Malformed Protein Structure and Proteostasis in Lung Diseases

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Abstract

Recent discoveries indicate that disorders of protein folding and degradation play a particularly important role in the development of lung diseases and their associated complications. The overarching purpose of the National Heart, Lung, and Blood Institute workshop on “Malformed Protein Structure and Proteostasis in Lung Diseases” was to identify mechanistic and clinical research opportunities indicated by these recent discoveries in proteostasis science that will advance our molecular understanding of lung pathobiology and facilitate the development of new diagnostic and therapeutic strategies for the prevention and treatment of lung disease. The workshop’s discussion focused on identifying gaps in scientific knowledge with respect to proteostasis and lung disease, discussing new research advances and opportunities in protein folding science, and highlighting novel technologies with potential therapeutic applications for diagnosis and treatment.

Keywords: protein misfolding; post-translational processing; proteosome; ubiquitination; pulmonary health

At a Glance Commentary

Scientific Knowledge on the Subject: Proteostasis refers to a collection of cellular processes handling protein folding, misfolding, unfolding, and degradation. Recent studies have indicated an important role for proteostasis in the development of lung diseases and their associated complications.

What This Study Adds to the Field: The overarching purpose of the National Heart, Lung, and Blood Institute workshop on “Malformed Proteins and Proteostasis in Lung Diseases” was to identify research opportunities to advance our mechanistic and clinical understanding of proteostasis in lung pathobiology.

The complex cell biology and physiology of the human lung and its integration with neural, vascular, muscle, and immune organ systems represents a remarkable juxtaposition of protein folding forces that manage the cellular, tissue, and organismal

response to the environment. The conducting airways and alveoli remove oxygen and excrete carbon dioxide from nearly one-quarter of a billion liters of air and a similar volume of blood over a normal lifespan to dynamically maintain

mitochondrial energy homeostasis throughout the body (1). This function of the lung requires that a large airway and epithelial surface area is directly exposed to the environment (1). The epithelium must not only protect itself from oxidative

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stress imposed by the very substrate (oxygen) that is so essential to life but also detect, remove, and coordinate an immune response to airborne particulate matter, toxins, and pathogens. It is, therefore, not surprising that lung diseases collectively represented the third most common cause of mortality in the United States in 2011, with an unacceptably high associated morbidity (2). Overall, 6.3% of U.S. adults and 11.6% of those older than 65 years of age have chronic obstructive pulmonary disease (COPD). Lung cancers are the most common cause of cancer-related death, and pneumonia was the eighth leading cause of death in the United States in 2011 (3).

Constant mechanical stress and diverse forms of environmental stress represent a constant threat to normal protein folding in the lung. To meet this challenge, the lung is equipped with a robust proteostasis network that prevents protein misfolding and repairs or recycles damaged proteins. Proteostasis refers to a collection of cellular processes handling protein folding, misfolding, unfolding, and degradation (4). Normal proteostasis is achieved by a complex, highly dynamic, and interactive set of cellular systems in subcellular compartments. Alterations in proteostasis can result in loss of protein function (e.g., insufficient amounts of active protein) and/or gain of toxic function (e.g., aggregation of misfolded proteins) (5). Proteostasis is fundamental to cell survival and function and critical for all organs and tissues. The ability to handle proteostatic stress declines with age, making it likely that the public health and economic impact of proteostasis dysfunction will increase as the population ages (6).

To explore proteostasis as a new therapeutic paradigm for pulmonary disease, research is needed to delineate mechanisms coupling protein folding and degradation to the development of lung diseases and their associated complications (4). In response to this need in lung research, the Division of Lung Diseases in the National Heart, Lung, and Blood Institute (NHLBI) convened a workshop, “Malformed Protein Structure and Proteostasis in Lung Diseases,” on November 19 and 20, 2012, focused on identifying gaps in scientific knowledge with respect to proteostasis and lung disease, discussing new research advances and opportunities in protein folding science, and highlighting novel technologies with potential

therapeutic applications for the diagnosis and treatment of lung diseases.

