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Oxidative Stress in Lung Diseases

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This book is dedicated to Prof. Amartya Sen (Nobel Prize winner in Economic Sciences in the year 1998) for his outstanding contribution in human welfare economics devoted to ameliorate inequality and poverty. Prof. Sen was born in Santiniketan on 3rd November 1933 on the campus of Rabindranath Tagore's Visva-Bharati University. He spent some of his childhood in Dhaka (now in Bangladesh) and was educated there at the St. Gregory School. His father, Prof. Asutosh Sen, taught chemistry at the Dhaka University. However, it was at Santiniketan where his educational attitudes were formed. After schooling at Santiniketan, Prof. Sen studied at Presidency University in Calcutta, where his intellectual horizon was

radically broaden. After graduation, he moved to Trinity College in Cambridge. He later taught at both these universities, and also at Delhi University, London School of Economics, Oxford University, and Harvard University, and on a visiting basis at MIT, Stanford, Cornell, and the University of California at Berkeley.

Prof. Sen has devoted his career mostly on the well-being of the community. In several writings, he addressed problems such as individual rights, majority rule, and the availability of information about individual lives, which inspired many researchers to turn their attention to basic welfare of common people. His views eventually prompted policy-makers of different developing countries to find ways to enhance well-being of the poor through public works project. He is a vigorous defender of academic freedom, possible education, and public health system.

Prof. Sen undoubtedly is a legendary international figure, who devoted most part of his academic and social life for the development of the poor. He has excellent ability to motivate young researchers. His novel insight to explore and amalgamate philosophy with economics for human welfare is amazing. He is truly a genius. We feel honoured to dedicate this book to Prof. Amartya Sen and wish him good health in his long fruitful activities.

Kalyani, West Bengal
India

Sajal Chakraborti

Preface

> **"Queen:** O Hamlet, thou hast cleft my heart in twain.
> **Hamlet**: Oh, throw away the worser half, and live a purer life with the other!"
>
> (*William Shakespeare: Hamlet: Act 3; scene-4*)

The numbers of diseases in which detrimental oxidation processes play aggravating roles have grown steadily over the past two decades. Among the diseases, oxidant-induced lung diseases are the most prevalent in humans.

"Oxidative stress" indicates a disturbance in the pro-oxidant/antioxidant balance and swings it in favour of the pro-oxidants, leading to potential damage to various components of cells and tissues. The novel roles of oxidants and antioxidants as mediators in signalling cascades have opened new areas of active research. This book provides chapters with evidence for crucial roles of oxidants and antioxidants in regulating different types of lung diseases.

This book focuses on some new strategies of antioxidant defence counting new pharmacologically active agents, presents current knowledge of known agents, and provides possible therapeutics of different lung diseases. It is hoped that the book will serve as a potential stimulus for further research.

Considering the progression of a plethora of research in this area, it is possible that some of the propositions made by the contributors may eventually turn out to be otherwise. A Harvard biochemist once said to his graduate students that "half of what we taught is probably wrong, but at this stage, we do not know which half". Gottfried Schatz (former Secretary General of EMBO, former President of Swiss Science and Technology Council, and former Editor of *The FEBS Letters*) once said that "the uncertainty of scientific knowledge is not weakness, but strength. The scientific vision of the world has dynamic stability. It is not chained to facts, but in a way of looking at them. Most institutions demand absolute faith, but science makes scepticism a virtue. Scientists see the world as it is and not as they want it to be".

This book is an outcome of enthusiasm of various renowned experts in their relevant research areas and contains four subdivisions. *Part 1* describes the general aspects of reactive oxygen species-mediated lung diseases; *Part II* enumerates chronic lung diseases like asthma, COPD, inflammatory lung diseases, and lung fibrosis; *Part III* provides notable information on respiratory syncytial virus (RSV)-induced lung diseases and different aspects of lung cancer; and *Part IV* deals with

prevention and therapeutics. Each chapter in this book raises many questions that need to be addressed for finding appropriate solutions in the area of oxidant-induced lung diseases.

We are greatly indebted to all contributors for their considerable energy, time, and effort to accomplish a complete chapter with no quid pro quo benefit. We would like to thank Mr. Lenold Esithor and Dr. Madhurima Kahali (Springer Nature) for their cooperation and support during the preparation of the book.

Kalyani, West Bengal, India Sajal Chakraborti

About the Book

This book is intended to provide multidisciplinary approach demonstrating cellular and molecular mechanisms associated with ROS-induced initiation and progression of a variety of lung diseases such as COPD, emphysema, asthma, cystic fibrosis, occupational pulmonary diseases, and importantly lung cancer. The book also covers translational research on lung diseases and recent research on the prevention and therapeutics of different types of lung diseases. Considering the depth and plethora of information to be covered, each article of this book are immensely useful for the researchers working on understanding the mechanisms associated with different types of lung diseases and to identify targets for drug development. With this multidisciplinary scope, this book will bridge the gap between fundamental and translational research with its application in biomedical and pharmaceutical industry, making it a thought-provoking reading for basic and applied scientists engaged in biomedical research.

Contents

About the Editors

Dr. Sajal Chakraborti is a Professor of Biochemistry at the University of Kalyani, West Bengal, India. His research focus is on the role of proteases, oxidant, and Ca^{2+} signaling phenomena in the pathogenesis of lung diseases. He received Ph.D. from the Calcutta University in the year 1982 and his D.Sc. from the Kalyani University in the year 2004. He did postdoctoral research at the Johns Hopkins University, University of Utah, and New York Medical College as a Fulbright Fellow in 1987–1990. He received DBT-Senior Overseas Research Award for his research at the Brain Institute, University of Florida, Gainesville, Florida, in 1999. He has been involved in teaching and research in Biochemistry for the past 40 years. He has published over 110 original research papers, 22 book chapters, and 15 review articles and also edited 12 books published by Springer.

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Dr. Nirmal K. Ganguly was the Director General, Indian Council of Medical Research (ICMR), New Delhi. He is currently associated with the Translational Health Science and Technology Institute, Faridabad, India. He has published over 700 research papers and edited several book chapters and review articles.

Dr. Tapati Chakraborti is a Professor of Biochemistry at the University of Kalyani, West Bengal, India. She did Ph.D degree at the Jadavpur University, Kolkata, in 1992, and did postdoctoral research at the Brain Institute, University of Florida, Gainesville, Florida as a Fellow of the American Heart Association in 1999–2002. She has been actively involved in teaching and research for the past 30 years, and one of the important aspects of her research focuses on the regulation of pulmonary vascular tone under stimulation of vasoactive agents. She has published over 80 original research papers, 18 book chapters, and 12 review articles and also edited 3 books entitled published by Springer.

Part I

General Aspects of Reactive Oxygen Species-Mediated Lung Diseases

The Effects of Free Radicals on Pulmonary Surfactant Lipids and Proteins

1

Mustafa Al-Saiedy, Francis Green, and Matthias Amrein

Abstract

The pulmonary surfactant forms a mixed protein–lipid film at the air–lung interface. It plays a dual role of surface tension reduction and host defense against inhaled pathogens. In acute lung injury (ALI) and its more severe form of acute respiratory distress syndrome (ARDS), high surface tension throughout the lung results in intrapulmonary shunts and edema leading to atelectasis and hypoxemia. Pulmonary surfactant inhibition is associated with various pulmonary diseases. ALI/ARDS is common (150,000 new cases per year in the United States) with mortality ranging from 30% to 60% depending on disease stage. High surface tension can result from an absence of a surfactant film over significant portions of this interface, or from the presence of dysfunctional layer of surfactant. Elevated cholesterol levels are shown to be a potent surfactant inhibitor. Oxidative damage to both phospholipids and proteins is shown to inhibit surfactant function. The pulmonary surfactant may be degraded by reactive oxygen and nitrogen (RONS) species in the inflamed lung in the presence of physiological cholesterol levels. The inhibitory mechanism of oxidative damage on the surfactant film is outlined in this chapter. Lipid-sequestering therapies, including cyclodextrins, may offer a potential treatment to restore surfactant function and reduce pulmonary inflammation.

Keywords

Pulmonary surfactant dysfunction · Oxidation · Cholesterol · Surfactant protein · Phospholipids · Peroxidation · Epoxidation · Peroxynitrites · Cyclodextrins

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1.1 Introduction

The pulmonary surfactant is a protein and lipid mixture, secreted by the epithelium into the alveolar lining fluid, which spreads into a surface-active film at the air–lung interface. The film reduces surface tension of the air–water interface (about 70 mN/m) to <1 mN/m [1]. The small airways and the alveoli are unsupported by a collagenous scaffold and depend on low surface tension to maintain their structure. In inflammatory lung diseases, the film is damaged and collapses before the surface tension is sufficiently reduced. This leads to alveolar collapse (atelectasis) and reduces the patency of small airways [2], reducing gas exchange in the lung and further driving inflammation. Failure of surfactant is ascribed to the effects of exudative plasma proteins and, more importantly, the damage to surfactant lipids and proteins in the oxidative milieu of the inflamed lung [3, 4].

In this chapter, we review the structure–function relationship of surfactant and how pathological changes in surfactant composition and oxidative damage cause dysfunction. We list the susceptibility to oxidation for the relevant classes of surfactant lipids and the surfactant-specific proteins. We discuss the implications of a recent observation that the oxidative damage of the film is cholesterol dependent in the context of treatment options for surfactant dysfunction.

1.2 The Function–Structure Relationship of the Pulmonary Surfactant

For a film of molecular dimensions, pulmonary surfactant (PS) is exceptionally strong, a function that depends on its unique composition and structure. The pulmonary surfactant consists of 75–90% (by weight) phospholipids, with a large proportion (~30–45%) being 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC) [5–7] (i.e., both acyl chains of this lipid contain no double bonds), whereas mono- and polyunsaturated phospholipids account for the remaining 50–60%. Polyunsaturated species are reported as >10% of total surfactant phospholipids [8, 9]. Neutral lipids, primarily cholesterol, are 2–10% of pulmonary surfactant by weight. PS also contains four surfactant-associated proteins – SP-A, SP-B, SP-C, and SP-D [8]. There are two forms of surfactant aggregates, a highly surface-active form, referred to as the large aggregates, which is enriched in the hydrophilic protein SP-A and hydrophobic proteins SP-B and SP-C, and inactivated form referred to as the small aggregates. SP-A and SP-D's main functions are innate host defense, whereas SP-C and SP-B are required for the surface-tension lowering property of surfactant [1, 10, 11]. The complex mixture of the surfactant film, which includes a high proportion of DPPC, is thought necessary for the surfactant system to achieve low minimum surface tension during the film compression occurring at expiration. Additionally, it appears that the surfactant film requires unsaturated phospholipids to act as liquefiers for efficient surface adsorption and reinsertion of material during film expansion during inspiration [12].

Surfactant function has been studied in situ, in the lung, and fluctuates during the breathing cycle between about 10 mN/m and 0 [13, 14]. The early work on surfactant function and stability showed the surfactant film in a functional state in lipid monolayer and multilayer regions at the air–lung interface. This structure can be reproduced in vitro to study the structure–function relationship in details [1, 15].

To assess surfactant function in vitro, captive bubble surfactometer (CBS) may be used. CBS comes close to mimicking lung function as determined in vivo from pressure–volume studies [16, 17]. The CBS resembles near-physiological conditions and includes temperature (37 °C), dynamic cycling rates (20 cycles/min), and interfacial adsorption from minute volumes of concentrated (27 mg/mL) surfactant containing dense aggregates [18]. Surfactant film functional assessment begins with measurements of film formation, indicated by the fall of surface tension upon surfactant spreading. Subsequently, the measurement of surface tension upon dynamic compression–expansion cycling is performed. The minimal surface tension (MST) reached during film compression is the primary indicator of surfactant function in this test. The amount of area reduction required to reach MST is another indicator of function. Surfactants tested to date include a clinically used, animal-derived surfactant, such as bovine lipid extract surfactant (BLES) that contains both hydrophobic surfactant proteins and the lipids, surfactants extracted from bronchoalveolar lavage (BAL) of animal models of health and disease, as well as patient surfactants.

To study the structure–function relationship, surfactants may be spread to the air–water interphase of a Langmuir trough, surface tension lowered by adjusting the film area, the film lifted off the surface by the Langmuir–Blodgett technique and imaged in an atomic force microscope. These studies show that the lipids of the surfactant accumulate in the interface with the hydrophilic head groups exposed to the water and the hydrophilic tail groups stretched out toward the air in a tightly packed film. Functional surfactant, in addition to this monomolecular lipid film, shows multilayered regions scattered over the surface. The multilayer regions of the film are 5 nm or multiples thereof high, consistent with lipid bilayer stacks. By comparing the structure of functional (Fig. 1.1c–e) and dysfunctional surfactants ([1, 16, 19], Fig. 1.2) as well as theoretical considerations ([15], Fig. 1.1f), stacks act as reinforcing elements that prevent the film from buckling at low surface tension. Buckling is the out-of-plane deflection of the film that leads to film collapse [1]. The surfactant proteins SP-B and SP-C cross-link the bilayers to the monolayer. This attachment is essential (Fig. 1.1b). Otherwise, they would glide over the monolayer and have a little mechanical effect (Fig. 1.1c demonstrates cross-linking).

Surfactant-associated proteins B and C (SP-B and SP-C) independently enhance film stability and spreading by facilitating the recruitment of saturated and unsaturated phospholipids into the expanding film (Fig. 1.1e [20]). The positive charges of SP-B and SP-C proteins are essential for their activity. The positive charge allows for interactions with PG and other anionic surfactant phospholipids, critical to film adsorption. Additionally, SP-B and SP-C are essential for the formation of tubular myelin, the tubular meshwork the surfactant unfolds into upon release from the type II cells. Tubular myelin then promotes the rapid phospholipid insertion into the

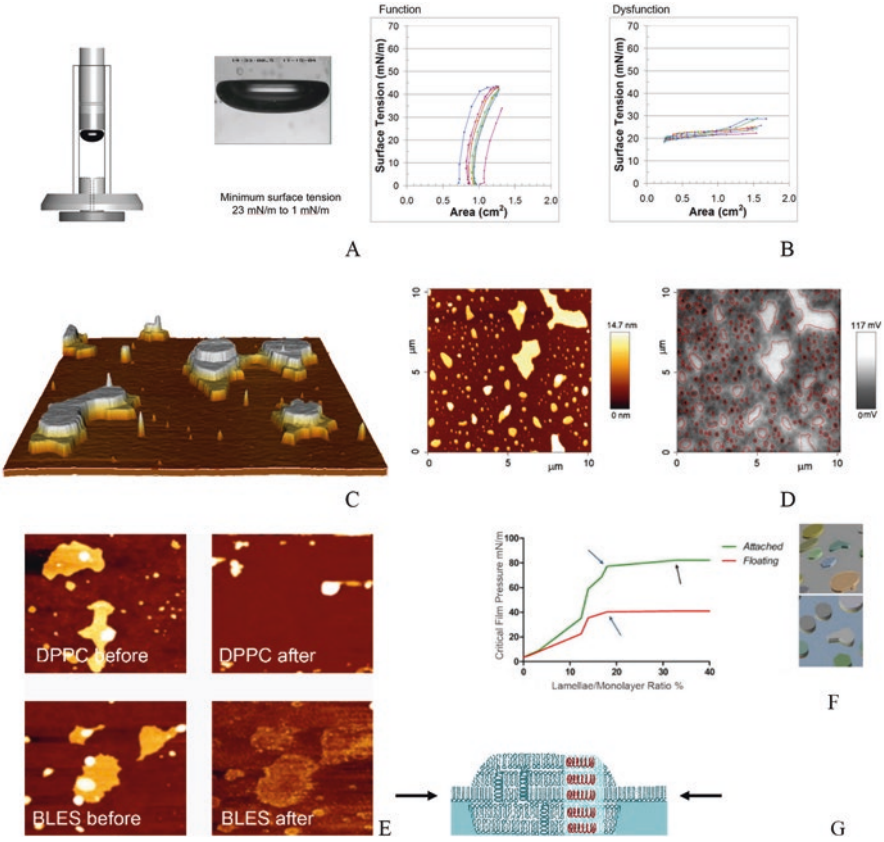

Fig. 1.1 (**a**) Captive bubble surfactometer. The surfactant is spread at the air–buffer interface of a trapped bubble and its size varied while measuring surface tension (surface tension is calculated from the shape of the bubble). This procedure reproduces surfactant performance in the lung during the breathing cycle [19]. (**b**) Surface tension during cycles (color coded). Functional surfactant drops to a near-zero value upon area reduction, where a dysfunctional surfactant stays at about 20 mN/m (i.e., the equilibrium surface tension of most lipids). (**c**) AFM micrograph in three-dimensional representation of surfactant (5 μm × 5 μm). The film shows stacks of bilayers. Each layer is five nanometers high. (**d**) AFM topography of the bovine-derived BLES (left) and electrical surface potential of the region (right). SP-C is a strong molecular dipole that gives rise to the high surface potential in the lamellae [22]. (**e**) Lamellae of surfactant are cross-linked to the monolayer, whereas lamellae of pure lipid films are not. Films of pure lipids (DPPC, top row) and pulmonary surfactant (BLES, bottom row) were imaged by AFM (left column). At this point, partially collapsed lipid films appear no different from surfactant films. However, lamellae of pure lipids can be scraped off from the monolayer in an AFM without trace (top right). Lamellae of surfactant cannot be easily scraped off and leave a trace, indicating that monolayer and lamellae are cross-linked (bottom right). (**f**) Surfactant film may fail by buckling (i.e., moving out of plane). Computational finite element analysis of the critical buckling load as a function of the coverage of the interface by lamellae (the role of multilayer structures in preventing premature surfactant film buckling) explains the stabilizing effect of the lamellae. For a film to resist surface tension without collapse, about 20% of the area or more needs to be covered by lamellae. The lamellae need to be cross-linked to the film. (**g**) sketch of the cross-linking function of SP-C. Surface tension (arrows) excerpts lateral pressure on the film. The film withstands the pressure. The multilayers locally distribute the load (symbolized by springs) and prevent buckling

air–liquid interface, regulating the molecular ordering of the film, and the formation of multilayer structures (Fig. 1.1g) [21].

