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NADPH oxidase-4: Pathophysiology and therapeutic potential in Idiopathic Pulmonary Fibrosis

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is the most common form of lung disease with unknown etiology that causes scarring and stiffness in the tissue. The disease represents 50% 3-5-year survival post-diagnosis. Mounting evidence indicates that oxidative stress is a significant player in the pathogenesis of IPF. Oxidative stress is characterized by an increase in the production of reactive oxygen species (ROS) sourced predominantly from NADPH oxidases (NOXes) and mitochondria. Several Studies have unveiled the role of NOXes esp NOX1, NOX2 and NOX4 in the IPF pathogenesis in addition to the role of mitochondrial ROS. Among the other NOX-isoforms, NOX4 is particularly noted for its inducibility by transforming growth factor-beta (TGF-β, known for its high levels in IPF) and its ability to generate H2O2 after induction.

Studies have shown that NOX4 may be an important catalyst in the maintenance of a profibrogenic environment, mainly acting through fibroblasts and extra-cellular matrix (ECM) deposition.

As such, several therapeutic strategies have been proposed to reduce the production of ROS and attenuate its role in fibrosis. Some of the most notable modalities aim to selectively target and inhibit NOX4 and its concomitant dynamic interplay with other related signaling molecules in the fibrotic pathway. Many emerging therapeutic options are currently being investigated, however, the search for the ideal specific mode of treatment remains elusive.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common form of interstitial lung disease of unknown cause, characterized by thickening, scarring and stiffening of the lung tissue. Individuals with IPF develops life-threatening complications due to shortness of breath and respiratory failure with mean survival of 2-5 years post-diagnosis [1,2] . Histologically, IPF is characterized by subpleural reticulation associated with honeycomb changes and fibroblast foci consisting of collections of myofibroblasts and collagen [3,4].

This disease is associated with several risk factors such as: cigarette smoking, exposure to metal fumes and wood dust, certain occupations such as farming, hair-dressing, and stone-polishing [5–10].

Although, IPF is usually a sporadic condition but also seen in families known as Familial Pulmonary fibrosis (FPF)- which often seen in two or more family members. However the prevalence of FPF is roughly represent about 5% of the total IPF cases [11] [12] and radiological, clinical and histological features of FPF are found indistinguishable from sporadic IPF. Genetic variants in FPF-related genes, such as germline loss of function mutations in the “telomerase genes” like TERT, TERC, and recently associated RTEL and PARN genes, are most frequent mutations found with FPF-patients.

Mutations in some alveolar-epithelial cells specific genes like surfactant protein-C (SFTPC) and A2 (SFTPA2) genes have also been found in some distinct cohort of FPF-subjects[13][14]. One study on early-senescent fibroblast cells senescence (SES)-reported that mitochondrial-ROS is a critical determinant of telomere-dependent senescence[15] and an imbalance in oxidative- and anti-oxidative mechanism can’t be ignored.

NADPH oxidases have been found to produce the ROS in lung tissue, and its dynamic interplay with other cytokines and growth factors, notably transforming growth factor-Beta (TGF-β). TGF-β is a primary drivers in the development of fibrosis, which involves a cascade of injury, inflammation, myofibroblastic differentiation, extracellular matrix deposition, and fibrotic foci formation. This review aims to discuss the molecular pathways responsible for the pathogenetic events that result in IPF-

disease, as well as the different therapeutic modalities and strategies employed for its treatment.

OXIDATIVE STRESS-A MASTER REGULATOR IN PULMONARY FIBROSIS (PF)

An increasing amount of evidence shows that the pathogenic mechanism of IPF is triggered due to repeated injury to the alveolar epithelial lining cells. Several predisposing risk factors result in alveolar injury, one of which is the formation of reactive oxygen species (ROS). ROS are a type of oxygen-derived free radical which are produced generally in cells during mitochondrial respiration. In pathologic conditions and states of oxidative stress, there may be increased production or decreased scavenging of these substances leading to an excess of free radicals [16][17].