Proteostasis: The Underappreciated Second Half of Protein Biology

The protein life cycle is defined by two stages. In the first stage, protein synthesis is regulated at the level of transcription, RNA stability, and translation. In the second and less well-studied stage, proteostasis mechanisms are required for protein folding, processing, trafficking, and degradation (5, 7, 8). Unless this second stage is successful, the protein will not be available in sufficient quantities or will be improperly folded, potentially affecting its function or localization within the cell. A major task of the proteostasis network is to ensure protein quality control. Up to 30% of newly synthesized proteins are not properly folded and are targeted for immediate degradation (9–11). Disruption of the proteostasis program inevitably results in the aggregation of misfolded proteins and proteotoxicity in cells that can trigger disease and cell death (12–15). Even more importantly, proteostasis must function as a true network to maintain functional conformation of all proteins, because misfolding of one protein can trigger the mishandling of hundreds of seemingly unrelated proteins in the cell (16). A more

complete understanding of proteostasis is essential for defining normal cellular function and the cellular response to environmental stress (Figure 1). The proteostatic network includes approximately 2,500 genes (>10% of the total number of protein coding genes), organized into regulatory networks, each of which serves a specific function in maintaining the stability and functional conformation of each protein in different types of cells (17). How most proteins interact with the proteostasis machinery during the process of folding is poorly understood, and even less is known with respect to the changes in proteostasis in response to stress. Research is needed to understand how proteostasis networks maintain cellular functions in response to internal and external perturbations over the lifetime in different types of lung cells.

Proteostasis includes an extensive array of protein synthesis, folding, and fold-management mechanisms that begin during translation of the protein and persist through post-translational processing and degradation (5). Proteostasis machinery can be found in the nucleus, cytosol, and numerous membrane trafficking compartments, including the exocytic–endocytic membrane trafficking axes (7), autophagy–lysosome axis (13), and mitochondria (18). Proteostasis includes an extensive system of molecular chaperones that ensures proteins are maintained in an optimal functional state. Cellular or environmental perturbations cause

Figure 1. Key processes in the second half of the protein life cycle. (1) About 70% of newly synthesized proteins fold into functional proteins (assisted by chaperones); (2) a major knowledge gap exists about the role of misfolded or unfolded proteins in lung diseases; (3) genetic mutations or environmental risk factors can cause protein misfolding or unfolding; (4) the unfolded protein response refolds unfolded proteins using chaperones; (5) proteins that fail to refold are degraded by the ubiquitin proteasome system and autophagy–lysosome pathways.

protein unfolding or misfolding that will push healthy cells toward disease if misfolded proteins cannot be repaired or degraded by adaptive responses within the proteostatic network. These response mechanisms are aimed at preserving proteostasis and include activation of the heat-shock response (16), antioxidant and redox signaling (ARS) (19), unfolded protein response (UPR) (20), mitochondrial response (mitoUPR) (18), and histone acetylation or deacetylation pathways (21, 22). Recent work highlights the importance of the latter pathways in modifying the behavior of the protein fold through acetylation of surface lysine residues, which compete with degradation tagging pathways, including ubiquitination. This is just one example of how profolding pathways are tightly coupled to protective degradative pathways, which include the ubiquitin–proteasome system (UPS) (23) and the autophagy–lysosome pathways (ALPs) (13). Proteostasis, therefore, is a complex adaptive system (Figure 2), which regulates protein

folding, misfolding, transport, and degradation. Environmental, inflammatory, or genetic perturbations in the lung can result in enhanced physical, oxidative, and metabolic stress, presenting a major challenge to the signaling pathways that generate and maintain the proteostasis network in the airways and alveoli (24, 25).