1.3 Diseases That Are Associated with Surfactant Dysfunction

Pulmonary surfactant function is impaired in acute respiratory distress syndrome (ARDS), impacting lung health and survival. With up to 34 cases per 100,000 population per year [16], ARDS is common and constitutes a high burden for the healthcare system and society with a cost of well over $100,000 per patient in Canada for acute care, for example. In ARDS, a defect in pulmonary surfactant leads to high surface tension, causing alveolar collapse, intrapulmonary shunts, and edema leading to hypoxemia, a dominant factor in the morbidity and high mortality. Direct respiratory failure accounts for about 15% of deaths [23]. In addition, mechanical stress between overinflated and collapsed lung regions strongly amplifies local and systemic inflammation and may help explain the high incidence of multi-organ dysfunction (MODS). ARDS with MODS has much higher mortality, up to 80% [23]. Surfactant dysfunction as a major pathogenic factor for ARDS has not been treatable to date [23–27].

Bronchiolitis associated with cystic fibrosis (CF) is characterized by inflammation in the distal airways and impaired surfactant function [28]. In vitro testing of pediatric CF surfactant samples, obtained largely from medium and small airways, revealed that the ability of the pulmonary surfactant to maintain patency of a capillary tube was markedly reduced, a finding that may explain obstructed airflow in CF [28, 29]. Surfactant dysfunction correlates with the severity of pulmonary impairment as reflected by FEV1.

Other conditions associated with surfactant impairment include pneumonia [30, 31], non-CF bronchiolitis [28], ventilator-induced lung injury (VILI) [32], common complication of mechanical ventilation [7], asthma [33], chronic obstructive pulmonary disease (COPD [33]), neonatal respiratory distress syndrome due to meconium aspiration [34], and Niemann–Pick disease [35].

1.4 Mechanisms of Surfactant Inhibition

Surfactant inhibition refers to the processes that reduce or abolish surfactant surface activity. These processes interfere with surfactant unfolding and adsorption, and film formation interferes with film compression and its ability to reach low surface tensions, or affect surfactant film respreading during expansion [8]. In neonates, however, Avery has shown that impaired lung function in neonatal respiratory distress syndrome (NRDS) is caused by a lack of pulmonary surfactant [36]. This led to the well-established and successful treatment by intratracheal administration of exogenous surfactant. ARDS, on the other hand, is associated with dysfunction, rather than lack of surfactant. According to the standard model, exudative proteins

and/or other surface-active substances adsorb to the air–lung interface. High surface tension would then result from an absence of a surfactant film over significant portions of this interface [37]. However, exudative proteins are readily displaced by surfactant at the interface and do not result in lasting inhibition [8, 38], and high surface tension is best attributed to impairment of the film itself [26, 39]. Small amphiphilic molecules such as cholesterol, lysolipids, and free fatty acids in the surfactant film may render it dysfunctional [1, 32, 38]. The detrimental effect of surfactant proteins and lipid oxidation by reactive oxygen species (ROS) produced in the inflamed lung in high levels also falls into this category [2, 18].

1.5 The Combined Role of Oxidation and Cholesterol in Surfactant Inhibition

The oxidative milieu of the inflamed lung leads to oxidation that renders the surfactant dysfunctional. Surfactant degradation by reactive oxygen species (ROS) is well established and explained by the influx of inflammatory cells in the injured lung, direct and indirect environmental insults to the lung [2, 40, 41]. Free radicals have been implicated in the pathology of pulmonary disease: asthma, bronchiolitis, cystic fibrosis [28, 44], acute respiratory distress syndrome, chronic obstructive pulmonary disease [42, 43], and acute lung injury [32].

Free radicals are continuously formed in the human body [45]. Oxidative damage results from an imbalance in oxidant–antioxidant equilibrium. There are (i) endogenous and (ii) exogenous sources of radicals in the lung. Endogenous oxidants are mainly formed by enzymatic reactions, such as cyclooxygenase-dependent and xanthine oxidase peroxidation, or they are produced and secreted by activated inflammatory cells [47], whereas exogenous oxidants (e.g., NO_2, O_3, and O_2) occur naturally from direct exposure to environmental gasses and particles [46]. A balance between oxidants and antioxidants is vital for function, homeostasis of physiological systems. Oxidative damage may originate from the chemical property of oxygen to break up into unstable metabolites (radicals). These radicals can then react with various biomolecules and inactivate their properties [46, 48].

The oxidative dysfunction of surfactant is strictly dependent on the presence of cholesterol, insofar, that its removal reverses surfactant dysfunction for a broad range of diseases [15, 18, 28]. Cholesterol may be removed from surfactant by adding methyl-β-cyclodextrin (MβCD) to the aqueous phase when testing surfactant in vitro or by delivering this substance by inhalation in mice. This restores the normal function of the film [32, 49, 50]. The relatively hydrophobic interior of the toroid-shaped MβCD molecule can host various hydrophobic molecules, including cholesterol. High levels of MβCD can extract notable amounts of cholesterol from interfacial surfactant films [30], cell membranes [29], and unilamellar cholesterol/phospholipid vesicles [30] into water-soluble cholesterol–cyclodextrin complex.

Cholesterol on its own abolishes surfactant function [19] when highly elevated to levels, such as published for some incidences in ARDS [50]. Interestingly, for most pathologies of surfactant, cholesterol is not elevated to a level that is in and of itself

enough to explain dysfunction, and, yet, removal of cholesterol reverses dysfunction. For these cases, the surfactant oxidation has rendered the surfactant susceptible to mildly elevated or even normal levels of cholesterol. In a recent study with pediatric cystic fibrosis, surfactant samples were unable to sustain normal low surface tensions in the CBS (CF, 13.54 ± 1.37 mN/m versus 2.50 ± 0.88 mN/m for lung-healthy controls) ($P < 0.001$) [28]. After MβCD treatment, the function for 14/16 CF and 2/2 NCFB samples was restored [28, 50]. The proportion of cholesterol in surfactant was significantly higher in CF (13.00 ± 1.44 wt. %) compared to lung-healthy controls (4.96 ± 0.70 wt. %) ($P = 0.008$) and non-CF bronchiolitis (4.87 ± 0.48 wt. %) ($P = 0.017$). While elevated, these cholesterol levels are only inhibitory in the presence of oxidation [19]. Non-CF bronchiolitis surfactant is dysfunctional too [50]. This surfactant shows normal cholesterol levels (5.6 ± 0.5 wt%), and, yet, a function is restored upon treatment by MβCD. Other examples where surfactant dysfunction was reversible by MβCD despite only moderately increased cholesterol are a murine model of acute lung injury as well as a murine model ventilator-induced lung injury, where Vockeroth et al. showed that surfactant inhibition could be reversed by methyl-β-cyclodextrin (MβCD) treatment in vitro, despite only a relatively moderate increase in surfactant cholesterol [7, 50].

Another strict correlation relates to film architecture in health and disease. Functional surfactant exhibits well-defined lipid bilayer stacks on a smooth lipid monolayer. Dysfunctional surfactant forms either no- or ill-formed stacks on a rough monolayer. Removal of cholesterol from such films always restores the normal structure. For films containing 20% cholesterol, the surface tension is not reduced much below the equilibrium value, no matter how large the area reduction (Fig. 1.1b right). For these dysfunctional films, the reinforcing lamellar structures are absent [1]. When now methyl-β-cyclodextrin (MβCD) is added to the aqueous phase, the normal function and the normal structure of the film are restored [32, 49, 50]. For a murine model of acute lung injury, a dysfunctional surfactant film showed ill-formed multilayers. There too, treatment with MβCD restored both the normal function and structure of the surfactant (Fig. 1.2e, f) [10, 50].

1.6 Oxidative Stress and Surfactant Inhibition

1.6.1 The Role of Oxidation on Surfactant Proteins

There are many potential inhibitory mechanisms for surfactant function in acute lung injury [18, 19]. These include lipid peroxidation of mono- and polyunsaturated fatty acids [20], denaturation of surfactant apoproteins [21, 22], protein cross-linking and inhibition, [23] and alterations in surfactant recycling [24]. Several proteins have been shown to inhibit the activity of pulmonary surfactant, particularly serum proteins, such as fibrin and its associated degradation products which are found in high concentration in the lungs of patients with ARDS [5–7, 25]. Studies with SP-A and other soluble proteins suggest that methionine and tryptophan oxidation can occur [42] and that proteins are preferred targets in protein surfactant

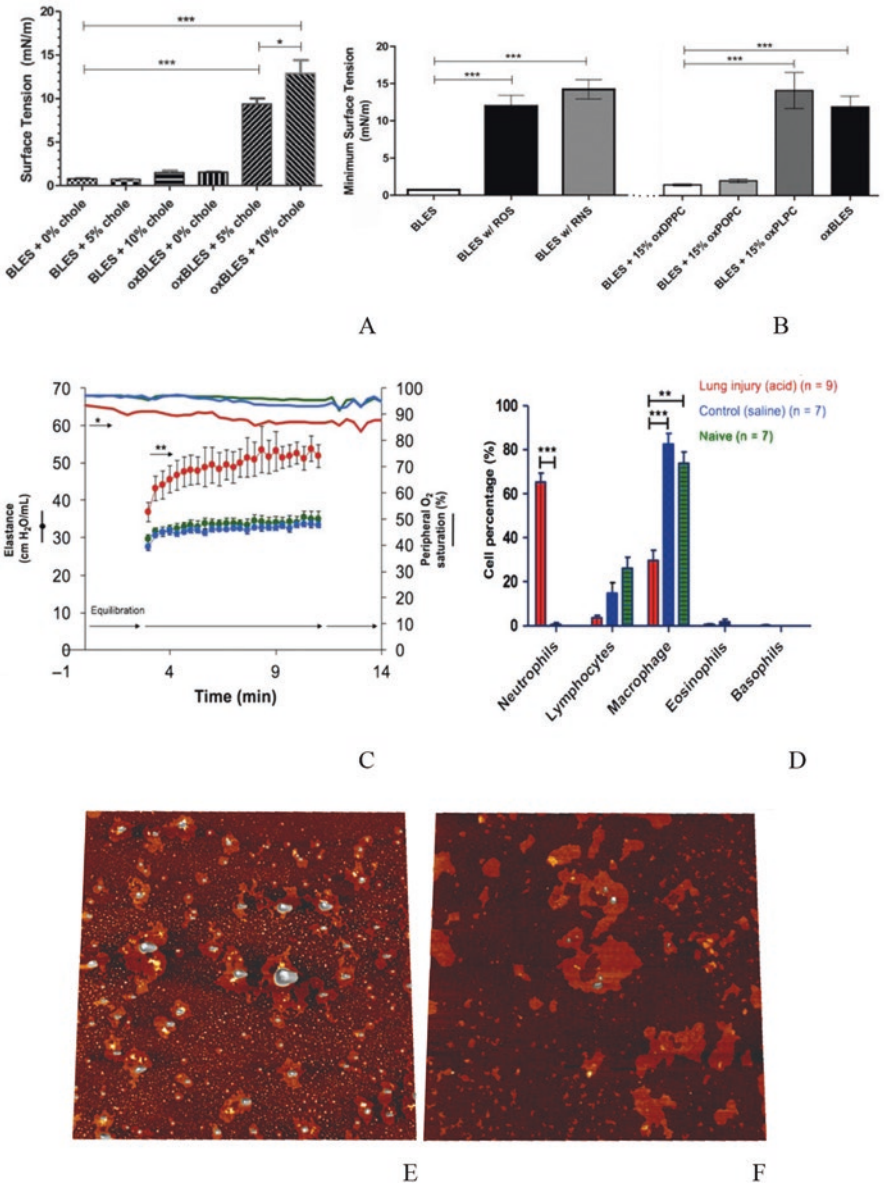

Fig. 1.2 (**a**) Minimum surface tensions during compression–expansion cycles of BLES and oxidized BLES with 0%, 5%, or 10% w/w added cholesterol ($*p < 0.5$, $***p < 0.001$). Both oxidation and cholesterol need to be present to cause the surfactant to fail. (**b**) Oxidative damage is caused by either reactive oxygen species (ROS) or reactive nitrogen species (RNS). Upon analyzing the effect of oxidation on saturated phospholipids (DPPC), monounsaturated (POPC) and polyunsaturated phospholipids, only the latter leads to surfactant dysfunction in the presence of physiological cholesterol levels [5]. (**c**) Murine model of ALI (acid injury), lung function. Mean values (±SE) for elastance (H) and peripheral oxygen saturation are plotted against time in saline-exposed C57BL/6

mixtures [43]. Other studies explained the role of SP-A inhibition on SP-B and SP-C stability. Thus, it appears likely that ROS and RNS could inhibit surfactant adsorption by affecting SP-B and/or SP-C [2, 40].

Hydrophilic SP-A is the most abundant surfactant protein by mass, contributing about 50% of total surfactant protein. It is made up of six trimers (octadecamer) belonging to the Ca^{2+}=dependent carbohydrate-binding collectin family. SP-A is comprised of 248 amino acid residues with a monomeric molecular weight (MW) of 26–38 kDa. SP-A contains four domains: a carbohydrate recognition domain (CRD), a neck, a collagenous domain, and an amino-terminal domain. The structure of SP-A may explain its importance in innate immunity and surfactant stability. It is immunologically active, whereby it enhances superoxide production via macrophage activation. Additionally, SP-A has been proposed to influence film formation and adsorption, compression–expansion cycling surfactant, and film respreading. SP-A's interaction with SP-B, forming SP-A/SP-B complexes at the boundaries of condensed DPPC domains, has been shown important in promoting adsorption and film stability.

