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Reactive Oxygen Species as Signaling Molecules in the Development of Lung Fibrosis

Francisco J Gonzalez-Gonzalez¹, Navdeep Chandel¹, Manu Jain¹, and GR Scott Budinger¹

¹Division of Pulmonary and Critical Care Medicine, Feinberg School of Medicine, Northwestern University, Chicago, USA

Abstract

Pulmonary fibrosis is a relatively rare but devastating disease characterized by the excessive deposition of extracellular matrix. The increased matrix results in reduced lung compliance and increased work of breathing, while the obliteration of alveolar-capillary structures can result in hypoxemia and pulmonary hypertension, which manifests clinically as worsening shortness of breath, respiratory failure and death. Unbiased genome wide association studies combined with animal models suggest that damage to the alveolar epithelium is the initiating factor in pulmonary fibrosis. This epithelial injury leads to the activation and proliferation of myofibroblasts that secrete extracellular matrix proteins characteristic of fibrosis. The best described molecular link between alveolar epithelial dysfunction and myofibroblast activation and proliferation is the profibrotic cytokine transforming growth factor- β (TGF- β). We and others have found that mitochondrial and NADPH oxidase-generated reactive oxygen species (ROS) play a signaling role to enhance TGF- β signaling and promote fibrosis. This purpose of this review is to review ROS signaling downstream of the activation of TGF- β . We suggest that an improved understanding of these pathways might explain the failure of nonselective antioxidants to improve outcomes in patients with pulmonary fibrosis and might identify novel targets for therapy.

Introduction

Pulmonary fibrosis is a relatively rare but devastating disease characterized by the excessive deposition of extracellular matrix (1). The increased matrix results in reduced lung compliance and increased work of breathing, while the obliteration of alveolar-capillary structures can result in hypoxemia and pulmonary hypertension. Together these findings contribute to a progressive decline in lung function, which manifests clinically as worsening shortness of breath, respiratory failure and death. While newer therapeutics can slow disease progression through mechanisms that remain obscure, virtually all patients continue to suffer from progressive disease that eventually results in death or lung transplantation (2–4).

Correspondence: GRS Budinger, 240 E Huron, M300, Chicago, IL 60657, Phone: 312 908 7737, s-buding@northwestern.edu.

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Clinical features of pulmonary fibrosis provide clues to the underlying pathophysiology. First, there appear to be robust mechanisms that prevent the development of fibrosis in most individuals. For example, mutations in the promoter region of MUC5b that confer a 30-fold increase in the risk of pulmonary fibrosis are present in ~20% of the population, but the population incidence of pulmonary fibrosis is much lower (~16.3 in 100,000) (5, 6). Similarly, 15–20% of the US population are current or former smokers, another risk factor for pulmonary fibrosis, but only a small fraction will develop the disorder (7). Secondly, pulmonary fibrosis is a disease of aging suggesting an overlap between pathways implicated in biologic aging and in those favoring the development of fibrosis. The incidence of pulmonary fibrosis in patients over the age of 75 is nearly 8 times higher than patients under 55 years of age (5). Indeed, even in patients with strong genetic predisposition to disease, pulmonary fibrosis is variably penetrant and is usually delayed until later in life (8, 9). Finally, in at least some patients with pulmonary fibrosis, the disease is characterized by step-wise declines in lung function that may coincide with “exacerbations”, brief periods of severe lung inflammation followed by worsening fibrosis, rather than a continuous decline in lung function (10–12). These data suggest the development of fibrosis is mediated by complex interactions between immune cells, the epithelium, and matrix secreting myofibroblasts.

