Respiratory protease/antiprotease balance determines susceptibility to viral infection and can be modified by nutritional antioxidants

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1Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; 2Center for Environmental Medicine, Asthma and Lung Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; 3Curriculum in Toxicology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; and 4Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

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Meyer M, Jaspers I. Respiratory protease/antiprotease balance determines susceptibility to viral infection and can be modified by nutritional antioxidants. Am J Physiol Lung Cell Mol Physiol 308: L1189–L1201, 2015. First published April 17, 2015; doi:10.1152/ajplung.00028.2015.—The respiratory epithelium functions as a central orchestrator to initiate and organize responses to inhaled stimuli. Proteases and antiproteases are secreted from the respiratory epithelium and are involved in respiratory homeostasis. Modifications to the protease/antiprotease balance can lead to the development of lung diseases such as emphysema or chronic obstructive pulmonary disease. Furthermore, altered protease/antiprotease balance, in favor for increased protease activity, is associated with increased susceptibility to respiratory viral infections such as influenza virus. However, nutritional antioxidants induce antiprotease expression/secretion and decrease protease expression/activity, to protect against viral infection. As such, this review will elucidate the impact of this balance in the context of respiratory viral infection and lung disease, to further highlight the role epithelial cell-derived proteases and antiproteases contribute to respiratory immune function. Furthermore, this review will offer the use of nutritional antioxidants as possible therapeutics to boost respiratory mucosal responses and/or protect against infection.

protease/antiprotease; viral infection; nutritional antioxidants

NEARLY 40 DIFFERENT TYPES of cells have been identified in the respiratory tract, including neutrophils, dendritic cells, macrophages, natural killer (NK) cells, B cells, and T cells (125). Because of this diverse population of cells, a central “orchestrator” is necessary for coordinating an appropriate immune response to inhaled stimuli. The respiratory epithelium is one of the first sites within the respiratory tract to be exposed to inhaled stimuli and functions as a focal orchestrator to organize and initiate appropriate responses in three distinct ways (Fig. 1).

First, the respiratory epithelium forms a physical barrier through tightly regulated cell-cell interactions. These interactions promote basic biological functions, protect against invading pathogens, and establish mechanical strength. Second, epithelial cells express receptors and ligands that coordinate cellular responses and activate immune cells. These processes are important for detecting foreign stimuli, communicating among different cells, and guiding immune responses. Third, respiratory epithelial cells secrete soluble factors such as cytokines, chemokines, and host defense mediators such as proteases and antiproteases. These secreted, soluble factors are crucial for the overall host defense response. In the context of antimicrobial host defense responses, epithelial function can be modulated by intrinsic factors, such as age, and extrinsic factors, such as microbial infection, exposure to inhaled toxicants, and supplementation with nutritional antioxidants. As such, this review will examine the role of epithelial cell-derived proteases and antiproteases, in the context of lung disease and viral infection and offer a potential strategy to modulate this balance with nutritional antioxidants.

Respiratory Viruses Infect Respiratory Epithelial Cells and Cause Exacerbations of Lung Disease

Respiratory epithelial cells are targets for respiratory viruses and differ in protease and/or antiprotease sensitivity. Respiratory epithelial cells are the primary targets for respiratory viral infection based on extracellular ligands and residues that are found on these cells. More than 200 antigenically different viruses can infect respiratory epithelial cells (110). These viruses include coronaviruses, rhinoviruses, metapneumoviruses, enteroviruses, adenoviruses, respiratory syncytial viruses, measles virus, parainfluenza viruses, and influenza viruses. These respiratory viruses differ in genome composition, viral structure, specific target cell type, and sensitivity to components of the respiratory protease/antiprotease balance (Table 1).

Upon viral infection, respiratory epithelial cells replicate viral genomes and propagate infectious virions for productive viral infection. Additionally, respiratory epithelial cells mount intracellular immune responses to warn neighboring cells as well as orchestrate recruitment of immune cells to sites of

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infection (152). The respiratory epithelium also secretes soluble mediators such as type II transmembrane serine proteases (TTSPs) and antiproteases, such as secretory leukocyte protease inhibitor (SLPI), which are implicated in viral infection. Many respiratory viruses, such as coronaviruses, metapneumoviruses, parainfluenza viruses, and influenza viruses, are sensitive to TTSP-dependent cleavage (Table 1). However, whereas some respiratory viruses, such as coronaviruses, induce antiprotease expression, only parainfluenza and influenza are sensitive to SLPI activity (Table 1). Thus investigating the role of the protease/antiprotease balance in the context of a parainfluenza or influenza infection could elucidate mechanisms and strategies to prevent viral infections.