Surfactant-associated protein-B (SP-B) is a hydrophobic protein found in surfactant as a disulfide-dependent dimer of 18 kDa. SP-B deficiency in humans results in lethal neonatal respiratory distress syndrome (NRDS), characterized by disorganized surfactant secretory organelles (lamellar bodies) found in type II alveolar cells and the absence of lung compliance [52, 53]. SP-B is a member of the saposin-like family (SAPLIP) that plays an important role in surfactant stability by interacting with the phospholipid bilayers by amphipathic helices. The formation of surface-active film requires quick adsorption and film spreading at the air–water interface. Furthermore, the pulmonary surfactant film must resist collapse and reach near-zero surface tension, during respiration. To achieve low surface tension, the film needs to be enriched in saturated phospholipids (DPPC), whereas rapid adsorption requires the heterogeneous lipid–protein mixtures [54, 55]. Although SP-B and SP-C influence the formation and maintenance of the surface film, surfactant-associated proteins lose their functional integrity in the presence of oxidation. Various studies showed the production of protein modifications, MDA and HNE, conjugated dienes, and carbonyl derivatives [40]. Further, intrinsic fluorescence measurements indicated Fenton (peroxidation reaction), but not HOCl, induced conversion of Trp9 of SP-B to hydroxyTrp (OHTrp), kynurenine (Kyn), and N-formylkynurenine (NFKyn) [2, 40]. Electrospray ionization mass spectrometry revealed molecular weight changes consistent with oxidation of Trp (Fenton) residues and Met (HOCl, Fenton). Oxidative modification to Met29 and Met65 (Fenton, HOCl) and to Trp9 (HOCL

Fig. 1.2 (continued) ($n = 6$), baseline C57BL/6 ($n = 6$), and acid-exposed C57BL/6 ($n = 7$) mice. (**d**) Differential cell count percentage on BAL cytospin films for the ALI model. Enhanced neutrophils correlate with impaired lung function ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). (**e**) AFM topography of the ALI mouse model (20 μm × 20 μm). Being untreated, this dysfunctional surfactant showed an abnormal film structure with multilayers and monolayer regions both showing large and small extrusions of lipids. (**f**) The mouse surfactant after adding MβCD to the aqueous phase upon which the film was spread. The normal film function and structure were restored [50, 51]

with OHTrp and NFKyn plus Kyn with Fenton) was confirmed by matrix-assisted laser desorption mass spectrometry (MALDI-MS) studies on SP-B tryptic fragments [2].

SP-C is a hydrophobic transmembrane protein. SP-C is a single, alpha-helix, bilayer-spanning, confined to the multilayer regions of the surfactant film. The human SP-C gene consists of six exons located on the short arm of human chromosome 8, and its transcription is regulated by thyroid transcription factor-1 (TTF-1). TTF-1 is expressed in type II pneumocytes and was shown to regulate the transcriptional targets of lung maturation and surfactant synthesis. SP-C is synthesized as aproprotein and is cleaved into its mature form in the multivesicular bodies by removing the C- and N-terminal peptides. Further, SP-C is only expressed by the alveolar type II cells postnatally. The deficiency or lack of SP-C leads to severe clinical effects. SP-C-deficient mice developed progressive, severe pulmonary disease associated with epithelial cell dysplasia, acute respiratory distress syndrome (ARDS), and monocytic infiltrates in conducting and respiratory airways [20, 56].

In summary, SP-B and SP-C undergo oxidative changes that significantly hinder their biophysical properties. The oxidative inhibition of the pulmonary surfactant was also related to phospholipid inhibition. The surface activity impairment studies found in the reconstituted phospholipid–protein mixtures that both protein and unsaturated phospholipid oxidation are both responsible for the impaired activity of oxidized surfactants. The oxidative damage to either SP-B or SP-C can hinder surfactant function. Damaging surfactant proteins may play a consequential role in surfactant inhibition that arises during oxidative stress-related disorders like ALI, ARDS, and cystic fibrosis [40].

1.6.1.1 Mechanism of Lipid Peroxidation

Lipid peroxides are present in many pathological conditions [57]. In the lungs, reactive oxygen/nitrogen/species are produced by macrophages, endothelial cells, and neutrophils. Reactive radicals are unstable metabolites of oxygen/nitrogen with oxidizing properties. They are responsible for lipid autoxidation in various biological processes and are associated with the processes that lead to cell death and tissue damage. The degree of tissue injury due to oxidation is dependent on the balance between oxidants and antioxidants. Lipid autoxidation does not only increase lipid peroxidation levels; it may also form epoxide-containing lipids. Among the targets of oxidative damage are phospholipids containing unsaturated fatty acyl and cholesterol. Lipid autoxidation is a process in which molecular oxygen/nitrogen and phospholipids react via free-radical-mediated chain reaction [57], resulting in the formation of lipid epoxidation and/or peroxidation.

Lipid peroxidation pertains to nonenzymatic and autocatalytic autoperoxidation process, leading to perpetual breakdown and formation of dioxygen adducts of unsaturated lipids and lipid hydroperoxides. The enzymatically driven lipid peroxidation is also evident in biological systems, such as cyclooxygenase-dependent peroxidation of arachidonic acid and xanthine oxidase induced. The mechanism of lipid peroxidation is well studied, whereby different oxidative mediators are investigated in various biological processes. Particularly, hydroxyl radicals are shown to

be highly reactive with unsaturated fatty acids under oxidative stress. Saturated fatty acid chains, such as 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC), are shown to be more resistant to oxidative modification as they do not contain allylic hydrogen atoms. In mono- and polyunsaturated fatty acyl chains, monounsaturated (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and polyunsaturated (1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC)), the allylic hydrogen atoms on methylene groups adjacent to the double bonds exhibit low hydrogen–carbon (C–H) bond energies, whereas bis-allylic hydrogens positioned between two double bonds have even lower C–H bond energies. The initiation phase of lipid peroxidation begins with the initial hydrogen abstraction and then followed by the introduction of molecular oxygen (O2). In polyunsaturated fatty acid (PUFA) chains, the addition of O2 could occur multiple times to a single acyl chain due to the rearrangements of a peroxyl radical to a new carbon center or due to the presence of other bis-allylic hydrogen atoms. The intermediate stage of lipid peroxidation results in the formation of additional carbon- or oxygen-centered radicals. Peroxidation intermediate is responsible for perpetuating the damage by radicals. Hydrogen abstraction by radical groups on the initial α-carbon converts it to a non-radical center. The process whereby a single free radical attack damages multiple lipid molecules is known as the propagation phase. Termination phase is the final step in lipid peroxidation; this phase is mediated by either increased radical–radical interactions which forms non-radical products or antioxidants.

1.6.1.2 The Mechanism of Reactive Nitrogen Species

The pulmonary surfactant is exposed to several insults that alter its molecules: reactive nitrogen and oxygen species (RNOS), peroxidases secreted by activated inflammatory cells, and proteases. $O^{\bullet 2-}$ and •NO react to form peroxynitrite in the epithelial lining fluid [26, 58], which ultimately result in surfactant inhibition as a result of lipid peroxidation and damaged surfactant-specific proteins. Exposure of surfactant contents to peroxynitrite damages unsaturated phospholipids and small hydrophobic surfactant proteins SP-B and SP-C [5, 6]. In vitro studies showed that the addition of peroxynitrite to clinical surfactant is capable of decreasing the surface activity, inducing lipid peroxidation, inducing protein-associated nitrotyrosine, and decreasing the function of surfactant proteins, SP-A, SP-B, and SP-C [59].

During edema and inflammation, vasoconstriction is often observed, whereby considerable levels of oxidants are produced ($O\bullet^{2-}$ and H_2O_2). Superoxide ($O\bullet^{2-}$) can react with nitric oxide to produce powerful oxidant peroxynitrite (ONOO-), which can nitrate lipids and proteins, or is converted into hydrogen peroxide (H_2O_2) under the influence of superoxide dismutase [60].

ONOO- is an important biological oxidant in various oxidation-mediated inflammatory lung diseases. ONOO- may indirectly contribute to the generation of ARDS by depleting antioxidants and inactivating antiproteases. Although free radicals are indiscriminate, PUFA-rich membranes are particularly susceptible [61]. Peroxynitrite anions are formed by the rapid reaction of nitric oxide with superoxide (k = 6.7 × 109 M-1s-1). Additionally, nitric oxide is able to outcompete

superoxide dismutase (SOD) for superoxide, resulting in the formation of peroxynitrites. The amount and activity of nitric oxide synthase, the activity of downstream metabolic enzymes, and the redox balance influence the rate of nitric oxide reactions. ONOO- easily oxidizes biological molecules including proteins, lipids, and DNA. Furthermore, ONOO- can modulate various oxidative molecular pathways; it can result in irreversible NO-induced modification of proteins and nitrosylate amines through nitrosative deamination of DNA bases. Peroxynitrous acid (ONOOH) and ONOO- conjugates are also formed in inflamed environment [62]. Under favorable conditions, ONOOH can undergo hemolysis, producing hydroxyl radicals and nitrogen dioxide (•NO_2). HO•can diffuse freely through lipid membranes and react with the unsaturated lipid component (Fig. 1.3). This process is responsible for surfactant film inhibition.

Additionally, ROS can also form from abnormal oxidative levels. Inflammatory cells release peroxynitrite ($ONOO^-$), hypochlorous acid (OCl^-), and hydrogen peroxides in concentrations that may equal 0.1 mM/min. In the presence of ferrous iron, they can induce Fenton chemistry in the epithelial lining fluid [63, 64]. Disease

Fig. 1.3 Oxidative pathway of RNS-mediated phospholipid damage. This illustrates the molecular level the three autoxidation stages (initiation, propagation, and termination)

state is also dependent on the magnitude of the oxidative levels [64]. Protein oxidation-mediated surfactant inhibition has also been shown to be a model of surfactant dysfunction [41, 65].

There is involvement of ONOO-, NO, and other RNSs in the generation and propagation of lung epithelial injury in a variety of pathological conditions [60]. First, induction of immune complex alveolitis in rat lungs resulted in significant elevation of decomposition products of NO (nitrate and nitrite) and albumin levels in the bronchoalveolar lavage, indicating the presence of increased alveolar permeability to solute and suggesting that a surfactant-deficient state may be present [60]. Instillation of NG-monomethyl-L-arginine, a specific inhibitor of the oxidative L-arginine pathway responsible for the production of NO, resulted in a significant diminution of nitrite, nitrate, and alveolar albumin levels, without altering neutrophil recruitment in the lung [60]. Second, exposure of rats to 5 and 10 ppm NO resulted in a significant degree of lung injury and alteration in the physiological function of SP-A [66]. Third, peroxynitrite inactivated αl-proteinase (αl-PI) inhibitor, the most abundant extracellular antiprotease in the epithelial lining fluid, by oxidizing the methionine residue of this peptide [60, 67]. Inactivation of α1-PI may render the alveolar epithelium susceptible to neutrophil-induced injury [60]. Since alveolar macrophages remain activated for several hours, peroxynitrite levels may continue to rise. This may explain the lack of clinical response to exogenous surfactant replacement therapy observed in patients with ALI and ARDS. These patients suffer from an inflammatory response and increased reactive radical species released by the alveolar macrophages, leading to oxidizing exogenous surfactant and damaging its function.

1.6.1.3 Epoxide-Mediated Surfactant Inhibition

It is known that oxidative stress results in degradation and loss of function of biological membranes. Direct cellular damage of lipid moiety due to lipid peroxide accumulation on both alveolar surfaces and lung parenchymal tissues has been shown in various studies. Epoxides are cyclic ether with a three-atom ring. Normally, the strained ring and polarized carbon–oxygen bond are responsible for the high reactivity of epoxides [68, 69]. However, nonactivated aliphatic epoxides are relatively stable under physiological conditions. Epoxidation of unsaturated phospholipids begins with the abstraction of allylic hydrogen from unsaturated fatty acid tails. The peroxy radical introduction to neighboring unsaturated fatty acids is then followed by rapid rearrangement of fatty acids. Subsequently, the O–O bond is broken, and the nucleophilic π bond donates its electrons to the oxygen, forming the new carbonyl bond. The electrons from the old O–H bond make up the second new C–O bond, whereas the protons are picked up by the original carbonyl group. This oxidative reaction produces transient radical center that is able to attack the adjacent double bond and/or decompose to form epoxides (Fig. 1.4). During peroxide and epoxide transition, alkoxy intermediate formation plays a significant role in the conversion process, as they act as hemolytic scission products of organic nitrates and peroxides. Alkoxy intermediates also play a role in autoxidation processes. In vitro olefin epoxidation may be accomplished by transition metal catalysis or peracidic

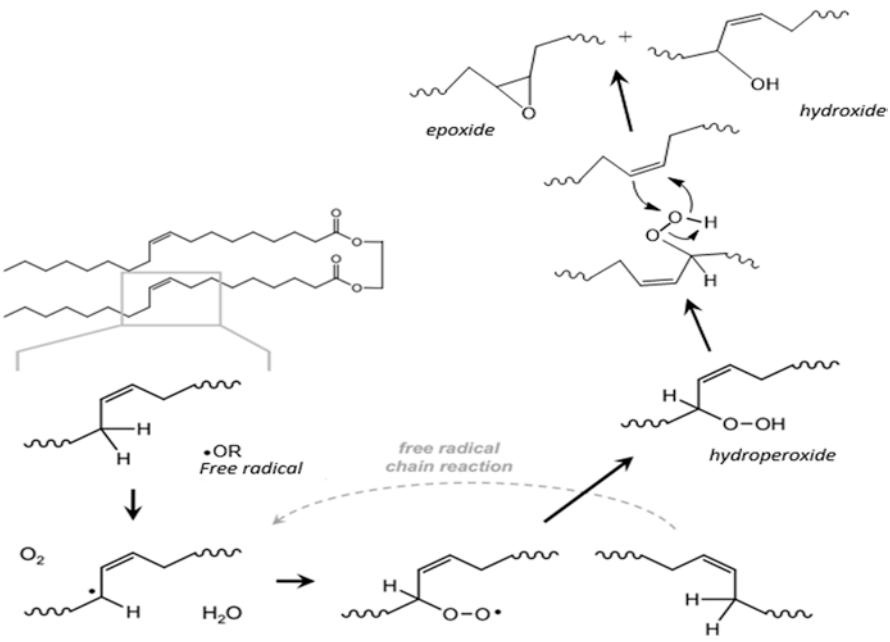

Fig. 1.4 Free radical-mediated phospholipid oxidative reaction. This illustrates at the molecular level the three stages of autoxidation (initiation, propagation, and termination)

acids. Peracidic acids have an extra oxygen atom between the acidic hydrogen and their carbonyl group, forming an electrophilic carbon center at the oxygen-rich zone. This proton transfer process is the product of an electrophilic attack from the epoxide oxygen to the carboxylic acid by-product.

The role of epoxides in pulmonary surfactant damage has been studied to a lesser extent. Here we outline the underlying mechanism of epoxide-mediated surfactant dysfunction. Various studies had emphasized the role of hydrogen peroxide on surfactant dysfunction; however, based on mass spectrometry (MS) studies of oxidized pulmonary surfactant, epoxides appear to play a role in surfactant pathophysiology. It is important to mention that MS bears certain limitations. The challenge with MS lies with distinguishing between two similar mass-to-charge (*m/z*) species.

Saturated fatty lipids are resistant to oxidative damage, that is, dipalmitoylphosphatidylcholine (DPPC). However, the monounsaturated (POPC) and polyunsaturated (PLPC) (*m/z* 794.55, *m/z* 792.53, *m/z* 828.51, and *m/z* 844.50, respectively) phospholipids indicate the presence of an oxidative process (Fig. 1.5). Furthermore, the polyunsaturated phospholipids expressed a variety of oxidative species in their fatty acyl tails. This is due to the degree of available weak allylic hydrogen susceptible to radical attack. The function of pulmonary surfactant is disrupted by Prilezhaev chemistry exposure, producing pure epoxides into the hydrocarbon chain. However, in vitro studies showed that the introduction of epoxidation into BLES in the absence of cholesterol does not result in film inactivation [51].