The term “oxidative stress” pertains to the entire molecular, cellular, and tissue abnormalities resulting from excess ROS production and depleted antioxidant defenses[16]. The lung is postulated to be particularly sensitive to oxidative stress due to its exposure to relatively higher oxygen tensions when compared to other tissues and organs [18].

These free radicals exert cellular injury by binding to and modifying lipid, protein, and nucleic acid components, and thus eventually destroying cells. ROS can also activate or mediate the effects of several growth-regulating cytokines. There is growing evidence that these play a significant role in the pathogenesis of IPF, which is characterized by an increase in the production of reactive oxygen species sourced predominantly from NADPH oxidases (NOXes) and mitochondria[16]. However, the endogenous sources for oxidants in this disease are still largely unknown [19].

SIGNIFICANCE OF MITOCHONDRIAL-ROS IN THE PATHOGENESIS OF IPF

Mitochondria are double-membrane organelles of the cell which function primarily to produce energy in the form of ATP through oxidative phosphorylation. Also, this organelle serves a function in other processes involving cellular signaling in differentiation or apoptosis. These are typically up to 3 µm in diameter, but studies have shown that they are approximately three times larger in type II alveolar epithelial cells (AECs) compared to other lung cell types. In times of cellular stress or injury, type II AECs can differentiate into type-I AECs by reducing the number and size of mitochondria by mitochondria fusion [20]. Conversely, mitochondria can also adapt to stress by increased fission.

Mitochondria from Type-II AECs and fibroblast from IPF-lungs display reduced electron-transport chain complex-I, IV activity and ATP content with reduced rate of oxygen consumption respectively [21,22]. Mice that harboring mitochondrial-targeted human catalase gene displayed low AEC-mtROS production, mtDNA damage and mitochondrial fragmentation and an improved fibrotic profile upon crocidolite (blue-asbestos) or bleomycin treatment than their non-carrier counterparts [23]. Dysfunctioned mitochondria from lung-macrophage is a common feature observed in IPF-subjects [24] and in fibrotic-experimental mice displaying increased production of ROS, and mitochondrial fragmentation (mitophagy)[25]. Mitochondrial dysfunction is characterized by a loss of efficiency in the electron transport chain resulting in altered mitochondrial function, reduced membrane potential, and increased ROS formation. In patients with IPF, studies have demonstrated that alveolar cells like epithelial cells, macrophages as well as fibroblasts show signatures of dysfunctional mitochondria and disturbed mitochondrial dynamics.

NADPH Oxidases ROLE

The oxidative burst is an immune system response dedicated towards the eradication of pathogenic microbes, which involves the NADPH oxidase (NOX) -mediated release of ROS by neutrophils and macrophages. Extensively, it was believed that superoxide generation through NOX could occur only in phagocytes, but it has already been identified that ROS production by other enzymes could also occur in varying tissues. These other enzymes are similar to that found in inflammatory cells. Thus, they are conjointly referred to as the NOX family [26].

NOX enzymes catalyze several functions through redox signalling in various tissues such as in the vascular endothelium, renal tubules glomeruli, lung alveoli, colon mucosa, and brain microglia. In the lung, several cell types have been noted to generate NOX-dependent ROS, which is implicated in the pathogenesis of IPF.

At the alveolar-vascular interface, a substantial amount of stroma exists in the space between the alveolar epithelium and the vascular endothelium, which is composed predominantly of fibroblasts. In the event of tissue damage, these fibroblasts can migrate to the site of injury and differentiate into myofibroblasts. Together with the undifferentiated fibroblasts, these myofibroblasts are responsible for the production and deposition of the extracellular matrix, specifically fibronectin and collagen, which are vital components of fibrosis. In healthy lung tissue, the cellular components of these fibrotic foci undergo apoptosis, and the extracellular matrix undergoes clearance, both of which result in resolution. On the other hand, in lung tissues of those with IPF, myofibroblasts are characteristically increased and clustered in the injured lung parenchyma, giving rise to the fibrotic focus, which is a critical histopathological hallmark and prognostic marker of IPF[27].

