from the LPO gene are alternatively spliced to generate mRNA that lacks the LPO propeptide thus providing a means to synthesize a protein similar to that found after proteolytic processing. The data also showed that the propeptide containing LPO variant is differently distributed inside gland cells. Finally the data show that LPO mRNA is only slightly upregulated in response the bacterial products, but is strongly upregulated by pro-inflammatory cytokines and in airway epithelial cell cultures from asthmatics.

Although alternative splice forms of thyroid peroxidase have been reported (Ferrand et al., 2003; Le Fourn et al., 2004), no variants of MPO or eosinophil peroxidase, that are more similar to LPO, have been seen. Both MPO and eosinophil peroxidase are
synthesized as proforms but the exact function of the propeptide is not understood (Hansson et al., 2006). Unprocessed MPO is active and thus propeptide removal is not necessary to generate a functional enzyme. The LPO propeptide has not been investigated in a similar way. Recombinant LPO was completely processed in our insect cells expression system effectively preventing assessment of enzyme activity of proLPO.

Recombinant MPO synthesized without the propeptide is less likely to acquire heme and be delivered to granules and more likely to be degraded, depending on the cell type used for expression. Thus the MPO propeptide may play a role in correct folding and maturation. Our data suggested that LPO containing the propeptide is differently distributed in submucosal glands suggesting that it may also play a role in targeting LPO to a different intracellular pathway.

To date, no in vitro cell culture systems have been described that synthesize and secrete endogenous LPO in the amounts expected from its levels in milk, saliva, or airway secretions. Despite cytokine induction of LPO mRNA in the system, Western blot analysis of IFNγ and TNFα treated cultures did not show any induction of LPO in secretions or cell lysates. Development of a system that synthesizes LPO in vitro and resembling glandular epithelia will be invaluable in further investigation of the LPO propeptide’s biological role.

The presence of the LPO splice variants in other tissues where LPO is expressed showed that these variants are not airway specific. Surprisingly, LPO mRNA variants were also detected in testes, although the presence of the LPO in this tissue has not been previously reported. LPO is constitutively present at high levels on epithelial surfaces
(Salathe et al., 1997) although a previous study suggested that the enzyme is upregulated in response to bacterial stimuli (Kinbara et al., 1992). Our investigation of LPO mRNA in a model in vitro system suggested that classical bacterial products, only slightly altered LPO mRNA levels even though they are known to stimulate robust increases in other host defense products such as mucins (e.g. Li et al., 1997; Dohrman et al., 1998). The earlier studies measured increases in LPO protein in rats raised in a pathogen-free environment and thus may not reflect normal regulation in non-sterile environments (Kinbara et al., 1992). Alternatively, our in vitro culture system may not replicate the mechanism at work in whole animals that might require signaling through accessory cells or perhaps, translational control instead of transcriptional changes could explain the difference in our studies.

Consistent with the idea that LPO may be upregulated via accessory cells, cytokines secreted by cells of the immune system stimulated increased LPO mRNA. IFNγ increases were transient decreasing after 48 h. This increase in LPO mRNA levels contrasts with the decrease in MPO mRNA levels reported after exposure to IFNγ (Kawano et al., 1993), and no effect was seen on thyroid peroxidase unless IFNγ and TNFα were used together (Tang et al., 1995).

A computational model of LPO activity in airway secretions (Conner et al., 2007) suggested that Duox-mediated H2O2 production was the rate-limiting component of the system. Thus, these data are consistent with the idea that acute upregulation of LPO host defense functions does not rely on acute increases in mRNA.
IFNγ was previously shown to increase mRNA levels of Duox2 that may provide H$_2$O$_2$ substrate for LPO (Harper et al., 2005; Harper et al., 2006). Thus, LPO might to be coordinately regulated with other protein components of the antibacterial system. In contrast to our studies, LPO mRNA was not detected in the cell line (HBE1) used to explore IFNγ regulation of Duox and IFNγ upregulation of LPO could not be observed (Harper et al., 2006). Thus, it seems that primary airway epithelial cells redifferentiated at the ALI demonstrate different responses when compared to immortalized cells. Since previous studies suggested that LPO is a major scavenger of H$_2$O$_2$ in human and sheep airway secretions, increases in LPO may have a functional role during inflammatory responses that are associated with increases in H$_2$O$_2$. Thus LPO may have functions in addition to its known role in host defense against infection.
**Figures**

**Figure 3.1. PCR Identification of LPO variants in human airway.** PCR with primers flanking the entire LPO coding region (see Figure 2) identified three variants in a human tracheal cDNA library (arrows, lane L). Markers are λ DNA/Hind III (lane M).
Figure 3.2. Diagram of LPO mRNA splice variants.

