



Review

Airway Smooth Muscle Regulated by Oxidative Stress in COPD

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Abstract: Since COPD is a heterogeneous disease, a specific anti-inflammatory therapy for this disease has not been established yet. Oxidative stress is recognized as a major predisposing factor to COPD related inflammatory responses, resulting in pathological features of small airway fibrosis and emphysema. However, little is known about effects of oxidative stress on airway smooth muscle. Cigarette smoke increases intracellular Ca^{2+} concentration and enhances response to muscarinic agonists in human airway smooth muscle. Cigarette smoke also enhances proliferation of these cells with altered mitochondrial protein. Hydrogen peroxide and 8-isoprostans are increased in the exhaled breath condensate in COPD. These endogenous oxidants cause contraction of tracheal smooth muscle with Ca^{2+} dynamics through Ca^{2+} channels and with Ca^{2+} sensitization through Rho-kinase. $\text{TNF-}\alpha$ and growth factors potentiate proliferation of these cells by synthesis of ROS. Oxidative stress can alter the function of airway smooth muscle through Ca^{2+} signaling. These phenotype changes are associated with manifestations (dyspnea, wheezing) and pathophysiology (airflow limitation, airway remodeling, airway hyperresponsiveness). Therefore, airway smooth muscle is a therapeutic target against COPD; oxidative stress should be included in treatable traits for COPD to advance precision medicine. Research into Ca^{2+} signaling related to ROS may contribute to the development of a novel agent for COPD.

Keywords: oxidants; antioxidants; Ca^{2+} dynamics; Ca^{2+} sensitization; tracheal smooth muscle; phenotype changes; reactive oxygen species



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1. Introduction

Although chronic obstructive pulmonary disease (COPD) is simply diagnosed based on persistent air flow limitation that will not return to the normal range, using spirometric measurements, this disease is heterogenous and complex in symptoms, disease progression, lung function and response to therapies [1]. The pathogenesis of this disease results from chronic lung inflammation due to cigarette smoke and other environmental exposures (biomass fuel etc.); and this chronic inflammation is associated with activation not only of neutrophils and macrophages but also of eosinophils. While these responses to lung inflammation are normal in many healthy subjects, in contrast, the response is potentiated in patients who develop COPD. This chronic lung inflammation affects distal airways, leading to emphysema and small airway fibrosis (pathological characteristics of this disease) [2,3]; and these pathological alterations in COPD are progressive in most cases [1]. The mechanisms of this modified inflammation are not understood well. Moreover, a wide variety of inflammatory mediators are related to this chronic lung inflammation. For this reason, specific treatment for inflammation is not well established in this disease. Oxidative stress is defined as a state in which oxidation exceeds the capacity of antioxidant systems in the body secondary to a loss of the balance between them. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Oxidative stress from oxidative metabolism causes base damage, as well as strand breaks in DNA. Base damage is mostly indirect and caused by reactive oxygen species (ROS) generation,

e.g., $O_2^{\bullet-}$ (superoxide anion radical), $\bullet OH$ (hydroxyl radical), H_2O_2 (hydrogen peroxide) and O_3 (ozone) (Figure 1) [4]. Inflammatory cells as described above are recruited into the lungs in COPD [5]; and structural cells in the respiratory system (airway epithelial cells, fibroblasts, and endothelial cells) also contribute to the lung inflammation. These cells generate multiple mediators, including cytokines that perpetuate and amplify the inflammation in the lungs. These cells are also important sources of ROS, leading to oxidative stress in the lungs (Figure 1). Oxidative stress in the lungs due to exogenous oxidants (cigarette smoke, biomass fuel, air pollution) and endogenous oxidants (ROS generated by inflammatory cells, epithelium) are associated with clinical and pathophysiological characteristics of COPD (Figure 1) [6]. Mitochondrial respiration is an important source of ROS, and cigarette smoke produces excessive ROS via mitochondrial dysfunction (Figure 1) [7]. It is now generally considered that COPD results from an acceleration of lung ageing with the accumulation of senescent cells [8–10]. Senescent cells secrete high levels of inflammatory cytokines, immune modulators, growth factors, and proteases, referred to as senescence-associated secretory phenotype (SASP) [10]. This phenotype change is perhaps an essential mechanism in the chronic lung inflammation of COPD [11]. Since senescent cells also release ROS more than intact cells, this chronic lung inflammation potentiates oxidative stress in COPD (Figure 2). Therefore, oxidative stress is probably a major driving mechanism of many of the pathophysiological changes in COPD [12].

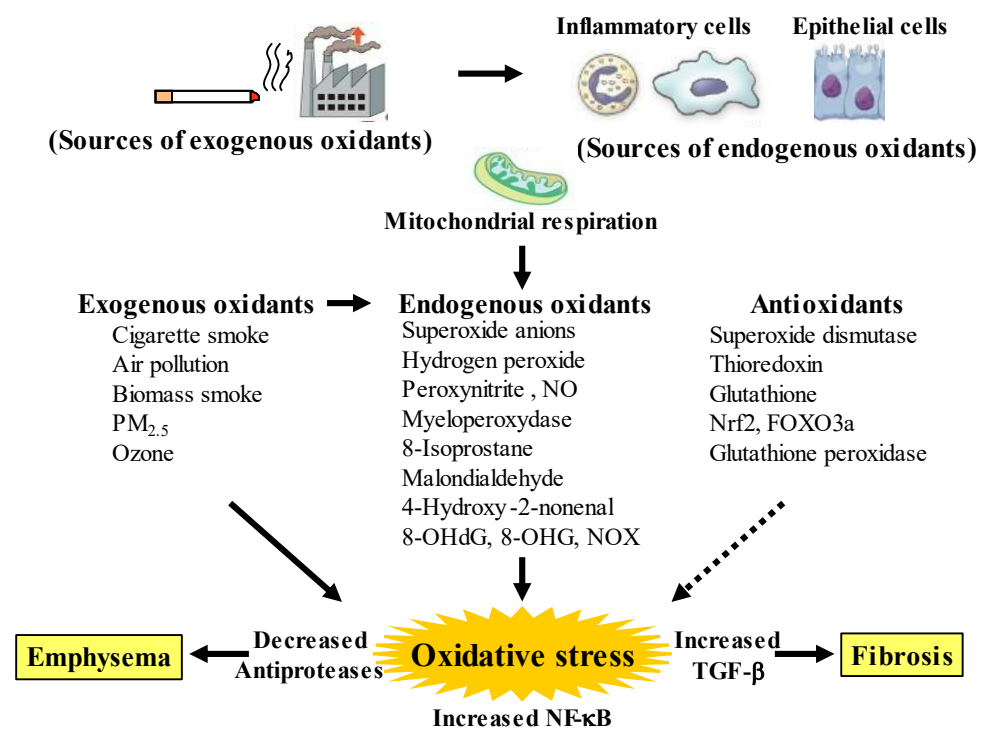


Figure 1. Oxidants and antioxidants involved in COPD, and relationships between oxidative stress and the pathology related to this disease. Oxidative stress in the lungs results from increased exogenous and endogenous oxidants, and from reduced antioxidants. Endogenous oxidants are generated by mitochondrial respiration. Elevated production of endogenous oxidants continues after stopping smoking. Increased oxidative stress is caused by a lack of balance between oxidants and antioxidants. Exogenous oxidants are derived from cigarette smoke, air pollution and biomass smoke, etc.; endogenous oxidants are derived from inflammatory cells (macrophages, neutrophils) and airway epithelial cells. Oxidative stress results in emphysema in alveolar areas with decreased antiproteases, and in fibrosis in the small airways with increased transforming growth factor (TGF)- β . PM: small particulate matter, 8-OHdG: 8-hydroxy-2'-deoxyguanosine, 8-OHG: 8-oxo-7,8-dihydroguanosine, NOX: membrane-bound NADPH oxidases, Nrf2: nuclear erythroid-2 related factor 2, FOXO3a: forkhead box O3a. Arrows: activation, dotted arrows: inactivation.