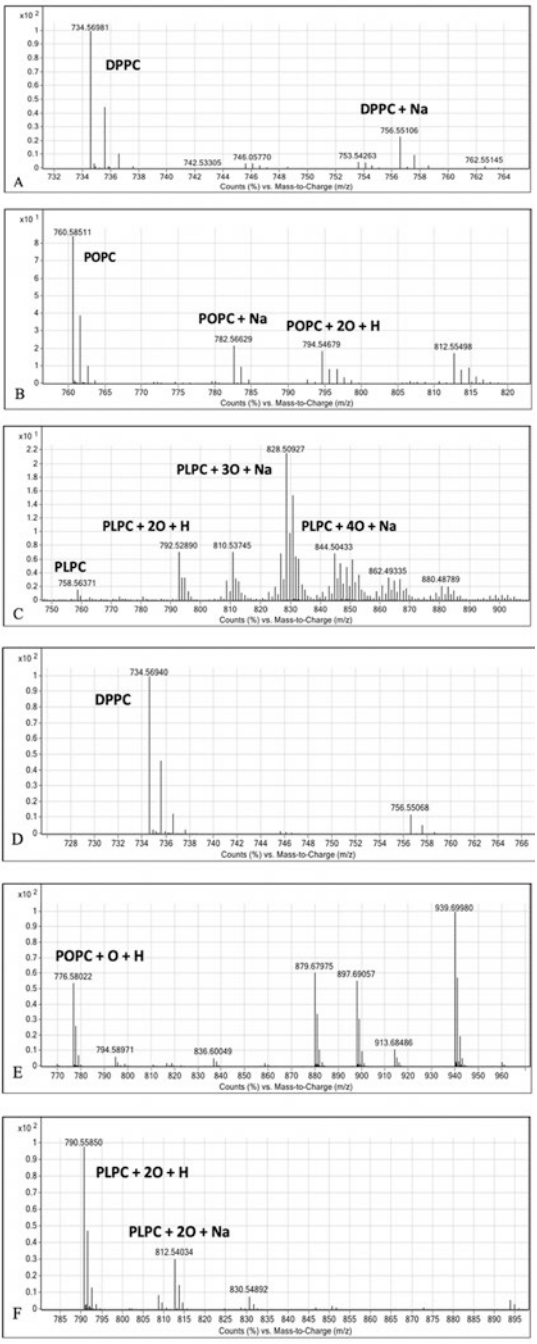

Fig. 1.5 Mass spectra of oxidized phospholipids. (**a**) Saturated (DPPC), (**b**) monounsaturated (POPC), and (**c**) polyunsaturated (PLPC) phospholipids. These mass spectra illustrate whether or not oxidation was introduced into the investigated samples as a result of the Fenton chemistry

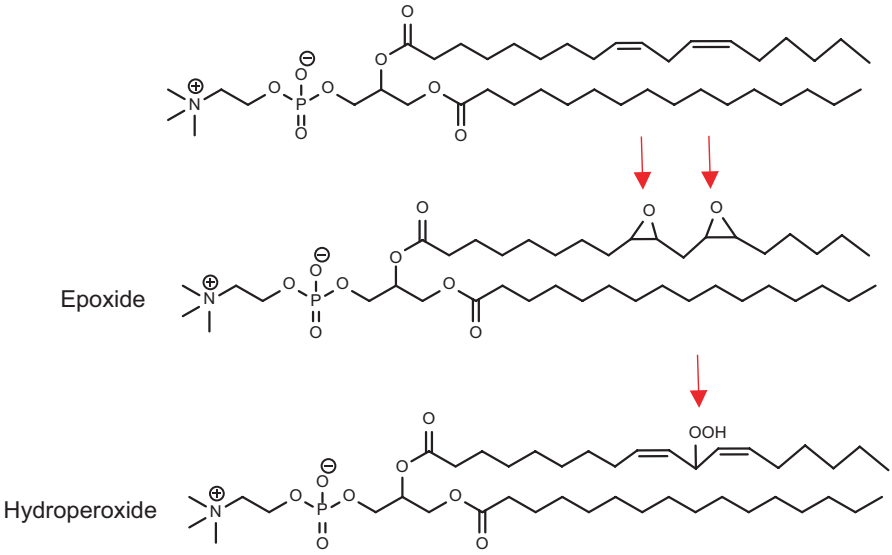

Fig. 1.6 Molecular illustration of PLPC, epoxy PLPC, and hydroperoxy PLPC. Red arrow denotes the difference between hydroperoxy PLPC and epoxy PLPC

To appreciate this difficulty of identifying the exact molecule, Fig. 1.6 is an illustration of PLPC, epoxy PLPC, and hydroperoxy PLPC. The difference between two epoxide rings and one hydroperoxide group attached to the hydrocarbon tail is 1 *m/z*. The mass difference is rather small between the two species, causing difficulties in accurate molecular identification.

Lipid epoxides mediate surfactant dysfunction. Some observations indicate that phospholipid peroxidation in lung tissues may be the origin of lipid epoxides [70]. Despite the origin, lipid epoxides appear to be traceable products of lipid peroxidation. In this chapter, we have discussed importance of cholesterol in mediating the inhibition of oxidized pulmonary surfactant. However, further investigation of the inhibitory mechanism of oxidized cholesterol on the pulmonary surfactant system is imperative, as it may provide stronger evidence of the importance of direct phospholipid oxidation in surfactant inhibition.

1.7 Conclusion

This chapter explains the detrimental role of oxidative environments on the molecular alteration of surfactant, leading to reduced surface activity at normal cholesterol levels. The modification of surfactant mono- and polyunsaturated phospholipid constituents yields in measurable changes to surfactant film molecular architecture.

The molecular mechanism of surfactant inhibition indicated in this chapter is relevant to disease. Studies where oxidized surfactants are instilled into animal

models have shown good associations between in vivo activity and in vitro performance. The combination of oxidation and normal cholesterol levels abolishes the essential biophysical aspect of surfactant, that is, the ability to reduce the minimum surface tension. With the increased knowledge of pulmonary surfactant inhibition, several avenues may be used to repair impaired surfactant system. In ALI, CF, and ARDS, elevated cholesterol levels can explain the dysfunction on its own [51]. Therefore, reducing cholesterol levels in the surfactant film may be a possible therapeutic target. Identifying viable treatments for pulmonary surfactant dysfunction remains to be an ongoing field of research.

The mechanism of surfactant inhibition differs in respiratory distress syndrome (NRDS) in premature infants and acute respiratory distress syndrome (ARDS). NRDS is caused by a lack of pulmonary surfactant which is successfully treated by intratracheal administration of exogenous surfactant [25], whereas in ARDS the surfactant is damaged but is not lacking; therefore, replacement surfactant therapy in ARDS has led to improved oxygenation and increased lung compliance in some clinical trials, but these effects have been transient [25, 71]. None of the many controlled double-blind studies was successful. It is important that surfactant research continues, as improved patient outcome still appears to hinge on a functioning surfactant. Although surfactant replacement therapy may be the wrong approach as it addresses a deactivation mechanism that might not be relevant, various in vitro and in vivo experiments show that this type of surfactant deactivation will not likely respond to surfactant replacement therapy because damaged surfactant at the air–water interface is not readily displaced. Moreover, exogenous surfactant may rapidly be rendered dysfunctional by the same mechanism that degraded the endogenous surfactant [26, 72, 73]. Introduction of exogenous surfactant may also strain the catabolic and recycling system of the peripheral lung. Based on the reversibility of surfactant dysfunction by MβCD for a broad range of inflammatory lung diseases, cholesterol-dependent oxidative surfactant inhibition appears to be a generic inhibitory pathway, applicable to most situations. MβCD may indeed be a potential treatment for surfactant dysfunction in inflammatory lung diseases. Cyclodextrins are nontoxic simple sugars and widely used as drug carriers. When inhaled they are readily cleared from the lung, leaving the body through renal secretion. Cyclodextrins have low toxicity, used as vehicles for delivering many drugs and sequestration of toxic compounds [74]. MβCD was used for its ability to efficiently sequester cholesterol. Additionally, MβCD was shown to take up linoleic acid in vitro, an important lipid mediator of inflammation in ALI/ARDS, as well as suppress acute inflammation in an animal model of ALI [75]. Thus, inhaled cyclodextrins may have two important properties for treating acute lung injury: an anti-inflammatory effect and the ability to repair dysfunctional surfactant.

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Oxidative Stress in Experimental Models of Acute Lung Injury

2

Daniela Mokra and Juraj Mokry

Abstract

Overproduction of oxidants within the lung leads to acute lung injury (ALI) which may progress to irreversible lung fibrosis. The sources of oxidants may be (1) intrinsic, that is, derived from phagocytic macrophages and neutrophils and endothelial and alveolar epithelial cells, or (2) extrinsic, that is, caused by inhaled pollutants or high concentrations of oxygen. The complex antioxidant system of the body includes reduction–oxidation enzymatic systems, nonenzymatic scavengers, and dietary components which balance the concentrations of both antioxidant and oxidant substances with dominance of a reducing state. However, decreasing levels of antioxidants and/or increasing levels of oxidants disturb the stability of the antioxidant–oxidant system and lead to oxidative lung injury. Several groups of therapeutic agents, including N-acetylcysteine, flavonoids, corticosteroids, phosphodiesterase inhibitors, etc., appeared to be beneficial in the treatment of various forms of experimentally induced ALI where they significantly reduced the oxidative damage. This chapter reviews the pathophysiology and mechanisms of ALI with respect to oxidative and inflammatory changes and critically evaluates perspectives of promising treatments to prevent or to minimize

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the oxidative changes in experimental models of ALI reflecting possibilities of their use also in the treatment of patients with acutely damaged lung.

Keywords
Acute lung injury · Oxidative stress · Lung · Inflammation · Animal model · Antioxidants · Anti-inflammatory treatment

Abbreviations

ALI	acute lung injury
AP-1	activator protein-1
ARDS	acute respiratory distress syndrome
BALF	bronchoalveolar lavage fluid
cAMP	cyclic adenosine monophosphate
CAPE	caffeic acid phenethyl ester
CAT	catalase
cGCR	cytosolic glucocorticoid receptor
cGMP	cyclic guanosine monophosphate
CMV	conventional mechanical ventilation
COX-2	cyclooxygenase-2
CXCL	chemokine (C-X-C motif) ligand
DAMPs	danger-associated molecular patterns
DNA	deoxyribonucleic acid
ET-1	endothelin-1
FiO2	fraction of inspired oxygen
GCs	glucocorticoids
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRE	glucocorticoid response elements
GSH/GSSG	reduced and oxidized states of glutathione
HCl	hydrochloric acid
HFOV	high-frequency oscillatory ventilation
HMGB1	high-mobility group box 1 protein
4HNE	4-hydroxy-2-nonenal
HO-1	heme oxygenase-1
HTV	high tidal volume ventilation
ICAM-1	intercellular adhesion molecule-1
IFNγ	interferon γ
IL	interleukin
iNO	inhaled nitric oxide
iNOS	inducible NO synthase
LPS	lipopolysaccharide

LT	leukotriene
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MDA	malondialdehyde
MIP-2	macrophage inflammatory protein-2
MMP	matrix metalloproteinase
MODS	multiple organ dysfunction syndrome
MPO	myeloperoxidase
MSAF	meconium-stained amniotic fluid
NAC	N-acetylcysteine
NETs	neutrophil extracellular traps
NF-κB	nuclear factor kappa B
NO	nitric oxide
8-OH-dG	8-hydroxydeoxyguanosine
PaO_2	arterial partial pressure of oxygen
PDE	phosphodiesterase
PMN	polymorphonuclears
RAGE	receptor for advanced glycation end products
RNS	reactive nitrogen species
ROS	reactive oxygen species
SIRS	systemic inflammatory response syndrome
SNAP	S-nitroso-N-acetyl-penicillamine
SOD	superoxide dismutase
SP	surfactant protein
TAP	total antioxidant performance
TAS	total antioxidant status
TBARS	thiobarbituric acid reactive substances
TGF-β	transforming growth factor-β
TLR	Toll-like receptors
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VCAM-1	vascular cell adhesion molecule-1
VILI	ventilator-induced lung injury
VT	tidal volume
vWf	von Willebrand factor
W/D ratio	wet-dry lung weight ratio

2.1 Introduction

Oxygen is vitally important for human existence. Oxidation processes supply energy for biological actions, and free radicals generated in these processes participate in cell regulation. However, the lung as an organ responsible for uptake and

utilization of appropriate quantities of oxygen is highly susceptible to oxidative damage. Therefore, it contains very effective system of antioxidants located in the cells and in the epithelial lining fluid to minimize detrimental effects of oxidants. Under physiological conditions, lung concentrations of antioxidants and oxidants are well balanced with dominance of a reducing state. When the ratio between oxidants and antioxidants shifts toward oxidants, oxidative processes result into protein, lipid, and DNA deterioration [1]. In acute lung injury (ALI), oxidative stress is associated with both overproduction of oxidants and malfunction of antioxidant systems [2]. As the oxidative damage of the lung tissue significantly contributes to pathophysiology of ALI, an intensive research in the preclinical and clinical conditions has to find out treatments which may positively influence changes in the injured lung. This chapter reviews inflammatory and oxidative changes in various animal models of ALI and points out several treatments which may be potentially beneficial in this situation.

2.2 Acute Lung Injury

2.2.1 Definitions, Incidence, and Etiology

Acute lung damage represents a life-threatening situation which can occur in all age groups. Diffuse alveolar injury, formation of lung edema, inflammation, and ventilation-perfusion mismatch may finally lead to decrease in lung compliance and into profound hypoxemia [3].

American–European Consensus Conference in 1994 [4] postulated diagnostic criteria for acute respiratory distress syndrome (ARDS) or ALI in patients with acute lung damage: (1) acute hypoxemia, defined as a ratio of arterial partial pressure of oxygen (PaO_2) and fraction of inspired oxygen (FiO_2) – for ARDS, PaO_2/FiO_2 is <200 mmHg (26.7 kPa), for ALI, PaO_2/FiO_2 is between 200 mmHg (26.7 kPa) and 300 mmHg (40 kPa); (2) bilateral infiltrates on chest X-ray; and (3) no increase in pulmonary artery wedge pressure. Newer, so-called Berlin definition defined three categories of ARDS according to severity of hypoxemia: mild (PaO_2/FiO_2, 200–300 mmHg), moderate (PaO_2/FiO_2, 100–200 mmHg), and severe (PaO_2/FiO_2, less than 100 mmHg) forms of ARDS [5]. The term "acute lung injury" that in the older definition expressed the milder form of lung damage was omitted, and nowadays it is used for general expression of the situation or for experimental studies where respiratory insufficiency is induced artificially and other clinically relevant signs except of hypoxemia cannot be determined.

Despite better understanding of the pathophysiology and wider use of lung-protective ventilations, incidence of ARDS remains high, about 30–80 cases per 100,000 population [6].

ALI may develop from direct reasons, for example, in pneumonia, aspiration of gastric content, near-drowning, inhalation of toxic gases, etc., or from indirect reasons as an accompanying situation in serious systemic disorder, for example, in sepsis, severe trauma, or pancreatitis [3, 7].

2.2.2 Pathophysiology of ALI/ARDS

The hallmarks of ALI/ARDS pathophysiology include ongoing neutrophil-mediated inflammation, excessive transmigration and activation of leukocytes and platelets, increased activation of coagulation pathways, and enhanced permeability through alveolar–capillary membrane [8]. Changes in ALI/ARDS develop in three stages which overlap. The initial or exudative phase (day 1–7) is defined by diffuse alveolar damage of lung epithelium and/or endothelium which is linked with release of various factors causing injury and cell death. The loss of integrity of alveolar–capillary barrier leads to flooding of the alveoli with proteinaceous fluid and dilution of pulmonary surfactant. These changes result into generation of lung edema, decreased lung compliance, and impairment of gas exchange [9]. Damage to the lung tissue is associated with massive transmigration of immune cells into impaired lung. Activated neutrophils, alveolar macrophages, and fixed lung cells produce vast quantities of pro-inflammatory substances, for example, interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)α, proteases, and reactive oxygen species (ROS), further potentiating the lung tissue injury [9, 10]. Within several days, the exudative phase fluently passes to proliferative phase that is characterized by resolution of pulmonary edema and regeneration of damaged tissue by proliferation and phenotypic changes in type II alveolar cells, myofibroblasts and fibroblasts, and new matrix deposition. In the absence of recovery, the situation in some patients may progress to a fibrotic stage characterized by diffuse fibrosis and irreversible change of lung architecture [9, 11].

In direct lung injury, noxious stimuli hit primarily the lung structures. The complex immune response is activated by linking of microbial products or cell injury–associated endogenous molecules (danger-associated molecular patterns, DAMPs) to pattern recognition receptors (e.g., Toll-like receptors, TLR) on the lung epithelial cells and alveolar macrophages which results in inflammation [8]. As additional immune effector mechanisms contributing to the tissue injury, neutrophil extracellular traps (NETs) and extracellular histones have been identified. NETs are formed by dying neutrophils that release DNA, histones, and granular proteins (e.g., neutrophil elastase and myeloperoxidase). The released histones, major proteins of chromosomes, are highly cytotoxic. They act as DAMPs and further induce epithelial and endothelial cell death. After entering the circulation, histones stimulate platelet aggregation, promote recruitment of neutrophils, and aggravate systemic inflammation [12, 13]. The systemic leukocyte activation can progress to systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), and multiple organ failure [15].