Diagram of LPO mRNA splice variants. The LPO transcription unit is shown in the top line. Un-translated regions are shown as solid boxes and invariant exons in open boxes. The signal peptide is contained in a single exon (arrow) with the 5’ splice site at the ATG initiation codon. Exon 3 is removed in V2 while removal of coding exon 3 and 4 generates V3 and removes one potential glycosylation site that may contribute to the complexity of the published sizes of human LPO in milk, saliva, and airway secretions. Proteolytic processing of LPO in milk generates an N-terminus in exon 4. Primers designed to amplify the full coding region are shown as arrowheads. Primers flanking Exons 3 and 4 are shown as arrows.
Figure 3.3. LPO variants in airway epithelial cell cultures. PCR with primers located in exon 2 and 5 that flank the spliced regions identified three bands (401, 312, and 128 bp) in a human tracheal cDNA library (lane L) and in airway epithelial cell cultures (lanes ALI: 1 and 2, two separate lung donors). Additional bands at ~380 bp and 275 bp were observed (arrowheads) that likely correspond to heteroduplexes of V1, 2 and 3. Markers (lane M) were Hae III digested φX174.
Figure 3.4. LPO variants are present in other tissues. PCR analysis (35 cycles) utilizing primers flanking the spliced regions identified three bands (401, 312, and 128 bp) in a human tracheal cDNA library (lane Trach), mammary gland (lane MG), and salivary gland (SG) RNA. Bands of similar size were also observed in testes except for the 128bp band (lane T) but not in liver RNA (lane L). Markers (lane M) were Hae III digested φX174. Lanes containing no RT controls showed no amplimer.
**Figure 3.5. Western blot analysis of proLPO in secretions.** Antibodies against LPO (Anti-rhLPO); LPO propeptide (Anti-Ex4p1) and control (non-immune IgG), were used to probe human tracheal aspirates (100 µg, lane 1), human saliva (30 µg, lane 2), recombinant human LPO (0.1 µg, lane 3), bovine LPO (0.1 µg, lane 4), human MPO (0.3 µg, lane 5). Blots were visualized using alkaline phosphatase conjugated goat anti-rabbit Fc fragment antibody.
Figure 3.6. Immunolocalization of LPO in human airway mucosa within serous cells. Panels A & E show phase contrast images of human tracheal sections which were double labeled with affinity purified rabbit antibodies against human recombinant LPO (panel B) and mouse anti-secretory leukocyte protease inhibitor (SLPI), utilized as a marker for serous cells, (panels C & G). Non-immune rabbit IgG was utilized as a control (panel F). Labeling was visualized by utilizing either anti-rabbit IgG coupled to AlexaFluor555 (red) or anti-mouse IgG coupled to Alexa 488 (green). LPO was primarily observed in submucosal glands and localized to the basal surface of gland acinar cells (panel B). Panels D & H show the merge of Alexa 555 and Alexa 488 labeling. Bar = 100µm
**Figure 3.7. LPO is not expressed in mucous gland cells.** Sections of human trachea (phase contrast, panels A and E) were double labeled with affinity purified rabbit antibodies against hrLPO (panel B, red) and mouse anti-Muc5AC utilized as a marker for mucous secreting cells (panels C & G, green). Non-immune Rabbit IgG was utilized as a negative control (panel F). Labeling was visualized with either anti-rabbit IgG coupled to AlexaFluor555 (red) or anti-mouse IgG coupled to Alexa 488 (green). In normal airways, LPO did not colocalize with Muc5AC (panel D). Anti rhLPO cross reacted with smooth muscle (*). Panels D & H show the merge of Alexa 555 and Alexa 488 labeling. Bar = 100µm
Figure 3.8. Subcellular distribution of proLPO. Panels A, E, and I show phase contrast images of human tracheal sections double labeled with antibodies against rhLPO (Panels B and N) or anti-Ex4p1 (Panels F and Q) and mouse anti-SLPI, (Panels C, G, K, O, R, and U). Peptide competed anti-Ex4P1 (Panel J) or nonimmune IgG were negative controls (Panel T), followed by either anti-rabbit IgG coupled to AlexaFluor555 (red) or anti-mouse IgG coupled to Alexa488 (green). SLPI was used as a marker for serous cells. Anti hrLPO detected LPO primarily in submucosal glands and localized to the basal region of gland acinar cells (Panel B). When anti Ex4p1 antibody was used, LPO was observed homogeneously localized throughout the glands (Panel F). Both antibodies detected LPO coexpressed with SLPI (panels D and H), suggesting that LPO is produced in serous gland cells. Panels D, H and L show the merge of Alexa 555 and Alexa 488 labeling. Panels A-L (Bar = 100µm. Panels M-U, Bar = 50µm.)
Figure 3.9. Immunolocalization of LPO in airway surface epithelium. Sections were incubated with anti-Ex4p1antibodies (Panel A) followed by anti-rabbit IgG coupled to AlexaFluor555. Non-immune rabbit IgG was utilized as a control (Panel C). Panels B & D show phase contrast images of the epithelium in human tracheal sections. Bar = 100µm.
Figure 3.10. Induction of LPO mRNA levels after treatment with cytokines. Time course experiment of airway epithelial cultures stimulated basolaterally with TNFα (□, 50ng/ml) and IFNγ (●, 100 ng/ml) for 12, 48 and 72hrs. Each time point represent the average LPO mRNA fold change of 6 cultures from two different donors relative to control. Values are means ± S.E.M. and n = 2 lungs, 3 cultures each (error bars are within the symbols for some points).
CHAPTER 4