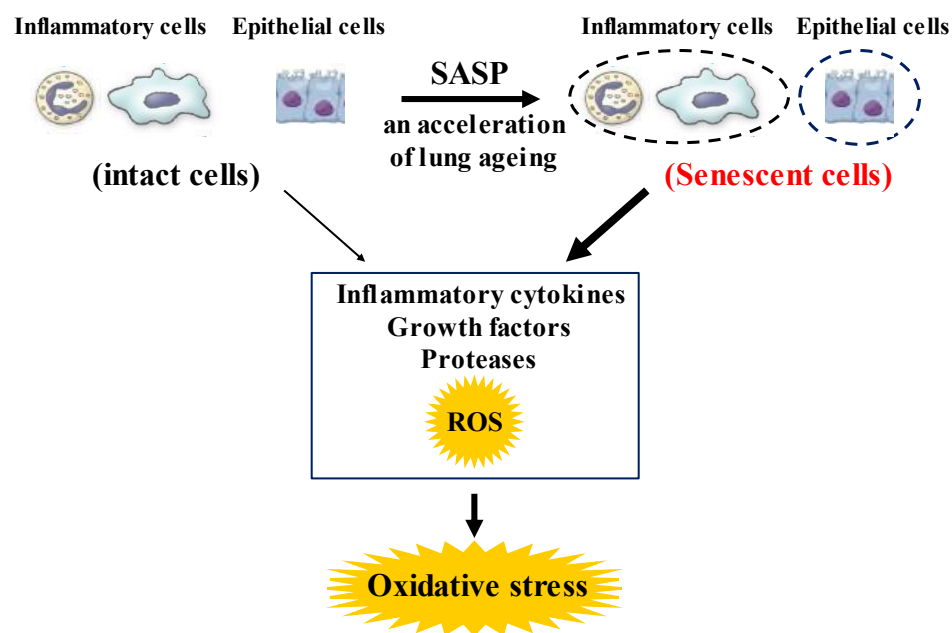


Figure 2. Roles of senescence in inflammatory and airway epithelial cells to enhance oxidative stress in COPD. These senescent cells in the lungs synthesize inflammatory cytokines, growth factors, proteases, and ROS more than intact cells in them, referred to as senescence-associated secretory phenotype (SASP). These phenotype changes in these cells perhaps potentiate not only the lung inflammation but also oxidative stress in COPD. ROS: Reactive oxygen species.

To improve the management and treatment for COPD, patients with COPD should be classified by grouping according to distinct clinical phenotypes. These groupings, based on multiple dimensions (clinical, physiological, imaging, and endotyping) determine clusters of patients with common characteristics, which are associated with clinically meaningful outcomes such as symptoms, exacerbations, response to therapy, and disease progression (stratified medicine). Moreover, since several phenotypes can coexist in individual patients with COPD, an approach due to therapeutic target identified phenotypes and endotypes (treatable traits) has been proposed as an advanced therapy recently (precision medicine) [13]. Although oxidative stress perhaps plays an important role in amplifying the chronic lung inflammation in COPD [14], little is currently known about the involvement of oxidative stress in the pathogenesis of COPD. Therefore, research for clinical phenotype classification focused on oxidative stress is needed to establish precision medicine for development of the therapeutic management for COPD.

In this chapter, roles of oxidative stress on airway smooth muscle are described with focuses on functional alterations that bring about phenotype changes related to the characteristic pathophysiology in COPD. Involvement of Ca^{2+} signaling due to Ca^{2+} dynamics and Ca^{2+} sensitization is also reviewed as a mechanism of the functional alterations in airway smooth muscle induced by oxidative stress in COPD.

2. Oxidative Stress in COPD

2.1. Pathological Features

Oxidative stress occurs in the lungs during COPD, leading to characteristic pathological changes in this disease (Figure 1). It is well proven by data derived from bronchial biopsy [15], sputum examination [16], and in vitro studies [17] that inflammatory cells such as neutrophils, macrophages and T lymphocytes infiltrate and various proinflammatory molecules are present at increased levels in smokers' lungs. Inflammatory cells, particularly neutrophils and macrophages that are recruited into the lungs, as well as structural cells, such as airway epithelial cells and fibroblasts, generate endogenous oxidants (ROS) in the lungs, leading to destruction of peripheral airways and alveoli. Mitochondrial respiration

in these related cells is a key source of ROS, and cigarette smoke enhances generation of ROS through mitochondrial dysfunction, supporting the pathophysiological characteristics in COPD [18,19]. These destructive processes overcome the local protective mechanisms, and cause tissue damage without manifestations. The inflammatory tissue damage may be perpetuated for a long time after smoking cessation in patients with COPD [20]. Cigarette smoke causes the chronic lung inflammation; however, only about 20% of smokers develop COPD, indicating that there are factors that increase susceptibility and amplify the normal inflammatory response to cigarette smoke. Although these mechanisms are still unknown in detail, this phenomenon is probably involved in oxidative stress due to synthesis of ROS and imbalance of local proteolysis/antiproteolysis states that are related to oxidative stress (imbalance of oxidants/antioxidants).

2.2. Oxidants Related to COPD

Oxidative stress is recognized as a major predisposing factor of the inflammatory response related to COPD. Oxidative stress is probably associated with the pathology and severity of COPD. Oxidative stress is potentiated in patients with COPD, especially when acute exacerbations occur. Cigarette smoke, air pollution and biomass smoke are major exogenous oxidants related to COPD in the lungs, referred to as exogenous oxidative stress, but oxidative stress also arises from endogenous processes due to endogenous oxidants, after stop smoking, referred to as endogenous oxidative stress (Figure 1). The number of activated alveolar macrophages is markedly increased in the lungs of patients with COPD, compared to healthy subjects; and a large amount of ROS is released from these activated macrophages as superoxide anions and hydrogen peroxide (H_2O_2) [21]. This phenomenon is more potentiated during COPD exacerbations. Activated neutrophils also infiltrate to the lungs in patients with COPD, and activated neutrophils release a large amount of ROS, especially during COPD exacerbations [22]. In patients with COPD, generation of 4-hydroxy-2-nonenal (4HNE) is increased in the lungs, indicating that lipid peroxidation, a marker of oxidative stress, occurs on endogenous lipids [23]. Clinical studies have demonstrated that H_2O_2 , 8-isoprostane, 4HNE, myeloperoxidase (MPO) and malondialdehyde (MDA) (endogenous oxidants as biomarkers of oxidative stress) are increased in exhaled breath condensate in patients with COPD [13,24–27], compared to healthy individuals; and these markers are more elevated during exacerbations [28]. These markers, such as MDA, 8-isoprostane, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and MPO, are also elevated in sputum from patients with COPD [29,30]. Furthermore, nucleic acid oxidation, 8-oxo-7,8-dihydroguanosine (8-OHG) in RNA and 8-OHdG in DNA are elevated in alveolar lung fibroblasts from patients with emphysematous COPD [18,31]. These augmented biomarkers of oxidative stress do not decrease, and remain elevated in ex-smokers after the cessation of smoking, suggesting that persistent lung inflammation is caused by endogenous oxidative stress [25].