If the primary cause of ALI/ARDS is located in other tissues than the lung, lung inflammation and edema formation may be triggered by high concentrations of histones to which the lung is highly susceptible [12, 14]. However, many other substances including pro-inflammatory cytokines TNFα and IL-1β, high-mobility group box 1 (HMGB1) protein, or mitochondrial DNA may also act as DAMPs and induce the lung inflammation and ALI/ARDS [16].

However, there are other differences between direct and indirect ALI/ARDS, as well. In the direct ALI/ARDS, injury is more localized to alveolar epithelial cells, with alveolar collapse; accumulation of polymorphonuclears (PMN), particularly of neutrophils; fibrin deposition; hyaline membranes; and alveolar wall edema. In indirect ALI/ARDS, diffuse injury to endothelial cells is more prominent, and typical is the finding of interstitial edema and smaller lung accumulation of neutrophils than in the direct form. Moreover, in the direct form of ALI/ARDS, concentrations of pro-inflammatory cytokines TNFα, IL-1β, IL-6, and IL-8 increase in the bronchoalveolar lavage fluid (BALF) or lung tissue homogenates. In the indirect form of ALI/ARDS, increased levels of these cytokines are detected predominantly in the plasma indicating that the lung injury is secondary due to reaction of substances released from the extrapulmonary sites of injury into the circulation [17, 18]. Regarding the primary injury to epithelial cells in direct ALI/ARDS, surfactant protein (SP)-D has been identified as a valuable marker of injury to type II alveolar cells and receptor for advanced glycation end products (RAGE) as an indicator of deterioration of type I alveolar cells. The damage of endothelial cells and systemic inflammation which are more prominent in the indirect ALI/ARDS can be confirmed by increased concentrations of von Willebrand factor (vWf), IL-6, IL-8, and angiopoietin-2 in the plasma [9, 19].

2.3 Oxidative Stress in Acute Lung Injury

Dysregulated acute inflammatory response in ALI/ARDS is linked with higher level of lung oxidative stress due to an overproduction of oxidants and reduced activity of antioxidants. In normal situation, lung oxidants and antioxidants are kept in balance with little dominance of a reducing state. In pathological situations, such as ALI/ARDS, increases in oxidants and decreases in antioxidants disrupt the equilibrium and lead to oxidative changes. However, the oxidative stress is not responsible only for direct lung injury, but it can also influence the control of lung inflammation [20].

Oxidants can be of intrinsic or extrinsic origin. Intrinsically generated oxidants can be derived from mitochondria, but mainly from phagocytic cells (recruited neutrophils and residential lung macrophages) and from alveolar epithelial and endothelial cells [21]. Exogenous sources of oxidants represent inhaled oxidant gases as well as medicinal oxygen in supraphysiological concentrations which is used for artificial ventilation of patients with severe ARDS [21].

Reactive oxygen species (ROS – superoxide, hydrogen peroxide, hydroxyl radicals, etc.) are formed in sequential reduction of oxygen (equivalent to sequential addition of electrons)[2]. Reactive nitrogen species (RNS), particularly nitric oxide (NO) and peroxynitrite, also contribute to pathophysiology of ALI/ARDS. Nitric oxide (NO) released in small amounts has important neurotransmitter and regulatory functions. However, high concentrations of NO generated due to increased activity of inducible NO synthase (iNOS) in inflammation exert deleterious effects when combine with superoxide and form highly toxic peroxynitrite [2].

To counterbalance the detrimental action of free radicals, endogenous antioxidant systems, such as superoxide dismutase (SOD), catalase (CAT), glutathione, and glutathione peroxidase, are expressed in high concentrations in the lung lining fluids [21]. ROS and RNS cause the injury by several ways: (a) strand breaks and point mutations of DNA, (b) peroxidation of lipids and generation of vasoactive and pro-inflammatory substances, (c) oxidation of sulfhydryl and other protein groups altering the activity of proteins, and (d) alteration of transcription factors nuclear factor (NF)-κB and activator protein (AP)-1 resulting in increased expression of pro-inflammatory genes [22]. Oxidation and cross-linking of proteins, lipids, DNA, and saccharides lead to changes of cellular structures and function, increased vascular endothelial permeability, and lung edema formation as well as pulmonary epithelial dysfunction with impaired sodium transport and fluid reabsorption from the alveoli [2].

There are several possibilities to detect free oxygen and nitrogen species and their by-products to evaluate the extent of oxidative changes in the lung [20]. Some fluorogenic markers of ROS may be detected in live cells [23]. However, more studies demonstrate changes in the concentrations of markers of lipoperoxidation, for example, thiobarbituric acid reactive substances (TBARS), including malondialdehyde (MDA), 4-hydroxy-2-nonenal (4HNE), isoprostanes, etc.; markers of protein oxidation, such as nitrotyrosine, dityrosine, lysine–lipoperoxidation products, etc.; markers of DNA damage, such as 8-hydroxydeoxyguanosine (8-OH-dG), hydrogen peroxide and superoxide products, nitrites/nitrates, etc.; and markers of antioxidant status, such as levels of SOD, CAT, reduced (GSH) and oxidized (GSSG) states of glutathione, or total antioxidant status (TAS) in the samples of BALF, plasma or serum of patients with ARDS or in the BALF, plasma, serum, and lung homogenates of experimental animals with models of ALI. Further information on nitroxide free radicals, carbon monoxide, and other markers of oxidative stress can be obtained from analysis of expired air or exhaled air condensate [20].

2.4 Animal Models of Acute Lung Injury

Experimental models of ALI/ARDS provide an additional knowledge on the pathophysiology of respiratory distress and enable to determine effectiveness of novel therapeutic approaches [24]. Nevertheless, while in humans the criteria for ARDS are well defined, these cannot be completely accepted for animal studies. Official American Thoracic Society Workshop has considered for fundamental signs of experimental ALI: (1) lung tissue damage verified by histopathological investigation, (2) increased permeability of alveolar–capillary membrane, (3) acute lung inflammation, and (4) deterioration in physiological parameters (lung functions, oxygenation, etc.). To estimate whether ALI was successfully induced, at least three of the mentioned "signs" need to be identified [25].

When modeling human lung injury, species differences should be considered. There are unique species characteristics, that is, differences in innate immune response (differences in TLR, mononuclear phagocytic system, NO production,

chemokines and chemokine receptors, etc.) and differences in animal size which can limit a direct applicability of results obtained from the animal models [24]. Furthermore, models of ALI are artificially induced in healthy animals, while ARDS often occurs in older patients with concomitant chronic diseases.

According to the primary target, experimental models of ALI can be assorted into three groups:

(a) Models with primary dysfunction of epithelium (i.e., models of direct ALI), such as model of surfactant depletion induced by saline lung lavage, model induced by intratracheal instillation of lipopolysaccharide (LPS), neonatal meconium, or hydrochloric acid, model induced by exposure to hyperoxia or mechanical ventilation, etc.
(b) Models with primary dysfunction of endothelium (i.e., models of indirect ALI), such as model induced by intravenous LPS administration or instillation of oleic acid.
(c) Models with dysfunction of both epithelium and endothelium [24, 26].

2.5 Evidence of Lung Damage and Oxidative Stress in Animal Models of "Direct ALI"

2.5.1 Model of ALI Induced by Intratracheal LPS/Bacteria

Shortly after instillation of bacterial endotoxin LPS or bacteria into the lung, these are recognized by alveolar macrophages via their pattern recognition receptors. Identification of pathogenic bacteria initiates an inflammatory response with generation of pro-inflammatory cytokines (TNFα, IL-1β, etc.) and chemokines, such as macrophage inflammatory protein (MIP)-2. These mediators then activate epithelial cells to express various TLR receptors including TLR4 and via stimulation of NF-κB-dependent pathways to produce additional chemokines attracting neutrophils into the lung [27]. Transendothelial migration of PMN into the interstitium began 1 h after exposure to LPS and reached a maximum of 12–24 h, while transmigration of PMN into the alveoli appeared more than 2 h after LPS, with a maximum at 24 h [28]. In other studies, aerosolized or intratracheal LPS increased counts of neutrophils in BALF and lung tissue, elevated pro-inflammatory cytokines and neutrophil chemoattractants, and evoked production of lung edema [29–31]. The influx of inflammatory cells after LPS inhalation correlated well with lung function impairment, and histopathological changes peaked at 48 h [32]. In the recent study, LPS increased expression of TNFα, iNOS, and cyclooxygenase (COX)-2 and generation of ROS in cell line. In addition, LPS activated mitogen-activated protein kinase (MAPK) pathways, enhancing NF-κB activation and iNOS expression [33]. Similarly in mice, LPS elevated counts of total cells, neutrophils, and macrophages; induced lung edema formation due to increased lung cell apoptosis and damaged epithelium barrier function; increased TNFα, IL-1β, glutamate, and myeloperoxidase (MPO) activity; and reduced endogenous lung antioxidants [33].

2.5.2 Model of Surfactant Depletion Induced by Saline Lung Lavage

Repetitive saline lung lavage partially removes the lung surfactant leading to increased alveolar surface tension, alveolar collapse, and impairment of local host defense [24, 34]. These changes result in hypoxemia, edema production, PMN migration into the alveolar spaces, and elevated expression of cytokines TNFα, IL-1β, IL-6, and IL-8 [35–41].

Injury to cells and inflammation are accompanied with oxidative changes. In saline-injured and oxygen-ventilated rabbits, total antioxidant performance (TAP) in the lung tissue and plasma decreased, and oxidative DNA damage of the lung increased already 4 hours after induction of the model [42]. In a similar model, MDA as a marker of peroxidation of lipids, 3-nitrotyrosine as an indicator of oxidation of proteins, and levels of nitrites/nitrates as markers of nitrosative stress elevated in the lung tissue [38–41]. Histological investigation showed peribronchial edema, thickened alveolar–capillary barrier, destruction and desquamation of epithelial and endothelial cells, type I cell necrosis, and injury to basement membrane [43] as well as significantly increased apoptosis of lung epithelial cells verified by TUNEL methods and detection of caspase-3 already 4 h after induction of the model [38–41].

2.5.3 Model of ALI Induced by Neonatal Meconium

Meconium and meconium-stained amniotic fluid (MSAF) which can be aspirated due to intrauterine stress or during the labor contain many substances potentially toxic for the lung, such as bile acids, bilirubin, cholesterol, free fatty acids, pancreatic phospholipase A_2, etc. [44]. In animal models, intratracheal instillation of neonatal meconium suspension caused dose-dependent airway obstruction, drop in lung compliance, and alveolar atelectasis due to dysfunction of pulmonary surfactant, right-to-left pulmonary shunting, lung cell damage and death due to apoptosis and necrosis, and severe hypoxemia, hypercapnia, and acidosis [45–52]. In addition, meconium-induced, neutrophil-mediated inflammation was verified by massive PMN transmigration into the lung, increased pro-inflammatory cytokines in the lung homogenate and plasma, and increased protein content in BALF and wet–dry (W/D) lung weight ratio as markers of lung edema formation [52, 53]. Exposure to meconium also increased neutrophil-derived production of ROS, which in pigs correlated with meconium concentration [54]. In rabbits, meconium instillation triggered oxidative changes leading to significant peroxidation of lipids and proteins in the lung tissue and plasma, as confirmed by higher concentrations of TBARS, 3-nitrotyrosine, SH groups, dityrosine, and lipoperoxidation products and lower total antioxidant status (TAS) and activity of cytochrome c oxidase in the lung mitochondria [52, 53]. The experimental results have been recently confirmed by the results of the clinical study showing elevated concentrations of MDA and 8-OH-dG in the cord blood of full-term and late preterm newborns which were delivered

through MSAF than in the newborns not delivered through MSAF [55]. Meconium aspiration-associated changes can be also responsible for oxidative changes in distant organs, for example, in the hippocampus [56, 57].

2.5.4 Model of ALI Induced by Hyperoxia

Exposure to high oxygen concentrations for longer time can induce primary hyperoxic lung injury, or can exacerbate changes in the lung injured from other primary causes [58]. Under hyperoxia, excessive ROS are directly toxic for cells. In addition, ROS enhance pro-inflammatory pathways leading to dysfunction of alveolar–capillary membrane and influx of PMN into the alveolar spaces [58, 59].

Exposure to oxygen lasting 3–4 days triggered damage to type I cells, necrotic changes of endothelial cells, formation of interstitial and intra-alveolar edema, and increased platelet adhesion and PMN accumulation [60]. Exposure of rats and mice to 100% oxygen for 40–50 h resulted in lung deterioration, and exposure for 60–70 h caused death [24, 60]. Exposure of mice to 95% oxygen for more than 8 h caused DNA base damage in the isolated lung cells in comparison to room air [61]. In rats with 7-day exposure to hyperoxia (≥90% oxygen), weight gain slowed down, and lung tissue was severely injured as indicated by hematoxylin-eosin staining, higher W/D ratio, and higher protein and mRNA expression of HMGB1 and RAGE than in the control group [62].

2.5.5 Model of ALI Induced by Mechanical Ventilation

Mechanical ventilation is an important tool for support of critically ill patients with ARDS. However, inappropriate ventilation strategy aggravates the effects of a "first hit" to the lung due to alveolar overdistension caused by high inspiratory pressure of tidal volume at the end of inspiration ("volutrauma") or due to injury from repetitive closing and opening the alveolar units in insufficient value of positive end-expiratory pressure ("atelectrauma") [63]. Thus, this so-called ventilator-induced lung injury (VILI) produces additional lung damage [64, 65].

In animals, already 30 min of excessive ventilation led to detachment and death of endothelial cells and denudation of underlying basement membrane [66]. These changes were associated with increased microvascular permeability, formation of hyaline membranes, stimulated transmigration and activation of PMN, and increased synthesis of pro-inflammatory cytokines [67–69], particularly of TNFα [65].

While large-volume ventilation caused alveolar hemorrhage, hyaline membrane formation, neutrophilic infiltration, decline in lung compliance, and worsened gas exchange [70], small-volume ventilation mitigated inflammation and lung tissue damage [37]. Ventilation with high tidal volumes (HTV, 18 ml/kg) compared to low tidal volume ventilation (8 ml/kg) in rats increased oxidative stress in the lung expressed by elevated concentrations of methylguanidine and MDA in BALF and lower antioxidative activity expressed by protein expression of glutathione

peroxidase-1. In addition, HTV ventilation increased W/D ratio, leukocyte count in BALF; protein expression of pro-inflammatory markers, such as NF-κB, vascular cell adhesion molecule (VCAM)-1; and TNFα and matrix metalloproteinase (MMP)-9 and decreased expressions of specific surfactant proteins SP-A and SP-D in the pulmonary tissue [71].

2.5.6 Model of ALI Induced by Acid Aspiration

Intratracheal instillation of hydrochloric acid (HCl) within a short time caused surfactant dysfunction, increase in vascular permeability and intrapulmonary shunts, rapid deterioration of gas exchange, and neutrophil-mediated acute inflammatory response [72–74]. Acid-induced sequestration of PMN and increased W/D ratio and concentration of proteins in BALF were associated with overproduction of ROS and elastase, whereas IV administration of SOD and CAT prevented an edema formation, but did not reduce PMN sequestration [75]. Recruitment and activation of neutrophils correlated with high levels of serine proteases. However, the authors found proteases to be the primary mechanism of this injury, whereas administration of deferoxamine, causing chelation of iron necessary for production of hydroxyl radicals, or of catalase, promoting enzymatic breakdown of hydrogen peroxide, did not protect the lung against injury [76]. Due to abundant production of oxidants and serine proteinases degrading certain superoxide dismutases, the capacity of antioxidants declined [77, 78]. Acid aspiration also triggered generation of oxidized phospholipids in the lung, stimulating production of cytokines via TLR-4 receptor [79, 80]. Systemic inflammation can be additionally promoted by increased release of nucleosomes and histones [81]. As a result, acid aspiration models were associated with rapid increase in levels of TNFα, IL-8, IL-1β, IL-6, and IL-10, MIP-2, and eicosanoids and activation of complement [74, 82].

Nevertheless, in addition to HCl making low pH, gastric content consists of food particles, bacterial cell wall products, cytokines, etc. which may participate in aspiration-induced lung injury in humans [83–85]. Intratracheal instillation of both acid and small gastric particles evoked more evident lung injury as verified by finding of worsened surfactant dysfunction and oxygenation and elevated counts of erythrocytes, total leukocytes, and neutrophils and higher concentrations of total protein and albumin in BALF than in the group with instillation of only HCl or small nonacidified gastric particles [86].