Discussion

The human airway epithelium provides an efficient innate host defense system, which prevents microbial infection in the airways by diverse mechanisms which include the production of antimicrobial substances by the epithelium. One of these antimicrobial substances is the enzyme LPO which together with its substrates, hydrogen peroxide and SCN\(^{-}\), form the LPO system. This antimicrobial system works in harmony with other antimicrobial systems found in the airway. LPO is thought to play an important role in maintaining airway sterility based on the knowledge that LPO inhibition decreases the ability to clear live bacteria in sheep airways (Gerson \textit{et al.}, 2000) as well as its abundance in airway secretions (1% of total protein) (Salathe \textit{et al.}, 1997b) and its relevance to diseases characterized by chronic infection such as cystic fibrosis (Conner \textit{et al.}, 2007b; Moskwa \textit{et al.}, 2007).

Characterization of SCN\(^{-}\) transport

Our study on the characterization of SCN\(^{-}\) transport in human airway epithelial cells showed that SCN\(^{-}\) transport takes place in a transcellular manner (Fragoso \textit{et al.}, 2004). Our data suggested that SCN\(^{-}\) transport across the basolateral membrane took place through NIS. Our study also provided evidence for the involvement of CFTR in SCN\(^{-}\) transport from the cytosol into the airway lumen due to the pharmacological profile of SCN\(^{-}\) efflux in airway epithelial cells. Based on this data we proposed a model for SCN\(^{-}\) transport in the airway (Fig. 4.1). In this model NIS or in the case of the airway Sodium
Thiocyanate Symporter (NTS) moves Na\(^+\) along with SCN\(^-\) across the basolateral membrane into the cytosol. SCN\(^-\) then, as suggested by our data, moves from the cytosol across the apical membrane and is released at the apical surface by means of a CFTR regulated mechanism.

**Figure 4.1. Proposed model for SCN\(^-\) transport**

Other studies published after our report (Fragoso et al., 2004) supported our findings of CFTR’s involvement in SCN\(^-\) transport across the apical membrane (Conner et al., 2007b; Moskwa et al., 2007; Pedemonte et al., 2007) Pedemonte et al. suggested Pendrin, to be an additional channel for SCN\(^-\) transport in the airway. Although their work provided evidence that Pendrin-associated SCN\(^-\) transport was higher that that observed through CFTR, this was only observed when cultures were treated with IL-4 for 24 hours. Presence of IL-4 in the airway has been observed in CF airways when compared to
normal individuals. (Brazova 2005, Wojnarowski 1999, Sobol 2002), but the expression of pendrin in CF airways remains to be determined. Although Pedemonte’s study suggests that Pendrin might be compensating for the lack of a functional CFTR in CF airways there is evidence that suggests that SCN⁻ transport depends mainly on a functional CFTR. A study currently being performed at the University of Florida by a group collaborating with our lab indicates that the [SCN⁻] is very low in exhaled breath condensates from cystic fibrosis patients compared to normal individuals (unpublished data). Recently Banfi et al. reported that [SCN⁻] in nasal secretions of CF patients were about 30% of levels reported for normal patients and suggested that the levels found (~0.25 mM) were too low to support LPO antibacterial activity (Cystic Fibrosis Conference 2007, Anheim CA). Conner et al also reported low SCN⁻ transport rates in CF airway epithelial cell cultures Conner et al. This study showed that the apical [SCN⁻] present in cultures was not sufficient for a functional LPO system. This study also showed that reduced transport rate cannot be compensated by increases in LPO or H₂O₂ and suggest a link between SCN⁻ transport and host defense in cystic fibrosis (Conner et al., 2007b).