The respiratory system is constantly exposed to oxidative stress due to sources of endogenous ROS generated by mitochondrial respiration and inflammatory responses to bacterial and viral infections. The persistent oxidative stress in COPD results not only from activated neutrophils and macrophages but also from epithelial cells in the respiratory system. Oxidative stress is associated with mitochondrial respiration in these structural cells [32]. Other sources of intracellular ROS include the cytoplasmic ROS generating enzymes, such as membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) and the xanthine/xanthine oxidase system, as well as neutrophil derived MPO [33]. Superoxide anions, relatively weak oxidizing agents, are mainly produced endogenously by NOX; and are rapidly converted to more damaging ROS, such as the hydroxyl radical and H_2O_2 , or the powerful and damaging peroxy radical in the presence of nitric oxide (NO) [34]. MPO is released from activated neutrophils which are recruited into the lungs of patients with COPD; MPO also produces very destructive hypochlorous acid such as 3-chlorotyrosine [35]. However, in healthy adults, intracellular antioxidant defenses can overcome these damaging ROS, thus limiting their cellular effects;

on the other hand, in patients with COPD these antioxidant defenses are overwhelmed. Increased oxidative stress has great effects on driving the pathophysiology of COPD as described below [33]. Oxidative stress causes activation of the proinflammatory transcription factor nuclear factor- κ B (NF- κ B) pathways; expression of NF- κ B is augmented in COPD in airway epithelium and macrophages in patients with COPD. Oxidative stress also causes activation of the transforming growth factor (TGF)- β 1 pathways, which acts on the lung epithelium, and induces fibrotic repair via driving epithelial-to-mesenchymal transition (EMT) [36], leading to small airway fibrosis. The inhibitory effects of TGF- β 1 on nuclear erythroid-2 related factor 2 (Nrf2) bring about reduced expression of endogenous antioxidants [37]. Oxidative stress increases the expression of matrix metalloproteinase 9 (MMP9), an elastolytic enzyme, related to the development of emphysema.

2.3. Antioxidants Related to COPD

Increased oxidative stress may be potentiated with a reduction in endogenous antioxidant-induced defenses in patients with COPD (Figure 1). A clinical trial has demonstrated that concentrations of glutathione are lower in bronchoalveolar lavage fluid from unstable COPD patients with frequent exacerbations than in that from stable COPD [38]. Extracellular superoxide dismutase (SOD3) is decreased around small airways in patients with COPD [39]. Thioredoxin, which is an important regulator of redox balance, is decreased in COPD [38]. Nrf2 and forkhead box O3a (FOXO3a) are decreased in the lungs of patients with COPD [40,41]. Nrf2 and FOXO3a, which are key transcription factors that regulate multiple antioxidant genes, protect the respiratory system against oxidative damage [42]. Nrf2 is activated in healthy smokers, but its activation is impaired by oxidative stress in patients with COPD, resulting in reduced antioxidant gene expression [42]. Glutathione peroxidase is decreased in the lungs of COPD patients [43]. Glutathione peroxidase transgenic mice are protected against the development of inflammation and emphysema after cigarette smoke exposure, whereas glutathione peroxidase gene knockout increases the tissue destruction in the lung's response to cigarette smoke [44]. Imbalance between oxidants and antioxidants probably plays an essential role in the chronic inflammation related to the pathology of COPD (Figures 1 and 2).

2.4. Reduced Responsiveness to Corticosteroids Caused by Oxidative Stress

Oxidative stress probably causes corticosteroid resistance in COPD. Oxidative stress reduces activity and expression of histone deacetylase-2 (HDAC2), which is required for inflammatory gene suppression [45], by activation of phosphoinositide-3-kinase (PI3K)- δ [46]. This phenomenon prevents the acetylation of glucocorticoid receptors, which is necessary for the inhibition of NF- κ B that mediates the anti-inflammatory effects of corticosteroids, leading to reduced responsiveness to corticosteroids. Therefore, chronic lung inflammation is not fully inhibited by corticosteroids in COPD, different from mild asthma [6,33]. Recent preclinical studies have indicated that improvement of the redox balance by the administration of antioxidants or the stimulation of endogenous antioxidant response may overcome the corticosteroid resistance in COPD [47,48]. Nrf2 is known to act as an antioxidant. Sulforaphate, an activator of Nrf2, improves reduced responsiveness to corticosteroids mediated by upregulation of Nrf2 and enhancement of HDAC2 expression and activity in the allergen challenged mice that were exposed to cigarette smoke [49]. Nrf2 may be a potential molecular target for cigarette smoke-related resistance to corticosteroids in COPD.

3. Dysfunction of Airway Smooth Muscle in COPD

3.1. Phenotype Changes

Airway smooth muscle cells in culture have the ability to change the degree of various functions such as contractility, proliferation, migration, and the synthesis of inflammatory mediators [50–52]. Alterations of airway smooth muscle cells from a contractile to a synthetic or a proliferative phenotype is involved in the pathophysiology of asthma and COPD,

such as in airflow limitation, airway hyperresponsiveness, β_2 -adrenergic desensitization, and airway remodeling. These phenotype changes cause an abnormality in the function of airway smooth muscle. The dysfunction of airway smooth muscle occurs in asthma and COPD; and this phenomenon is associated with symptoms, a decline in lung function and the pathophysiology characterized by these diseases, and brings about contractile abnormality, release of inflammatory mediators, and hypertrophy in airway smooth muscle [53,54]. Airway smooth muscle cells can alter the degree of a variety of functions, including contraction, proliferation, migration, and the secretion of inflammatory mediators, referred to as phenotype plasticity. Characteristic features (major pathophysiology) of asthma and COPD, such as airflow limitation, airway hyperresponsiveness, β_2 -adrenergic desensitization, and airway remodeling, probably occur through phenotype changes in airway smooth muscle cells [50–52]. Changes between contractile and hyper-contractile, synthetic/proliferative phenotypes result from Ca^{2+} dynamics and Ca^{2+} sensitization, which are associated with the pathophysiology of these diseases [50–52]. Ca^{2+} dynamics through the large-conductance Ca^{2+} -activated K^+ (K_{Ca}) channel/L-type voltage dependent Ca^{2+} (VDC) channel linkage, and Ca^{2+} sensitization through the RhoA (a monomeric G protein)/Rho-kinase (a target molecule of RhoA) pathway is involved not only in alterations in the contractile phenotype related to airflow limitation, airway hyperresponsiveness and β_2 -adrenergic desensitization but also in alteration of the synthetic/proliferative phenotype related to airway remodeling [50,52].

3.2. Airway Hyperresponsiveness

Responsiveness to contractile and relaxant agents in airway smooth muscle is modified not only by inflammatory response related to asthma and COPD but also by excessive exposure to β_2 -adrenergic agonists (Figure 3). Airway hyperresponsiveness is clinically shown as augmented responsiveness to muscarinic agonists or histamine. This pathophysiological alteration is a hallmark of asthma; but is observed in some cases of COPD [55]. Dysregulation of contractility is not as widely documented in airway smooth muscle of COPD, however patients with airflow limitation are shown to be very sensitive to inhaled methacholine [56], and the tissues of airway smooth muscle from patients with obstructive lung disease (with all but one characterized as having COPD) demonstrate significantly increased maximal isometric force and isometric stress, which is correlated to decline in lung function [57]. Increased contractility in airway smooth muscle causes manifestations (dyspnea, wheezing), airflow limitation and airway hyper-responsiveness. Airway hyperresponsiveness is associated with inflammation related to the pathogenesis of asthma and COPD. Airway hyperresponsiveness may have a harmful effect on therapy for asthma-COPD overlap, since response to inhaled corticosteroids with bronchodilators is reduced in patients with COPD who have eosinophilia and hyperresponsiveness in the airways [55].