These clinical and pathophysiologic features of pulmonary fibrosis have been recapitulated in murine models. While stimuli capable of inducing acute lung injury are legion, only a handful of stimuli have been reported to result in lung fibrosis, and a large number of genetic mutations and pharmacologic interventions have been shown to prevent or attenuate lung fibrosis in mice (13). We interpret these findings to suggest that persistent lung fibrosis is a kind of “perfect storm” requiring the failure of multiple biologic pathways designed to prevent it. Secondly, older animals show an increased predisposition to fibrosis, developing more severe and persistent fibrosis in response to a fibrotic stimulus when compared with younger animals (5, 14, 15). Finally, almost all of the described animal models of fibrosis show a temporal pattern of a mild or moderate acute lung injury, characterized by the influx of inflammatory cells into the lung, followed by a period of fibrosis, perhaps modeling the exacerbation phenotype observed in humans (16, 17).

Unbiased genome wide association studies combined with animal models suggest that damage to the alveolar epithelium is the initiating factor in pulmonary fibrosis. For example, a mutation in the MUC5b promoter that confers a risk of pulmonary fibrosis is associated with increased expression of an abundantly expressed mucin expressed exclusively in the airway and alveolar epithelium (6, 18, 19). A folding mutation in Surfactant Protein C, the expression of which is largely restricted to alveolar type II cells in the adult underlies a rare familial cause of IPF (20, 21). Epithelial specific loss of telomerase function in mice is associated with the development of spontaneous lethal pulmonary fibrosis (22, 23). There is consensus among investigators that this epithelial injury leads to the activation and proliferation of “myofibroblasts”, fibroblast like cells expressing α -smooth muscle actin, that secrete extracellular matrix proteins characteristic of fibrosis (24). These cells likely originate from resident lung fibroblasts, although their ontogeny remains an active area of study (25). The best described molecular link between alveolar epithelial dysfunction and myofibroblast activation and proliferation is the profibrotic cytokine Transforming Growth

Factor- β (TGF- β) (26, 27). We and others have found that mitochondrial and NADPH oxidase-generated ROS can enhance TGF- β signaling to promote fibrosis (14, 28, 29). This purpose of this review is to review ROS signaling with a goal of understanding the molecular mechanisms that underlie their role in TGF- β signaling. Readers interested in a more comprehensive discussion of pulmonary fibrosis mechanisms are referred to other excellent reviews (30).

TGF- β , oxidant signaling and fibrosis

In murine models, TGF- β is required and sufficient for the development of fibrosis; genetic loss of components of the TGF- β pathway prevent the development of lung fibrosis, and adenoviral infection of the lung epithelium with a virus expressing an active form of TGF- β is sufficient to induce fibrosis (31, 32). TGF- β is constitutively present in the lung interstitium in a latent form, and upon injury to the epithelium or endothelium, is converted from to an active form via a process that requires the $\alpha_v\beta_6$ integrin (33–37). Alternatively, active TGF- β can be released by macrophages, myofibroblasts or other cells including platelets (38, 39). Active TGF- β binds to one of a family of TGF- β receptors present on the cell surface, initiating an intracellular signaling cascade, which results in the phosphorylation of transcription factors referred to as small mothers against decapentaplegic (Smads). This induces the binding of Smad2 and Smad3 to Smad4 and the resulting Smad complex translocates into the nucleus. Within the nucleus the Smad complex interacts with transcriptional co-activators and co-repressors as well as a nuclear scaffolding proteins and histone acetyltransferases, which facilitate binding with canonical Smad-binding elements of target genes to activate a pro-fibrotic program of transcription (31).

We have shown that TGF- β binding to its receptor induces mitochondrial ROS, which augment TGF- β transcriptional responses, including NOX4 (29). As the generation of ROS by NOX4 is transcriptionally regulated, this might set up a positive feedback loop of ROS signaling that drives fibrosis (Figure 1). We found that signaling by mitochondrial ROS is not required for Smad phosphorylation or nuclear translocation, suggesting that non-canonical pathways contribute to TGF- β target gene expression, and might play a role in this positive feedback loop. For example, activation of the mitogen-activated protein kinase (MAPK) pathways including p38, extracellular signal-regulated protein kinase (ERK), or the c-Jun N-terminal kinase (JNK) augment TGF- β -induced gene expression (31). Sustained activation of these pathways by mitochondrial or NOX4-derived ROS, for example through the inactivation of phosphatases, may provide the link between TGF- β induced mitochondrial ROS and Smad-mediated gene expression (40). In support of oxidant signaling during fibrosis, several genes that directly regulate redox metabolism have been shown to be differentially regulated during fibrosis including NOX4, NOX1, ROMO1, SRXN1, DECR1, SOD2, KEAP1, and NMNAT1 (41).