According to Conner et al., modeling of airway LPO activity suggested that H₂O₂ seems to be the limiting reagent in the LPO antimicrobial activity in in vitro studies because H₂O₂ is rapidly consumed and not replenished (Conner et al., 2007b). This model does not take into account the production of H₂O₂ produced by pathogens during infection in vivo and the fact that MPO utilizes SCN⁻ as its substrate as well. Both of which might contribute to the low [SCN⁻] in the airway lumen of CF airways. Thus the
presence of a nonfunctional CFTR in CF airways may result in a defective host defense provided by the LPO system.

**Identification of LPO mRNA variants**

LPO mRNA splice variants were identified in a tracheal cDNA library. The data showed that transcripts from the LPO gene are alternatively spliced to generate mRNA that lacks the LPO propeptide thus providing a means to synthesize a protein similar to that found after proteolytic processing. The data also showed that the propeptide containing LPO variant is differently distributed inside gland cells. It is not clear whether the previously reported salivary LPO polymorphisms are related to the splice variants described here since the former depend on disulfide bonding to other salivary proteins (Azen, 1977). Although alternative splice forms of thyroid peroxidase have been reported (Ferrand *et al*., 2003; Le Fourn *et al*., 2004), no variants of MPO or EPO, which are peroxidases more similar to LPO, have been reported.

We expect that the LPO mRNA variant missing the two coding exons will give rise to a functional protein since the location of the active site, which is highly conserved in the peroxidase family, is outside the region comprised by the missing exons. Similar to LPO both MPO and EPO are synthesized as proforms but the function of the propeptide remains to be determined (Hansson *et al*., 2006) since unprocessed MPO is active and thus propeptide removal is not necessary to generate a functional enzyme. The role of the LPO propeptide has not been studied. Based on reports that suggest that MPO propeptide may play a role in correct folding and maturation we hypothesize that the LPO propeptide might have a similar function due to our findings, which suggested that LPO containing the propeptide is differently distributed in submucosal glands.
The presence of the LPO mRNA splice variants in other tissues where LPO is expressed showed that these variants are not airway specific. We hypothesize that expression of LPO in testes, which has not been previously reported, is involved in maintaining this tissue infection-free, which is imperative for the viability of sperm.

**Effect of cytokines and bacterial products on LPO mRNA**

LPO is constitutively present at high levels on epithelial surfaces (Salathe *et al.*, 1997a). The data in the stimulation studies presented in this study also showed that LPO mRNA was only slightly upregulated in response to bacterial products (LPS and flagellin), but significantly upregulated by pro-inflammatory cytokines. Although contrary to our results a previous study suggested that the enzyme is up-regulated in response to bacterial stimuli (Kinbara *et al.*, 1992). One explanation for the differences in these results is that the earlier studies measured increases in LPO protein in rats raised in a pathogen-free environment and thus may not reflect normal regulation in non-sterile environments. (Kinbara *et al.*, 1992). An alternate explanation for our results is that the *in vitro* culture system utilized in this study may not replicate the conditions present *in vivo*. To date, no *in vitro* cell culture systems have been described that synthesize and secrete endogenous LPO and this could explain why Western blot analysis of IFNγ and TNFα treated cultures did not show any induction of LPO in secretions or cell lysates even though there was a significant up-regulation in LPO mRNA. More experiments need to be done to determine why there was a transient increase in LPO mRNA due to IFNγ challenge followed by a down regulation whereas when cultures were challenged with TNFα LPO mRNA increase was sustained up to 72 hrs. This increase in LPO mRNA levels is similar to that observed by Caturegli *et al.* 2000 who showed TPO mRNA was
slightly up-regulated in the thyroid after stimulation with IFNγ (Caturegli et al., 2000). Whereas another study reported that no effect was observed unless IFNγ and TNFα were used together (Tang et al., 1995). These findings contrast to the decrease in MPO mRNA levels reported after exposure to IFNγ (Kawano et al., 1993). Thus, some of the members of the peroxidase family may be regulated in a similar manner or possibly share the same promoter elements regulating their expression.

In order to understand how the LPO gene is regulated and which pathways are involved in its modulation, more studies need to be done to determine the relationship of IFNγ to other inflammatory modulators such as TNFα in the airway, and to determine if there is an effect in human airway similar to that observed in human epithelial keratinocytes in which TNFα expression was up-regulated by IFNγ (Matsuura et al., 1998). This would also provide information to explain the results observed in the time course experiment which showed a delay in the LPO mRNA up-regulation by IFNγ and would provide an insight as to whether LPO might be indirectly up-regulated by cytokines like IFNγ which up-regulates expression of other cytokines.