In 2009, Hecker et al identified a specific variant of the NOX gene (NOX4 gene) in human fetal lung mesenchymal cells as one of the most highly induced genes when stimulated by TGF-β[28]. This study also established that NOX4 plays a crucial role in generating H2O2 for attributing contractile and synthetic properties to myofibroblasts that differentiated under the influence of TGF-β. Furthermore, with the use of immuno-histochemical staining, the study demonstrated that NOX4 is highly expressed in myofibroblastic foci of the lungs affected by IPF, similar result detected in bleomycin-induced lung injury in mice[24].

Another cell type is the alveolar epithelial cell, which consists of two types, the alveolar type 1 cell (AT1) and the alveolar type

2 cells (AT2), each covering about 96% and 4% of the internal surface area of the lung respectively. AT1 cells are squamous cells that primarily serve as capillary-alveolar barriers for gas exchange. In contrast, AT2 cells are cuboidal secretory cells that produce surfactants and function as progenitor cells which can repopulate injured lung epithelium. In lung tissue of those with IPF, increased expression of NOX4 has been demonstrated in atypical hyperplastic AT2 cells. It suggests that NOX4 increases ROS generation in abnormal alveolar epithelial cells and therefore contributes to pathologic repair[27].

Depletion of antioxidant defense mechanism

Homeostasis in the pulmonary environment requires maintenance of balance between oxidant and antioxidant substances in the intracellular and interstitial milieu. In order to preserve this dynamic equilibrium, there are antioxidant defense mechanisms that include the following: small-molecular-weight antioxidants (ex. glutathione), mucins, metal-binding proteins (ex. transferrin), intracellular and extracellular superoxide dismutases (SODs), reducing enzymes, detoxification enzyme systems, and other redox regulatory thiol proteins [18,29].

Disregulated or uncontrolled ROS formation is commonly thought to contribute to the pathology of diseases. Overproduction of ROS, either by increased oxidase enzyme activation or mitochondrial dysfunction, or compromised metabolism of ROS by antioxidant systems can result in an increased free radical burden which can lead to pervasive damage to molecules such as DNA, lipids and proteins[30].

Several studies have suggested that oxidant-antioxidant imbalances in the lower respiratory tract play a significant role in the pathogenesis of IPF [19][29,30]. Additionally, it has been established that patients with IPF showed increased markers of oxidative damage[18], an increased levels of Oxidative stress marker 8-isoprostane were reported in half of IPF patient [31,32] and in exhaled breath condensate in asthmatic subjects[33]. Therefore, a deficiency of antioxidant defense mechanisms can give rise to oxidative stress and further aid in the development of fibrosis. A number of studies have determined that several of these systems are impaired in IPF[34,35].

Catalase as an antioxidant in pulmonary fibrosis

Catalase is widely expressed within the pulmonary alveolar epithelium and in lung inflammatory cells. Since it is a valuable scavenger of H2O2, it can inhibit H2O2-mediated activation of fibroblasts in IPF lungs[36]. In one study, it was determined that continuous intravenous infusion of polyethylene glycol catalase could block asbestosis in animal models [37]. Another study supported this finding by demonstrating that catalase administered intra-tracheally to animal models with asbestos-induced lung injury was able to prevent pulmonary fibrosis by inhibiting H2O2 production [38]. Lastly, low levels of catalase with reduced activity in the lungs of human patients with IPF were seen. Similar result reproduced in bleomycin-exposed mice [39], suggesting a high significance of catalase.