Understanding the role of the protease/antiprotease balance in the context of an influenza infection is of public health importance, since influenza infects 2–5 million people worldwide each year and can cause worldwide pandemics, as seen with the recent 2009 H1N1 strain (162). Moreover, in the US alone, over 20,000 people die because of influenza infection and related complications (29a). Influenza remains a significant public health burden due to yearly virus mutations and the ability of the virus to infect a wide range of hosts. Despite large-scale vaccination programs and the development of antiviral therapeutics, influenza-associated morbidity and mortality rates have not changed in recent years (99). This is of particular importance in years when the strains used in the influenza vaccine

Table 1. Genome and structure, target cell type, and sensitivity to protease/antiprotease balance of respiratory viruses

<table>
<thead>
<tr>
<th>Respiratory Virus</th>
<th>Genome; Structure</th>
<th>Target Cell</th>
<th>Require Proteolytic Cleavage by Secreted Respiratory Proteases?</th>
<th>Sensitive to Secreted Respiratory Antiproteases?</th>
</tr>
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<tr>
<td>Adenovirus</td>
<td>dsDNA; Nonenveloped</td>
<td>Epithelial cells using clathrin-dependent entry mechanisms (119)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Rhinoviruses</td>
<td>+ssRNA; Nonenveloped</td>
<td>Epithelial cells of the upper airway expressing ICAM-1 (69)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Enteroviruses</td>
<td>+ssRNA; Nonenveloped</td>
<td>Epithelial cells expressing ICAM-1 or DAF (129)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Coronaviruses</td>
<td>+ssRNA; Enveloped</td>
<td>Epithelial cells expressing ACE2 or DPP4 (107, 139)</td>
<td>Yes- cleave S protein (17, 19, 20, 64, 77, 157, 194)</td>
<td>Yes (96, 142)</td>
</tr>
<tr>
<td>Measles viruses</td>
<td>-ssRNA; Enveloped</td>
<td>Epithelial cells expressing nectin 4 (132, 133)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Metapneumoviruses</td>
<td>-ssRNA; Enveloped</td>
<td>Upper and lower airway epithelial cells expressing αVβ1 integrin (39)</td>
<td>Yes- cleave F protein (158)</td>
<td>No</td>
</tr>
<tr>
<td>Respiratory syncytial viruses</td>
<td>-ssRNA; Enveloped</td>
<td>Epithelial cells expressing sulfated glycosaminoglycans (70)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Parainfluenza viruses</td>
<td>-ssRNA; Enveloped</td>
<td>Epithelial cells expressing 2,3 or 2,6 linked sialic acid residues (55)</td>
<td>Yes- cleave F protein (3, 94)</td>
<td>Yes (52, 93, 94)</td>
</tr>
<tr>
<td>Influenza viruses</td>
<td>-ssRNA; Enveloped</td>
<td>Epithelial cells expressing 2,3 or 2,6 linked sialic acid residues (36)</td>
<td>Yes- cleave HA protein (10, 11, 16, 19, 22, 23, 56, 75, 148, 178, 194)</td>
<td>Yes (14, 91, 93, 95)</td>
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do not antigenically match the circulating influenza virus strains, as is the case in the current 2014–2015 influenza epidemic (54). Therefore, examining strategies such as modulating the protease/antiprotease balance may serve as alternative targets to prevent influenza infection.

**HA must be proteolytically cleaved for productive IAV infection.** For influenza A virus (IAV) to infect cells, the viral membrane protein hemagglutinin (HA) must be proteolytically cleaved and activated by respiratory serine proteases. HA resides on the virion as a fusion-inactive precursor (HA0), and upon proteolytic cleavage a fusion-active trimer with disulfide-linked HA1 and HA2 subunits is generated (16, 18, 22, 23). After entry into the cell, the virion enters endosomal compartments, and upon acidification of the endosome viral and host membranes fuse together. The membrane fusion allows for viral entry into the cell, so viral replication can initiate (98).

Most HA0 will be cleaved at either a single arginine or lysine residue to generate active HA1 and HA2. For low-pathogenic IAV strains, respiratory trypsin-like serine proteases cleave at monobasic arginine or lysine residues (21, 102). However, highly pathogenic avian influenza viruses possess multibasic cleavage sites that can be cleaved by ubiquitous intracellular furin-like serine proteases (166, 182). This difference in HA cleavage motifs increases susceptibility for systemic, widespread infection to sites such as the central nervous system rather than localized, restricted infection to sites within the respiratory tract. Consequently, investigating strategies to block protease-induced HA activation and increase antiprotease activity may serve as a potential approach to protect against IAV infection.