Mechanisms of signaling by ROS

Shortly after discovering molecular oxygen, Joseph Priestly noted that several days of exposure to 100% oxygen resulted in death of mice, a finding subsequently reproduced in many animals and in humans. Several decades later, Freeman and Crapo provided evidence

that oxygen toxicity was caused by the formation of reactive oxygen species derived from the partial reduction of molecular oxygen (42). These and subsequent findings informed an understanding of reactive oxygen species as cellular toxins capable of nonselectively oxidizing cellular proteins, lipids and DNA. While this paradigm is likely true at pharmacologic levels of oxygen, we now recognize that low levels of ROS produced as a consequence of the redox reactions that sustain life can serve as intracellular signaling molecules regulating a diverse array of cellular functions. Recent work from our group and others have implicated ROS as signaling molecules important for the development of fibrosis in multiple organs, including the lung(43, 44).

There are only a handful of examples where investigators have defined the precise molecular mechanisms by which ROS signal. Ligand binding to EGFR stimulates the assembly of plasma membrane NAD(P)H oxidases that generate O_2^- and H_2O_2 , which oxidize susceptible cysteines in protein phosphatases amplifying the phosphorylation cascade(45). In addition, NOX4 has been shown to inactivate protein phosphatases in the ER that regulate trafficking of EGFR to the plasma membrane(46). In another example, thioredoxin, which is primarily responsible for the reduction of oxidized peroxiredoxins (see below), also functions as an oxidant sensor to activate cellular stress response pathways. In its reduced state, thioredoxin binds to the MAP kinase kinase kinase ASK1. This interaction is inhibited by cysteine oxidation of thioredoxin, releasing ASK1, which then undergoes auto-phosphorylation, eventually leading to the activation of the MAPK pathways c-jun-N terminal Kinase (JNK) and p38, both of which are key to the metabolic adaptation to cellular stress(47). As MAPK signaling has been shown to augment TGF- β signaling, and we have found that the augmentation of TGF- β signaling by mitochondrial ROS is independent of Smad phosphorylation or nuclear translocation, both of these mechanisms may be important for ROS signaling in response to TGF- β .

Two other signaling pathways by ROS that might impact TGF- β signaling during fibrosis have been well-defined. The transcription factor nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) binds to consensus sequences in DNA to induce the transcription of genes involved in antioxidant defenses, including many of the enzymes responsible for ROS metabolism described below. In the absence of oxidant stress, Nrf2 is bound to Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1 (KEAP1), which targets Nrf2 for ubiquitin-mediated degradation in the cytosol. During oxidant stress, H_2O_2 oxidizes cysteine residues in KEAP-1 (and Nrf2) that disrupt their interaction, thereby stabilizing Nrf2 and allowing its translocation to the nucleus to induce gene transcription. Failure of oxidant mediated induction of Nrf2 target genes, as might occur in aging, could therefore enhance TGF- β signaling. In a redox relay, electrons are transferred from H_2O_2 to the target protein indirectly through intermediary oxidant sensors. These relays were first identified in yeast where oxidation of the glutathione peroxidase-like enzyme oxidant receptor peroxidase-1 (ORP-1) activates the transcription factor Yap1 to induce the expression of antioxidant genes. Sobatta and colleagues identified a redox relay in mammals in which H_2O_2 -induced thiol oxidation of peroxiredoxin-2 oxidizes specific thiols in STAT3 to inhibit its transcriptional activity(48). A redox relay could provide a mechanism to explain how nanomolar concentrations of H_2O_2 generated in the mitochondria can target cytosolic proteins involved in TGF- β signaling.