2.5.7 Model of ALI Induced by Mustards

Sulfur mustard, nitrogen mustard, and their analogs are used as chemical warfare agents, targeting respiratory system [87]. Mustards as lipophilic agents rapidly penetrating the tissues and cells cause alkylation and cross-linking of cell structures, resulting in oxidative and nitrosative stress, DNA breaks, deterioration of cell function, and cell death by apoptosis or autophagy [87, 88].

Exposure to sulfur mustard in rats resulted in fast local damage, thickening of alveolar walls, increased transmigration of inflammatory cells into the lung, evidence of autophagy and apoptosis processes, elevated protein in BALF, and elevated expression of COX-2, TNFα, and iNOS in the alveoli and of MMP-9 in airway epithelium. In contrary, expressions of antioxidant heme oxygenase (HO)-1 and anti-inflammatory collectin SP-D lowered after exposure to sulfur mustard [89]. Exposure to nitrogen mustard altered lung mechanics, caused marked histological changes, increased protein content and cell counts in BALF, elevated expression of pro-inflammatory enzymes iNOS and COX-2, implicated in lung injury, and enhanced expression of substances controlling turnover of extracellular matrix [90]. Similarly, other authors reported higher levels of pro-inflammatory and profibrotic mediators including ROS and RNS, cytokines, and transforming growth factor (TGF)-β [88, 91, 92].

2.5.8 Model of ALI Induced by Halogen Inhalation

Exposures to halogens, chlorine or bromine, can appear due to accidental release in industry or use of chemical weapons [93]. Inhaled chlorine gas is extremely toxic and caused dyspnea, hypoxemia, airway obstruction, and pulmonary inflammation and edema and in severe cases led to ARDS and death of exposed patients [94]. Subacute effects include abnormal epithelial repair, mucus overproduction, airway obstruction and fibrosis, and worsened lung function [93, 95] as well as systemic vascular dysfunction and cardiac injury [96, 97]. Similar signs were observed in animals, where chlorine inhalation altered breathing pattern, caused hypoxemia, inflammation, mitochondrial damage, formation of lung edema, pulmonary hypertension, airway hyperresponsiveness, lipid peroxidation, surfactant dysfunction, and lung cell apoptosis or necrosis [93, 98, 99]. Mild hypoxemia, airway hyperresponsiveness, and deterioration of small airways verified histologically were observed 7 days after chlorine inhalation in rabbits [99].

Oxidative stress is the principal mechanism contributing to chlorine toxicity [93]. Inhaled chlorine firstly interacts with antioxidants in lung epithelial lining fluid. Chlorine in reaction with water generates hypochlorous acid and HCl, followed by oxidation of plasmalogens in the lung and surfactant. Products of these reactions (chlorinated lipids, 2-chloropalmitaldehyde, and 2-chlorostearaldehyde) can (a) stimulate neutrophils; (b) react with protein side chains, DNA, and lipids of the airway epithelial cells leading to injury; (c) inhibit Na^+-dependent alveolar fluid clearance that results into lung edema formation; or (d) can be further oxidized to intermediates triggering inflammation via stimulation of MAPK and NF-κB pathways [100, 101].

Bromine is less reactive than chlorine, but also induces airway hyperresponsiveness and ARDS. Similarly to chlorine, inhaled bromine reacts with antioxidants in the epithelial lining fluid. When the local antioxidant stores are depleted, bromine and hypobromous acid react with epithelial cell membranes to form reactive

brominated lipids causing disruption of airway epithelium, inflammation, and lung edema [93].

2.5.9 Model of ALI Induced by Phosgene Inhalation

Phosgene is an industrial gas generated as an intermediate in production of various chemicals: dyes, pesticides, plastics, polyurethanes, pharmaceuticals, etc. Inhaled phosgene causes the pulmonary damage and edema formation which are dependent on the time and intensity of exposure. These changes are the consequences of acylation and oxidant-mediated reactions resulting in protein and phospholipid dysfunction and generation of ROS and RNS [102].

In animal models, exposure to phosgene increased permeability of alveolar–capillary barrier, damaged type I epithelial cells [103], and altered energy metabolism [104], and expression of proteins contributed to glutathione redox cycle [105], increased lipid peroxidation [106], decreased levels of 3′-5′-cyclic adenosine monophosphate (cAMP)[107], enhanced production of leukotrienes (LT)[108], and stimulated release of endothelin (ET)-1 [109].

2.6 Evidence of Lung Damage and Oxidative Stress in Animal Models of "Indirect ALI"

2.6.1 Model Induced by Intravenous/Intraperitoneal Lipopolysaccharide (LPS)

An early phase after intravenous/intraperitoneal LPS is characterized by monophasic thermic response (LPS-induced fever), increased minute ventilation due to changes in breathing rate and tidal volume [110, 111], lower cardiac output and arterial pressure, and higher pulmonary artery pressure due to increased resistance in postcapillary veins [24], tachycardia [112], and changes in heart rate variability (an index of cardiac autonomic control), accompanied with increased IL-6 in the heart tissue [113], intravascular coagulation, and increased risk of death [24]. If LPS-exposed animal survives, the acute phase is followed by hemodynamic stabilization with microvascular injury and PMN lung sequestration, increased permeability, shunt fraction and pulmonary artery pressure, hypoxemia and higher alveolar–arterial oxygen differences, and intravascular thrombosis [24, 114].

In contrast to intratracheal LPS delivery, PMN infiltration into the lung, epithelial damage, and formation of hyaline membranes are relatively small [24]. Intravenous/intraperitoneal LPS triggers apoptotic changes of capillary endothelium preceding other tissue damage [115] and lung edema formation [110, 116]. In addition, leukopenia and release of various pro-inflammatory cytokines, adhesion molecules, and tissue factors can be detected [110, 112, 116–118] as well as changes in concentrations of surfactant proteins [111].

Intravenous/intraperitoneal administration of LPS also led to serious oxidative changes as indicated by higher levels of oxidants and lung MPO activity and lower levels of antioxidants [116, 118–121]. Similar changes have been found in patients with sepsis, too [122–124].

2.6.2 Model of Oleic Acid-Induced Lung Injury

Deleterious effects of intravenous oleic acid administration can be detected early after the administration, with maximum changes at 12 h [24]. There are several presumed mechanisms contributing to this type of the lung injury [125]. Oleic acid directly binds to biological membranes triggering intracellular pathways leading to lung cell death due to necrosis [126]. In addition, increased lung cell apoptosis is indicated by an increase in pro-apoptotic markers including caspase-3 and caspase-6 and decrease in antiapoptotic markers [125, 127]. Other presumed mechanisms participating in oleic acid-induced lung injury are a covalent binding of oleic acid to sodium channels and Na^+-K^+ ATPase in the epithelial cells leading to impairment of sodium transport and enhanced edema formation [128]. Furthermore, fatty acids participate in many processes, such as generation of lipid mediators, ROS and RNS, influencing activity of various intracellular signaling pathways, linking to TLR receptors, regulation of gene expression, activation of transcription factors, etc. [129].

2.7 Therapeutic Options

2.7.1 Antioxidants

Antioxidants represent rather heterogenous group of compounds that produce direct or indirect antioxidant effects. Directly acting antioxidants act as scavengers of ROS/RNS. As by-products, they can generate further reactive substances, for example, hydrogen peroxide is produced in dismutation of superoxide by SOD. Some agents can serve as cofactors or substrates for other endogenous antioxidants. For instance, a direct scavenger ***N-acetylcysteine*** (NAC) can act also as a source of cysteine for GSH synthesis. Thiol group containing antioxidants can interact with carbon-centered radicals. Effects of other substances, for example, polyphenolic compounds, are based on generation of a mild oxidative stress response, which can evoke an antioxidant response leading to suppressed oxidative stress [130, 131].

Administration of NAC successfully diminished oxidative and inflammatory changes in various forms of ALI. NAC reduced chlorine inhalation toxicity [132] and attenuated pulmonary edema, decreased markers of lung oxidative stress, and increased Nrf2 activation in phosgene-induced ALI models [133, 134]. In other models of chemical-induced injury, NAC preserved cell viability [135] and protected the cells from GSH depletion and lipid peroxidation [136, 137]. In sulfur mustard-induced lung injury, NAC improved survival, enhanced gas exchange,

decreased neutrophils and protein content in BALF, and decreased inflammatory markers [138–140]. In hyperoxia-exposed rats, nebulized NAC given once a day for 7 consecutive days decreased W/D ratio, alleviated lung histopathological changes, and reduced protein and mRNA expressions of HMGB1 and RAGE [62]. In meconium-induced lung injury in rabbits, intravenous NAC significantly decreased markers of lipid and protein oxidation and prevented a decrease in TAS [52]. Additional improvement in this model was found when intravenous NAC was delivered together with exogenous surfactant treatment [141, 142].

From other antioxidants, intravenous Cu/Zn ***SOD*** prevented impairment of the lung function induced in rats by ventilation with high tidal volumes (18 ml/kg) compared to low tidal volume ventilation (8 ml/kg), effectively reversed pulmonary oxidative stress and inflammation, preserved SP-A and SP-D expressions in the lung, and increased serum NO level enhancing NO bioavailability [71].

Glutathione, the most frequent thiol in cells of nonprotein structure, can have lower effectiveness compared to other antioxidants administered orally because of its limited gastric absorption and degradation [131]; however, it can reduce the oxidative damage [143].

Melatonin (N-acetyl-5-methoxytryptamine), a hormone regulating circadian rhythms, also acts as an antioxidant [144]. Melatonin treatment following nitrogen mustard injection restored oxidative and nitrosative stress indicators in the rat lungs to levels comparable with controls, probably due to iNOS inhibition [145]. Melatonin protected the cell culture models against chlorine and phosgene [146] and a rat phosphine-induced model from peroxidation of lipids, depletion of GSH, and DNA breaks [147].

Metal-containing catalytic antioxidants represent synthetic complexes mimicking the activity of endogenous antioxidant enzymes (SOD, CAT, etc.)[148]. For instance, catalytic antioxidant AEOL 10150 diminished oxidative lung damage in in vivo model of sulfur mustard analog inhalation [149] and protected from negative effects of chlorine as it was suggested by suppressed airway hyperresponsiveness, inflammation, and oxidative lung injury [150].

Other potentially beneficial antioxidants in ALI/ARDS are certain ***vitamins and their analogs***. Vitamin D delivered to hyperoxia-injured neonatal rats reduced upregulation of TLR4 by hyperoxia and alleviated lung deterioration by preventing the loss of integrity of the lung structures, decreasing deposition of compounds of extracellular matrix, and reducing inflammation and lung cell apoptosis [151, 152]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as a water-soluble analog of vitamin E enhanced survival and suppressed oxidative changes in models of sulfur mustard ALI [143, 153]. Atypical synthetic adamantyl retinoid ST1926 given to LPS-injured mice decreased cell counts in BALF and concentrations of IL-1β, IL-18, IL-6, and TNFα in serum and lung; suppressed NF-κB, inhibitor-κB, and IκB kinase α, as well as TLR4 receptor induced by LPS; and thereby suppressed ROS production [154].

Positive results were observed also for ***bioflavonoids***. For example, quercetin protected against sulfur mustard toxicity likely because of detoxication of intermediates and breaking of lipid peroxidation reaction [143, 153, 155]. Pretreatment

with quercetin 1 h before LPS challenge prevented interstitial edema and infiltration of PMN into the lung; attenuated elevation in the BALF total protein and count of neutrophils, W/D ratio, and levels of TNFα, IL-6, MDA, and MPO activity; and stimulated antioxidant activities of SOD, CAT, and glutathione peroxidase in LPS-challenged rats [156]. Other flavonoid fisetins decreased concentrations of lung MPO and expressions of IL-6, TNFα, IL-1β, MIP-1α, and MIP-2 and reduced upregulation in gene transcription of HO-1 and SOD2 in a mouse model of LPS-induced lung inflammation [157]. Anti-inflammatory and antioxidative effects in models of ALI have been recently published also for other compounds from medical plants, such as isovitexin [158] or vitexin [159]. Anti-inflammatory effects of resveratrol, a natural phytoalexin, in intratracheal LPS-induced ALI are probably related to suppression of oxidation, leukocyte activation, and generation of inflammatory mediators. Furthermore, resveratrol mitigated the LPS-induced histopathological changes, edema, and PMN infiltration, prevented an increase of IL-1β and IL-18 in BALF, inhibited expressions of NLRP3 and caspase-1 mRNA, and suppressed activation of NLRP3 inflammasome in the lung and nuclear translocation of NF-κB p65 and reduced activity of NF-κB and generation of ROS [160]. Similarly, resveratrol improved lung histopathological changes during endotoxemia, decreased prooxidant biomarkers MDA and H_2O_2, increased activity of antioxidants (GSH/GSSG ratio, TAC, CAT, and SOD), and suppressed expression of iNOS, NO, and peroxynitrite in intraperitoneal LPS-induced ALI [161].

Other antioxidants, for example, ***caffeic acid phenethyl ester (CAPE)***, reduced phosgene-evoked elevation of MDA and SOD activity, reversed the decline of GSH concentrations in BALF and lung tissue, and partially inhibited nuclear translocation of NF-κB p65 [162].

2.7.2 Glucocorticoids

Glucocorticoids (GCs) act via both genomic and nongenomic mechanisms. The genomic mechanisms are mediated by interaction with cytosolic glucocorticoid receptor (cGCR). After passing through cell membrane, GCs link to ligand-binding domain of cGCR. The glucocorticoid–cGCR complex translocates to nucleus and binds to DNA-binding sites or glucocorticoid response elements (GRE). Linking to GRE^+, the glucocorticoid–cGCR complex activates transcription of anti-inflammatory proteins (IL-10, annexin 1, inhibitor of NF-κB, etc.) and other proteins regulating metabolism ("transactivation") [163, 164]. Binding to GRE^-, the glucocorticoid–cGCR complex inhibits transcription of NF-κB and AP-1 which leads to suppression of synthesis of pro-inflammatory cytokines (IL-1, TNFα, interferon (IFN)γ, etc.) [165], expression of iNOS and production of NO [166], etc. ("transrepression").

The nongenomic mechanisms of GCs act through specific membrane-associated and cytosol-associated receptors and second messengers [164]. These effects are, for example, involved in rapid T-cell immunosuppressive action [167], decreasing

arachidonic acid production [168], and activation of endothelial NO synthase leading to NO-dependent vasorelaxation [163].

Treatment with GCs in ALI/ARDS led to controversial results according to the type of injury. In intravenous endotoxin-induced ALI model, intratracheal ***budesonide*** enhanced lung compliance and PaO_2/FiO_2; decreased W/D ratio, protein content, concentration of neutrophil elastase, and leukocyte and neutrophil counts in BALF, lowered TNFα, IL-1β, and IL-8; and increased IL-10 in BALF, reduced lung injury, and improved survival [169]. Similar improvement was found in ALI model induced by large-volume ventilation, where intratracheal budesonide besides the abovementioned benefits decreased neutrophil elastase level, intercellular adhesion molecule (ICAM)-1, and MIP-2 and increased IL-10 in BALF and plasma, decreased phosphorylated NF-kB lung levels, and reduced lung histological changes and apoptosis [170]. Intratracheal budesonide also ameliorated combined VILI+endotoxin-induced ALI model which enhanced oxygenation, ameliorated inflammation, reduced lung histopathological changes and apoptosis, and enhanced survival [171]. Potent anti-inflammatory and antioxidant effects of intravenous ***dexamethasone*** [172] and intratracheal budesonide [47] were observed also in meconium-induced ALI, where these agents significantly decreased markers of both protein and lipid peroxidation in the lung. Positive impact of budesonide was enhanced when administered intratracheally together with exogenous surfactant [51, 53, 173]. Intratracheal budesonide alleviated inflammation and lung cell apoptosis also in saline lavage-induced ALI [39]. In chlorine-induced ALI, high-dose dexamethasone reduced acute inflammation and airway hyperresponsiveness [174], ***mometasone*** and budesonide showed a dose-dependent inhibition of neutrophil influx into the lung [175], and aerosolized budesonide improved lung functions [176]. In acid-injured lung, dexamethasone reduced neutrophil recruitment, edema formation, and oxygenation [177, 178]. In contrary, in ALI caused by phosgene inhalation, no benefit of GCs in the acute phase of injury was found, and, in the recovery phase, their effect was even harmful [179–182].