**Proteases and antiproteases of the Lung: a Delicate Enzymatic Balance**

**Epithelial cell-derived proteases.** Proteases are key components of respiratory host defense. In the healthy lung, proteases maintain tissue homeostasis and their activities are regulated by antiproteases. Elevated net protease activity is associated with lung destruction and the development of chronic lung diseases such as emphysema and chronic obstructive pulmonary disease (COPD) (1, 90). Lung destruction is induced by proteolytic degradation of extracellular matrix (ECM) components, including collagens, laminins, and elastin, with type I collagen serving as the major target (164). Cysteine proteases, matrix metalloproteinases (MMPs), and serine proteases are prevalent proteases found in the lung, with serine proteases serving as the most predominant class. Moreover, each family has unique target substrates, cellular sources, and active sites (Table 2).

**Cathepsins.** Cysteine proteases, or cathepsins, are synthesized as proenzymes, require cleavage for activation, and contain conserved cysteine and histidine residues, with the cysteine serving as a nucleophile for target motifs. Cathepsin B, K, L, and S are highly abundant in the lung; cathepsin K, mainly produced in bronchial epithelial cells, is one of the most potent matrix-degrading proteases identified to date (26). Cathepsins cleave elastin, laminin, and collagen as well as antiproteases such as SLPI and are important for major histocompatibility complex II formation and antigen presentation (60, 144, 174). Cathepsins have been linked to respiratory virus infection since cathepsin L cleaves and activates the spike protein of severe acute respiratory syndrome coronavirus (SARS-CoV), and cathepsin B is induced during IAV infection in mice (20, 27).

**MMPs.** MMPs are a family of metal endopeptidases that target ECM components. Secreted by immune cells and epithelial cells, MMPs possess a common prodomain and catalytic domain. Because MMPs are synthesized as inactivezymogens, the signal domain must be cleaved for MMPs activity. The catalytic domain contains a conserved zinc binding site that confers protease activity. MMP1, 2, 3, 7, 8, 9, 12, and 13 are the main MMPs found in the respiratory tract and are induced by growth factors, reactive oxygen species, and proinflammatory cytokines (42, 68, 100). MMP1, 8, and 13 cleave collagen, whereas MMP2 and 9 act as gelatinases and also degrade elastin and type IV collagen (46, 84).

Additionally, increased MMP activity is associated with inflammation. Baseline MMP levels such as MMP1, 2, 3, 8, and 9 are elevated in smokers and patients with COPD (40). Moreover, MMP9 cleaves proinflammatory cytokines such as interleukin-8 (IL-8), resulting in the release of a modified, more potent neutrophil chemotactic mediator (134). MMP12 degrades elastin but also inactivates key antiproteases in the lung such as SLPI (140). Although MMP activity has not been implicated in viral activation, MMPs are involved in virus-induced inflammation. In epithelial cell lines and mouse models, MMP2 and 9 are induced during IAV infection and contribute to the IAV-associated lung destruction (105, 185, 190). Tacon et al. (171) demonstrated that MMP levels are

<table>
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<th>Table 2. Target substrates, sources, and activity of respiratory proteases</th>
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<tr>
<td>Proteases in the Respiratory Tract</td>
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<tr>
<td>Cathepsin B, K, L, and S</td>
</tr>
<tr>
<td>MMP1, 2, 3, 7, 8, 9, 12, and 13</td>
</tr>
<tr>
<td>Neutrophil-derived proteases (NE, cathepsin G, proteinase 3)</td>
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<tr>
<td>TTSPs</td>
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elevated in vitro and in vivo during human rhinovirus (HRV) infection, which was dependent on NF-κB, further illustrating the role of MMPs in virus-induced respiratory inflammation.

**TTSPs.** Serine proteases make up the largest portion of respiratory proteases (66). Whereas neutrophil elastase (NE), proteinase 3, and cathepsins G are neutrophil-derived proteases, other serine proteases such as TTSPs are produced and secreted by the respiratory epithelium. TTSPs are a family of proteases that possess an NH2-terminal transmembrane domain and are secreted by the respiratory epithelium. TTSPs are produced as a single-chain, inactive proenzyme and require cleavage following a basic arginine or lysine (82). The catalytic domains are located on the terminal extracellular region to permit direct exposure to the extracellular environment.

TTSPs are comprised of four subfamilies: the human airway trypsin-like protease (HAT)/differentially expressed in squamous cell carcinoma (DESC) subfamily that include HAT, DESC1, and HAT-like 1–5; the hepsin/transmembrane protease serine (TMPRSS) subfamily contains hepsin, TMPRSS2-5 and 13, mosaic serine protease large-form, and enteropontidase; the corin protease; and the matriptase subfamily that includes matriptide 1–3 and polyserase-1 (5). Although TTSPs are synthesized as a single-chain, inactive proenzyme and require cleavage following a basic arginine or lysine (82). The catalytic domains are located on the terminal extracellular region to permit direct exposure to the extracellular environment.