Delineating the Role of Proteostasis in Lung Disease

Genetic mutations and many physiologic and pathologic stresses can induce protein misfolding in cells (4, 26). Genetic mutations within protein coding regions cause inappropriate folding and localization of proteins in cystic fibrosis (CF), α_1 -antitrypsin deficiency (α_1 AD) (27–29), pulmonary fibrosis (surfactant protein C) (30, 31), and three subtypes of Hermansky-Pudlak syndrome (32). Genetic mutations outside the MUC5B protein coding region

alter secretion of MUC5B into the airway lumen and may contribute to or result from stress in the proteostasis network of the airway epithelium in patients with CF, idiopathic pulmonary fibrosis (IPF), and COPD (33–35). We now appreciate that mutations within a protein coding region can alter protein synthesis, folding, conformation, and function. Recently, NHLBI's exome sequencing project reported the discovery of more than 1 million single nucleotide variants in more than 15,000 protein coding regions (36). Although nonsynonymous (missense and nonsense) mutations may simply overwhelm the cell's capacity for folding and/or degradation, synonymous mutations could also affect protein biogenesis and function due to altered splicing, transcription, or translation. They may thereby increase the risk for early- and late-onset disorders, depending on the allele and the cell's proteostasis capacity. It will be important to delineate how single nucleotide variants are coupled to

Figure 2. Proteostasis management of pulmonary health at a glance. Pulmonary health (inner circle) requires that constant pulmonary stresses (listed in the second layer) are accurately handled by proteostasis management pathways (listed in the third layer). The workshop identified knowledge gaps, timely scientific questions, and research tool needs to catalyze the application of proteostasis science to improve our understanding of lung disease pathophysiology and new approaches for treatment/management (outer layer and left panel).

mechanisms of lung disease through alterations in protein synthesis, folding conformation, and/or protein degradation pathways.

Genetic mutations are not the only cause of protein misfolding/unfolding. Environmental, physiologic, and pathologic stresses relevant to lung cells, including perturbations in pH, temperature, osmotic pressure, mechanical strain, infection, inflammation, oxidative stress, particulate matter, hypercapnia, hyperoxia, and hypoxia, may also induce general protein misfolding. Oxidative stress and immune dysfunction each induce proteostatic stress and are hypothesized to contribute to the development of COPD and muscle wasting observed in those patients (37, 38). Multiple factors contribute to oxidative stress in the injured lung, which can impair proteostasis (39). Inappropriate activation of the degradative UPS and ALPs has been associated with excessive degradation of skeletal muscle fibers resulting in muscular dysfunction, the major cause of morbidity in survivors of acute lung injury (ALI) and sepsis and an important problem in patients with COPD (40–43). Autophagy has been linked to lysosomal pathways that play a critical role in the cellular defense against proteostatic challenges by buffering metabolic stress and allowing cellular reorganization and remodeling required for tissue repair after injury during ALI and sepsis (44).

Recent studies suggest that proteotoxic stress is a common feature of normal human physiology and is exacerbated in many late-onset complex diseases, including COPD, IPF, pulmonary arterial hypertension, vascular injury, and muscle dysfunction. In response to acute injury or chronic insult (e.g., cigarette smoking, pollution, infections, etc.), misfolded proteins not only challenge the biosynthetic pathways, thereby facilitating proteome genesis and repair, but also overwhelm the ubiquitin proteasome and autophagy pathways for protein degradation, thus exacerbating proteotoxic stress (45–47). Chronic activation of proteostasis responses to prolonged protein misfolding, as occurs during aging, may result in oxidative inflammation (chronic bronchitis associated with cigarette smoking and infection), cellular death (emphysema), protein aggregation (interstitial lung disease/diffuse lung disease), inflammation (asthma and COPD), and cellular dysfunction, all of which have been observed in patients with

