HHS Public Access

Author manuscript

Mol Aspects Med. Author manuscript; available in PMC 2019 October 01.

Published in final edited form as:

Mol Aspects Med. 2018 October ; 63: 59–69. doi:10.1016/j.mam.2018.08.001.

OXIDATIVE STRESS IN CHRONIC LUNG DISEASE: FROM MITOCHONDRIAL DYSFUNCTION TO DYSREGULATED REDOX SIGNALING

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Abstract

The lung is a delicate organ with a large surface area that is continuously exposed to the external environment, and is therefore highly vulnerable to exogenous sources of oxidative stress. In addition, each of its approximately 40 cell types can also generate reactive oxygen species (ROS), as byproducts of cellular metabolism and in a more regulated manner by NOX enzymes with functions in host defense, immune regulation, and cell proliferation or differentiation. To effectively regulate the biological actions of exogenous and endogenous ROS, various enzymatic and non-enzymatic antioxidant defense systems are present in all lung cell types to provide adequate protection against their injurious effects and to allow for appropriate ROS-mediated biological signaling. Acute and chronic lung diseases are commonly thought to be associated with increased oxidative stress, evidenced by altered cellular or extracellular redox status, increased irreversible oxidative modifications in proteins or DNA, mitochondrial dysfunction, and altered expression or activity of NOX enzymes and antioxidant enzyme systems. However, supplementation strategies with generic antioxidants has proven minimally successful in prevention or treatment of lung disease, most likely due to their inability to distinguish between harmful and beneficial actions of ROS. Recent studies have attempted to identify specific redox-based mechanisms that may mediate chronic lung disease, such as allergic asthma or pulmonary fibrosis, which provide opportunities for selective redox-based therapeutic strategies that may be useful in treatment of these diseases.

Keywords

epithelium; asthma; fibrosis; NOX; sulfenylation; S-glutathionylation; ER stress

1. Introduction

The lung represents the human body's largest interface with the external environment, with an estimated surface area of ~150 m^2. This large surface area is critical for effective uptake

and delivery of oxygen (O_2) to all organs, but also renders the lung vulnerable to airborne pathogens and pollutants, and the respiratory tract is therefore equipped with elaborate antimicrobial and antioxidant defense systems to minimize infection or injury and maintain appropriate lung function. Epithelial cells that coat the entire lung surface form a critical component of such defense, as they form a physical barrier that prevents entry of inhaled antigens and pathogens and also act as a source of antimicrobial factors, high-molecular weight glycoproteins (mucins), iron-binding proteins (e.g. lactoferrin and transferrin), and various secreted antioxidant enzymes and low-molecular weight antioxidant molecules (e.g. ascorbic acid, GSH), which are deposited into the airway surface fluids to provide a first-line defense network against inhaled pathogens or oxidant pollutants. While lung host defense against most airborne pathogens largely relies on resident inflammatory cell types, such as alveolar macrophages, dendritic cells, and innate lymphoid cells, the respiratory epithelium also plays a critical role in mediating acute responses to these pathogens by regulating innate or adaptive inflammatory responses to these common environmental challenges (Holgate, Holloway et al. 2004, Hammad and Lambrecht 2008). Chronic diseases of the lung, such as asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, have diverse etiologies but also share common traits in that they often involve repetitive cycles of injury to the respiratory epithelium (due to inhaled pollutants such as tobacco smoke or pathogens), combined with dysregulated epithelial repair pathways and chronic activation of inflammatory processes, collectively leading to inappropriate production of airway mucus, increased fibroblast activation, myofibroblast differentiation, smooth muscle proliferation, etc, thereby resulting in airway remodeling and decline in lung function. In addition, these various processes are further compromised by diverse genetic factors and chronic bacterial or viral infections due to loss of host defense pathways.

Many commonly encountered environmental pollutants, such as tobacco smoke, particulate matter, or ozone or nitrogen dioxide from photochemical smog, are oxidizing in nature and are thus thought to cause lung injury by oxidative stress. In addition, reactive oxygen species (ROS) are produced by phagocytic cells by activation of NADPH oxidase (NOX) enzymes in their efforts to kill pathogens, and inappropriate ROS production during acute or chronic lung inflammation is commonly thought to contribute to impaired lung cell function and progression of lung disease (Andreadis, Hazen et al. 2003, Jacobsen, Taranova et al. 2007, Shaw, Berry et al. 2007, Fahy 2009, Wedes, Khatri et al. 2009). Mitochondria, present in all lung cell types, also produce ROS during incomplete reduction of O_2 to water (H_2O) in the electron transport chain, and mitochondrial ROS formation is typically increased due to mitochondrial dysfunction during chronic disease, potentially contributing to disease pathology. Contrasting these presumed damaging effects of NOX- or mitochondria-derived ROS, NOX-family enzymes are widely expressed in other lung cells, and participate in other cell functions such as cell proliferation, differentiation, etc., illustrating more widespread physiological properties of ROS (van der Vliet 2011, Segal, Grimm et al. 2012). Similarly, mitochondrial ROS (mtROS) are also increasingly appreciated to serve important functions in cell biology, for example in basal and adaptive responses that control homeostasis and promote health span (Sena and Chandel 2012, Ristow 2014, Shadel and Horvath 2015). Based on these considerations, the mechanisms by which ROS contribute to lung disease (as well as diseases of other organs) may be related to dysregulated redox signaling rather than

by non-discriminate oxidative biomolecular damage ("oxidative stress"). Because of the diverse biological functions of ROS in many diverse cell types, in both physiological and pathological outcomes, it is perhaps not surprising that generic antioxidant-based approaches to quench ROS have so far been minimally effective in mitigating chronic disease, including lung disease, and have in some cases even promoted adverse outcomes (Nadeem, Masood et al. 2008, Fortmann, Burda et al. 2013, Kirkham and Barnes 2013, Ristow 2014, Sayin, Ibrahim et al. 2014, Ghezzi, Jaquet et al. 2017). Therefore, recent research efforts have focused on identifying specific redox-based post-translational mechanisms that may mediate lung disease pathology, by addressing the involvement of specific cellular ROS sources and by characterizing critical protein targets and their redox-dependent modifications that induce functional alterations and mediate adverse biological outcomes. This review will briefly summarize the current evidence supporting dysfunctional ROS homeostasis in lung disease, and discuss some recent developments illustrating how selective targeting of specific ROS sources or redox signaling events may be more beneficial in combating chronic lung disease.

2. Sources of ROS in the lung – Location matters

2.1. Mitochondria

All cells in the body derive at least some of their energy from oxidative phosphorylation (OXPHOS) in mitochondria, during which O_2 is reduced to H_2O, typically leading to production of intermediate ROS ($O_2^{\bullet -}$ and H_2O_2) at complex I and III in the mitochondrial respiratory chain, the major site of cellular O_2 consumption (Drose and Brandt 2012, Lenaz 2012, Wong, Dighe et al. 2017). Since the lung is the organ exposed to the highest O_2 concentrations, such mitochondrial ROS (mtROS) production may be especially relevant to lung biology. The lung contains over 40 different cell types, which all contain varying mitochondrial densities. Among these, ciliated epithelial cells and secretory club cells that line the airways and alveolar type II cells that secrete surfactant are highly metabolically active cells and rich in mitochondria (Agrawal and Mabalirajan 2016). Most lung cells depend on aerobic glycolysis to supply carbon in the form of pyruvate to support OXPHOS and provide ATP as a constant supply of energy. Oxygen consumption rates in the lung are comparable to those in most other organs, and ATP content is also similar to that of other organs and mostly dependent on mitochondrial sources (Cloonan and Choi 2016, Piantadosi and Suliman 2017). Well-oxygenated tissues such as lung contain high levels of a unique isoform of the electron transport chain cytochrome oxidase complex IV subunit, COX IV isoform 2, which affects oxygen sensitivity, although the precise mechanisms and consequences are still not clear (Huttemann, Lee et al. 2012, Pierron, Wildman et al. 2012, Sommer, Huttemann et al. 2017).