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Matriptase cleaves and activates protease-activated receptor 2 (PAR2), hepatocyte growth factor, and urokinase, indicating that matriptase plays a role not only in ECM degradation but also in epithelial cell differentiation and remodeling (104, 176). In the respiratory epithelium, matriptase is expressed on the basolateral side of the epithelial cells. Upon activation, matriptase migrates to the apical side of the epithelium, where it can be secreted into the lumen of the respiratory tract. Recent studies have revealed that intracellular matriptase localizes to the plasma membrane and endosomes, allowing for interaction and activation of the IAV virion (11, 73). Moreover, secreted matriptase cleaves HA protein on the IAV virion, including H1, but not H2 or H3, which activates the virion and allows for enhanced rates of replication (11, 73).

HAT cleaves fibrinogen, activates PAR2, and urokinase (11). In sputum samples from patients with chronic airway disorders such as bronchitis or asthma, secreted HAT levels are elevated (189). Moreover, Chokki et al. (34) demonstrated that HAT enhanced MUC5AC gene expression, resulting in increased mucus production. HAT is produced and secreted by ciliated epithelial cells and is not found in basal or goblet cells of the respiratory tract (175). HAT activates the SARS-CoV viral spike protein necessary for host cell entry (17). Moreover, HAT cleaves the monobasic site of the HA protein required for HA activation, enhancing IAV replication (21–23). Cleavage of HA by HAT has been shown to occur at the cell surface either during attachment and entry into host cells or during budding and shedding of virions from an infected cell (21).

TMPRSS2 possesses a wide range of functions specific to epithelial cell biology. Donaldson et al. (45) demonstrated that TMPRSS2 reduced epithelial sodium channel activity, indicating a role for TMPRSS2 in ion transport. TMPRSS2 can be expressed as a full-length 70-kDa protein or a variety of smaller, truncated forms, depending on tissue site and localization (3, 30). Additionally, TMPRSS2 is implicated in the activation of respiratory viruses. Cells expressing TMPRSS2 show enhanced replication of human metapneumovirus as well as increased activation and replication of SARS-CoV (19, 158). Bottcher et al. (22, 23) reported that cells stably expressing TMPRSS2 resulted in the monobasic cleavage of HA, which elevated IAV replication. In recent reports, TMPRSS2 has been shown to preferentially cleave H1N1, and to a lesser degree H3N2. Hatesuer et al. (75) revealed that viral titers in H1N1-infected TMPRSS2-deficient mice were significantly reduced compared with wild-type animals. Furthermore, this group reported that although H3N2 replicated at similar levels in both knockout and wild-type mice, the knockout mice had increased survival and body weight post-H3N2 infection (75).

Recent reports have further expanded on the HA specificity of TMPRSS2. One group demonstrated that TMPRSS2 knockout mice were protected from lethal H1N1 but not lethal H3N2 infection, whereas another group showed that TMPRSS2 knockout mice were protected against both H1N1 and H3N2 infection (148, 178). These opposing findings suggest that HA activation not only depends on HA structure but can vary among different viral strains bearing the same HA subtype. Despite these differences, there is substantial evidence implicating TTSPs in IAV pathogenesis.

**Epithelial cell-derived antiproteases.** Antiproteases are a broad class of proteins that inhibit proteases and modulate immune responses in the lung. Respiratory antiproteases are comprised of four families: tissue inhibitors of metalloproteases (TIMPs), serine protease inhibitors (serpins), trappin-2/elafin, and SLPI. Each antiprotease family has unique target enzymes, and TIMPs have been shown to be associated with chronic lung diseases such as asthma and COPD. Circulating granulocytes from patients with asthma have an impaired ability to release TIMP1, the main inhibitor of MMP9, after stimulation with phorbol 12-myristate 13-acetate (28). Additionally, although it has been shown that sputum samples from stable COPD patients contain elevated TIMP1 levels during a COPD exacerbation, others have reported that TIMP1 levels...
Table 3. Target substrates, sources, and activity of respiratory antiproteases

<table>
<thead>
<tr>
<th>Antiproteases in the Respiratory Tract</th>
<th>Target Substrates</th>
<th>Sources in the Lung</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMPs</td>
<td>MMPs (24)</td>
<td>Alveolar epithelial cells, alveolar macrophages, granulocytes (28, 97)</td>
<td>Insert reactive ridge NH2-terminal domain into MMP active site, inhibit apoptosis (103, 120)</td>
</tr>
<tr>
<td>Serpin</td>
<td>Serine and cysteine proteases, caspasas, bacteria, viruses (9, 57, 138, 141, 154)</td>
<td>Epithelial cells, alveolar macrophages (35, 115, 159)</td>
<td>Insert RCL into the active site of the targeted protease, inhibit LPS-induced inflammation, block microbial infection (101, 130)</td>
</tr>
<tr>
<td>Trappin/Elafin</td>
<td>NE and proteinase-3, enveloped viruses (193)</td>
<td>Tracheal epithelial cells, club cells, type II cells, alveolar macrophages (123)</td>
<td>Contain WAP domain for antiprotease activity, inhibit viral infection (59, 150, 156, 187)</td>
</tr>
<tr>
<td>SLPI</td>
<td>NE, cathepsin G, chymase, bacteria, and viruses (62, 184)</td>
<td>Epithelial cells, neutrophils, macrophages (48, 121)</td>
<td>Contain WAP domain for antiprotease activity, antimicrobial effects due to NH2-terminal domain and high cationicity, block inflammatory cascades, inhibit viral infection (67, 91, 93, 150, 156, 172, 173, 187)</td>
</tr>
</tbody>
</table>