Administration of GCs in patients with ARDS is still discussed [183]. ***Methylprednisolone*** given in early ARDS improved lung injury score, shortened the use of mechanical ventilation, enhanced survival, increased protein C levels, and decreased plasma IL-6 in patients with direct ARDS [184]. Recent meta-analysis of low-to-moderate dose of prolonged administration of GCs showed that GC delivery diminishes the time necessary for ventilation, extubation, and discharge from hospital and reduces mortality without obvious side effects. Therefore, the use of methylprednisolone in early moderate-to-severe ARDS (daily dose of 1 mg/kg) and late persistent ARDS (daily dose of 2 mg/kg) has been suggested [185]. Promising results were observed also in pediatric ARDS [186–188]. Methylprednisolone given as a loading dose of 2 mg/kg followed by a daily dose of 1mg/kg in infusion from days 1 to 7 decreased levels of IFN-α, IL-6, and IL-10, monocyte chemoattractant protein (MCP)-1, granulocyte-colony stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) and increased total leukocytes and platelet counts and IL-17α levels on day 7 [187]. On day 7, methylprednisolone reduced MMP-8 (indicating reduced activation of neutrophils), prevented increase

in soluble ICAM-1 (indicating decreased endothelial injury), and decreased soluble RAGE (indicating epithelial injury and recovery) which correlated well with respiratory functions [188]. On the other hand, high-dose GCs given in patients with ARDS within 7 days of admission increased mortality rates within 3 months [189]. Similarly, no benefit was observed for methylprednisolone in persistent ARDS or in starting methylprednisolone therapy later than 2 weeks after appearance of the first signs of ARDS [190, 191].

2.7.3 Phosphodiesterase Inhibitors

Phosphodisterase (PDE) inhibitors are enzymes hydrolyzing cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Each type of cells can produce different PDE isoforms; therefore, PDE inhibitors regulating the concentrations of cAMP and cGMP can modulate the function of various cells and organs. Because in the lung the PDE isoforms PDE3, PDE4, and PDE5 are produced at high concentrations, selective PDE3, PDE4, and PDE5 inhibitors and nonselective PDE inhibitors can be of benefit [192].

In isolated perfused rabbit lung model of phosgene exposure, administration of nonselective PDE inhibitor ***aminophylline*** mitigated peroxidation of lipids and lowered concentration of leukotrienes C(4)/D(4)/E(4) in perfusate, reduced lung weight gain, and prevented a decline in cAMP level in the lung tissue [133]. In meconium-induced ALI, intravenous aminophylline in higher dose (2 mg/kg) and lower dose (1 mg/kg) reduced inflammation, lung edema, and protein and lipid oxidative changes in the lung [193, 194]. Other nonselective PDE inhibitors, for example, ***pentoxifylline***, reduced signs of lung damage and inflammatory and oxidative changes in nitrogen mustard-injured rats, as verified by decreased indicators of oxidation lipocalin (Lcn)2 and HO-1 [195]. Pentoxifylline improved lung functions and survival also in rats with acid-induced ALI [196].

Positive effects were observed with selective PDE inhibitors, too. PDE3 inhibitor ***olprinone*** decreased markers of oxidation of lipids and proteins in the lung tissue, mitochondria, and blood plasma in rabbits with meconium-induced ALI [197]. PDE4 inhibitor ***rolipram*** inhibited degradation of cAMP alleviating lung edema, inflammation, and AHR in chlorine-induced ALI [198]. Other PDE4 inhibitor ***roflumilast*** effectively reduced inflammation, lung edema formation, oxidative changes, and lung cell apoptosis and improved respiratory parameters in saline lavage-induced ALI [199]. In phosgene-induced model of ALI, administration of selective PDE4 inhibitor ***CP-80633*** and PDE5 inhibitor ***sildenafil*** improved survival in mice [102]. In our recent study, PDE5 inhibitor sildenafil reduced leak of leukocytes (especially of neutrophils) into the alveoli; decreased pro-inflammatory cytokines (TNFα, IL-8 and IL-6), markers of oxidative damage (3-nitrotyrosine, MDA, and nitrites/nitrates), lung edema formation, and apoptosis of lung epithelial cells; and improved respiratory parameters in rabbits with surfactant depletion ALI model [41].

2.7.4 Inhaled NO and NO Donors

Despite that ***inhaled nitric oxide*** (iNO) can provide some benefits in ALI/ARDS as a potent vasodilator, participation of nitric oxide (NO) in pulmonary inflammatory response is controversial. NO modulates inflammation and regulates the immune responses by multiple ways. Low amounts of NO which are in normal conditions generated particularly by endothelial and neuronal NO synthases suppress expression of adhesion molecules, production of cytokines and chemokines, and adhesion and transmigration of leukocyte into the tissues. However, high concentrations of NO produced by iNOS, for example, in inflammation, can be deleterious and pro-inflammatory. Thus, effects of NO depend not only on the enzymatic source but especially on the cell context, NO level, and initial priming of inflammatory cells [200, 201].

Inhaled NO can react with superoxide and may form toxic compounds in the lung including peroxynitrite, which damages DNA, leads to lipid peroxidation and protein changes, and can stimulate a release of inflammatory mediators. In ARDS patients treated with iNO, increased concentrations of 3-nitrotyrosine, considered for marker of peroxynitrite production, and 3-chlorotyrosine, considered for marker of activation of neutrophils, were found in BALF [202]. In contrary, in porcine model of sepsis, iNO prevented an increase in protein content and neutrophil count in BALF [203]. iNO also positively influenced inflammatory markers in BALF of ARDS patients where iNO mitigated generation of hydrogen peroxide, decreased expression of β_2 integrin CD11b/CD18 in neutrophils, and lowered IL-8 and IL-6 [204]. In more recent studies, iNO mitigated DNA damage, histopathological lung score, and number of PMN in BALF and elevated TAP in the lung, but increased also plasma nitrites/nitrates in rabbits with surfactant depletion [205, 206].

In a similar model of ALI induced by saline lung lavage, intratracheal delivery of soluble NO donor ***S-nitroso-N-acetyl-penicillamine (SNAP)*** decreased TBARS and expression of iNOS in the lung and lowered plasma levels of nitrite/nitrate. Decrease in markers of oxidative stress was associated with decreased transmigration of neutrophils into the lung, decreased production of pro-inflammatory cytokines, and indicators of epithelial and endothelial damage and apoptosis of lung epithelial cells [38]. Nitric oxide-generating agents, such as ***sodium nitrite***, resulted in lower protein content in BALF, reduced lung cell apoptosis, restoration of normal lung W/D ratios, and improved survival [207, 208].

Additional benefits for iNO in ARDS patients have been recently published also regarding a posttreatment periodin which the ARDS survivors may suffer from long-term obstructive or restrictive pulmonary dysfunction. The ARDS patients who were treated with iNO given in low dose showed an improvement in selected pulmonary function tests at 6 months after the treatment than placebo-treated patients [209]. Similarly in pediatric ARDS, positive response to iNO was associated with fewer ventilator days [210].

2.7.5 Other Promising Pharmacological Approaches

In ALI/ARDS, molecular pathways including generation of ROS by NADPH oxidases and induction of phosphorylation of MAPK signal transduction pathways can also serve as therapeutic targets [58, 59]. In phosgene-induced model, ***specific inhibitors of MAPK*** exerted potent modulatory effect on the expression of pro-inflammatory enzymes COX-2 and iNOS which resulted in diminished lung edema, which was likely mediated by inhibition of MAPK activation and of lower generation of NO and prostaglandin E(2) [211]. In LPS-exposed A549 cells, synthetic ***N-methyl-d-aspartate receptor antagonist MN-08*** decreased the levels of TNFα, IL-1β, COX-2, iNOS, and ROS, upregulated expression of HO-1, and inhibited cell apoptosis. In ALI model evoked by intratracheal LPS, MN-08 lowered cells in BALF, attenuated lung edema, and reduced glutamate, MPO, and MDA, while it increased SOD and GSH activities and blocked MAPKs/nuclear translocation of NF-κB signaling pathways [33].

Promising results were also found on influencing iNOS, enzyme mediating production of RNS by macrophages. ***Blocker of iNOS aminoguanidine*** reduced changes in nitrogen mustard-injured lung and suppressed oxidative stress and inflammation [87].

Anticoagulants and ***antithrombotics*** exhibiting potent anti-inflammatory effects could also be beneficial. For instance, ***heparin*** reduced disturbance in pulmonary coagulopathy and inflammation in chlorine-induced ALI [212, 213], and ***activated protein C*** decreased pro-inflammatory cytokines and lung edema formation in HCl-induced ALI [214].

Effective in reduction of oxidative stress and inflammation was also ***lidocaine***. In rabbits with HCl-induced ALI, lidocaine attenuated HCl-induced increase in superoxide production by neutrophils and improved PaO_2 and lung mechanics [215].

Some improvements were found for ***dexmedetomidine***, ***a specific agonist of α2-adrenoreceptors***. In neonatal rat model of hyperoxia-induced ALI, dexmedetomidine treatment attenuated lung injury by decreasing W/D ratio, mitigating oxidative and inflammatory changes, and reducing lung cell apoptosis [216].

Positive effects were observed after administration of ***β-adrenoreceptor agonists***, too. Nonselective β-agonist ***isoproterenol*** lowered pulmonary vasoconstriction, decreased requirement for tracheal pressure, reduced lung weight gain, diminished leukotriene C(4)/D(4)/E(4)-mediated permeability of lung capillaries, and kept the GSH redox states in the lung tissue of the phosgene-induced ALI [133]. Nebulized short-acting β2-agonist ***salbutamol*** reduced neutrophil influx into the lung but did not improve survival in pigs after phosgene exposure [217]. Long-acting β2-agonist ***arformoterol*** mitigated chlorine toxicity on airway reactivity and alveolar fluid clearance by increasing lung cAMP [218].

Positive effects on survival rates in phosgene-injured mice showed also ***inhibitors of GABA transaminase*** (e.g., valproic acid, vigabatrin) and ***anticholinergic/antiserotonergic compounds*** (e.g., cyproheptadine) indicating participation of

neurogenic signaling mechanisms, as well as administration of nonselective ***TRPA antagonist*** (RR) and ***TRPA1 inhibitor*** (HC-030031) confirming a role of TRP channels in airway irritation and inflammation [102]. ***Inhibitor of TRP vanilloid***, an ion channel expressed in lung endothelial cells, reduced vascular permeability and airway hyperreactivity and enhanced gas exchange in mice with chlorine exposure [219].

Nonsteroidal anti-inflammatory drug ***ibuprofen*** can improve some markers of ALI, as well. For instance, in phosgene-injured animals, ibuprofen increased the survival reducing lipid peroxidation and GSH depletion [133] and reduced lung edema production [220].

Favorable effects were observed also for ***inhibitors of cytokines and other inflammatory mediators***. In nitrogen mustard-injured rats, ***anti-TNFα antibody*** reduced lung histopathologic alterations and protein and cell contents in BALF, decreased expression of oxidative stress marker HO-1, and suppressed collection of M1 macrophages with pro-inflammatory and cytotoxic effects in the lung and reduced profibrotic TGF-β [221]. In acid-injured rats, ***IL-8 antibody*** reduced lung edema formation and neutrophil transmigration, decreased IL-8, and improved oxygenation [222], ***inhibitor of complement pathway*** ameliorated increase in TNFα and subsequent neutrophil lung sequestration [223], and ***monoclonal antibody to adhesion molecule anti-CD18*** inhibited binding of neutrophils to the endothelium [224].

Another promising approach is ***sirtuin 3*** (SIRT3). This mitochondria-specific protein is necessary for deacetylation of metabolic enzymes and oxidative phosphorylation. The recent study has shown that overexpression of SIRT3 may reduce oxidative lung damage and edema [225].

Serine protease inhibitor ulinastatin decreased synthesis of pro-inflammatory cytokines via regulation of MAPK/NF-κB signaling pathway in intratracheal LPS-induced ALI [226]. Decrease in BALF cells and concentrations of IL-15 and ICAM-1 in blood serum and improved lung structure after ulinastatin were also found in phosgene-induced model of ALI [227].

Pretreatment with ***nilotinib, a tyrosine kinase inhibitor,*** attenuated lung edema, histopathological marks of lung injury, and accumulation of cells in BALF, increased SOD and GSH activities, decreased MDA and nitrites/nitrates, and decreased lung levels of TNFα, TGF-β1, and iNOS in rats with aerosolized LPS-induced model [228].

2.7.6 Lung-Protective Ventilation Strategies

Ventilation with high tidal volumes leads to more severe lung changes than ventilation with smaller volumes. Therefore, current guidelines suggest lung-protective ventilation modes, such as small-volume conventional mechanical ventilation (CMV) with small tidal volumes (VT) (<6 ml/kg body weight) or high-frequency

oscillatory ventilation (HFOV), using limited airway pressure (<30 cm H_2O) and appropriate value of positive end-expiratory pressure [229, 230].

Lung-protective ventilation can reduce oxidative changes which are dependent on the extent and severity of lung injury. CMV with VT of 6 or 8 ml/kg in a pig model of phosgene-induced ALI improved oxygenation, decreased shunt fraction and mortality, and reduced hemorrhage, neutrophilic infiltration, and intra-alveolar edema compared to CMV with VT of 10 ml/kg [231]. Similarly, in gastric juice-induced ALI, MPO activity, lung injury score, and edema formation were lower in three types of lung-protective ventilation compared to aggressive high-volume CMV [232]. In saline lavage-induced ALI, lung-protective strategies (low tidal volume CMV and HFOV) attenuated lipid peroxidation expressed by MDA and other markers of inflammation and lung injury, protein content, leukocytes, elastase, and TNFα in tracheal fluid [35]. TAP in the lung and plasma of saline lavage-injured rabbits elevated, and oxidative DNA damage declined in animals ventilated with HFOV compared to CMV [42]. In rabbits with meconium-induced ALI, plasma levels of TBARS gradually increased in both CMV- and HFOV-ventilated animals during experiment; however, no between-group differences in the plasma or lung TBARS were found [233].

2.8 Conclusions

Oxidative changes represent a key mechanism in the acute lung damage. Although there are no pharmacological approaches generally approved for the treatment of ALI/ARDS, there are several groups of medicaments which can mitigate neutrophil transmigration, alleviate damage to alveolar–capillary barrier, and reduce generation of ROS/RNS and pro-inflammatory cytokines, proteases, etc. However, positive as well as potentially adverse effects of these agents should be carefully studied in various animal models of ALI before they could be recommended for administration in patients with ARDS.

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3 Potential of Mesenchymal Stem Cells in Modulating Oxidative Stress in Management of Lung Diseases

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Abstract

The current therapies for oxidative stress-induced lung diseases are majorly based on the reduction of airway obstruction and improved exacerbations. However, none of the available treatments have been proven to avoid disease progression or reduce mortality. In this context, mesenchymal stem cell (MSC) therapy has become a strong contender for better therapeutic strategies for several pulmonary diseases. MSCs can be readily harvested from various tissues and efficiently propagated and possess strong immunomodulatory/suppressive properties. Animal studies have shown encouraging outcomes with MSC therapy for various lung disorders, like COPD and emphysema. These studies have inspired research groups to understand the mechanisms by which MSCs may bring about their beneficial effects upon transplantation; however, clinical trials have not been as successful. This chapter summarizes and highlights the various aspects of MSC therapy in cellular, preclinical, and clinical settings.