Glutathione as an antioxidant in pulmonary fibrosis

Glutathione (GSH) is one of the most abundant low molecular weight antioxidants found in healthy human lungs and airway secretions. In those with IPF, reduced glutathione levels were found in the epithelial lining fluid of the lower respiratory tract, as well as in the blood, sputum, and most importantly in fibrotic foci of the lungs[29]. It was supported by studies investigating the anti-fibrotic effects of N-Acetylcysteine (NAC), which is a glutathione precursor, in animal models with substance-induced fibrosis [40,41], which demonstrated that administration of NAC increased lung glutathione levels and attenuated fibrotic development.

Superoxide dismutase role in pulmonary fibrosis

Superoxide dismutase (SOD) exists in three isoforms in mammals: intracellular copper-zinc SOD; mitochondrial manganese SOD; and extracellular SOD. These exist in lung tissue and primarily decompose superoxide radicals to H2O2. Extracellular SOD is expressed in high concentrations in the lung and is the isoform that is implicated in the pathogenesis of ROS-induced pulmonary diseases. It is thought that this enzyme wields effects against fibrosis by preventing degradation of the extracellular matrix and release of these degeneration products[16]. Additionally, the mitochondrial SOD isoform was found to have higher expression in alveolar macrophages, but it has low concentrations in late fibrotic lung lesions of IPF. It implies that SOD activity may be impaired in fibrogenesis[29].

NADPH-Oxidases- STRUCTURE, ACTIVATION, FUNCTION and implication in IPF

The NADPH Oxidase (NOX) enzymes are a family of oxido-reductase enzymes consisting of seven members in mammals (NOX 1-5 and DUOX 1-2). The different isoforms of these enzymes have six alpha-helices spanning the cellular membrane with C- and N-terminals[16,26,27,30]. All NOX members contain a homologous C-terminal flavoprotein domain consisting of both an NADPH-binding region and a flavin adenine dinucleotide (FAD) binding region. On the other hand, NOX 1-5 isoforms possess a hydrophobic N-terminal domain composed of six transmembrane α-helices that contain two heme-binding sites. The only known function of NOX is the generation of ROS by catalyzing one to two-electron reductions to form O2- and H2O2, which are employed for cellular signaling, microbial death, and host tissue damage when these are released primarily by inflammatory cells and to some extent by non-inflammatory cells.

In the most recent paper, the enzymes NOX1, NOX2, and NOX4 were implicated in the pathogenesis of IPF in addition to mitochondrial ROS. However, the exact mechanism by which NOX and mitochondrial ROS contribute and cooperate in this pathology has not been demonstrated [27].

NOX1

NOX1 is expressed in epithelial, endothelial, and smooth muscle cells. Although it is widely expressed in different tissues, this enzyme is most enriched in colon mucosal epithelium, where it plays a principal role in ROS-generation by innate immune defense mechanisms[26,27,30]. The specific

participation of NOX1 in IPF pathophysiology is still unclear, however, in healthy lung tissue; it is expressed in pulmonary vascular smooth muscle cells, pulmonary artery endothelial cells, pulmonary epithelial cells, and bronchial epithelial cells. Carnesecchi et al demonstrated that human pulmonary artery endothelial cells showed decreased levels of intracellular ROS and fibrotic markers (vimentin, a-SMA, and CD31) when these were transfected with NOX1-targeted shRNA [42]. Further, it was determined that these resulted in decreased collagen deposition in animal models (mouse lungs) with radiation-induced fibrosis.

NOX2

NOX2 was the first discovered isoform of NOX and was studied primarily in the phagocytic cells immune response. Expression of this enzyme is most abundant in neutrophils and macrophages, where it plays a well-defined role in mediating inflammation and microbial killing. Activation of the NOX2 complex requires translocation of four cytosolic subunits p47phox, p67phox, p40phox, and either Rac1 or Rac2 to the enzyme. Once p47phox is phosphorylated, a conformational change occurs which promotes interaction of other cytosolic factors with NOX2 [27].