MMPs, matrix metalloproteinases; NE, neutrophil elastase; RCL, reactive center loops; SLPI, secretory leukocyte protease inhibitor; TIMPs, tissue inhibitors of metalloproteinases; WAP, whey acidic protein domain.

are decreased (40, 120). Moreover, Mercer et al. (120) demonstrated that during a COPD exacerbation there was a shift in the protease/antiprotease molar balance, with an increase in MMP9 and a decrease in TIMP1. In the context of viral infection, overexpressing TIMP-1 blocks respiratory syncytial virus syncytia formation (191). However, since MMP activity is directly implicated in viral activation and viral entry/fusion, there are limited studies that investigate the direct antiviral effects of TIMPs.

Serpins. Serpins are the largest and most broadly distributed superfamily of protease inhibitors. The majority of serpins inactivate serine proteases, but a small portion of serpins also inhibits caspase and cysteine proteases (141, 154). Serpins exert their antiprotease activity by undergoing irreversible conformational changes that allow for the interaction and inactivation of serine proteases. To inhibit protease activity, serpins form long, flexible reactive center loops (RCL) that insert into the active site of the targeted protease. These RCL structures form above the serpin framework and allow for the NH2-terminal portion of the RCL to insert into the protease active site. At this time, the protease and serpin remain covalently linked, the protease structure is conformationally changed, and the protease activity is significantly reduced (101). This action leads to the permanent deactivation of both serpin and protease, often described as a ‘suicide’ event.

SerpinA1 encodes the most abundant antiprotease in the lung, alpha-1 antitrypsin (α-1AT). Point mutations of α-1AT generate a misfolded version of this protein, resulting in an α-1AT deficiency (109). α-1AT-deficient individuals are at a high risk for developing emphysema and COPD (168, 169). Respiratory epithelial cells secrete α-1AT in both the apical and basolateral compartments of the cell (159). Additionally, serpins have been shown to block viral entry of enveloped viruses such as human immunodeficiency virus (HIV) and herpes simplex virus (9, 138). Sme et al. (161) showed that in vitro serpin antithrombin III treatment inhibited IAV infection nearly 100-fold more than ribavirin, a nucleoside inhibitor used to halt IAV replication. The authors found that this effect was dependent on viral HA with decreasing efficacy in order of H1N1>H3N2>H5N1 (161), suggesting that serpin activity may parallel the TTSP activity in the context of IAV infections, which is dependent on amino acid sequence, protein structure, and/or HA substrate accessibility. A recent study demonstrated that plasminogen activator inhibitor 1 (PAI-1), encoded by SerpinE1, inhibited HA cleavage and reduced infectivity of progeny virions (44). Specifically, these studies demonstrated that antiproteases, such as PAI-1, regulate the spread of IAV infection by blocking proteolytic maturation of newly formed virus particles and propagation of the infection. Thus protease/antiprotease balances may be important mucosal regulators of IAV spreading.

Trappin-2 and elafin function as alarm antiproteases, as both are produced in response to inflammatory stimuli (136). In respiratory epithelial cells, elevated elafin levels are protective against recombinant NE treatment and products derived from activated human neutrophils in vitro (160). In addition to functioning as an alarm antiprotease, elafin possesses antimicrobial effects. Ghosh et al. (59) revealed that recombinant elafin was protective during HIV infection only when elafin was preincubated with HIV, suggesting that elafin directly interacts with HIV virion and prevents viral infection. Interestingly, this group found that secreted elafin levels were elevated in the cervicovaginal lavages of HIV-negative women compared with HIV-positive women, albeit not statistically significant, suggesting that elafin secretions may be decreased after HIV infection. Similarly, after HRV infection, elafin is downregulated in subjects with COPD (113). These findings suggest that, despite elafin functioning as an alarm mediator, elafin secretions may be decreased after viral infection, thus enhancing susceptibility to viral infection.