Airway hyperresponsiveness is brought from Th2 cytokines such as interleukin (IL)-4, IL-13 that generate IgE [58,59]; involvement of IL-5 in this pathophysiology is still controversial. Adenosine triphosphate (ATP), which is released from injured epithelium in the airway by activated eosinophils, causes airway hyperresponsiveness with no change in concentration of intracellular Ca^{2+} [60] (Figure 3). Mast cells infiltrate airway smooth muscle in asthma, referred to as mast cell myositis. Exposure to tryptase and sphingosine 1-phosphate (S1P), which are released from mast cells, also causes airway smooth muscle contraction with an increase in concentration of intra-cellular Ca^{2+} , and airway hyperresponsiveness with no change in Ca^{2+} (Figure 3) [61,62]. Ca^{2+} sensitization due to the RhoA/Rho-kinase processes is involved in airway hyperresponsiveness, which reflects a relationship between inflammatory cells and airway smooth muscle cells [50,52,60,62,63].

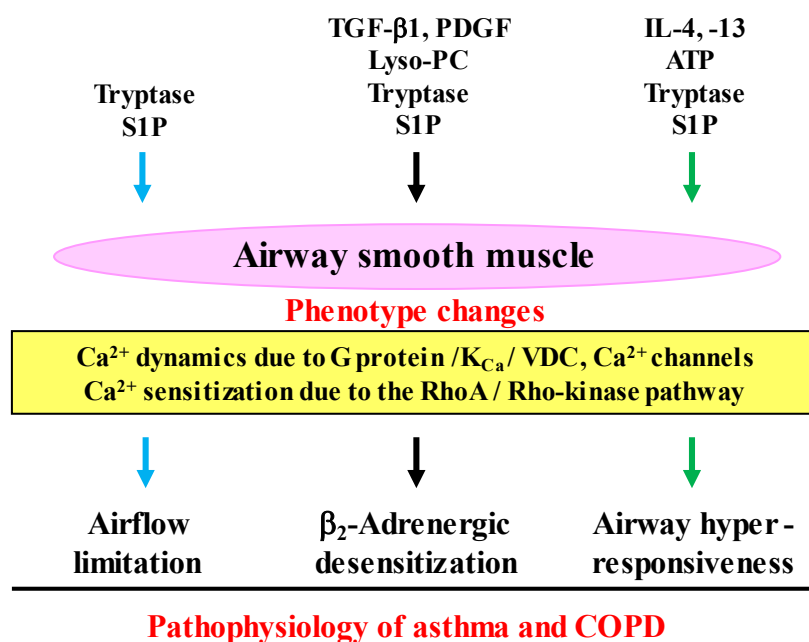


Figure 3. Interactions between inflammatory cells and airway smooth muscle cells in the pathophysiology of COPD. Functions (tension generation and response to contractile agents) of airway smooth muscle cells are altered (phenotype changes) by inflammatory substances (cytokines, growth factors, serine proteinases, phospholipids), which are synthesized in inflammatory cells (mast cells, eosinophils, etc.). Ca²⁺ signaling (Ca²⁺ dynamics and Ca²⁺ sensitization) is involved in the dysfunction of airway smooth muscle cells, leading to airflow limitation, β₂-adrenergic desensitization and airway hyperresponsiveness (the pathophysiology features of asthma and COPD). Lyso-PC: lysophosphatidylcholine, TGF-β1: transforming growth factors-β1, PDGF: platelet-derived growth factor, ATP: adenosine triphosphate, S1P: sphingosine 1-phosphate, K_{Ca}: Ca²⁺-activated K⁺ channel, VDC: L-type voltage-dependent Ca²⁺ channel. Arrows: activation.

3.3. β₂-Adrenergic Desensitization

Short- and long-acting β₂-adrenergic agonists are widely used as bronchodilators as reliever and controller therapy for both asthma and COPD. Reduced responsiveness to β₂-adrenergic agonists occurs in airway smooth muscle after excessive (repeated or sustained) exposure to these agonists and after persistent inflammation related to asthma and COPD, referred to as β₂-adrenergic desensitization (tachyphylaxis) [64–66]. Repeated application of β₂-adrenergic agonists results in a gradual reduction of their relaxant effects on muscarinic airway contraction. When isoprenaline, a full agonist, is repeatedly applied eight times every 30 min, relaxant effects of isoprenaline disappear almost completely in airway smooth muscle [66]. In contrast, relaxant effects of partial agonists such as formoterol are just slightly reduced under the same experimental conditions [67]. This phenomenon is not observed after excessive exposure to other cAMP-related agents bypassing β₂-adrenergic receptors, such as forskolin or theophylline [64,65]. Agonist-induced dysfunction of β₂-adrenergic receptors results from homologous desensitization in airway smooth muscle, not heterologous [64,65]. This phenomenon is caused by uncoupling the stimulatory G protein of adenylyl cyclase (G_s) from phosphorylated β₂-adrenergic receptors, not by down regulation, because of exposure time within 30 min [64,65].

Intrinsic efficacy, which is related to allosteric effects, is involved in homologous β₂-adrenergic desensitization, which is associated with Ca²⁺ dynamics due to inhibitory linkage between G_s and large conductance Ca²⁺-activated K⁺ (K_{Ca}) channels (Figure 3) [52,64–68]. The linkage of G_s/K_{Ca} channels is deeply involved in the functional antagonism between muscarinic and β₂-adrenergic action in airway smooth muscle [69–71]. Reduced responsiveness to β₂-adrenergic agonists in airway smooth muscle also occur after persistent exposure to cytokines (IL-1β, tumor necrosis factor-α: TNF-α) [72], growth factors (Transforming

Growth Factor- β 1: TGF- β 1, platelet-derived growth factor: PDGF [73,74], phospholipids (S1P, lysophosphatidylcholine: Lyso-PC) [75,76], and mast cell tryptase [77], which are deeply involved in the pathogenesis of asthma and COPD (Figure 3). Pre-exposure to Lyso-PC, tryptase and S1P cause homologous β ₂-adrenergic desensitization mediated by Ca²⁺ sensitization due to RhoA/Rho-kinase processes. In contrast, pre-exposure to TGF- β 1 and PDGF cause heterologous β ₂-adrenergic desensitization mediated by Ca²⁺ dynamics due to K_{Ca} channel inhibition. β ₂-Adrenergic desensitization in airway smooth muscle is caused not only by therapy but also pathogenesis related to asthma and COPD.

3.4. Airway Remodeling

Airway smooth muscle can change to synthetic and proliferative phenotypes after exposure to various exogenous stimuli, such as matrix (ECM, in particular, collagen type 1 and fibronectin) and growth factors (PDGF and TGF- β), leading to airway remodeling [51,78]. Airway smooth muscle cells derived from patients with asthma show alterations towards a more proliferative phenotype than when derived from healthy subjects [79]. Exposure to IL-13 and PDGF-BB causes an increase in recapitulation of a more secretory and proliferative phenotype, resulted from a decrease in expression of the SR Ca²⁺ ATPase (a Ca²⁺ transporter) [80]. A synthetic phenotype is caused by an increase in synthetic organelles for protein and lipid synthesis (the Golgi apparatus and numerous mitochondria); and proliferative capacity is induced. Modulation towards proliferative and synthetic phenotypes is associated with an increase in non-muscle MHC, I-caldesmon, vimentin, α / β -PKC and CD44 homing cellular adhesion molecule [44]. In airway smooth muscle cell culture, 20–60% of the cells express secretory capacity; on the other hand, approximately 50% of the cells express proliferative capacity. Cytokine production and proliferation may be overlapping and not independent functions [81]. Hence, airway smooth muscle contributes to the inflammatory environments in both asthma and COPD, because of releasing various cytokines and chemokines in response to asthma and COPD related stimuli [82]. An increase in the amount of airway smooth muscle resulting from hypertrophy and hyperplasia is observed surrounding the central and peripheral airways in asthma and COPD (airway remodeling) [83,84].