Although the exact mechanism by which inflammation propagates fibrosis in IPF is unclear, studies in patients with this disease have shown that alveolar macrophages and neutrophils produce ROS and contribute to alveolar epithelial cell death, which could be regulated through NOX2 activity. To support this, a study by Wang, et al. showed that bronchoalveolar lavage fluid obtained from patients with IPF possessed neutrophils with higher p47phox and p67phox expression compared to those without, suggesting specific participation of NOX2 in alveolar neutrophils [43]. Additionally, studies employing animal models noted that NOX2-deficient mice were protected from substance-induced pulmonary fibrosis [44,45].

NOX3

NOX3 has been identified primarily in fetal and adult kidneys, or small amounts in the liver, lung, and spleen [26]. In healthy kidneys, ROS production by NOX3 is the method by which renal function is moderated through the control of sodium transport, tubuloglomerular feedback, and renal oxygenation. Additionally, sodium chloride absorption in the loop of Henle is increased by free radicals, culminating in the regulation of the sodium-hydrogen exchange.

The study by Yasuoka et al. investigated whether the production of free radicals would be induced by Insulin-like Growth Factor Binding Protein-5 (IGFBP-5) [46]. Although NOX3 is present only in small amounts in lung tissue, this study showed that NOX3 primarily regulates ROS production by IGFBP-5 in human lung fibroblasts and that this resulted to the secretion of extracellular components which further participated in the fibrotic process. However, the authors concluded that the effect of IGFBP-5 on cellular antioxidant defenses and transduction of the fibrotic signal contributing to lung fibrosis remains to be completely elucidated[46].

NOX4

NOX4 was initially discovered in high concentrations in the human and mouse kidney. Its composite amino acid sequence is homologous to that of NOX2 in only about 39% [27]. This enzyme is expressed abundantly in tissues of the kidney, liver, ovary, and eye. Although it is not as copious in the lung, it is ubiquitously demonstrated in its cells such as macrophages, smooth muscle cells, endothelial cells, mesenchymal cells, and epithelial cells [19,24,47,48]. Among the other NOX isoforms, NOX4 is particularly noted for its inducibility by transforming growth factor- beta (TGFβ) and its ability to generate H2O2 upon induction. Moreover, the isoform is considered constitutively active because of its independence from cytosolic adaptor proteins. It implies that ROS production in the form of H2O2 by this enzyme is predominantly controlled by the level of NOX4 expression or by post-translational modification.

Recent studies have implicated NOX4 as an essential catalyst in the maintenance of a profibrogenic environment, mainly acting through fibroblasts and ECM deposition [46]. Its main contribution to the pathogenesis of IPF is discussed further below.

NOX5 and DUOX 1-2

NOX5 is a unique NADPH-oxidase isoform in that within its N-terminal cytosolic domain there are four calcium-binding motifs. It is commonly found in small amounts in tissues of the spleen, testis, mammary glands and cerebrum, and it produces free radical primarily in the form of O2-. NOX5 has also been isolated in the endoplasmic reticulum of human endothelial cells [49]; however, its exact participation in the mechanism of ROS-dependent vascular remodeling is unclear.

The dual oxidases Duox1 and Duox2 were initially isolated from the thyroid and are otherwise also known as thyroid oxidases. They possess a homologous domain similar to that of NOX1-4 and share approximately 50% identical amino acids with NOX2. Both enzymes mainly produce H2O2 and are glycosylated proteins which can be found in the cell membrane and endoplasmic reticulum. Although, the study by Little et al have shown that a loss of lung epithelial DUOX1 can promote mesenchymal differentiation by alveolar epithelial cells, its relevance in the pathology of fibrotic diseases has not been established [50].

Not much is known about how other NOX enzymes participate in the pathogenesis of IPF [30]. In contrast to the NOX1-4 enzyme isoforms, which require heterodimer formation with the transmembrane protein p22 for their activation, NOX5 and the DUOX1-2 enzymes require phosphorylation and calcium-binding for activation. Therefore, since these enzymes require activation by calcium signaling, calcium channel antagonism is an essential method in reducing this activity.