Production of mtROS has typically been considered as a leak resulting from incomplete reduction of O_2, but more recent studies indicate that such mtROS release may function as a cytosolic signal to support mitochondrial integrity and organismal homeostasis (Sena and Chandel 2012, Shadel and Horvath 2015, Topf, Suppanz et al. 2018). The diverse nature of mtROS production, which originates from up to 11 different sites depending on bioenergetic conditions (Wong, Dighe et al. 2017), further illustrates the complex and variable biological functions of mtROS. Moreover, mitochondria are engaged in dynamic networks throughout

the cell, and factors that impact on mitochondrial fission/fusion dynamics or subcellular mitochondrial trafficking dictate the subcellular location of mitochondrial ATP or ROS to support specific cell functions (Schuler, Lewandowska et al. 2017, Lopez-Domenech, Covill-Cooke et al. 2018). Indeed, mitochondria in airway epithelial cells are not distributed randomly, but are localized primarily near the apical surface, and also in the basolateral region, indicating their specific functions in localized responses to e.g. external (apical) triggers (Ribeiro, Paradiso et al. 2003, Xu, Janocha et al. 2014). Thus, production of mtROS is a highly localized event, and likely effects cellular outcomes in a specific manner depending on location.

2.2. NADPH oxidases

A well-known major source of ROS during conditions of infection and inflammation is the activation of the so-called respiratory burst in phagocytic cells and other immune cells, due to assembly and activation of the NADPH oxidase complex within their phagosomes to generate intraphagosomal ROS to kill ingested microorganisms by oxidative mechanisms (Babior, Lambeth et al. 2002, Winterbourn, Hampton et al. 2006, Bedard and Krause 2007). We now know that multiple homologs of NADPH oxidase (NOX) exist that are more widely distributed across virtually all cell types, with a variety of biological functions beyond host defense (Bedard and Krause 2007, van der Vliet 2008). Similarly, NOX enzymes are widely distributed throughout the lung, with major roles in inflammatory/immune cells (alveolar macrophages, dendritic cells, T and B lymphocytes) as well as structural cell types (airway and alveolar epithelial cells, endothelial cells, fibroblasts, smooth muscle cells), each with distinct functional properties (van der Vliet 2011, Bernard, Hecker et al. 2014). Activation of NOX enzymes generates superoxide anion ($O_2^{\bullet -}$) and/or hydrogen peroxide (H_2O_2) as primary products (Geiszt and Leto 2004, Ameziane-El-Hassani, Morand et al. 2005, Martyn, Frederick et al. 2006), which in some specialized cases interact with locally secreted heme peroxidases (e.g. myeloperoxidase, lactoperoxidase) to generate secondary oxidants (e.g. nitrogen dioxide, hypochlorous acid, hypobromous acid) (Wijkstrom-Frei, El-Chemaly et al. 2003, Geiszt and Leto 2004, El Hassani, Benfares et al. 2005). In some cases, $O_2^{\bullet -}$ or H_2O_2 can also react directly with alternative susceptible cell targets, including redox-sensitive proteins, by which NOX enzymes control a variety of biological processes ranging from cell proliferation, differentiation, to inflammatory signaling and immune regulation. NOX-mediated signaling mechanisms are often related to regulation of common signaling processes involving protein phosphorylation and Ca^{2+} signaling (Finkel 2003, Forman, Fukuto et al. 2004, Terada 2006, Janssen-Heininger, Mossman et al. 2008, van der Vliet 2008). Specificity in such oxidative signaling is typically achieved by strict spatial localization of NOX activation and its target proteins, e.g. in endosomes or membrane lipid rafts (Terada 2006, van der Vliet 2008, Ushio-Fukai 2009). In fact, ROS can have opposing effects on biological processes, depending on the location or extent of ROS production, as illustrated by the ability of ROS to promote as well as inhibit activation of transcription factors such as nuclear factor (NF)-κB or HIF-1 (Brar, Kennedy et al. 2003, Li, Harraz et al. 2006, Janssen-Heininger, Mossman et al. 2008).

Recent observations also indicate the presence of reciprocal interactions between different NOX enzymes (e.g. (Heppner, Hristova et al. 2016, Kim, Kim et al. 2017) and also between

NOX enzymes and mitochondria (Dikalov 2011, Kroller-Schon, Steven et al. 2014), as examples of the more broadly known concept of ROS-induced ROS release (Zandalinas and Mittler 2017). These interactions imply that site-specific actions of specific NOX enzymes or mtROS can also induce more widespread oxidative events by more indirect mechanisms.

2.3. Other ROS sources

Other cellular sources of endogenous ROS production include cytochrome P450 enzymes, nitric oxide ($NO^{\bullet}$) synthases, as well as various metabolic enzymes involved in e.g. lipid biosynthesis and β-oxidation of amino acids (Schrader and Fahimi 2006, Antonenkov, Grunau et al. 2010), oxidative lipid metabolism by cyclooxygenases and lipoxygenases (Bae, Oh et al. 2011), enzymes involved in oxidative protein folding within the ER (e.g. endoplasmic reticulum oxireductin 1; ERO1) (Zito 2015), or the family of MICAL (for "molecule interacting with CasL") oxidoreductases (Fremont, Romet-Lemonne et al. 2017), and are typically countered by (organelle-specific) antioxidant enzymes, such as e.g. catalase (CAT) in peroxisomes (Fig. 1). The biological significance of ROS production from these latter sources is much less appreciated and studies of biological ROS-mediated signaling have focused primarily on regulated ROS production by NOX enzymes or mitochondria. Nevertheless, it should be evident from Figure 1 that cellular ROS-mediated events are highly localized and likely have widely distinct cellular consequences. Also, this implies that generic antioxidant-based therapeutic approaches lacking specificity towards specific cellular ROS events are not necessarily useful.

3. Oxidative stress and chronic lung disease – What is the evidence?

3.1. Direct evidence of oxidative stress in lung disease

Over the years, a large volume of studies has attempted to associate various measures of oxidative stress with disease progression, and this is also true for various chronic lung diseases such as asthma, COPD, pulmonary fibrosis, etc. Such approaches have largely been based on measurements of overall redox status (e.g. GSH redox status), lipid oxidation products, or analysis of stable oxidation products in proteins (e.g. protein carbonyls, nitrotyrosine, etc.) or DNA (e.g. 8-oxo-guanine), and have often shown an association of these outcomes with disease status (Andreadis, Hazen et al. 2003, Rahman, Biswas et al. 2006, Fitzpatrick, Park et al. 2014). However, clinical studies of antioxidant supplementation typically do not assess any of these oxidative markers. Therefore, evidence that any of these stable oxidative modifications actually contribute to disease progression is sparse.