Intracellular Sources of ROS

In cultured human lung fibroblasts from normal individuals and those with pulmonary fibrosis, our group discovered that the administration of TGF- β induced the generation of ROS from the mitochondria, and that mitochondrially-targeted antioxidants inhibited TGF- β -induced gene transcription (29). The mitochondria are highly abundant double membrane organelles. Indeed in some tissues over 50% of expressed RNA transcripts are comprised of mitochondrial DNA (49). Superoxide anions are generated from the mitochondrial electron transport chain, a set of multiprotein complexes that link the free energy released from the oxidation of glucose, lipids or proteins to the reduction of molecular oxygen to water (50). During electron transport, electrons generated from substrate oxidation are used to produce NADH and FADH₂ and then transferred to a series of multiprotein complexes that couple the progressive loss of free energy to the transport of protons across the inner mitochondrial membrane, generating a chemiosmotic gradient that is used to phosphorylate ADP to ATP. At virtually any step in this process, an electron can be inadvertently transferred to molecular oxygen, generating a superoxide anion, however, the bulk of the superoxide anions produced during electron transport are generated at Complex I and III of the mitochondrial electron transport chain (51).

Complex I is comprised of 14 conserved core units and some 30 additional proteins all located within the mitochondrial matrix (52, 53). In Complex I, electrons from NADH are transferred from a flavin mononucleotide to a series of Fe-S clusters resulting in the reduction of ubiquinone to ubiquinol in the mitochondrial matrix. This process provides energy for the transport of protons across the inner mitochondrial membrane, and electrons to the remaining complexes in the electron transport chain (52). While the precise mechanisms by which superoxide is formed in complex I are not known, a reaction between the flavin mononucleotide and molecular oxygen is thought to be the most likely cause. Because high levels of NADH promote reduction of the flavin mononucleotide, ROS from complex I are increased when the NADH/NAD⁺ ratio is high, as occurs when metabolic substrates are abundant.

The formation of ROS at mitochondrial complex III in mammals occurs during the sequential transfer of electrons through highly conserved proteins located within the mitochondrial inner membrane including the Rieske Fe-S protein, the proteins comprising the cytochrome *b* complex and cytochrome *c*₁ (54). The two electrons coming from complex I/II must be transferred to cytochrome *c*, which can only accept one electron at a time. This is accomplished by transferring the electrons to ubiquinol (the “Q cycle”), resulting in the transient formation of ubisemiquinone, a radical capable of transferring electrons directly or indirectly to molecular oxygen to produce superoxide anions. We found that knockdown of the Rieske Fe-S protein (upstream of the formation of ubisemiquinone in complex III) inhibited TGF- β -mediated transcription of fibrotic genes, while knockdown of a protein in the cytochrome *bc*₁ complex downstream of the formation of a ubisemiquinone radical did not. These results suggest that mitochondrial ROS generated from complex III are required for TGF- β induced transcription of profibrotic genes. While the importance of this pathway in lung fibrosis has not yet been determined, investigators have observed that the administration of an antioxidant targeted to the mitochondria attenuated the severity of

airway fibrosis in a TGF- β mediated murine asthma model and the development of nephropathy in a murine model of diabetic nephropathy (55, 56).

It is worth remarking that the negative charge of O_2^- likely prevents it from traversing cell membranes in the absence of an anionic transporter, and, even if one existed, overcoming the large negative charge of the mitochondrial inner membrane would likely prevent this from happening. As complex I O_2^- is completely generated in the matrix, it is less likely that it escapes from the mitochondria without being dismutated to H_2O_2 (57). In contrast complex III induced O_2^- can be generated in either the mitochondrial matrix or intermembrane space and therefore might escape the mitochondria prior to dismutation, particularly when ROS generation is high (58). These observations are also important to consider when interpreting experimental studies in which the SOD enzymes have been genetically deleted or overexpressed; SOD1 is located in the cytosol or intermembrane space, while SOD2 is localized to the matrix and SOD3 is localized to the extracellular space (54).