NOX4 EXPRESSION IN DIFFERENT CELLS, TISSUES AND ROLE IN FIBROTIC PROCESS

Pathogenesis of IPF

The pathogenesis of IPF is thought to develop with the onset of alveolar epithelial cell. Once injured, the alveolar epithelial cells release the pro-inflammatory substances TGF-β and TNF-a.

TNF-a, along with other cytokines, signals recruitment of other inflammatory cells, while TGF-β, along with other growth factors, stimulates the migration and proliferation of fibroblasts increasing collagen deposition. Additionally, other growth factors induce the emergence of myofibroblasts from resident cells. Over time, these myofibroblasts organize into clusters known as fibroblastic foci, where they continue to deposit extracellular matrices. This process of healing and repair eventually becomes uncontrolled and dysregulated as matrix accumulates and the fibrotic foci become established. The dynamic interplay between matrix synthesis, degradation, and resorption becomes imbalanced, further contributing to the fibrotic process. The injured alveolar epithelium eventually becomes unable to repair itself and thus becomes vulnerable to apoptosis. Without the layer of lining epithelium, the underlying basement membrane is exposed and breached, allowing fibroblasts to enter the alveolar space. The infiltrating cells also proliferate and fail to undergo apoptosis, therefore resulting in continuous extracellular matrix deposition, alveolar invasion, and eventual loss of standard lung parenchyma architecture.

Role of NOX4 in the fibrotic process

Alveolar epithelial damage is thought to initiate the fibrotic response in IPF, but how this occurs precisely is unknown. Increased oxidative stress is observed in IPF and postulated as a significant driving cause in IPF pathogenesis.

Data from recent studies have shown that both NOX2 and NOX4 are involved in the propagation of fibrosis. More specifically, in lung tissue, NOX4 was demonstrated in pulmonary fibroblasts and macrophages, with an upregulation of expression in those with IPF. This increase in NOX4 expression is accompanied by an increased intracellular ROS production, which predisposes the alveolar epithelial cells to injury, cell damage and mitochondrial biogenesis [19,24]. Cellular injury then results in the release of cytokines and growth factors, the most notable of which is TGFβ.

Aside from increased expression, several recent studies have demonstrated that TGFβ induces NOX4 in different tissues such as in the liver, heart, and kidney, and most importantly, in lung fibroblasts [51]. Hecker et al. demonstrated this occurrence in pulmonary fibroblasts that were cultured from lungs with IPF and further showed that induction occurs via ALK5 activation and Smad-3 phosphorylation[28] . In many fibrotic diseases, TGF-β is a multi-function protein that exists in different isoforms (TGF-β1, 2, and 3) and is the most potent pro-fibrogenic cytokine which plays a vital role in extracellular matrix deposition. TGF-β stimulates the production of ROS, resulting in propagation of oxidative stress, which in turn can activate latent TGF-β, setting up a vicious profibrogenic circle [16].

TGFβ is also interrelated with NOX4 such that TGFβ-induced ROS generation contributes to myofibroblast differentiation, extracellular matrix production, and increased fiber contractility, resulting in the formation of fibrotic foci. The main event involved in the downstream signal of the NOX fibrogenic pathway is Smad phosphorylation, probably through an autocrine loop which involves ROS produced by activated NOX4 [51]. Also, NOX4 mRNA expression correlated with mRNA expression of a-SMA and procollagen-I. While the change in SMA and procollagen expression in response to TGFβ required NOX4-generated ROS, NOX4 modulated SMA and procollagen-I expression by controlling the Smad phosphorylation pathway. It is supported by several studies which showed that silencing NOX4 expression in fibroblasts nullifies TGFβ-induced Smad phosphorylation and myofibroblast differentiation in fibroblasts of both lung and cardiac tissue [19,52]. Platelet-Derived Growth Factor (PDGF) also drive increase in NOX4 expression in lung fibroblast, which in turn may stimulate the migration and differentiation of fibroblast to myo-fibroblast and contribute in ROS levels. ROS mediate Smad phosphorylation-a main contributor in the activation of transcription programs regulating the myofibroblast differentiation. Also, ROS can activates mitogen-activated protein kinase (MAPK) pathways viz c-Jun N-terminal kinase (JNK) pathway, p38MAP kinase pathway, and the MAPK/ERK pathway which are also involved in the transcriptional activation of NOX4. Hence, activity of NOX4 during fibrosis required pharmacological inhibition to prevent pathologic progression [53,54].