SLPI. SLPI is an additional alarm antiprotease that is highly expressed by epithelial cells, neutrophils, and macrophages. SLPI can be detected in respiratory secretions, saliva, seminal fluid, cervical mucus, tears, and cerebral spinal fluid (78, 151).
Comprised of two closely related domains, the tertiary structure of SLPI resembles a boomerang (48). Similar to the trappin family, SLPI contains a cysteine-rich WAP COOH-terminal domain that is responsible for protease inhibition as well as the NH2-terminal domain that stabilizes the protease/antiprotease complex and exerts antimicrobial activity (192). The COOH-terminal active site, which confers antiprotease activity, is comprised of leucine and methionine residues (50, 181).

The main function of SLPI is to inhibit serine proteases such as NE (its main target), cathepsin G, elastase, and chymase (62, 184). SLPI further contributes to respiratory host defense by modulating inflammation and inducing wound healing. SLPI exerts cellular anti-inflammatory effects by preventing the degradation of regulatory components of the NF-κB pathway and by directly competing for NF-κB binding sites in the promoter regions of proinflammatory cytokines such as IL-8 and tumor necrosis factor-α (TNF-α) (67, 172, 173). SLPI is also implicated in wound healing as reported by Ashcroft et al. (8), who investigated the role of SLPI in a mouse model of dermal wounds. Using this model, the authors found that SLPI knockout mice had impaired wound healing due to increased NE activity.

SLPI possesses broad antimicrobial effects. The NH2-terminal domain of SLPI possesses extracellular antibacterial activity by disrupting Staphylococcus aureus and Escherichia coli membranes (79). Additionally, SLPI binds to Opa protein of Neisseria gonorrhoeae, which leads to the lysis of bacterial membranes (37). Fahey et al. (53) reported that increased extracellular SLPI secretion was correlated with elevated bactericidal activity and treatment with an anti-SLPI antibody significantly diminished bactericidal activity. SLPI has been shown to exert intracellular antibacterial effects by disrupting translation of E. coli during bacterial replication (124). Furthermore, SLPI exerts fungicidal and fungistatic activity toward Aspergillus fumigatus and Candida albicans that is also dependent on the NH2-terminal domain of SLPI (179).

In the context of viral infections, SLPI inhibits HIV, human papillomavirus (HPV), and respiratory viruses. McNeely et al. (117) first detailed the anti-HIV activity of SLPI in human saliva. The authors demonstrated that SLPI was highly expressed in the saliva and inhibited HIV infection in monocytes and T cells in dose-dependent fashion. Further studies revealed that SLPI exerts anti-HIV properties not through direct interaction with the virus, but rather through interacting with the target host cell (86). However, this group found that, despite decreasing viral load, SLPI did not alter the infectivity of progeny virions, suggesting that SLPI inhibits HIV at the early stages of viral infection (118).

SLPI is also associated with protection from human papillomavirus, with two recent studies demonstrating that high levels of SLPI, possibly induced by cigarette smoking, correlate with protection against HPV infection (81, 137). Additionally, SLPI is induced and protective during respiratory virus infection. Kido et al. (93) first demonstrated that SLPI decreased infectivity of IAV and Sendai viruses. Moreover, reports have revealed that intranasal administration with recombinant SLPI (rSLPI) decreased progeny virus nearly 3,000-fold compared with vehicle-treated mice infected with mouse-adapted IAV (95). Furthermore, the authors also reported that in mice administered rSLPI nearly all the progeny viruses released from infected cells were secreted as noninfectious HA0 precursor virions (95). Additionally, we have shown that recombinant human SLPI inhibits IAV replication in vitro (91).

Although SLPI contributes to respiratory host defense by decreasing inflammation and infection, SLPI can also induce proinflammatory responses. Mulligan et al. (126) demonstrated that posttranslational modifications of SLPI increased IL-8 secretion, neutrophil recruitment, and vascular permeability. Extracellular SLPI can be posttranslationally modified by respiratory proteases such as cathepsin L, MMP12, chymase, and NE, resulting in cleavage of SLPI (12, 116, 140, 174). Cleaved SLPI levels are elevated in COPD patients during respiratory infection, which renders SLPI nonfunctional (1, 113, 135). Moreover, we have shown that in nasal lavage fluid from smokers, SLPI is posttranslationally processed and cleaved (121). Reactive oxygen intermediates generated in the context of environmental exposures to oxidant pollutants, such as cigarette smoke, modify the active site and decrease the antiprotease properties of SLPI (29, 183). Whereas SLPI exerts powerful protective effects in the respiratory tract, posttranslational alterations and/or oxidative modifications can drastically shift the activity of SLPI toward potentially damaging effects and may reduce its antiviral properties.