3.2. Mitochondrial dysfunction

Increasing evidence indicates that mitochondrial integrity and function is impaired or altered in various chronic lung diseases, including asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, and lung cancer (Cloonan and Choi 2016, Piantadosi and Suliman 2017). Electron microscopy has revealed ultrastructural changes in airway epithelial mitochondria in mouse models of asthma or biopsies from human subjects with asthma, associated with biochemical features of mitochondrial dysfunction such as increased oxidative damage or activation of apoptotic pathways (Agrawal and Mabalirajan 2016). More recent studies also indicated increased mitochondrial number in airway epithelia of

asthmatics, which has been linked with metabolic alterations (Xu, Ghosh et al. 2016). Mitochondrial abnormality has also been described in epithelial cells and airway smooth muscle cells from patients with COPD (Agrawal and Mabalirajan 2016, Cloonan and Choi 2016), and leukocytes of smokers with COPD have almost 50% lower mtDNA copy number compared to healthy controls (Liu, Kuo et al. 2015). Mitochondrial dysfunction in alveolar type II cells from patients with idiopathic pulmonary fibrosis (IPF) was found to be associated with upregulation of ER stress markers and reduced expression of PTEN-induced putative kinase 1 (PINK1), an enzyme that regulates mitochondrial maintenance by mitophagy of damaged mitochondria (Bueno, Lai et al. 2015). The importance of mitochondria in lung disease is also supported by genetic evidence, for example by reported associations of the mitochondrial haplogroup U with increased IgE and asthma (Zifa, Daniil et al. 2012, Flaquer, Heinzmann et al. 2014), and genetic associations of genes of mitochondrially targeted proteins involved in metabolism or ROS regulation with COPD (Agrawal and Mabalirajan 2016). For example, the iron-regulatory protein IRP2 (critical in regulating cellular iron homeostasis) was identified as a major COPD susceptibility gene, and its overexpression in the lungs of COPD patients was associated with mitochondrial dysfunction (Cloonan, Glass et al. 2016). Enhanced production of mtROS is often considered a central feature of mitochondrial dysfunction. Therefore, in attempts to address the specific role of mtROS in lung pathology, various experimental approaches to target mtROS (e.g. mitoQ, mitoTEMPO) have been used in animal studies of allergic airways disease or COPD (Jaffer, Carter et al. 2015, Wiegman, Michaeloudes et al. 2015, Agrawal and Mabalirajan 2016). Mitochondrially-targeted iron chelators have also been shown to attenuate inflammation and mucociliary defects in experimental models of COPD (Cloonan, Glass et al. 2016), which may further point to a role for mtROS in COPD pathology given the importance of iron overload in ROS biochemistry. Administration of the mitochondria-targeted antioxidant mitoQ has also been shown to attenuate hypoxia-induced pulmonary vasoconstriction, thus suggesting a role for mtROS in pulmonary hypertension (Pak, Scheibe et al. 2018).

3.3. Dysregulated NOX expression or activation

The most widely appreciated source of ROS in the context of acute or chronic lung inflammation is NOX2 present in infiltrating monocytes, macrophages, and/or granulocytes following their activation. In addition to their direct antimicrobial roles, NOX2-derived ROS also contribute to pro-inflammatory signaling (Forman and Torres 2002, Rinna, Torres et al. 2006, Cruz, Rinna et al. 2007), antigen processing and cross-presentation (Jancic, Savina et al. 2007, Rybicka, Balce et al. 2010, Rybicka, Balce et al. 2012), and regulation of e.g. neutrophil apoptosis (Carneiro, Roma et al. 2018). The latter also explains why genetic deficiency of NOX2 or its cofactors often results in enhanced inflammation and injury in response to infection or other stimuli (Morgenstern, Gifford et al. 1997, Gao, Standiford et al. 2002, Kassim, Fu et al. 2005, Segal, Davidson et al. 2007), and leads to chronic granulomatous disease (CGD), illustrating the importance of NOX2 in regulating inflammation *and* its resolution (Singel and Segal 2016). The respiratory epithelium also represents an important source for extracellular ROS in response to inhaled pathogens or allergens or inflammatory stimuli, originating primarily from the main epithelial NADPH oxidase isoforms DUOX1 and DUOX2 (Geiszt, Witta et al. 2003, Harper, Xu et al. 2006,

Moskwa, Lorentzen et al. 2007, Wesley, Bove et al. 2007, Gattas, Forteza et al. 2009). DUOX1 is mostly localized at the apical surface of tracheobronchial and alveolar epithelial cells, and DUOX2 is primarily found in salivary and submucosal glands (Geiszt, Witta et al. 2003, Schwarzer, Machen et al. 2004, Forteza, Salathe et al. 2005, Harper, Xu et al. 2005, Fischer, Gonzales et al. 2007). Airway DUOX2 is highly inducible by various bacterial and viral stimuli (Harper, Xu et al. 2005, Gattas, Forteza et al. 2009), and was also found to be elevated in severe asthma (Voraphani, Gladwin et al. 2014). DUOX1 is the main constitutive isoform in airway and alveolar epithelia, is inducible by Th2 cytokines, and is enhanced in allergic asthma (Hristova, Habibovic et al. 2016, Wan, Hollins et al. 2016). In contrast, airway DUOX1 (and to a lesser extent DUOX2) are suppressed in lung epithelial cells from smokers with COPD (Nagai, Betsuyaku et al. 2008) and in various lung cancers (Little, Sulovari et al. 2017). Airway and alveolar epithelial cells also express other NOX isoforms such as NOX1 and NOX4 which may be enhanced during acute lung injury (Carnesecchi, Deffert et al. 2009), neutrophilic asthma (Wan, Hollins et al. 2016), or pulmonary fibrosis (Carnesecchi, Deffert et al. 2011). NOX4 is also expressed in smooth muscle cells and pulmonary fibroblasts, and is enhanced in lung tissues from patients with pulmonary arterial hypertension (Mittal, Roth et al. 2007) and pulmonary fibrosis (Hecker, Vittal et al. 2009, Amara, Goven et al. 2010). Collectively, lung pathologies are associated with variable changes in different NOX isoforms, which potentially unique contributing roles to disease pathology. Selective NOX inhibitors are largely lacking (van der Vliet, Danyal et al. 2018), but a semiselective NOX1/NOX4 inhibitor was found to attenuate and reverse pulmonary fibrosis in experimental models (Hecker, Logsdon et al. 2014).

3.4. Alterations in antioxidant systems

As a fourth line of evidence for ROS imbalance as a potential causative factor in lung pathology, several studies indicate altered expression or activity of various ROS-metabolizing enzymes during chronic lung disease. For example, the cytosolic isoform of superoxide dismutase (Cu,Zn-SOD; SOD1), abundantly expressed in most lung cell types (Zelko, Mariani et al. 2002, Kinnula and Crapo 2003), is inactivated in asthmatic airways (based on loss of overall SOD activity, which is primarily accounted for by SOD1) (Smith, Shamsuddin et al. 1997, Comhair, Xu et al. 2005, Ghosh, Willard et al. 2013). The mitochondrial MnSOD isoform SOD2 is induced in the bronchial and alveolar epithelium of e.g. smokers with or without COPD (Harju, Kaarteenaho-Wiik et al. 2004), but oxidative modifications within SOD2 have been detected in asthmatic airways, which may contribute to the loss of SOD activity in these patients (Comhair, Xu et al. 2005). The third isoform, extracellular (EC)-SOD (SOD3) is also induced in the lungs of COPD patients (Folz, Guan et al. 1997), and SOD3 haplotypes have been associated with development of COPD (Young, Hopkins et al. 2006, Bentley, Emrani et al. 2008). Evidence also suggests that oxidative mechanisms during asthma contribute to loss of lung activity of the H_2O_2-metabolizing enzyme catalase (Ghosh, Janocha et al. 2006, Comhair and Erzurum 2010). In contrast, some isoforms of GSH peroxidase (GPX), such as GPX2 and the extracellular isoform GPX3, are enhanced during allergic airway inflammation (Comhair and Erzurum 2005, Dittrich, Meyer et al. 2010). Finally, several isoforms of peroxiredoxin (PRDX), especially PDRX1, PRDX5, and PRDX6, are inducible by oxidative stress or during inflammation and are often elevated in several lung diseases including lung cancer,

mesothelioma and sarcoidosis (Kinnula, Lehtonen et al. 2002, Aracena-Parks, Goonasekera et al. 2006, Rostila, Puustinen et al. 2012). Overall, the various changes in antioxidant enzyme expression or activity can be expected to contribute to dysregulated cellular responses to ROS. In fact, upregulation of mitochondrial thioredoxin (TRX) and PRDX3 in cancer cells is thought to contribute to dysregulated signaling and energy metabolism, and may represent attractive targets for therapeutic management of various intractable human cancers (Cunniff, Newick et al. 2015).