THERAPEUTIC STRATEGY IN PULMONARY FIBROSIS

Acetylcysteine as a treatment modality for IPF

N-Acetylcysteine (NAC), a prodrug to L-cysteine, is a precursor to the biologic antioxidant glutathione, which has been found to have reduced levels in the epithelial lining fluid, serum, sputum, and fibrotic foci of the lungs in patients with IPF [29]. Theoretically, the administration of acetylcysteine replenishes glutathione stores, and this hypothesis is central to the studies which aim to investigate acetylcysteine as a treatment modality for IPF.

Several studies investigated using acetylcysteine as a monotherapy for patients with IPF. These showed that high doses of acetylcysteine restored depleted glutathione levels and that treatment with this regimen had favorable corollary effects on lung function [55]. In 2005, Demedts et al conducted the IFIGENIA (Idiopathic Pulmonary Fibrosis International Group Exploring N-Acetylcysteine I Annual) trial to test the hypothesis that the administration of a high dose of acetylcysteine over one year in addition to prednisone and azathioprine, would slow the functional deterioration in patients with idiopathic pulmonary fibrosis [56]. This study showed that the addition of acetylcysteine to standard therapy with prednisone and azathioprine in patients with IPF significantly slows the rate of deterioration of the primary pulmonary surrogate endpoints vital capacity and single-breath carbon monoxide diffusing capacity (DLCO) than standard therapy alone. However, significant effects in the clinical setting remained to be seen, and thus Behr et al (2009) recommended in their analysis that a long-term clinical trial testing NAC in IPF was warranted [57].

To answer this, several randomized control trials investigated NAC in IPF, and these studies showed that there was no significant benefit on mortality when using NAC monotherapy. Additionally, no significant differences in lung function, quality of life, or adverse outcomes were noted. However, no evidence of significant harm was found. Therefore, the recommendation to discontinue NAC monotherapy in patients already receiving

this regimen was not made. Furthermore, it was stated that if there is no benefit from starting this therapy, then it is unlikely that there is a benefit from continuing. To support these recommendations, a review from meta-analysis studies was conducted by Sun and associates showed that treatment with NAC failed to provide statistically significant benefits in terms of lung function, adverse events, or death [58]. However, no significant difference in adverse events or mortality was found as well, which suggests that this medication is still relatively safe for patients with IPF.

Clinical Practice Guidelines for treatment of IPF

Additional studies then aimed to combine NAC with immuno-modulators. The PANTHER Study was conducted in 2012, and this randomized controlled trial showed that a combination of prednisone, azathioprine, and NAC (triple- therapy) was associated with an increased risk of death and hospitalization, as well as treatment-related severe adverse events when compared with placebo.

In 2011, the American Thoracic Society (ATS), European Respiratory Society (ERS), Japanese Respiratory Society (JRS), and the Latin American Thoracic Association (ALAT) released a joint Clinical Practice Guideline on the Treatment of Idiopathic Pulmonary Fibrosis, which was updated in 2015 [59]. The guidelines no longer recommended the combination therapy of N-acetylcysteine, azathioprine, and prednisone because of the negative effects demonstrated by the PANTHER Study. However, there was no consensus on how to manage patients who have already been receiving long-term combination therapy long-term

Emerging NOX4/1-targeted Therapies

Although, accumulated data have supported the concept that NOX enzymes play critical roles in fibrogenesis, specific NOX inhibitors still have not been identified or tested in animal models [60][61]. The study by Jarman et al. in 2014 investigated this further using a small molecule NOX4/NOX1 inhibitor, where targeting NOX4 resulted in a reduction of fibrotic response [61]. Further, this study demonstrated that NOX4 antagonists attenuate TGF-β–mediated up-regulation of profibrotic genes and also inhibit myofibroblastic differentiation.