4. Airway Smooth Muscles Regulated by Oxidative Stress

4.1. Expression of Oxidants in Airway Smooth Muscle

It is generally considered that oxidative stress influences the function of airway smooth muscle in COPD [85]. Cigarette smoke is the most encountered risk factor for COPD across the world. Cigarette smoke contributes to oxidative stress by induction of ROS production in COPD [86,87], leading to the development of COPD. Since airway smooth muscle plays an important role in tension and inflammation related to the pathophysiology of asthma and COPD, oxidative stress probably affects the function of airway smooth muscle in these diseases (Figure 4). Expression of the ROS generating enzyme NADPH oxidase (NOX)-4 is enhanced in airway smooth muscle from patients with COPD; increased expression of NOX-4 is correlated with disease severity and lung function decline [88]. H₂O₂ stimulated ROS production is completely abolished by an inhibition of NOX-4 in airway smooth muscle [89]. Therefore, oxidative stress causes the dysfunction of airway smooth muscle in COPD; and NOX-4 is probably a potential therapeutic target for COPD. Apocynin, a non-selective inhibitor of NOX, inhibits the inflammatory response to cigarette smoke in mice [90], although clinical trials have not been reported yet in COPD.

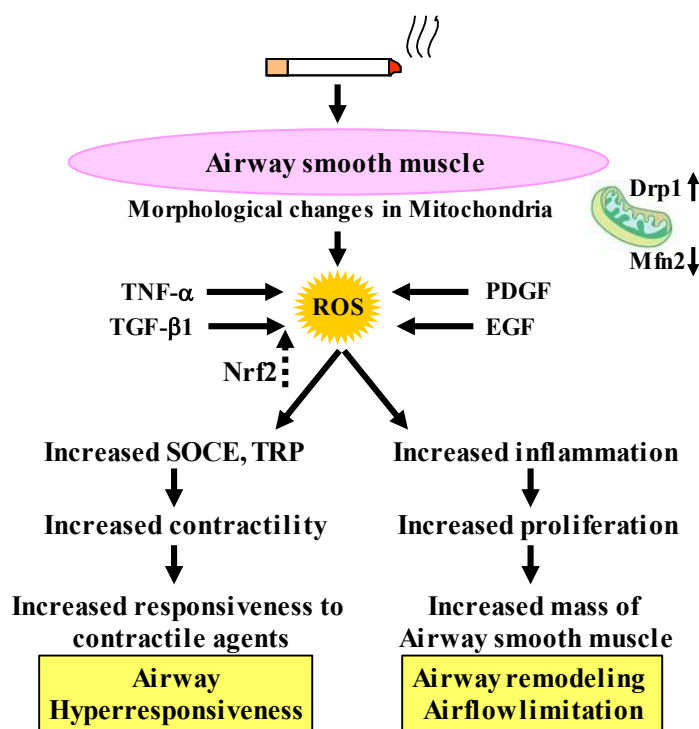


Figure 4. Involvement of oxidative stress in the dysfunctions of airway smooth muscle cells in COPD. Cigarette smoke enhances contractility caused by Ca^{2+} dynamics through TRP and SOCE. TNF- α and growth factors (TGF- β 1, PDGF, and EGF) synthesize ROS, resulting in amplified cell proliferation through mitochondrial morphological changes, resulting in potentiated response to contractile agents (airway hyperresponsiveness) and increased mass of airway smooth muscle (airway remodeling, airflow limitation), which are pathological and pathophysiological characteristics of COPD. Nrf2 inhibits effects of TGF- β 1 on oxidative stress. TRP: transient receptor potential, SOCE: store-operated Ca^{2+} entry. TNF- α : tumor necrosis factor- α , TGF- β 1: transforming growth factor- β 1, PDGF: platelet-derived growth factor, EGF: epidermal growth factor, Nrf2: nuclear erythroid-2 related factor 2, ROS: reactive oxygen species. Drp1: dynamin-related protein 1, Mfn2: mitofusin 2. Arrows: activation; dotted arrows: inactivation.

4.2. Effects of Oxidative Stress on Contraction and Proliferation

Oxidative stress can induce changes in the degree of various functions such as contractility, proliferation, migration, and the synthesis of inflammatory mediators in airway smooth muscle cell [50–52,67,85]. These phenotype changes of airway smooth muscle are deeply involved in the symptoms (e.g., dyspnea, wheezing) and the pathophysiology (e.g., airflow limitation, airway hyperresponsiveness, airway remodeling) of COPD (Figures 3 and 4) [50–52,67,85]. It is unclear in detail whether cigarette smoke extract causes contraction of airway smooth muscle. However, H_2O_2 and 8-iso-PGF $_{2\alpha}$, which are oxidative stress markers that are elevated in exhaled breath (EB) from patients with COPD, cause contraction of tracheal smooth muscle in guinea pigs in a concentration-dependent manner [91,92], indicating that oxidative stress contributes to dyspnea, wheezing, and airflow limitation by an increase in the airway tension in COPD. Pre-exposure to cigarette smoke extract markedly enhances acetylcholine-induced force in human bronchial smooth muscle [93], indicating that cigarette smoke extract causes airway hyperresponsiveness. Nicotine, which can induce oxidative stress, also contributes to airway hyperresponsiveness (Figure 4) [94]. Nicotine-stimulated fibroblast-conditioned media increase expression of the contractile protein p-MLC in airway smooth muscle cells [95]; and nicotine also causes upregulation of nicotinic α 7 acetylcholine receptor (α 7nAChR) expression in airway smooth muscle cells [94], indicating that nicotine enhances contractility in airway smooth muscle. TNF- α generates ROS in airway smooth muscle cells [96,97]; and TNF- α

enhances contractile response to a muscarinic agonist (airway hyperresponsiveness) with ROS-dependent phosphorylation of MLC, which is the contractile protein [98].

Cigarette smoke increases cell numbers in bovine tracheal smooth muscle with cyclin D1 expression and DNA synthesis via activation of ERK 1/2 and p38 MAP kinase [99]. Exposure to cigarette smoke also causes cell proliferation on rat airway smooth muscle with expression of transient receptor potential cation channel subfamily M member 7 (TRPM7) which is activated by ROS [100,101]. Cigarette smoke causes mitochondrial fragmentation and disruption of mitochondrial networks through imbalance of the mitochondrial fission versus fusion. This phenomenon is due to an increase in dynamin-related protein 1 (Drp1) expression (fission) and a decrease in mitofusin 2 (Mfn2) expression (fusion), involving PI3K/Akt, PKC and ERK activation and transcriptional regulation via NF- κ B [7]. This morphological alteration in mitochondria (the imbalance of fission/fusion) is associated with mitochondrial function, and is involved in proliferation/survival, response to inflammation, extracellular matrix production, and Ca²⁺ regulation in airway smooth muscle cells [102]. Cigarette smoke may induce not only a contractile phenotype but also a proliferative phenotype of airway smooth muscle cells, which may be associated with airflow limitation, airway hyperresponsiveness and airway remodeling in COPD (Figure 4). Therefore, oxidative stress probably causes the dysfunction (phenotype changes) of airway smooth muscle cells with changes in mitochondrial morphology [7]. Growth factors, such as TGF- β 1, PDGF and EGF (epidermal growth factor), contribute to increased cell proliferation and dysfunction of airway smooth muscle with ROS production in COPD [85].