Collectively, these variable lines of evidence clearly establish that lung ROS-antioxidant status is frequently altered in chronic lung disease. However, given the diverse biological actions of ROS on both physiological processes and cellular dysfunction, it is difficult to translate these various findings into relevant mechanisms of disease. Indeed, one inherent problem is that reported ROS-antioxidant imbalances are typically based on global analyses of tissues, cells or body fluids, which does not offer insight into localized redox events within cells (e.g. in NOX-containing signaling complexes or near mitochondria) that are likely more relevant for cell signaling or biological outcomes.

4. Oxidative stress versus redox signaling – Basic concepts

4.1. Redox signaling is localized

The tendency to describe biological systems in terms of redox balance or oxidative status (ie. based on measurements of cell or tissue GSH/GSSG ratios or estimations of steady-state levels of ROS) has been helpful in appreciating how such parameters change e.g. with increasing age or during ongoing disease (Fitzpatrick, Park et al. 2014, Jones 2015), but such concepts fail to recognize the importance of subcellular location of specific and dynamic redox events occurring throughout a cell. It is also important to point out that the ability of ROS to diffuse freely throughout a cell (because of their small size) and cause global alterations of redox status within a cell is often overestimated. If one considers the complex architecture of a cell, containing dense networks of membrane-rich mitochondrial and ER structures, protein complexes such as actin and tubulin fibers, and a gel-like cytoplasm whose physicochemical properties are highly distinct from dilute aqueous buffer solutions, it should be easy to appreciate that even small molecules such as H_2O_2 (with physical properties similar to water) are unlikely to distribute randomly throughout a cell and may even require transport mechanisms (such as aquaporins that facilitate transmembrane transport of H_2O and also H_2O_2). It follows that biological actions of ROS must be dictated primarily by their site of production (e.g. activation of NOX, extracellular source, etc.), which is further assured by the widespread presence of diverse ROS-metabolizing enzymes within cells that help constrain locally generated ROS and prevent their ability to induce more widespread changes throughout the cell (Heppner, Janssen-Heininger et al. 2017). Observed changes in overall redox status in the cell due to e.g. metabolic alterations during aging or chronic disease, might reflect shifts in overall metabolic status determined by an imbalance between cellular ROS production and adaptive responses leading to induction of antioxidant enzyme, but may not necessarily affect local redox signaling events due to dynamic local fluctuations in e.g. H_2O_2 production by specific NOX enzymes. From this perspective, it is not difficult to understand why general antioxidant supplementation

strategies in an effort to alter global redox status may be relatively incapable of inhibiting such localized ROS production by e.g. NOX activation. This is fortunate, since such strategies would otherwise also interfere with beneficial effects of NOX in e.g. host defense functions and thus increase risk of infectious disease. In fact, supplementation with ascorbate has paradoxically been demonstrated to *enhance* ROS production and bacterial killing by phagocytes, rather than inhibit it (Carr and Maggini 2017), and might similarly affect activation of other NOX isoforms as well.

4.2. General mechanisms of redox signaling

The original concept of oxidative stress as leading to molecular and tissue damage as a causative factor in disease pathology has been largely supplanted by the phenomenon of redox signaling, which involves reversible redox modifications of susceptible protein targets as a mechanism to regulate protein function and affect many physiological aspects of cell biology, and the premise that pathology arises primarily from dysregulation of such redox signaling processes. The most common sites in proteins that are subject to such reversible redox modifications include redox-active transition metal ion centers (e.g. heme groups, iron-sulfur centers, zinc-thiolate centers) and oxidant-sensitive amino acid side chains (primarily cysteine, selenocysteine, and methionine residues). Oxidative modifications of these sites can either enhance or inhibit enzyme activity (e.g. through oxidation of catalytically active cysteines), but can also result in altered protein-protein interactions, subcellular trafficking, protein turnover, etc.

The field of redox-based signaling has mostly focused on reversible oxidation of cysteine residues, since cysteine residues are often highly conserved in proteins and the relative abundance of protein cysteines has increased with evolution of complex multicellular organisms, in apparent coordination with increased diversity of NOX enzymes in these higher organisms (van der Vliet 2008, Jones and Sies 2015). Protein tyrosine phosphatases were among the first proteins recognized to be subject to inactivation by reversible cysteine oxidation during e.g. growth factor-mediated signaling, but with development of improved detection tools and proteomics approaches many examples exist of proteins that are also oxidized on cysteines as a result of cellular ROS production (Janssen-Heininger, Mossman et al. 2008, Yang, Gupta et al. 2014, Topf, Suppanz et al. 2018). In addition to the fact that many protein phosphatases are subject to inhibition by reversible oxidative modification, more recent evidence indicates that various protein kinases (including both protein tyrosine kinases and serine/threonine-specific protein kinases) can be activated by oxidation of conserved cysteine residues, thus implying that redox mechanisms are closely intertwined with cell signaling pathways involving protein phosphorylation. An important consideration in this regard is the fact that oxidative cysteine modifications can be highly diverse (e.g. sulfenic acids vs. mixed disulfides with other proteins or small molecules such as GSH) (Figure 2A), with potentially variable functional or structural consequences (Heppner, Hristova et al. 2016, Truong, Ung et al. 2016, Moffett, Bender et al. 2017). Protein *S*-glutathionylation, the formation of mixed disulfides with GSH, has received special attention as a signaling mechanism, supported by the fact that enzymatic systems exist that can catalyze protein *S*-glutathionylation (e.g. glutathione *S*-transferases) and reverse it (glutaredoxins) (Janssen-Heininger, Mossman et al. 2008, Zhang, Ye et al. 2018). Also,

because of the rather bulky and charged nature of GSH, protein *S*-glutathionylation is thought to induce significant structural and functional alterations within proteins (e.g. (Moffett, Bender et al. 2017)). Protein glutathionylation is also believed to protect against irreversible cysteine oxidation of thioredoxins and peroxiredoxins, which can be restored by the action of glutaredoxins (Zhang, Du et al. 2014, Peskin, Pace et al. 2016), thus demonstrating complex and intricate interplay between these various redox systems. Dysregulation of these enzyme systems during disease can therefore impact on this important mode of redox signaling.