Metformin is a biguanide medication used primarily as a treatment for Type II Diabetes Mellitus. A study onto the role of metformin in attenuating fibrosis via NOX4 suppression. Their study found that TGF-β-induced myofibroblast differentiation was inhibited by metformin via activation of the AMPK pathway, which was responsible for inhibiting TGF-β-induced NOX4 expression [47]. Additionally, metformin reduced the expression levels of NOX4 and SMAD phosphorylation, with concurrent reduction of lung fibrosis. Hence, this study was able to establish an anti-fibrotic property of metformin, implying that it can be used as an alternative for IPF treatment.

Azithromycin is a macrolide antibiotic that is commonly used to treat respiratory infections, and it has been demonstrated to exhibit a pleiotropic effect on cellular processes, including antioxidant activity and immunomodulation. The study by Tsubouchi, et al., investigated the potential anti-fibrotic property of azithromycin using bleomycin (BLM)-induced lung fibrosis mouse models. This study demonstrated that azithromycin was able to suppress the development of lung fibrosis via concomitant reduction in the levels of NOX4, as well as increased proteasome activation. It results in the inhibition of TGF β-induced myofibroblast differentiation and a consequential prevention from lung fibrosis, thereby implying that azithromycin can be utilized in the treatment of IPF [62].

Bromodomain and extra-terminal (BET) proteins are promising therapeutic targets for inflammation and fibrosis since these proteins (Brd2, Brd3, Brd4, and BrdT), specifically Brd3 and Brd4, drive NOX4 expression in pulmonary fibroblasts. These proteins are epigenetic readers, which regulate gene expression by binding to acetylated lysines and recruiting transcriptional activators or repressors [63]. BET protein inhibitors have emerged as effective treatment options in reducing pathological fibrotic responses. JQ1, a small molecule inhibitor, has been shown to inhibit the binding of BET protein Brd4 to acetylated lysines, thereby blocking the progression of fibrosis by attenuating the TGF-β1-induced fibrotic gene expression and NOX4-dependent ROS generation [64]. The results of these studies revealed that Brd4 is a potential therapeutic target in the treatment of renal fibrosis.

NOX4/NOX1 inhibitors (GKT137831 and GKT136901) were developed by Genkyotex (Geneva, Switzerland). GKT137831 has recently been marketed as a dual Nox4/Nox1 small-molecule inhibitor drug candidate. In 2010, the European Commission granted orphan drug status to this medication for the treatment of IPF. However, whether it can specifically inhibit NOX4 remains controversial.

Several more studies are being done to investigate the development of NOX inhibitors. However, bona fide selective NOX inhibitors are not yet available in the market. Most recently, NOX inhibitors still do not have exact specificity for a single isoform, and no selective NOX inhibitors have been identified.

CONCLUSION

Idiopathic Pulmonary Fibrosis is a chronic progressive disease that is thought to develop with repeated alveolar epithelial injury brought about by states of oxidative stress due to increased concentrations of reactive oxygen species. These free radicals or ROS arise as products of the enzyme complex, NADPH Oxidase (NOX), of which NOX4 has garnered significant interest due to its participation in the fibrotic pathway of the lungs. Hence, targeted inhibition of NOX4 has become a focus of the investigation by several studies which aim to find safe and effective treatment for this debilitating disease. Although progress has been made at the forefront of this endeavor, the search for this ideal NOX4 inhibitor remains.

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