lung disease both within the lung parenchyma and in distally affected organs (e.g., skeletal muscle) (41, 45, 48–50). Indeed, prolonged protein folding stress has been hypothesized to play an important role in the global phenotypic changes of the lung associated with aging, particularly in response to the numerous environmental pulmonary stressors that occur during a normal and stressed health span (6). Sleep deficiency (e.g., obstructive sleep apnea, sleep deprivation) is a prevalent comorbidity in chronic lung disease and results in pathophysiology reflecting that involved in lung disease (e.g., oxidative stress, systemic inflammation, and autonomic dysregulation [51, 52]). Recent data indicate that sleep apnea-related intermittent hypoxia, sleep deprivation, and impaired sleep quality result in activation of endoplasmic reticular stress pathways in the brain, including chaperone function (BiP), ER-associated degradation (XBP1), protein translation inhibition (PERK, eIF2 α), and apoptosis (CHOP, GADD34) (51, 53). These findings point to the need for research to examine whether comorbid sleep deficiency is contributing to impaired proteostasis specifically in the lung, in the context of lung disease. If comorbid sleep deficiency emerges as a mechanistic modifier of lung pathophysiology, treating sleep deficiency could be tested for efficacy in the management of lung disease.

Age-related Loss of Proteostasis Capacity May Contribute to the Delayed Onset of Lung Disease

The lung has a large epithelial surface area ($\sim 100\text{ m}^2$), which is exposed to the environment throughout life (54). Environmental perturbations and mechanical stresses continuously trigger protein misfolding or unfolding that must be managed by adaptations in the proteostasis machinery. Interestingly, although disorders of proteostasis can occur at any age, it is more typical for these disorders to develop after a long lag time. For example, even well-described genetic mutations that cause severe protein misfolding, including mutations in CFTR and α_1 -antitrypsin, can take decades to manifest clinically (55, 56). Similarly, mutations in surfactant protein C or the MUC5B promoter associated with pulmonary fibrosis do not result in clinical

disease until the fifth or sixth decade of life (31, 33). It has been demonstrated in controlled experiments that smoking just one cigarette (10 puffs) interferes with oxidative protein folding in lung cells of mice and triggers down-regulation of CF transmembrane conductance regulator gene (CFTR), decreasing cell surface hydration and triggering mucous dysregulation (49). Despite this, COPD occurs after many years of cigarette exposure (57).

A potential explanation for these observations is that proteostatic capacity diminishes significantly during normal aging (6). The molecular mechanisms underlying this decline are not known; however, cumulative exposure to inflammatory, oxidative, or other environmental stress may be important. These findings suggest that enhanced proteostasis in younger individuals might compensate for some mutations associated with misfolding, whereas the combination of the progressive loss of proteostatic capacity over time and exposure to noxious factors overwhelms the system, and disease becomes evident. Research is needed to more clearly define the mechanisms that drive the loss of proteostasis capacity with age and in response to environmental stressors. Strategies that preserve or increase this capacity might delay the onset of lung disease even in individuals with unmodifiable risk factors (e.g., a history of cigarette smoking or high risk genetic mutations).

Applying Proteostasis Science to Advance Pulmonary Health

The workshop participants highlighted opportunities to elucidate mechanisms linking normal and disordered proteostasis to the pathophysiology of lung disease and to apply these discoveries to improvements in the diagnosis, management, and prevention of lung disease and associated complications. There is strong evidence showing involvement of the protective (molecular chaperones, protein folding, and trafficking) and degradative (ubiquitin proteasome degradation and autophagy) processes of proteostasis in COPD, asthma, interstitial lung disease/diffuse lung disease, IPF, ALI, and pulmonary arterial hypertension (4). Factors potentially contributing to altered proteostasis in the lung include cellular toxicity induced by misfolded native or mutant proteins, defects