Members of the NAD(P)H oxidase family can serve as important sources of intracellular ROS, and NOX4 in particular has been suggested to play a key role in the development of lung fibrosis (28, 59). Originally identified in granulocytes, NAD(P)H oxidases are multiprotein complexes comprised of two membrane components, one of five Nox proteins (Nox1–5) and p22phox and 4 cytosolic components, p47phox, p67phox, p40phox, and the small G protein Rac1/2. Activation of neutrophils results in the phosphorylation of p47phox, removing inhibition of formation of the functional complex in the membrane, which generates large amounts of superoxide (60). Nox4 differs from other NAD(P)H oxidases as it does not require a signaling event to induce its activation as its activity is predominantly transcriptionally regulated (60). The activity of Nox4 has been linked to the development of fibrosis induced by TGF- β in several organs (61, 62). Thannickal and colleagues demonstrated that the levels of Nox4 were increased in the lungs of patients with IPF and that knockdown of Nox4 mediated by the intratracheal delivery of a siRNA inhibited the development of fibrosis following the intratracheal administration of bleomycin (59). Collectively, these results suggest a multifaceted role for oxidants in the development of TGF- β induced fibrosis and suggest that different oxidant generating systems might act in concert to provide a permissive signal for the development of fibrosis. If so, targeted antioxidant therapies might be more effective if used in combination. Unfortunately, we know less about the importance of signaling levels of mitochondrial or NAD(P)H oxidase derived ROS in other cell populations in the lung.

Metabolism of ROS

The levels of mitochondrial or NADPH oxidase generated ROS in a resting or TGF- β -stimulated cell reflect a balance between production and metabolism. The superoxide anion is generated in cells when an electron is inadvertently or intentionally transferred to molecular oxygen. The superoxide anion can either react with cellular substrates or be metabolized by one of the three superoxide dismutases (63). All three SOD enzymes catalyze a similar reaction in which two protons are incorporated into the superoxide anion to form hydrogen peroxide (H_2O_2). The rapid kinetics of these dismutation reactions and the high concentrations of the SOD enzymes are likely to limit the concentrations of superoxide

anion during physiologic conditions. The product of this reaction, H_2O_2 , is primarily metabolized by the peroxiredoxins, a family of dimeric proteins that are expressed at micromolar concentrations in most cells (64). Peroxiredoxins act both as an electron donor and enzyme, transferring two electrons from a reduced cysteine residue to H_2O_2 , forming a sulfeinic acid, which then interacts with a cysteine in the dimeric partner to form a stable intraprotein disulfide bridge. The function of oxidized peroxiredoxins is restored upon reduction by the thioredoxins, which contain an active thiol that attacks the peroxiredoxin disulfide bond. Thioredoxins are in turn reduced by thioredoxin reductase at the expense of the oxidation of NADPH to $NADP^+$. A smaller fraction of H_2O_2 is metabolized by spontaneous decomposition into H_2O and O_2 , a reaction facilitated by catalase which is expressed predominantly in peroxisomes.

Glutathione metabolism operates in parallel with the peroxiredoxin/thioredoxin system to metabolize cellular oxidants (50, 65). Reduced glutathione can be oxidized by glutathione peroxidases in order to reduce H_2O_2 or lipid peroxides, or alternatively can be used to stabilize electrophiles through glutathionylation by Glutathione S-transferases. Reduced glutathione is then restored by glutathione reductase using electrons from NADPH. In addition, the systems connect through shared substrates of some of the reductases including the reduction of cysteines on intracellular proteins, which may be oxidized during more severe oxidant stress. Only when H_2O_2 escapes metabolism through these systems can it interact with cellular transition metals creating highly toxic hydroxyl radicals (Fenton chemistry), which can damage cellular proteins, lipids and DNA. It is clear from these reactions that maintenance of reduced pools of NADPH are essential to limit H_2O_2 -mediated signaling and oxidative damage in the cell, highlighting the inherent links between metabolism, redox signaling and oxidant-mediated injury, which are likely important to understand the role of ROS in the development of pulmonary fibrosis.