In contrast to the rapidly growing literature addressing cysteine oxidation, methionine oxidation has been less well recognized as a potential signaling mechanism. This is largely due to the fact that biochemical reagents are lacking to assess methionine oxidation in biological systems, and only MS approaches can successfully demonstrate methionine sulfoxidation. Sulfoxidation of methionine residues markedly increases the hydrophilic properties of methionine and can thus significantly alter the physicochemical properties of proteins, thereby either enhancing or inhibiting protein activity (Veredas, Canton et al. 2017). Also, similar to reversible cysteine oxidation, enzymatic systems have been identified that can both enhance and reverse methionine sulfoxidation (Manta and Gladyshev 2017) (Figure 2B). Intriguingly, recent proteomic analyses have revealed that methionine oxidation tends to be highly favorable in phosphorylation motifs, suggesting intricate crosstalk between methionine oxidation and protein phosphorylation pathways (Veredas, Canton et al. 2017).

5. Redox mechanisms in pulmonary disease

It should be evident from the previous sections that the field of redox biology has become exceedingly complex and continues to evolve. Indeed, reports are rapidly accumulating in the biomedical literature that address new aspects of redox-based signaling and its diverse impact on cell biology, and it would be beyond the scope of this review to adequately and cohesively summarize all available literature in this area. Indeed, a simple PubMed search using the title/abstract search terms “redox” and “lung” yields >1500 publications. Moreover, although ROS have been implicated in many diverse lung pathologies, our understanding of these cell-specific aspects of redox signaling in lung biology and diverse chronic lung disease is still rather various lung diseases. Therefore, rather than attempting to exhaustively cover the diverse literature that addresses redox-based mechanisms in various lung diseases, which has been covered in excellent recent reviews (e.g. (Sundar, Yao et al. 2013, Sommer, Strielkov et al. 2016, Boukhenouna, Wilson et al. 2018)), in the following paragraphs we will instead highlight some recent advances with respect to specific redox-based mechanisms in the context of two major lung pathologies, allergic asthma and idiopathic pulmonary fibrosis, which form the basis of new and unique redox-based therapeutic approaches that could be developed to treat these diseases. Given the fact that other chronic lung diseases, such as COPD, may also include overlapping features of chronic inflammation or remodeling similar to those seen in asthma or pulmonary fibrosis, these targeted approaches might also be applicable to other chronic lung pathologies.

5.1. NOX4 in pulmonary fibrosis

Pulmonary fibrosis is a debilitating disease characterized by progressive development of excess fibrous tissue, leading to lung “scarring” and rapidly diminishing lung function. In many cases, the cause of pulmonary fibrosis is unknown, which is known as idiopathic pulmonary fibrosis (IPF). Common features of pulmonary fibrosis are recurrent and persistent injury of the alveolar epithelium, combined with disordered deposition of extracellular matrix and accumulation of myofibroblasts in so-called fibroblastic foci. As mentioned previously, the NADPH oxidase homolog NOX4 was found to be overexpressed in the lungs of patients with IPF, primarily in myofibroblasts in fibroblastic foci and remodeled blood vessels, but also in epithelial cells associated with aberrant bronchiolization, and this NOX4 induction is largely mediated by production of the pro-fibrotic growth factor transforming growth factor beta (TGF-β) (Hecker, Vittal et al. 2009, Amara, Goven et al. 2010, Pache, Carnesecchi et al. 2011). Curiously, NOX4 apoptotic cell death in epithelial cells (Carnesecchi, Deffert et al. 2011) but inducing fibroblast differentiation towards and apoptosis-resistant myofibroblast phenotype (Bernard, Hecker et al. 2014), and thus appears to contribute to both critical features of progressive lung fibrosis. The molecular mechanisms by which NOX4-dependent production of H_2O_2 contributes to these functional outcomes are not clear, but various recent studies indicate an interaction of NOX4 with mitochondrial function. NOX4 contains an N-terminal mitochondrial localization signal, and can localize to the inner mitochondrial membrane and promote mtROS production (Block, Gorin et al. 2009, Graham, Kulawiec et al. 2010, Case, Li et al. 2013). Metabolic profiling of IPF lung tissues has suggested altered metabolic signatures in IPF consistent with increased glycolysis, reduced GSH biosynthesis, and increased ATP degradation (Kang, Lee et al. 2016), which may be associated with mitochondrial dysfunction. Studies in human or murine fibroblasts in which NOX4 was genetically deleted highlighted a suppressive effect of NOX4 on mitochondrial biogenesis and bioenergetics (Bernard, Logsdon et al. 2017), and indicated that NOX4 silencing resulted in upregulation of mitochondrial transcription factor A (TFAM) by nuclear factor erythroid-derived 2-like 2 (Nrf2). Intriguingly, ATP was recently identified as a negative allosteric regulator of NOX4 (Shanmugasundaram, Nayak et al. 2017) suggesting that ATP degradation during IPF may contribute to enhanced NOX4 activation, thus further perpetuating this progressive disease. Based on these various observations, pharmacologic inhibition of NOX4 has been proposed as a potential treatment for IPF, and preclinical studies have suggested efficacy of a recently development NOX inhibitor with some selectivity towards NOX4 (Hecker, Logsdon et al. 2014).

5.2. Redox regulation of epithelial apoptosis by *S*-glutathionylation in pulmonary fibrosis

IPF is often characterized by chronic injury and loss of bronchiolar and alveolar epithelial cells, and targeted injury to alveolar type II cells can induce fibrosis (Sisson, Mendez et al. 2010). One major contributor to such epithelial loss is the production of the ligand for the death receptor Fas (CD95) by myofibroblasts, which induces alveolar epithelial apoptosis (Golan-Gerstl, Wallach-Dayan et al. 2007) whereas myofibroblasts are typically resistant to Fas-induced apoptosis (Wynes, Edelman et al. 2011). It is widely known that apoptosis is associated with dramatic changes in cellular GSH status, and GSH status is also impaired in IPF (Anathy, Roberson et al. 2012), although approaches to restore GSH redox status using

e.g. *N*-acetylcysteine have not been successful in improving IPF in humans (Idiopathic Pulmonary Fibrosis Clinical Research, Martinez et al. 2014, Sun, Liu et al. 2016). Recent studies by our group indicated that Fas-induced apoptosis in epithelial cells is associated with *S*-glutathionylation of the Fas receptor on Cys294, and this *S*-glutathionylation was found to promote cell surface expression of Fas, as well as its aggregation and activation, leading to downstream caspase signaling (Anathy, Roberson et al. 2012). In addition, Fas-mediated caspase activation was found to promote cleavage and inactivation of glutaredoxin 1 (GLRX, the main cytoplasmic glutaredoxin isoform that reverses *S*-glutathionylation), and overexpression of GLRX could prevent Fas *S*-glutathionylation and attenuate apoptosis (Anathy, Aesif et al. 2009). Subsequent studies indicated that initial Fas stimulation promotes oxidative processing of ER-localized Fas, mediated by interactions with the ER-resident protein disulfide isomerase ERp57 and glutathione *S*-transferase P1 (GSTP1), a known catalyst of *S*-glutathionylation, and that such interactions between GSTP1 and Fas were also observed in experimental models of pulmonary fibrosis (Anathy, Roberson et al. 2012) as well as lung tissues from human IPF patients (McMillan, van der Velden et al. 2016). Since GSTP1 was also found to be increased in IPF lungs, pharmacological inhibition of GSTP1 *S*-glutathionylation could potentially inhibit the progression of pulmonary fibrosis by minimizing Fas *S*-glutathionylation and subsequent caspase activation and alveolar apoptosis. Indeed, experimentally-induced lung fibrosis in mice was strongly attenuated by genetic deletion of *Gstp*, and by administration of TLK177, the active metabolite of the clinically applicable GSTP inhibitor TLK199 (McMillan, van der Velden et al. 2016). Conversely, approaches to augment the activity of GLRX may also be similar efficacious in treating IPF. Indeed, we recently observed that increases in protein *S*-glutathionylation in fibrotic lung tissues are largely associated with significant decrease in GLRX activity, and that administration of catalytically active recombinant GRLX into the airways of mice with ongoing experimental pulmonary fibrosis can in fact reverse increases in collagen deposition, thus illustrating the therapeutic potential of exogenous GLRX in treating lung fibrosis (Anathy, Lahue et al. 2018). These various approaches to modify *S*-glutathionylation could affect fibrosis by attenuation Fas activation, but could also affect *S*-glutathionylation of other target proteins which may also contribute to disease pathology. Hence, further characterization of these additional *S*-glutathionylation events would be necessary to advance our understanding of this redox signaling axis in pulmonary fibrosis. An alternative redox mechanism involved in apoptosis involves the protein disulfide isomerase isoform PDIA3 (also known as ERp57), which is known to catalyze inter-molecular disulfide bonds in pro-apoptotic BAK and thereby enhances intrinsic apoptosis (Zhao, Lu et al. 2015), which was demonstrated in airway epithelial cells in the context of allergen induced peri-bronchiolar fibrosis (Hoffman, Tully et al. 2013, Hoffman, Chapman et al. 2016). Airway epithelial specific deletion of ERp57 was shown to decrease allergen induced apoptosis and peri-bronchiolar fibrosis in mice (Hoffman, Chapman et al. 2016).