**Regulation of the components of the LPO system by cytokines**

IFNγ was previously shown to increase mRNA levels of Duox2 that may provide increased H₂O₂ substrate for LPO (Forteza et al., 2005; Harper et al., 2005). Thus, LPO might be coordinately regulated with other protein components of the antibacterial system such as NIS and Duox (see figure 4.2). We hypothesize that NIS expression and activity in the airway is also regulated by cytokines in a similar manner to studies done with thyroid epithelial cells. In these studies NIS was shown to be down regulated by
IFNγ and TNF α (Ajjan et al., 1998), IL-1 (Ajjan et al., 1998), IFN β (Caraccio et al., 2005) and IFN α (Caraccio et al., 2005). It is interesting that a study in which a lower concentration of IFNγ was utilized reported an increase in both the expression and activity of NIS (Caturegli et al., 2000). The findings of these studies suggest NIS activity in the airway can be modulated by cytokines. A recent study reported that NIS activity can also be regulated by phosphorylation (Vadysirisack et al., 2007).

In the case of CF airways the effect of cytokines on NIS activity is expected not to be relevant due to the absence of a functional CFTR as suggested by Conner et al. (Conner et al 2007). An increase in CFTR mRNA has also been reported in the presence of cytokines but changes in activity were not reported (Skowron-zwarg et al., 2007). Since previous studies suggested that LPO is a major scavenger of H$_2$O$_2$ in human and sheep airway secretions, increases in LPO may have a functional role during inflammatory responses that are associated with increases in H$_2$O$_2$. Thus, the LPO system may be actively regulated to scavenge increased levels of H$_2$O$_2$, and have other functions in addition to its known role in host defense against infection.
Figure 4.2. Regulation of the LPO system. The availability of the components of the LPO system can be regulated by cytokines, cAMP and Ca$^{2+}$. An immune response takes place upon recognition of microbial products during infection. This leads to the secretion of cytokines. Cytokines activate signaling pathways which cause the modulation of the expression and/or activity of NIS, Duox and LPO. Duox activity can also be up-regulated by Ca$^{2+}$ causing an increased production of H$_2$O$_2$ which has been shown to activate signaling pathways and cause the modulation of other genes involved in inflammation. The activity of CFTR can be regulated by cAMP causing an increase transport of SCN$^-$. 

Possible regulatory mechanisms of the LPO system in the airway

The LPO system is one the first line non-immune defense mechanisms present in the airway and functions against viruses, fungi and bacteria. There are three possible mechanisms by which the antimicrobial activity of the LPO system can be regulated (figure 4.3). Each of these mechanisms involves the availability and expression of
components of the LPO system which are the following: the enzyme LPO and its substrates $\text{H}_2\text{O}_2$ and SCN$^-$. The first possible mechanism involves the regulation of LPO expression and secretion which could be affected by the presence of inflammatory cytokines and microbial products. The second mechanism involves the availability of SCN$^-$ in the airway lumen. The availability and concentration of this ion depends on the functionality of its transporters at both the apical and basolateral membranes. The third mechanism and which according to Conner et al, seems to be the limiting component in vitro studies is $\text{H}_2\text{O}_2$ production by Duox in the apical membrane of airway epithelium. It has been shown that if one of the components is not present at the appropriate concentration the LPO antimicrobial system will not be functional (Wijkstrom-Frei et al., 2003).
Figure 4.3. Possible regulatory mechanisms of the LPO system in the airway. The antimicrobial activity of the LPO system could be regulated by modulating the production and/or activity of the proteins responsible for allowing appropriate concentrations of the components of the LPO system in the airway lumen. The appropriate concentrations to have a functional LPO system are 3-10 μg/mL (LPO), 0.4mM (SCN⁻), 10⁻⁵-10⁻⁶M (H₂O₂).

Future directions

A more extensive study needs to be done to elucidate the effect of cytokines and other substances found in infected airways on the expression and activity of the proteins associated with the LPO system, as well as how this effect might cause the modulation of other host defense mechanisms.
Translational significance

The possibility that CFTR regulates SCN⁻ transport in airway epithelial cells raises important questions regarding the effect of impaired CFTR activity on LPO-mediated airway host defense, and other potential effects of a loss of LPO activity on airway homeostasis in cystic fibrosis since SCN⁻ transport has been shown to be deficient in CF cultures (Conner et al., 2007a; Moskwa et al., 2007). An insight on how the LPO system is regulated would provide information that could help to develop antimicrobial compounds or therapeutic strategies to compensate endogenous antimicrobial systems such as the LPO system and help control bacterial colonization in CF airways.
REFERENCES