Aging, ROS and the susceptibility to fibrosis

The incidence of IPF, increases exponentially with age, making older age one of the most important risk factors for the development of IPF (5). Indeed, even in patients harboring mutations that increase the risk for IPF, the disease seldom develops earlier than the third or fourth decade of life and some individuals are spared altogether. Collectively, these findings suggest a strong relationship between the biology of aging and the development of pulmonary fibrosis. While aging is often considered a risk factor that is not amenable to therapeutic targeting, recent research has identified several pathways that underlie the development of aging including mitochondrial dysfunction, which might increase the generation of signaling ROS or impair the protection against toxic levels of ROS (66). For example, Thannickal and colleagues linked cellular senescence with oxidant signalin and the failure to resolve fibrosis after lung injury during aging (14). They reported that while aged and young mice exhibited a similar level of peak lung fibrosis after the administration of bleomycin, fibrosis persisted in older mice. This was accompanied by an increase in the number of senescent myofibroblasts in lung tissue. Aged animals had reduced levels of Nrf2 target genes and higher levels of NOX4, and targeting NOX4 in older animals partially restored fibrosis resolution. Higher levels of ROS signaling with aging might also be induced by changes in the lung microenvironment. For example, we have shown that the

replacement of tissue-resident alveolar macrophages with monocyte-derived cells over the lifespan may adversely affect alveolar epithelial homeostasis in response to injury (67). A better understanding of the molecular events that affect ROS signaling during aging might identify pathways that can be targeted for therapy of pulmonary fibrosis.

Implications for therapy

Despite the importance of signaling levels of ROS in TGF- β -mediated profibrotic gene expression, a trial of N-acetyl cysteine (NAC) failed to improve outcomes when administered to patients with pulmonary fibrosis in a randomized clinical trial (68). While this could be interpreted as evidence that ROS are not required for pulmonary fibrosis, the failure of NAC might result from an incomplete understanding of ROS metabolism. NAC acts primarily by increasing stores of reduced glutathione, which may partially protect cells from lethal oxidant injury, but may have unpredictable effects on subcellular increases in local H₂O₂ concentrations that might drive pro-fibrotic signaling (69). Alternatively, or in addition, nonselective inhibition of ROS signaling might impair cellular processes that mitigate fibrosis severity. For example, we have shown that mitochondrial ROS generation is required for the stabilization of Hypoxia-Inducible Factor-1 during hypoxia, and that signaling levels of ROS are required for stem cell proliferation and differentiation (70). As an alternative, activation of endogenous antioxidant defense pathways, most importantly nuclear factor-erythroid-2-related factor 2 (Nrf2), might attenuate maladaptive oxidant signaling without interrupting homeostatic signaling to ROS (71). Perhaps the most promising areas of investigation are those that move beyond strategies to nonselectively limit ROS generation or enhance their metabolism, toward identification of the intracellular molecules that are oxidized or otherwise modified by signaling levels of ROS (72). Identifying these targets will necessitate careful consideration of their origin, normal metabolism and intracellular localization in distinct cell populations within the lung. Understanding the molecular mechanisms by which ROS contribute to TGF- β signaling, particularly in older individuals, might allow for the development of targeted therapies to treat fibrosis or improve the efficacy of those currently available.

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Figure 1. ROS signaling in response to TGF-β

In response to TGF-β, fibroblasts generate mitochondrial ROS from complex III of the electron transport chain. These ROS augment Smad-mediated transcription of TGF-β target genes through mechanisms that are not clear, but do not appear to involve ligand binding, Smad phosphorylation or Smad nuclear translocation. NOX4 is an important TGF-β target gene that also produces ROS and augments TGF-β target gene expression. Together, these systems suggest a positive feedback loop in which ROS signaling can sustain TGF-β mediated expression of profibrotic genes.