5.3. Redox-dependent activation of tyrosine kinases in allergic asthma

Asthma is a chronic inflammatory disease that is associated with hyperactive airways and variable airflow obstruction. Because asthma is typically characterized by activation of T-helper 2 (Th2) cells and eosinophilic inflammation, it is widely assumed that asthma pathology is largely driven by adaptive immune responses to allergens. However, a

significant subgroup of non-allergic asthmatics also exists, and increasing evidence points to the importance of innate responses to epithelial injury in asthma pathogenesis (Hammad and Lambrecht 2008, Holgate 2011). Indeed, the respiratory epithelium forms a major source of cytokines that can activate type 2 inflammation, such as IL-25, IL-33, and TSLP, and asthma is also characterized by epithelial remodeling in favor of secretory epithelial cells (Goblet cells) that generate mucus which contributes to airflow obstruction. Epithelial signaling through tyrosine kinases such as the epidermal growth factor receptor (EGFR) has been identified as a convergent pathway in innate epithelial responses to infectious and other noxious stimuli, and contributes to epithelial repair pathways, as well as production of mucins (such as MUC5AC) and the chemokine IL-8 (Burgel and Nadel 2008). Increased expression and activation of EGFR is observed in the airways of asthmatic subjects (Polosa, Puddicombe et al. 2002, Hamilton, Puddicombe et al. 2005, Habibovic, Hristova et al. 2016), and experimental studies have shown that pharmacological EGFR inhibition can prevent many features of allergic asthma (Le Cras, Acciani et al. 2011). Unfortunately, the clinical significance is limited, and recent studies showed poor tolerance of an inhaled EGFR inhibitor (BIBW 2948 BS) in COPD patients, which also had minimal effect on mucus secretion (Woodruff, Wolff et al. 2010). As mentioned previously, EGFR-dependent signaling is subject to redox-dependent regulation, through oxidative inactivation of protein tyrosine phosphatases (Lee, Kwon et al. 1998), but more recent studies indicate that EGFR itself can also be oxidized on a conserved cysteine within its kinase domain, which results in enhanced kinase activity (Paulsen, Truong et al. 2011, Heppner, Hristova et al. 2016, Truong, Ung et al. 2016). During ligand-mediated EGFR activation this oxidative mechanism relies on NOX2, which interacts directly with EGFR (Paulsen, Truong et al. 2011), but in the context of EGFR transactivation by diverse injurious stimuli, DUOX1 was found to be primarily responsible for EGFR activation, through initial redox-dependent activation of Src non-receptor tyrosine kinase (Sham, Wesley et al. 2013, Heppner, Hristova et al. 2016) (Figure 3). Molecular studies indicate that during EGFR/Src activation, both proteins are successively oxidized to a sulfenic acid and then *S*-glutathionylated, but that sulfenylation is primarily responsible for increasing kinase activity (Paulsen, Truong et al. 2011, Heppner, Hristova et al. 2016, Truong, Ung et al. 2016). Supporting the importance of these redox mechanisms, DUOX1 was found to be responsible for EGFR-mediated activation of MUC5AC as well as IL-8 in response to various external triggers (Shao and Nadel 2005, Boots, Hristova et al. 2009). Moreover, through similar EGFR-dependent mechanisms, DUOX1 was also found to mediate of epithelial production of IL-33 and IL-25 in response to common airborne allergens that are associated with allergic asthma (Hristova, Habibovic et al. 2016). Indeed, oxidative EGFR activation has been observed in nasal epithelial cells of asthmatic subjects, and in the airways of mice with experimental allergic asthma, and genetic or pharmacological inhibition of DUOX1 was found to suppress EGFR activation, mucus metaplasia and airway remodeling, features known to be related to enhanced epithelial EGFR signaling (Habibovic, Hristova et al. 2016). These and other findings implicating DUOX1 in asthma pathology (Chang, Linderholm et al. 2013) suggest that pharmacological targeting of DUOX1 may be an attractive strategy to treat severe asthma (van der Vliet, Danyal et al. 2018). It is important to note that other NOX isoforms can also contribute to asthma pathology, although their actions may be mostly involved in

other cell types such as T helper cell or dendritic cells (van der Vliet 2011, Sevin, Newcomb et al. 2013).

5.4. Redox-dependent activation of CaMKII by methionine oxidation

Another interesting example of oxidant-mediated enzyme activation is the calcium/calmodulin (Ca^{2+}/CaM)-dependent protein kinase II (CaMKII), which is typically activated by binding of Ca2+/calmodulin but can also be activated independently of Ca^{2+} by oxidation of a pair of methionine residues (Met281/Met282) (Erickson, Joiner et al. 2008). Oxidant-mediated CaMKII activation is also subject to reversal by methionine sulfoxide reductase A (MsrA). CaMKII is a multifunctional serine/threonine kinase that is ubiquitously expressed, but is most widely recognized for its role in cardiovascular biology (Hund and Mohler 2015). Recent studies highlighted the presence of oxidatively activated CaMKII in the bronchial epithelium of patients with asthma, suggesting a potential mechanism by which ROS contribute to asthma pathology. Indeed, the degree of oxidative CaMKII activation correlated with severity of allergic inflammation in animal models, based on studies with mice deficient in NOX activity or MsrA, and pharmacological targeting of CaMKII, using a small molecule inhibitor (KN-93) or an inhibitory peptide (AC3-I), was to inhibit inflammation and disease severity in a model of experimental asthma (Sanders, Koval et al. 2013). Subsequent studies indicated an association between oxidative CaMKII activation and mtROS production, suggesting that CaMKII is also localized in mitochondria and contributes to mtROS production in response to e.g. IL-13, an asthma-relevant cytokine. Moreover, a mitochondria-targeted CaMKII inhibitor was found to abrogate allergen-induced cytokine production, eosinophilic inflammation, and airways hyperresponsiveness in experimental models of allergic asthma (Sebag, Koval et al. 2017). The role of oxidative CaMKII activation in allergic asthma was also demonstrated by using a CaMKII knock-in mouse in which the two methionines were replaced with valine (Qu, Do et al. 2017). Also, these latter studies indicated an important function for oxidized CaMKII in mast cell accumulation during experimental asthma, suggesting wider roles for CaMKII oxidation in asthma pathology beyond functions in the airway epithelium (Qu, Do et al. 2017). While these studies suggest the potential use of CaMKII inhibitors in treatment of asthma, the ubiquitous presence of CaMKII and its importance in e.g. cardiac function (Hund and Mohler 2015) may complicate such approaches.