in components of the proteostasis machinery, and disruption of normal proteostatic signaling. These factors can be exacerbated by superimposed environmental and nutritional insults as well as physiological processes, such as the metabolic syndrome, sleep-disordered breathing and sleep deficiency, and aging. Research is needed to develop a more complete understanding of proteostasis in human airway and lung biology, including the delineation of proteostasis pathways directing lung function in health and disease, the development of tools to monitor proteostasis *in vivo* and *in vitro*, and the development of new cell-based and organismal models that recapitulate the regulatory processes of proteostasis in the lung. Experimental approaches could include the contribution of native and mutant human genes to proteostasis in model organisms (e.g., *Caenorhabditis elegans*, transgenic or knockout animal models), the development of genetically modified animals expressing “reporters” of proteostatic stress, and/or improved models of environmental stress. Improved cellular models might include the identification of cell populations particularly prone to proteostatic stress, the development of induced pluripotent stem cells from patients with lung disease, polarized primary human bronchial epithelial cultures, and the development of organoid (artificial lung) systems. In addition, bioinformatic and systems biology approaches are needed to understand the interactions within the proteostatic network during health and disease. It is apparent that these studies will require a cooperative integration of basic scientists, clinicians, and bioinformatics experts.

Proteostasis: A New Direction in Airway Therapeutics Drug Discovery

An improved understanding of the proteostasis regulatory networks in the lung will open multiple new areas for the discovery of drugs and public health strategies to reduce the burden of lung disease. Advances in therapy for CF provide a remarkable example of how an improved understanding of proteostasis can fuel drug discovery. Ivacaftor (trade name Kalydeco, developed as VX-770) acts as a potentiator to improve the function of the mutant CFTR protein (i.e., G551D mutation) at the cell membrane in patients with CF through modification of mutant channel function. Another drug,

VX-809, and the related compound VX-661 appear to restore function of the most common CFTR mutant ($\Delta F508$) by improving the proteostasis network to manage folding and trafficking of CFTR protein to the plasma membrane (58). Clinical trials have recently demonstrated that ivacaftor can be combined with VX-661 to treat patients with CF attributable to $\Delta F508$ mutations (59).

Therapies developed to treat specific genetic causes of disordered proteostasis might impact the care of patients with other lung diseases. For example, ivacaftor has been suggested to protect against cigarette smoke-induced COPD by promoting airway hydration and mucociliary clearance (60). Therapies targeting histone acetyltransferases, particularly HDAC2 (61), likely operate through proteostatic mechanisms, as illustrated in studies using mutant forms of CFTR and α_1 -antitrypsin (21, 27). Even crude manipulations of proteostasis with bortezomib, an inhibitor of the 26S proteasome, have proven remarkably effective for the treatment of multiple myeloma and fibrosis in lung and skin (62, 63). Substantial refinements to these types of therapies are on the horizon. For example, proprotein convertase subtilisin/kexin 9 (PCSK9) is a serine protease that binds to an extracellular domain of the low-density lipoprotein receptor, targeting it for degradation in the lysosome (64). In a recent clinical trial, an antibody to PCSK9 lowered low-density lipoprotein cholesterol levels by up to 61% in patients with persistent hypercholesterolemia, despite statin therapy (65). Similar approaches might identify one or more therapeutic targets in proteostasis networks if we have better understanding of proteostasis in lung. There are more than 2,500 proteins identified to participate in the proteostasis network in the lung, and their roles in lung disease are largely unknown. New technologies are needed to monitor protein unfolding and protein degradation in real time, *in vivo* or *in vitro*, in the lung. Using a systems-based bioinformatics approach, these primary data can be integrated to model the proteostasis interactome in the lung. In summary, we have reached a tipping point where an investment in proteostasis research in the lung could pave the way for the rapid development of novel treatments for common lung diseases for which there are few or no specific therapies, including COPD, IPF, ALI, critical illness myopathy,

and others. Mitigating the enormous human and economic burden of these diseases would represent a huge public health advance.