4.3. Inhibitory Effects of Antioxidants on Oxidative Stress Induced Proliferation

TGF- β 1 can induce expression of the ROS generating enzyme NOX-4 in airway smooth muscle [37,99] via decapentaplegic family member 3 (SMAD3)/phosphoinositide 3-kinase (PI3K) signaling, leading to increased proliferation in airway smooth muscle cells [37,103]. This TGF- β 1 induced dysfunction of airway smooth muscle cells is attenuated by activation of the antioxidant transcription factor Nrf2 [103]. In human tracheal smooth muscle cells, Nrf2 causes expression of the antioxidant genes heme-oxygenase 1 (HO-1), with activation of HO-1 reciprocally resulting in Nrf2 translocation from cytosol to nucleus [85]. The Nrf2/HO-1 signaling may potentially contribute to protecting against dysfunction of airway smooth muscle related to COPD since this process is involved in an inhibition not only of cell proliferation but also in inflammation and contraction of airway smooth muscle in the functional alterations caused by oxidative stress [85]. HO-1 activation causes an inhibition in TNF- α induced expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and generation of IL-6 via suppression of TNF- α induced superoxide and H₂O₂ generation [104]. The Nrf2 pathway is implicated in protecting against airway hyperresponsiveness in animal models [105]. The expression of HO-1 is more decreased and net contractile moment is more increased in airway smooth muscle from Nrf2 knockout mice compared to airway smooth muscle from wild type mice, suggesting that the Nrf2/HO-1 process prevents augmented contractility by ROS production [106]. Therefore, the Nrf2/HO-1 process is probably a therapeutic target against the dysfunction of airway smooth muscle related to oxidative stress.

4.4. Clinical Trials for Antioxidant Therapy

Although it is well known that oxidative stress is a major factor leading to the development of COPD, specific therapy using antioxidants has not been established yet in this disease. Various oxidants are mentioned as a candidate involved in the pathophysiology of COPD. However, much remains unclear. Related oxidants may be different for each patient with COPD, and multiple oxidants may be involved for each patient.

Dietary antioxidants (vitamin C, vitamin E, resveratrol, and flavonoids) do not have significant effects on lung function and manifestation in patients with COPD [107,108]. Resveratrol may reduce ROS released from the airway epithelium in COPD in vitro; but the clinical relevance of this result is unclear [109]. A retrospective study has indicated that a

Mediterranean diet that includes many dietary antioxidants may prevent the development of COPD [110]; but this result is still unclear because of confounding factors.

In clinical studies using chemical agents, thiol-based antioxidants (*N*-acetylcysteine, carbocysteine, erdosteine) were administered to patients with COPD. These agents that are used as expectorant to reduce mucus viscosity, act as antioxidants by elevating glutathione concentrations [111]. Some clinical studies on a small scale have indicated that these agents can decrease the number of exacerbations in COPD [112]. In contrast, large-scale trials have indicated that a high dose of *N*-Acetylcysteine has only modest efficacy in reducing exacerbation frequency [113], and that a low dose of that agent has no effect [114]. Nrf4 is generally considered to regulate multiple antioxidant genes. HO-1, which is an antioxidant gene regulated by Nrf2, can inhibit the development of emphysema, indicating that activation of Nrf2 may be effective in combatting oxidative stress in COPD. However, when sulforaphane, an activator of Nrf2, was administered to patients with COPD for 4 weeks, this agent increased an antioxidant gene related to Nrf2 and inhibited oxidative stress and inflammation [115]. Clinical studies using antioxidants for COPD have not been reported other than these trials. Hence, the clinical significance of antioxidants still remains unclear in this disease.

5. Calcium Signaling as Mechanisms of Oxidative Stress

5.1. Involvement of Ca^{2+} Dynamics in Oxidative Stress

It is considered that H_2O_2 and 8-isoprostane (8-iso-PG) $F_{2\alpha}$ could be useful biomarkers for oxidative stress in COPD [116,117], since these substances are elevated in exhaled breath from patients with COPD. To determine the intracellular mechanism underlining effects of oxidative stress on airway smooth muscle, H_2O_2 , 8-iso-PG $_{2\alpha}$, and ATP were cumulatively applied to the fura-2 loaded tissues of guinea pig tracheal smooth muscle; and isometric tension and F_{340}/F_{380} (an indicator for intracellular concentration of Ca^{2+}) were simultaneously recorded. H_2O_2 , 8-iso-PG $_{2\alpha}$, and ATP generated tension with an increase F_{340}/F_{380} in a concentration-dependent manner (Figure 5) [91,92]. SKF96365, a non-selective inhibitor of Ca^{2+} channels, markedly inhibited tension induced by H_2O_2 , 8-iso-PG $_{2\alpha}$, and ATP; in contrast, verapamil, an inhibitor of VDC channels, modestly inhibited them [91,92]. Since SKF96465 is an inhibitor of receptor-operated Ca^{2+} influx through transient receptor potential (TRP) channels and store-operated Ca^{2+} entry (SOCE) [118,119], Ca^{2+} dynamics due to TRP channels and/or SOCE are probably associated with this oxidant-induced contraction of tracheal smooth muscle; but, VDC channels may be less involved (Figure 4) [91,92]. When ligands connect to the GTP-binding (G) protein-coupled receptor (GPCR), Ca^{2+} is released from sarcoplasmic reticulum (SR), leading to SOCE, i.e., Ca^{2+} release-activated Ca^{2+} (CRAC) currents. SOCE is activated by stromal interaction molecule 1 (STIM 1), which is a Ca^{2+} sensor for store depletion in the SR (Figure 6). Although TRP channels may be related to the conduction of SOCE, it has recently been considered that the pore-forming protein Orai 1 is an essential component of the CRAC currents at the cell membrane [120] (Figure 6). This STIM 1/Orai 1 coupling contributes to SOCE in airway smooth muscle [121]. Although little is currently known about mechanisms of H_2O_2 -induced contraction, the effect of 8-iso-PG $_{2\alpha}$ and ATP are associated with thromboxane A2 receptors (TP receptors) [92] and P2X (ATP-activated purinergic receptors) [60], respectively (Figure 5). Endogenous oxidants such as H_2O_2 , 8-iso-PG $_{2\alpha}$, and ATP cause contraction of airway smooth muscle through Ca^{2+} dynamics, leading to dyspnea, wheezing and airflow limitation in COPD (Figure 5). The contractile effect of other oxidants in airway smooth muscle remains unclear. Although increased expression of NOX-4 is observed in airway smooth muscle from COPD patients [88], the potential involvement of NOX-4 in Ca^{2+} dynamics and contractility is still unknown.