6. Summary and future perspectives

The lung represents the largest surface in contact with the external environment, and thus needs to effectively cope with the often oxidative nature of environmental triggers. At the same time, all lung cells also utilize ROS derived from mitochondria and a family of NOX enzymes in many biological functions, such as appropriate immune responses, and maintenance of cell and tissue homeostasis. Therefore, lung pathologies cannot simply be attributed to general increases in ROS, but more likely result from dysregulated redox homeostasis or redox signaling pathways. Likewise, rather than non-discriminatory inhibition of ROS by supplementation with antioxidant molecules, more selective approaches that target specific dysfunctional redox processes in lung diseases are likely more effective in treating these diseases. This issue also applies to considerations of other

factors, such as aging or metabolic alterations associated with e.g. obesity, which are commonly associated with altered redox homeostasis and potentially unique redox alterations that may contribute to chronic lung disease. Recent efforts to develop immunotargeting approaches to deliver antioxidant enzymes to specific cell types (Han, Shuvaev et al. 2012) may be more effective, but since ROS are considered poor druggable targets (Ghezzi, Jaquet et al. 2017) it would be more sensible to develop approaches to target specific ROS sources or specific cellular redox events. Unfortunately, in spite of considerable progress, we still are lacking in specific pharmacological approaches that selectively inhibit individual NOX isoforms (van der Vliet, Danyal et al. 2018) or specific mitochondrial sources of ROS (in spite of encouraging results with mitochondria-targeted antioxidants), and further development of such specific inhibitors would be desirable. Alternatively, it is worthwhile to consider developing selective approaches to target other redox enzymes (e.g. GSTP1, ERp57, etc.) or specific redox-modifications in proteins, for example towards sulfenylated forms of EGFR, which would potentially avoid unwanted side effects of current EGFR tyrosine kinase inhibitors. The field of redox biology has relatively recently emerged. Judging from the newly discovered insights in reactions that govern redox biochemistry and new tools to measure and target redox perturbations in biological molecules, it is likely that important new information will be forthcoming about the mechanisms whereby redox-based processes regulate lung homeostasis, and their dysregulation facilitates disease. Such insights will be paramount towards the development of targeted redox-based drugs.

Acknowledgements:

The author wishes to thank NHLBI for their generous research support (grants R01 HL085646, R01 HL138708, R01 HL122383 and R35 HL135828).

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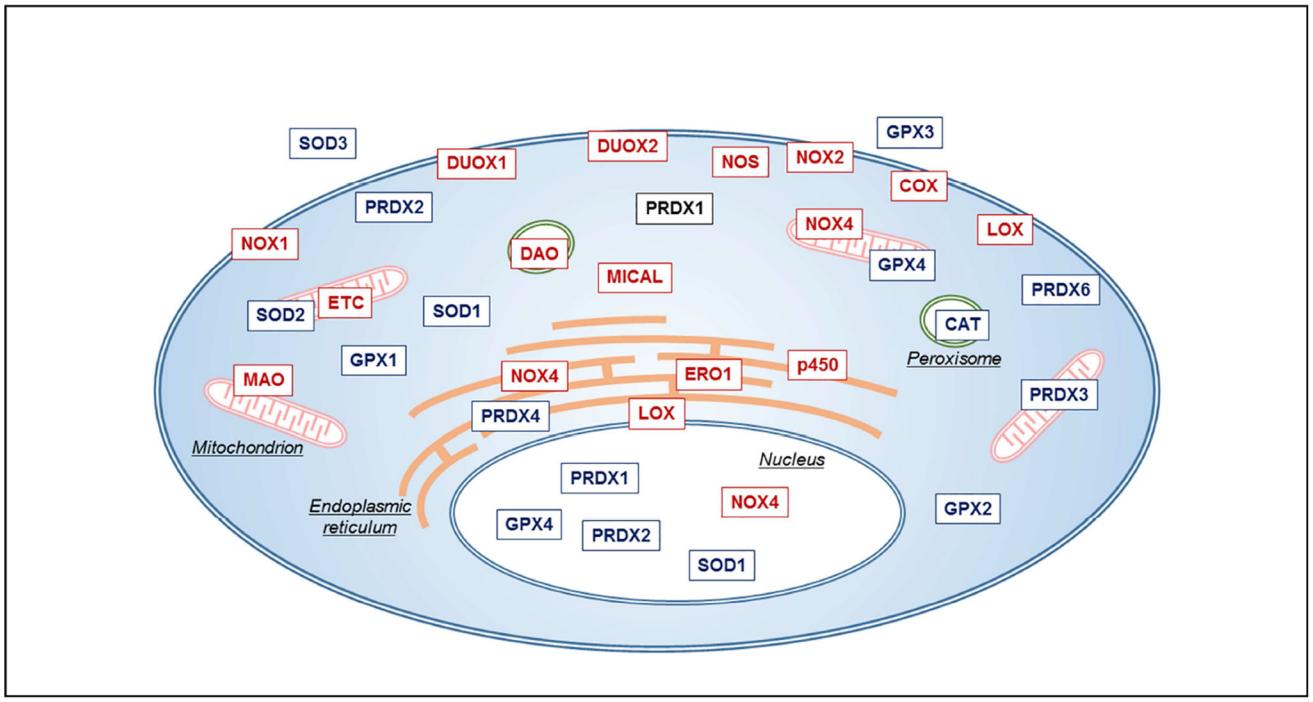

Figure 1: Distribution of various ROS sources and antioxidant systems throughout the cell.

All cells (as well as all lung cells) contain actively respiring mitochondria that generate ROS at their electron transfer chain (ETC), as well as various NOX/DUOX isoform distributed across different organelles. Other cytoplasmic or organelle-specific ROS sources include nitric oxide synthase (NOS), cyclooxygenase (COX), lipoxygenase (LOX), monoamine oxidase (MAO, D-amino acid oxidase (DAO), MICAL, ERO1, and others. Moreover, all cells contain various isoforms of (organelle-specific) antioxidant enzymes that metabolize ROS. Please refer to manuscript text for further clarifications.