Workshop Recommendations

In conclusion, the role of proteostasis in lung diseases is a novel and important area of investigation. This workshop acknowledged the timely opportunity to investigate the role of proteostasis networks in managing genetic mutations, especially those in Mendelian lung diseases, and to discover novel therapeutic targets within proteostasis networks. More importantly, it recognized that proteotoxic stress is a common feature of pathophysiology in many lung diseases and concluded that the lack of knowledge about the role of proteostasis in complex lung diseases is a major knowledge gap, which must be filled to have a better understanding of how environmental risk factors trigger the development of lung diseases in parallel with the aging process. The working group identified the following priority areas of research in lung proteostasis.

1. Define mechanisms leading to altered proteostasis in the lung (e.g., genetic, environmental, inflammation, comorbid conditions) and mechanisms coupling alterations in the proteostasis machinery to lung diseases (ALI, COPD, asthma, pulmonary fibrosis) and common associated complications (e.g., skeletal muscle wasting).
2. Investigate how the proteostasis network is affected by aging, obesity, and other comorbid factors in lung diseases. Develop a mechanistic understanding of how cumulative proteotoxic stress might accelerate or ameliorate age- or comorbidity-related changes in proteostasis capacity, including contributions to cell death and impaired lung regeneration. For example, do mechanisms underlying muscle dysfunction resulting from sepsis, pneumonia, or COPD differ in older or obese individuals, and do these individuals respond differently to therapies? Investigate proteostasis mechanisms in early aging of the lung and if these changes predispose to the development of chronic lung disease, such as COPD with and without lung cancer.
3. Develop pertinent cell- and animal-based experimental model systems to investigate proteostatic function

in vivo and in cell-based models of lung disease. For example, invertebrate models (e.g., *C. elegans*) could be used to investigate genetic mechanisms of oxidative stress in lung disease. Patient-derived cell lines (primary, induced pluripotent stem cells, and organoid cultures) could be used to apply and/or develop tools to assess the function of molecular chaperones and/or activation of signaling responses (heat-shock response, UPR, mitoUPR, UPS, ALP, ARS, histone acetyltransferase/histone deacetylase) critical to the etiology or exacerbation of lung disease. Mice expressing proteostasis reporters could be used to monitor the function of proteostasis networks in health and in models of lung disease.

4. Improve sampling from patients with lung diseases (blood, airway, and alveolar lining fluid and muscles) to assess proteostasis alterations and biomarkers in disease and response to therapy. Evaluate the potential of specific interventions on metagenomic, UPR, UPS, and autophagy mechanisms.
5. Delineate proteostasis-coupled mechanisms by which ALI, sepsis, and other critical illnesses that originate in the lung induce morbidity and mortality in extrapulmonary target organs (e.g., skeletal muscle, kidney, and heart).
6. Define how environmental stresses, including infection, hypoxia, hypercapnia, sleep-disordered breathing and sleep deficiency, and environmental contaminants modulate proteostasis

networks, as potential mechanisms of tissue damage, injury, and repair.

7. Develop new pharmacologic tools (e.g., small molecules) that target proteostatic pathways relevant to lung disease. Investigate the efficacy of targeted therapies aimed to manage proteostasis in the early and late stages of lung disease. Are there opportunities to slow or halt lung disease progression through proteostasis-targeted therapies?
8. Apply modern bioinformatic tools and approaches incorporating the proteasome into systems biology models of lung disease(s).
9. As a critical component of all of these research priorities, it is recommended that future efforts take an integrated approach:
 - a. Provide a platform to facilitate interdisciplinary interactions between experts in proteostasis and pre-clinical and clinical lung disease.
 - b. Develop resources for lung researchers to apply and/or develop new technologies in proteostasis science, including mass spectrometry, to monitor the onset and progression of diseases. A systems-based approach will be required to map these data to one another (developing a proteostatic network model) and to link these results to genetic and natural history of disease progression.
 - c. Use ongoing clinical trials, such as ARDSNet, IPFNet, and others, to assess the relationship between proteostasis health and related organ disorders, including muscle strength in intensive care unit patients. ■

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