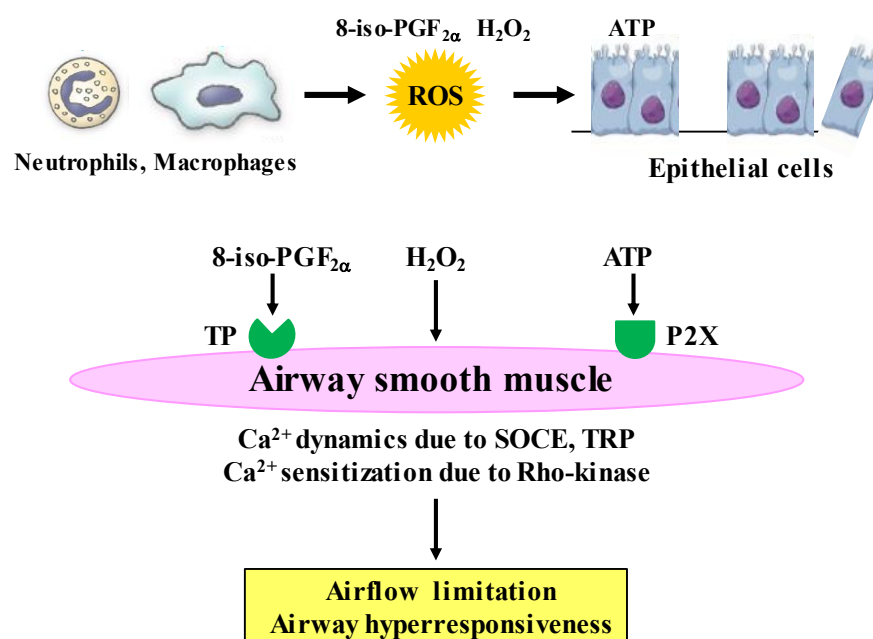


Figure 5. Roles of Ca²⁺ signaling in effects of oxidative stress related to COPD on airway smooth muscle. H₂O₂ and 8-iso-PGF_{2α}, which are endogenous oxidants (oxidative stress biomarkers) synthesized in inflammatory cells, cause contraction with Ca²⁺ dynamics through Ca²⁺ channels and Ca²⁺ sensitization through the RhoA/Rho-kinase pathway. ATP, which is released from injury to airway epithelium caused by ROS, generates tension with Ca²⁺ dynamics through Ca²⁺ channels, and enhances muscarinic contraction with Ca²⁺ sensitization through the RhoA/Rho-kinase pathway. The Ca²⁺ signaling (Ca²⁺ dynamics and Ca²⁺ sensitization) may contribute to airflow limitation and airway hyperresponsiveness (pathophysiological features of COPD) caused by oxidative stress. ROS: reactive oxygen species, ATP: adenosine triphosphate, TP: thromboxane A₂ receptors, P2X: P2X receptors (ATP-activated purinergic receptors), H₂O₂: hydrogen peroxide, 8-iso-PGF_{2α}: 8-isoprostaglandin F_{2α}.

Recent studies using human airway smooth muscle cells have indicated that cigarette smoke and cigarette smoke extract cause Ca²⁺ influx through TRP ankyrin 1 (TRPA1) related to myosin light-chain phosphorylation, not L-type voltage-dependent Ca²⁺ (VDC) channels [122], and enhance Ca²⁺ influx in responses to bradykinin and histamine, which cause airway smooth muscle contraction through SOCE (Figure 4) [93]. Although cigarette smoke does not cause force generation in airway smooth muscle, cigarette smoke enhances response to bradykinin and histamine with SOCE (an increase in contractility), probably leading to airway hyperresponsiveness. Cigarette smoke contributes to dysfunction involved in contractility and proliferation in airway smooth muscle cells. Since cigarette smoke enhances not only Ca²⁺ influx through TRP channels and SOCE but also expression of Ca²⁺ regulatory proteins such as TRPC3, CD38, STIM1 (a sensor for Ca²⁺ concentration in the SR), and/or Orai1 (Ca²⁺ channels in the plasma membrane) in human airway smooth muscle cells, Ca²⁺ dynamics due to these processes play a critical role in alterations of function and structure in the airways mediated by smoking-related oxidation [123]. Expression of TRPC3 and CD38 is also markedly increased in the airways of patients with long-term smoking history compared to lifelong never smokers [122]. Mitochondria also act on regulation of cytosolic Ca²⁺ concentration through interaction between STIM1 and Orai1. Cigarette smoke-induced mitochondrial dysfunction enhances Ca²⁺ dynamics through SOCE [124] (Figure 6).

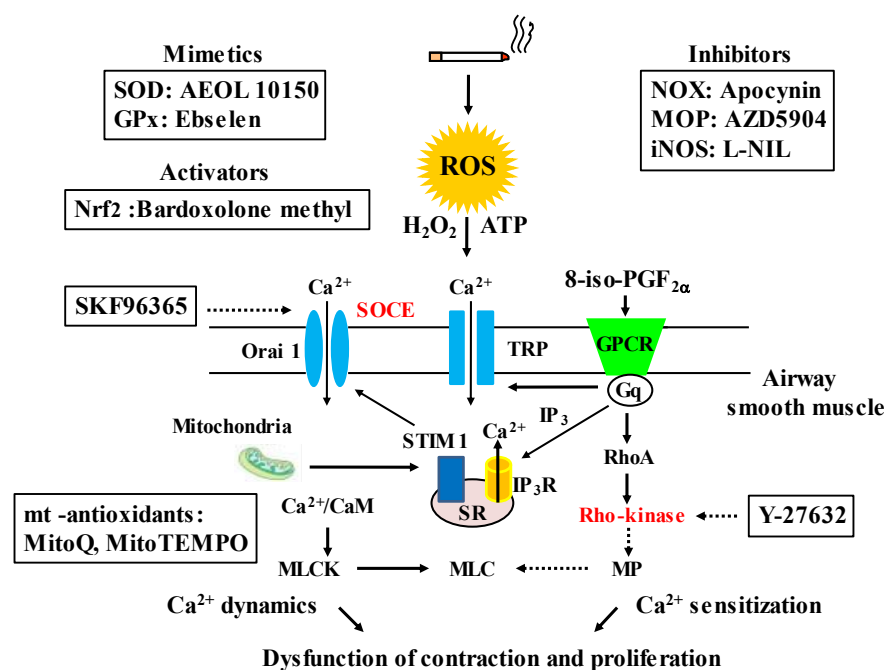


Figure 6. Chemical compounds that potentially act as antioxidants, and mechanisms (Ca^{2+} signaling) related to oxidative stress in COPD. The chemical compounds, which are mimetics of SOD, include: superoxide dismutase, GPx: glutathione peroxidase, activator of Nfr2: nuclear erythroid-2 related factor 2, inhibitors of NOX: NADPH oxidases, MOP: myeloperoxidase, iNOS: inducible nitric oxide synthase and mitochondria-related (mt)-related antioxidants; they are effective on oxidative stress related to COPD in animal models and vitro studies. Biomarkers of oxidative stress related to COPD (H_2O_2 , 8-iso-PGF $_{2\alpha}$) and external ATP which are released from injured airway epithelium cause contraction of airway smooth muscle via Ca^{2+} dynamics due to SOCE and Ca^{2+} sensitization due to Rho-kinase. These Ca^{2+} signaling pathways are also associated with proliferation of airway smooth muscle cell, and are inhibited by SKF96365 and Y-27632, respectively. ROS: reactive oxygen species, TRP: transient receptor potential, SOCE: store-operated Ca^{2+} entry, STIM 1: stromal interaction molecule 1, CaM: calmodulin, MLCK: myosin light chain kinase: MP: myosin phosphatase, MLC: myosin light chain, IP3: inositol trisphosphate, IP $_3$ R: IP $_3$ receptor, GPCR: G protein-coupled receptor. Arrows: activation; dotted arrows: inactivation.