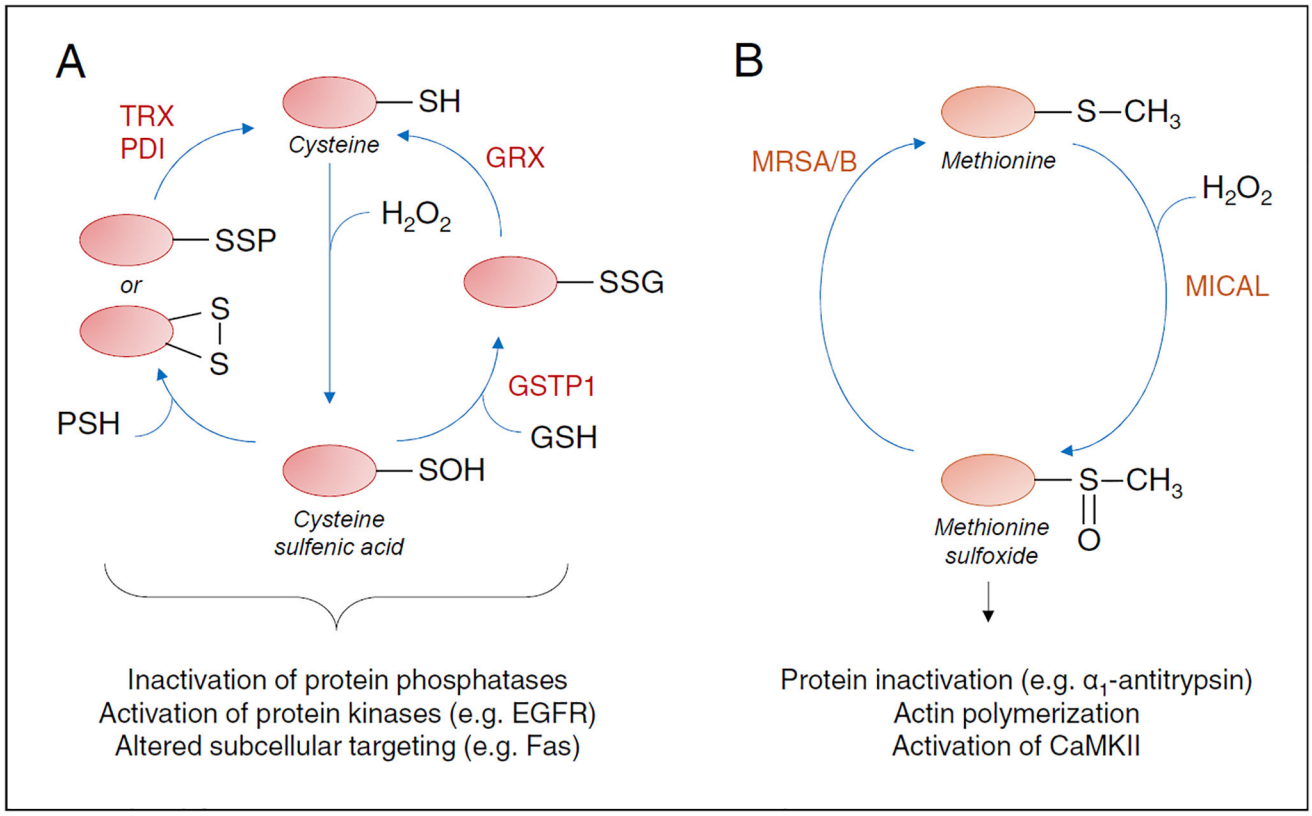

Figure 2: Schematic illustration of reversible oxidation of protein cysteine or methionine residues in redox-based signaling.

(A) Oxidation of protein cysteines (P-SH) by e.g. H_2O_2 results in initial formation of sulfenic acid (P-SOH), which can subsequently react with other protein cysteines to form inter- or intra-molecular disulfides, or with GSH (potentially catalyzed by GSTP1) to form *S*-glutationylated proteins (P-SSG). These disulfides can then be reduced by thioredoxins (TRX), protein disulfide isomerases (PDI) or glutaredoxins (GRX). **(B)** Oxidation of methionine by H_2O_2 (potentially catalyzed by MICAL) results in formation of methionine sulfoxide, which can be reduced by methionine sulfoxide reductases (MRSA and MRSB).

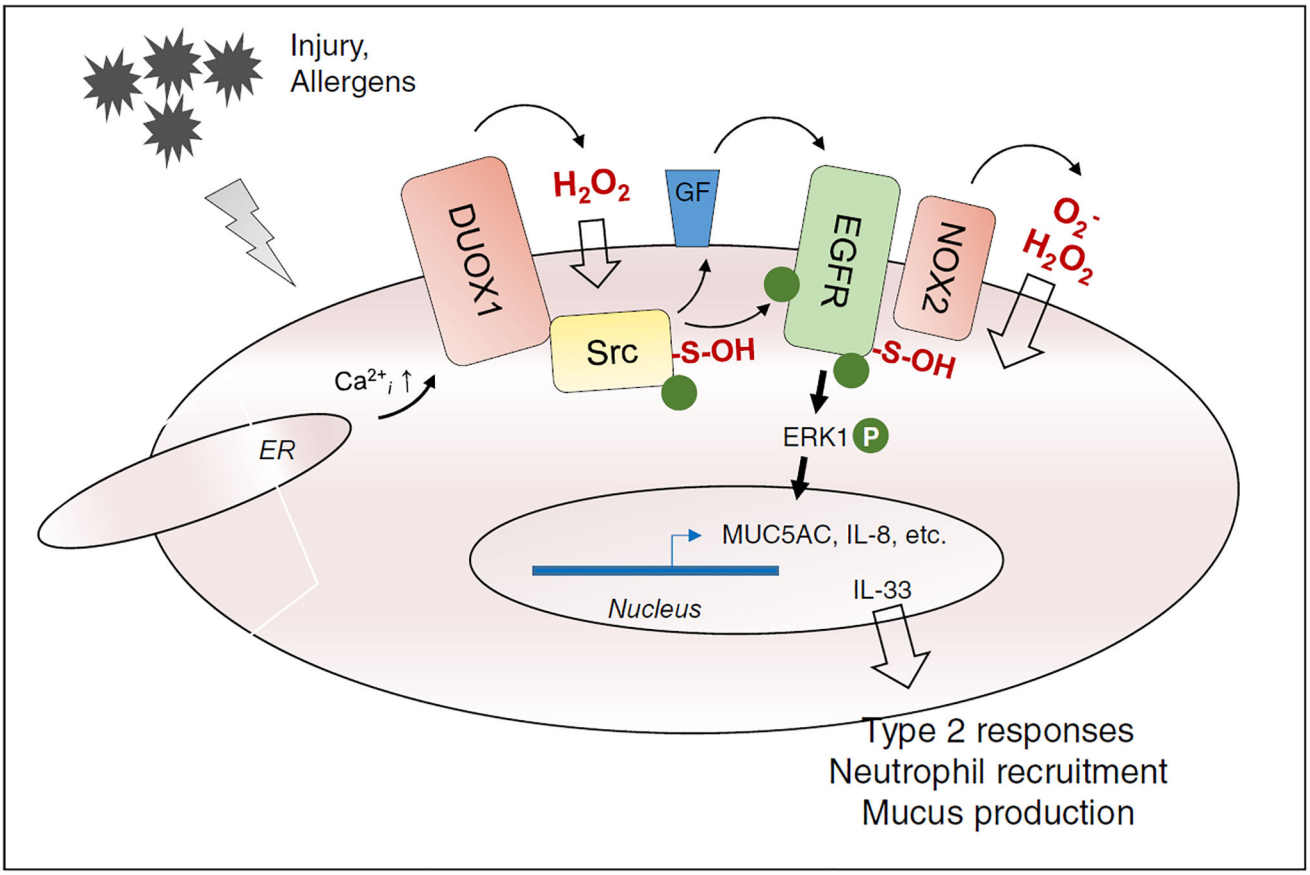

Figure 3: Schematic illustration of NOX-dependent activation of protein tyrosine kinases in epithelial responses to injury or allergens.

Injurious stimuli typically trigger Ca^{2+} mobilization or influx, which activates DUOX1 to produce H_2O_2. This contributes to oxidative activation of Src, and release of ligands for the epidermal growth factor receptor (EGFR). EGFR can also be oxidatively activated, which appears to depend on activation of NOX2. These collective events contribute to induction of mucus genes and IL-8, and release of alarmins such as IL-33.