The protease/antiprotease balance is a delicate interaction of enzymes and proteins that are involved in respiratory function. In the healthy lung, proteases are critical for cellular regeneration, cellular repair, and tissue homeostasis. Furthermore, antiproteases are important for protease inhibition and contribute to host defense. In the healthy lung, although there are many types of proteases and antiproteases expressed, a balance between proteases and antiproteases is established to ensure respiratory homeostasis. Balance will depend on the overall presence, timing of protease and antiprotease production/release, and activity. A shift in the balance toward increased protease expression and activity can lead to overt inflammation and the development of chronic lung disorders such as COPD and emphysema (1, 90). Moreover, respiratory serine proteases, such as the TTSP family, activate a variety of respiratory viruses for enhanced rates of viral infection. Additionally, antiproteases, such as SLPI, inhibit the function of serine proteases and block viral entry into the target cell. Hence the protease/antiprotease balance not only is a critical component for respiratory homeostasis but also is a powerful determinant of respiratory viral pathogenesis.

Nrf2 Decreases Oxidative Stress, Modifies Components of the Protease/antiprotease Balance, and Protects Against the Development of Lung Disease and Viral Infection

The respiratory epithelium is constantly being exposed to inhaled insults, which results in the generation of reactive oxygen species, free radicals, and peroxides (13). Oxidative stress has been associated with cancer, cardiovascular diseases, neurodegenerative diseases, and chronic inflammation (49, 89). Cells are equipped with various mechanisms, such as antioxidant responses, to block these damaging effects (71, 72). One mechanism involves nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a key transcription factor that regulates transcription of many antioxidant and detoxifying enzymes (32, 63).

Under baseline conditions, Nrf2 is sequestered in the cytoplasm by kelch-like ECH-associated protein 1 (Keap1), and
upon stimulation Nrf2 is released from Keap1 and Nrf2 translocates into the nucleus and binds to antioxidant response elements (ARE) (85). ARE binding sites are located on the promoter regions of phase II enzymes such as NADPH quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) (33). The Nrf2-dependent phase II response has been implicated in the development of chronic lung diseases since antioxidant capacity and Nrf2 protein levels are decreased in the lungs of COPD patients (112). Iizuka et al. (83) reported that Nrf2-deficient mice exposed to cigarette smoke developed emphysema-like qualities and were unable to induce antiproteases such as SLPI. SLPI contains ARE regions on its promoter and we have previously demonstrated that Nrf2 agonists enhance SLPI expression in airway epithelial cells in an Nrf2-dependent manner (122), suggesting that oxidative stress responses modulate SLPI regulation.

In addition to its role in maintaining oxidant/antioxidant homeostasis, we and others have previously shown that Nrf2 is critical for respiratory epithelial responses to respiratory viral infection (31, 92). Nrf2 has been shown to regulate and decrease TMPRSS2 expression in prostate cancer cell lines, suggesting that in addition to inhibiting respiratory oxidative stress, Nrf2 alters components of the protease/antiprotease balance and protects against respiratory infection (155). Thus strategies activating Nrf2 may be a potential mechanism to not only reduce oxidative stress, but also protect against viral infection.

Antioxidants are an attractive strategy to modulate the protease/antiprotease balance and protect against chronic lung disease and viral infection. Antioxidants have garnered much attention due to the inhibition of respiratory oxidative stress yet may also serve as therapies to modify the protease/antiprotease balance and inhibit viral infection (88). Antioxidants are a broad class of molecules that can be found in a variety of foods and have been shown to exert potent host defense effects. One potent class of antioxidants includes flavonoids, which are nonessential, polyphenolic phytomolecules that occur naturally in many plant-based foods and possess profound effects on human health (38). Over 8,000 flavonoid compounds have been identified, and they can be broken down into six groups: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavan-3-ols (43). Flavan-3-ols (or commonly referred to as flavanols or catechins) are the most common flavonoid consumed in the American diet and can be found in red wine, tea, apple, grapes, and chocolate (7).

Flavan-3-ols found in green tea, such as epigallocatechin-3-gallate (EGCG), have been studied extensively in the context of a viral infection. EGCG has been shown to bind to CD4 T cell receptors and prevent interaction with gp120, a HIV envelope protein (87). Nance et al. (128) showed that EGCG was able to inhibit HIV infection of human T cells and macrophages at physiological doses. Because of these effects, use of EGCG as an anti-HIV therapeutic has entered preclinical investigation. Furthermore, EGCG possesses potent anti-inflammatory properties. EGCG agglutinates influenza viruses and inhibits viral replication, which was dependent on influenza subtype (127, 163). Ling et al. (108) corroborated these findings, showing that oral administration of EGCG in mice infected with influenza had nearly a 50% decrease in viral titers and a 50% increase in survival rates. We have shown that EGCG induced SLPI secretion, reduced TMPRSS2 secretion, and decreased influenza replication (91). Furthermore, we previously reported that EGCG blocked influenza entry into target epithelial cells (92). Together, these results suggest that antioxidant supplementation with flavan-3-ols may serve as an attractive therapy to protect against lung disease and infection.