5.2. Involvement of Ca^{2+} Sensitization in Oxidative Stress

On the other hand, Y-27632, an inhibitor of Rho-kinase, inhibits H_2O_2 - and 8-iso-PGF $_{2\alpha}$ -induced contraction without a reduction in F_{340}/F_{380} in the fura-2 loaded tracheal smooth muscle of guinea pigs [91,92]. These results have demonstrated that Ca^{2+} sensitization (increased sensitivity to intracellular Ca^{2+}) related to Rho-kinase is involved in the airway tension induced by oxidant stress in COPD (Figure 5). When a contractile agonist connects to the GPCR, RhoA (a monomeric G protein) is activated by a trimeric G protein coupled to the GPCR. Rho-kinase, which is a target molecule of RhoA, inactivates myosin phosphatase acting on myosin phosphatase target subunit 1 (MYPT1) [62], leading to Ca^{2+} -independent contraction (Ca^{2+} sensitization) in airway smooth muscle. Ca^{2+} sensitization related to the RhoA/Rho-kinase pathway contributes not only to muscarinic contraction [125] but also to augmented response to muscarinic agonists [60,62,63], indicating that this Ca^{2+} sensitization is probably associated with symptoms (dyspnea, wheezing) and the pathophysiology (airflow limitation, airway hyperresponsiveness) in COPD. It is still unclear how oxidative stress induces airway epithelial disorder; however, ATP may be released from injured epithelium leading to the pathogenesis of COPD [126]. Extra-cellular ATP causes a modest increase in F_{340}/F_{380} without contractile response; and muscarinic contraction is significantly enhanced without an increase in F_{340}/F_{380} after exposure to ATP (Figure 5) [60]. Extracellular ATP acts on P2X (ATP-activated purinergic receptors, different

from GPCRs), and contributes to symptoms (dyspnea, wheezing) and the pathophysiology (airflow limitation, airway hyperresponsiveness) via Ca^{2+} sensitization related to the RhoA/Rho-kinase processes (Figure 5) [60]. Since 8-iso-PGF_{2α} acts on thromboxane receptors (GPCRs), oxidative stress due to 8-iso-PGF_{2α} causes airway contraction via a monomeric G protein. On the other hand, intracellular mechanisms are still unknown as regards Ca^{2+} sensitization by H₂O₂ and ATP.

6. Toward the Progress of Therapy for COPD

Previous clinical trials have shown that N-acetylcysteine (a thiol-based antioxidant, a precursor of glutathione) and sulforaphate (an activator of Nrf2) are not so effective in COPD, as noted above. In addition, previous studies using animal models and in vitro have indicated that several chemical compounds have effects against oxidants related to COPD, including: AEOL 10150 (a superoxide dismutase mimetic) [127], Ebselen (a glutathione peroxidase mimetic) [128], Apocynin (a NADPH oxidase inhibitor) [90], AZD 5904 (a myeloperoxidase inhibitor) [44], L-NIL (an inducible nitric oxide synthase inhibitor) [129], Bardoxolone methyl (a Nrf2 activator) [115], mitoQ, mitoTEMPO (mitochondria-targeted antioxidants) [130–132] (Figure 6). However, clinical trials using them still have not been carried out. Effects of setanaxib (a NADPH oxidase inhibitor) on COPD have not been proved yet even in preclinical studies. H₂O₂ and 8-iso-PGF_{2α} cause contraction in airway smooth muscle; these contractile actions are attenuated in the presence of SKF96365 and Y-27632 in a concentration-dependent manner [91,92]. These chemical compounds are agents for Ca^{2+} signaling, i.e., the former is an inhibitor of Ca^{2+} influx such as SOCE and TRP channels (Ca^{2+} dynamics), and the latter is an inhibitor of the Rho-kinase (Ca^{2+} sensitization) (Figure 6). Since Ca^{2+} signaling due to Ca^{2+} dynamics and Ca^{2+} sensitization is probably related not only to contractile but also proliferative responses in COPD, these Ca^{2+} signaling-related molecules such as Orai 1, TRP and Rho-kinase, could be treatable traits for this disease. Clinical trials using chemical compounds related to Ca^{2+} signaling are needed to establish more suitable precision medicine (personalized medicine) for COPD (Figure 7).

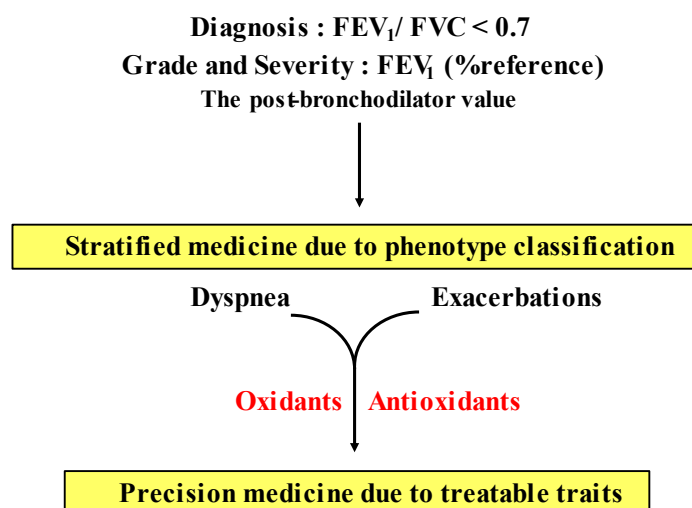


Figure 7. Precision medicine (personalized medicine) in COPD. The present guideline for COPD recommends that pharmacologic therapy for stable periods is carried out as a strategy based on symptoms (dyspnea) and frequency of exacerbations as treatable traits. Since COPD has heterogeneity, distinct phenotype classification is needed based on multidimensional approaches to advance from stratified medicine to personalized medicine in the management of COPD in near future. Although the clinical relevance of oxidative stress is still unclear, several oxidants can serve as treatable traits for development of precision medicine in COPD. See Section 6 in this text.

Although many previous reports have indicated that oxidative stress is perhaps involved in the pathophysiology of COPD, little is currently known about its clinical relevance in detail, as described above. There is still uncertainty regarding which is the most important oxidant in COPD, or how many oxidants are involved in each patient with COPD. Involved oxidants may be different for each patient with COPD. In the present COPD guideline (the 2023 GOLD report), dyspnea and exacerbations are shown as treatable traits, and pharmacological therapy for stable COPD is recommended according to degree of dyspnea and frequency of exacerbation as current precision medicine (individualized medicine) [1]. However, since COPD has heterogeneity, the present strategy based on only these two treatable traits may be not sufficient to guide stable long-term management for COPD. Patients with COPD need to be classified according to distinct clinical phenotypes based on multiple dimensions (clinical, physiological, imaging, endotypes) [13]. Although H₂O₂ and 8-isoprostane, which are stated as biomarkers for oxidative stress in the 2023 GOLD report [1], are increased in the airway in most patients with COPD, novel precision medicine including these oxidants has not been established yet. To advance the management and treatment for COPD, it is necessary to provide precision medicine using oxidants and antioxidants as treatable traits based on stratification of patients according to oxidants and antioxidants for specific clinical phenotypes (Figure 6 and 7).

7. Conclusions

The inflammatory responses related to oxidative stress in COPD have great effects on the function of airway smooth muscle. Because of this interaction between inflammatory cells and airway smooth muscle cells, the function for contraction and proliferation is altered in airway smooth muscle cells, leading to symptoms (dyspnea, wheezing), and the pathophysiology (airflow limitation, airway hyperresponsiveness, airway remodeling) in COPD. Ca²⁺ signaling (Ca²⁺ dynamics and Ca²⁺ sensitization) is involved in the mechanisms of ROS (oxidative stress)-induced dysfunction of airway smooth muscle. Therefore, oxidative stress should be included in treatable traits for COPD to establish precision medicine; airway smooth muscle can be a novel therapeutic target for this disease. Research into Ca²⁺ signaling in airway smooth muscle will also be important for the development of a novel agent for COPD.

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