SFN alters SLPI and TMPRSS2 levels, enhances lung host defense responses, and protects against infection. Another potent antioxidant is sulforaphane (SFN), an isothiocyanate that induces respiratory host defense by decreasing oxidative stress and inflammation (111). SFN also exerts broad antimicrobial effects. SFN supplementation of alveolar macrophages from COPD patients increases clearance of Pseudomonas aeruginosa (74). Furthermore, SFN elevates bacterial detection and induces phagocytosis in alveolar macrophages from COPD patients, which is dependent on Nrf2 activation (74).

SFN is derived from the hydrolysis of glucosinolates found in cruciferous vegetables such as broccoli, brussels sprouts, and cabbage. Using in vitro models of respiratory epithelial cells, SFN supplementation enhances Nrf2 activity, induces cellular antioxidants such as HO-1 and NQO1, and inhibits proinflammatory cytokine release (106, 145, 165, 177). Furthermore, Riedl et al. (143) reported that in vivo SFN supple-
mentation enhanced respiratory phase II enzyme expression, indicating that nutritional SFN supplementation induces respiratory antioxidant responses.

Beyond its antioxidant function, SFN may have important functions in modulating components of the protease/antiprotease balance. We have shown that SFN supplementation transcriptionally enhances SLPI expression, resulting in increased SLPI secretion in the nasal mucosa in an Nrf2-dependent fashion (122). SFN also inhibits MMP expression and inflammation (4, 114, 146). SFN decreases TTSPs, such as TMPRSS2, by downregulating androgen receptor (AR) signaling, a receptor involved regulation of TMPRSS2 expression (61). Recent reports from Schultz et al. (155) have confirmed and expanded these findings showing that Nrf2 negatively regulates AR transactivation of androgen response genes such as TMPRSS2. The authors found that nuclear factor (erythroid-derived 2)-like 1, a cytoplasmic transactivator of the AR, is sequestered in the nucleus when Nrf2 is induced. These studies reveal that SFN increases SLPI secretion and decreases TMPRSS2 expression in a variety of models, as summarized in Fig. 2.

Indeed, our studies indicate that SFN is protective against respiratory viruses such as IAV. We have shown that SFN decreases IAV replication by reducing IAV entry into respiratory epithelial cells (92). Furthermore, we and others have shown that SFN decreases TMPRSS2 expression, which we speculate is mediating the SFN-dependent protection against influenza (unpublished data) (61, 155). Furthermore, our group recently showed that sulforaphane-containing broccoli sprouts significantly decreased IL-6 and markers of viral replication in nasal lavage fluid from smokers in subjects inoculated with the live attenuated influenza virus vaccine (131). These results indicate that SFN may be a safe, low-cost intervention for decreasing influenza infection in susceptible populations such as smokers. On the basis of the data summarized above, we hypothesize that the anti-IAV effects of SFN may be mediated

Fig. 3. The protease/antiprotease balance regulates influenza A virus (IAV) infection and can be modulated by SFN supplementation. 1: For entry into target host cells, the hemagglutinin (HA) protein on the IAV virion must be proteolytically cleaved from HA0 (purple) into fusogenic HA1 and HA2 proteins (blue). Respiratory serine proteases such as TMPRSS2 (TM), the human airway trypsin-like protease (HAT), and matriptase have been implicated in HA activation and IAV infection, which are likely to strain specific. Although antiproteases such as SLPI inhibit serine protease activity and protect against IAV infection, it remains unknown what HA-activating protease is inhibited by SLPI and how SLPI mediates these effects. 2: Lastly, SFN supplementation can be used to increase SLPI secretion, reduce TMPRSS2 secretion, and decrease viral entry and downstream IAV infection.
in a two-pronged approach: 1) by inducing SLPI into the nasal mucosa, which protects against influenza entry into respiratory epithelial cells, and 2) by decreasing TMPRSS2 secretion, which decreases HA activation and IAV replication (Fig. 3).

Summary

It is evident that respiratory epithelial cells contribute to respiratory health beyond barrier function and initiating immune responses. Respiratory epithelial cells secrete a variety of host defense mediators, including protease/antiprotease balance, that are involved in respiratory homeostasis and have been implicated in inflammation and viral infection. This balance can be modified by inhaled oxidants, such as cigarette smoke, and nutritional antioxidants, such as SFN. Thus employing the use of nutritional antioxidants to decrease specific proteases and increase antiproteases could protect against airway inflammation, lung disease, and infection. In addition, nutritional antioxidants could be a potential therapy during influenza pandemics, especially in the context when the influenza vaccine strains do not offer adequate protection.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.M. and I.J. prepared figures; M.M. and I.J. drafted manuscript; M.M. and I.J. edited and revised manuscript; M.M. and I.J. approved final version of manuscript.

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