Keywords

Mesenchymal stromal cells · Asthma · COPD · Emphysema · Fibrosis

3.1 Introduction

The adult human lung has a surface area of 35–100 mm^2, depending on the lung capacity, and is continuously involved in the efficient exchange of gases for oxidative metabolism [1]. The lungs are exposed to a variety of chemicals, toxic gases, smoke, pollutants, airborne toxins and microorganisms. It is widely recognized that

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an altered balance between oxidant–antioxidant levels leads to an excessive accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the lungs [2]. A review article [3] in this context highlights how oxidative stress is implicated in the generation and progression of lung diseases. Lungs are continuously exposed to exogenous environmental pollutants in the air, for example cigarette smoke (CS), dust, smoke and ozone and ROS from xenobiotic compounds. The primary function of the lung is the exchange of gases across alveoli and capillaries. Adult human lungs exchange 10 k–20 k litres of air daily. Endogenous lung stem cells and progenitor cells are regenerative populations that are essential for the maintenance of cells and injury repair. These are the facultative progenitor cells – basal, Clara cells, Clara-like cells, pulmonary neuroendocrine cells and type II alveolar epithelial cells. Asthma is a chronic inflammatory airway disease, in which recruitment of inflammatory cells and excessive ROS production have been found in the airway of asthmatic patients. Airway inflammation-associated oxidative stress in asthma also induces oxidative modification of proteins and lipids in the airway. High levels of ROS lead to breakdown of antioxidant defences like changes in superoxide dismutase (SOD), catalase activity and reduced glutathione, leading to their deficiency and inactivation. Lung diseases like asthma, chronic obstructive pulmonary disorder (COPD) and pulmonary fibrosis (PF) are often induced by oxidative stress and are leading causes of morbidity and mortality. The major risk factors for these diseases are varied; however, even after the cessation of the factors, oxidative stress often persists and contributes to disease progression. Emphysema, an oxidative stress-induced lung disorder, is defined pathologically by airspace enlargement and destruction of alveolar septa. Important contributing factors to the pathobiology of emphysema are imbalances between proteases–antiproteases and oxidants–antioxidants. Inflammatory cells are recruited to the alveoli where they release elastases, cytokines and oxidants – cigarette smoke inhalation contributes to pathogenesis of emphysema. Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, irreversible lower respiratory disease characterized by diffuse alveolar inflammation and structural disorder, leading to pulmonary interstitial fibrosis [4, 5].

Treatments which are currently available for asthma and COPD are based on personalized estimation of symptoms and future risk of exacerbations. Available pharmacological approaches involve anti-inflammatory drugs like corticosteroids, bronchodilators and theophylline. These only help in minimizing acute exacerbations and airflow limitation, and, hence, improving the quality of life of patients with COPD [6]. There is no definitive therapy available that prevents disease progression or reduces mortality. Evidence suggests that antioxidant therapies have failed to improve therapeutic outcomes in oxidative stress-induced lung diseases. Various drawbacks like inadequate doses and short lives of antioxidants, unpredictable absorption of enteral antioxidants, difficulty in targeting a specific vulnerable tissue compartment, inability to deliver antioxidants during a viable therapeutic window [3], etc. have limited the effectiveness of antioxidant therapy. Thus, it is important to strategize newer therapies for lung diseases.

In this chapter, we focus on efficacy and safety of MSC therapy in experimental models of COPD, pulmonary emphysema and asthma of preclinical models and clinical trials that have brought up hopeful outcomes as well as exposed certain lacunae. We also highlight the main mechanisms through which MSCs exert their beneficial effects upon transplantation.

3.2 MSC Therapy in Lung Diseases

MSCs are multipotent progenitor cells that have been widely used in respiratory diseases as strong candidates for treatment of destructive disorders like COPD and emphysema due to their tissue repair abilities and immunosuppressive and anti-inflammatory properties. MSCs may be harvested from several adult tissues like the bone marrow, dental pulp, adipose tissue, lungs and foetal tissues, such as the umbilical cord, amniotic fluid, human tubal tissue, placenta etc. [6].

Various criteria need to be taken into account while choosing the optimum source for MSC transplantation:

1. *Tissue source* – Bone marrow is the most widely used source for MSC-based transplantation in lung diseases. MSCs behave differently depending on their tissue of origin, resulting in differences in tissue repair abilities [7, 8], immunogenicity and anti-inflammatory properties. These may impact the beneficial effects of MSCs in vivo. Thus, other MSC sources are also being considered and used increasingly these days. While all sources exhibit similar benefits in an experimental model of elastase emphysema, in some parameters like macrophage polarization to a more anti-inflammatory phenotype, BM-MSCs were found to be more effective in an experimental emphysema model [6].
2. *Dosage* – A broad range of MSC doses (e.g. 10^4–$6*10^6$ cells) has been used to treat lung disorders in mice under research settings. This has been particularly well-studied in case of emphysema, wherein in one such study, the authors suggested $5*10^4$ as the optimal dose using human umbilical cord blood-derived MSCs. The efficacy is usually measured in terms of their effect on the mean linear intercept. However, there were limitations of this study as the variations among the tested doses were small [6].
3. *Route of administration* – Two main ways of delivery have been tested for MSC administration – systemic (intravenous and intraperitoneal) and local (intrapleural, intratracheal, intranasal and intrabronchial). Generally in translational research, MSC delivery should be least invasive and with less contamination risks; thus, systemic delivery is considered better. However, for sufficient number of MSCs to reach the lungs and the extrapulmonary compartments in severe cases, very large quantities of MSCs will need to be administered, which is difficult to model in small animals, and the results may not be reproducible. Thus, local administration is often used in disease models nowadays. Evidence suggests that while intravenous administration is more effective in achieving beneficial immunomodulatory effects like production of vascular endothelial growth

factor (VEGF), macrophage polarization and endothelial cell proliferation, local administration confers more reparative benefits like reduction in lung hyperinflation and fibrosis in a chronic model of elastase-induced emphysema [6].

4. *Number of administrations* – Single or multiple administrations have been found to be more effective depending upon the degree of disease progression and model system [6].

3.2.1 Chronic Obstructive Pulmonary Disease (COPD) and Emphysema

Oxidants which contribute to the pathogenesis of COPD may originate endogenously (by metabolic reactions) or exogenously (i.e. through cigarette smoke). CS imposes oxidative stress which further activates oxidants released by inflammatory cells endogenously. Under normal conditions, a robust oxidant–antioxidant defence system is in place to maintain a balance. However, exposure to CS results in an excess of oxidants, thus creating oxidative stress, and is a pathogenic mechanism of COPD. Lung inflammation is enhanced via redox-sensitive inflammatory transcription factors, such as NFKB and activating protein-1, and subsequently by activating their downstream transcriptional pathways. Oxidative stress also increases neutrophil sequestration in the lung and enhances lung inflammation. Oxidative stress is also linked to stimulation of mucus release by airway epithelial cells and impaired mucociliary clearance.

Modulation of redox environment by MSCs is an emerging area of interest. Transplantation of MSCs in LPS-induced acute lung injury (ALI) rats has been shown to be accompanied by decreased oxidative stress. Malondialdehyde levels are found to be reduced in the lungs, along with increased synthesis of heme oxygenase-1 (enzyme that has strong antioxidative and cytoprotective effects). Transplantation of BM-MSCs has been shown to reduce oxidative stress in the brain of a rat model of spontaneous stroke, suggesting that this may be well the case in CS-induced emphysema.

Transplanted MSCs also differentiate into alveolar cells, thus benefitting the COPD mice. Differentiation of MSCs into type II alveolar epithelial cells has been shown to activate canonical Wnt signalling pathway [9]. A gene profiling study followed the expression profile in mouse lung post treatment of umbilical cord-MSCs in a 6-month-long cigarette smoke-induced emphysema model over time. Molecular level changes were observed in genes involved with oxidative stress, immune responses and transcription, soon after transplantation. MSCs display immunomodulatory action and interact with the immune system cells like the macrophages, rather than directly interacting with the lung. MSCs may induce macrophage polarization towards an anti-inflammatory phenotype (M2) while inhibiting pro-inflammatory phenotype (M1). MSCs have also been shown to interact with alveolar macrophages to promote their reprogramming via the cyclooxygenase 2/prostaglandin E2 pathway [6]. Li et al. [9] showed that induced pluripotent cell-derived MSCs

when co-cultured with CS-induced airway smooth muscle cells rescued mitochondrial membrane potential loss, cellular apoptosis and attenuated mitochondrial ROS (mtROS) in the latter. MSC-derived conditioned medium had the same effect on mtROS but not on membrane potential or apoptosis, indicating that paracrine effects did not affect these parameters. Mitochondrial transfer was seen in co-culture which was enhanced upon CS exposure. iPSC-MSCs had the same effect on airway hyperresponsiveness, ozone-induced mitochondrial dysfunction and inflammation mouse lungs under in vivo conditions.

Intravenous infusion of MSCs was found to suppress C-reactive protein levels in a clinical trial of COPD patients. MSCs were found to inhibit alveolar apoptosis through changes in expression of apoptotic and/or anti-apoptotic proteins. The apoptotic gene Bax and anti-apoptotic gene Bcl-2 were induced and repressed, respectively, upon administration of MSCs in a papain-induced model of emphysema in rats [11]. Another mechanism to ameliorate alveolar apoptosis could be through suppression of cleavage of caspase-3.

Several studies have evaluated the therapeutic effect of MSCs on COPD and acute respiratory distress syndrome (ARDS) through their immunomodulatory and anti-inflammatory effects. MSCs can migrate to tissue injury sites, and their immunosuppressive properties are useful for successful autologous and heterologous transplants. MSC administration to the lung in LPS-induced mouse ALI was shown to reduce pro-inflammatory factors like tumor necrosis factor α (TNF) and macrophage inflammatory protein (MIP-2) in the broncheolar lavage fluid (BALF) and plasma, and elevate the anti-inflammatory molecule IL-10. Increased survival rate of rats suffering from LPS-induced lung injury was observed upon injection of umbilical cord-MSCs. Pulmonary and systemic inflammation was also reduced to a significant extent along with reduced lung edema, lung wet–dry ratio, neutrophil counts, myeloperoxidase activity and protein concentration. In *E. coli*-induced ALI, intratracheal administration of MSCs led to higher survival of mice, and lung injury was reduced due to lower levels of pro-inflammatory molecules like IL-1β, IL-1 α, IL-6, TNFα and MIP-2. Myeloperoxidase (MPO) activity as well as lung water content were also reduced.

Mouse MSCs have been shown to ameliorate changes associated with emphysema. Destruction in elastase-induced emphysema model was seen through upregulation of hepatocyte growth factor, epithelial growth factor and secretory leukocyte protease inhibitor in the lung. Administration of MSCs was shown to revive emphysema and CS-mediated destruction through the decrease of pro-inflammatory mediators like TNFα, IL-1β, MCP-1 and IL-6. Protease expression like MMP9 and MMP12 were decreased, and VEGF, VEGF receptor 2 and TGFβ were found to be increased, thus reducing lung cell apoptosis [6].

Direct MSC administration to treat CS-induced emphysema model of COPD in rats reduced the mean pulmonary arterial pressure and cell apoptosis. In papain-induced pulmonary emphysema in rats, the protective effects of MSC transplantation were found to be partially mediated by upregulation of VEGF-A expression and inhibition of cell apoptosis [11].

3.2.2 Pulmonary Fibrosis (PF)

Ortiz et al. [12] reported that BM-MSC injection (0.5 lakh cells/mouse) through the jugular vein immediately after challenge with bleomycin (BLM) was found to significantly reduce pulmonary fibrosis. In silicon dioxide-induced PF mice model, hMSCs directly replaced fibrotic cells with normal lung cells and reduced PF symptoms like inflammation and collagen deposition. In a separate study, BM-MSCs were found to significantly reduce BLM-induced lung fibrosis by the downregulation of pro-inflammatory molecules, nitric oxide metabolites and angiogenic cytokines after 4 days of injection. Zhao et al. [13] showed that BLM-induced rats received protective effects from lung damage post BM-MSC engraftment. Combinatorial treatment of cyclophosphamide along with BM-MSCs was also found to protect mice from BLM-induced lung fibrosis. Data from MSC-based clinical trials were found to support the safety of single infusion in IPF patients. BM-MSCs were found to home to injured lungs after damage, exhibited epithelioid phenotype and reduced collagen deposition and inflammation in BLM-induced mice models. BM-MSCs were also found to migrate to airway epithelial cells in a 3D direct-contact wound repair model. These seem to be mediated by certain chemotactic factors and their receptors [10]. Stromal cell derived factor (SDF-1) is one such chemokine that has been shown to be crucial for migration via receptor CXCR-4. SDF-1 was found to promote the chemotactic migration of BM-MSCs. This effect was also seen in mice BLM lung extracts and inhibited by CXCR-4 antagonist (TN14003). SDF and CXCR-4 were found to be increased in IPF lungs compared to normal lungs. Concentration of SDF-1 in serum and BALF and expression level of CXCR-4 were found to be increased in BLM-induced animal models. SDF-1α mRNA levels in lungs were increased significantly compared to control groups, measured on both days 7 and 14. SDF-1 was also elevated in idiopathic interstitial pneumonia in patient lungs. Chemokine CXCL8 (interleukin 8) was also found to promote migration of hMSCs.

It has been often observed that MSCs show low engraftment and differentiation after administration, even though some beneficial effects are seen. This is believed to be largely due to the action of paracrine factors and immune adjustment. MSC-derived conditioned medium has been seen to exert protective effects in BLM-induced model. MSC-CM decreased pulmonary inflammation, fibrosis, collagen deposition and cell apoptosis. A549, alveolar epithelial cancer cells, were seen to be protected from cell apoptosis through MSC-mediated paracrine action. MSCs secrete a range of molecules like growth factors, chemokines and cytokines which regulate local immune responses that inhibit inflammation. Interestingly, MSCs pretreated with hypoxia had better healing effect in BLM-induced PF mice.

MSCs elicit their beneficial effects via reducing the expression of tissue inhibitor of metalloproteinase-1, matrix metalloproteinase MMP9, γ-interferon and TGF-β to reduce lung inflammation and fibrosis. MSCs have also been shown to increase gene expression levels of γ-glutamylcysteine synthetase, NADPH quinine oxidoreductase 1, nuclear factor erythroid 2-related factor 2 and heme oxygenase-1 [10].

3.3 Mechanisms Through Which MSCs Alleviate Oxidative Stress

3.3.1 Mitochondrial Transfer

Mesenchymal stem cells are known to transfer healthy mitochondria to stressed cells through tunnel nanotube formation. Islam et al. [14] showed that BM-MSCs transfer the mitochondria to pulmonary alveoli, thus protecting mice from LPS-induced ALI. MSCs were found to contribute to mitochondrial transfer through connexin 43, nanotube and microvesicles in a calcium-dependent manner. This was shown to rescue mitochondrial bioenergetics in the recipient cells. The attachment of MSCs to alveolar epithelium in ALI was found to be crucial for mitochondrial transfer from MSCs. Ahmad et al. [15] showed that MSCs transferred healhty mitochondria to injured cells in both ovalbumin and cockroach allergen mouse models and significantly alleviated symptoms. This was also shown in vitro in rotenone-induced lung injury model. BM-MSCs were also found to mitigate oxidative damage inflicted by CS-induced COPD mice. MSC-transferred mitochondria reduced inflammation, thus promoting rescue [16]. Chuang et al. [17] showed that WJ-MSCs are able to rescue cybrid cells from myoclonic epilepsy patients through transfer of healthy mitochondria thus improving mitochondrial bioenergetics. Paliwal et al. [7] have shown that MSCs from different sources like AD, BM, WJ and DP showed differential reduction of mtROS through mitochondrial transfer by nanotube formation.

3.3.2 Paracrine Mechanisms

It has been seen that MSCs from all sources act through paracrine actions without being necessarily present at the site of lesion. Most MSCs have been found to disappear within 1 day after injection. Although MSCs can spontaneously differentiate into bone, cartilage and adipose in vitro, they rarely differentiate into lung resident cells like epithelial cells. Thus, a large chunk of the MSCs' reparative abilities are believed to be through their paracrine actions [6].

Phase I clinical studies in newborn infants in case of neonatal lung diseases (bronchopulmonary dysplasia, severe intraventricular haemorrhage, hypoxic ischemic encephalopathy) have shown that MSC treatments are safe, feasible and possibly effective [18]. Very low rates of engraftment of MSCs upon transplantation and low differentiation in vivo, but high beneficial effects, indicate that long-term survival of cells on site may not be essential. Thus, large parts of the therapeutic effects are believed to be associated with their paracrine effects. MSC-conditioned media was shown to ameliorate hyperoxia-induced acute lung injury. MSC-derived exosomes have been shown to ameliorate oxidative damage in lung injury models. In ventilator-induced lung injury, MSC secretome has proved beneficial.

Protective effects of UC-MSCs were seen to be mediated through upregulation of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF)

in hyperoxic neonatal lung injury. Administration of VEGF siRNA-treated MSCs did not protect against impaired alveolarization, angiogenesis, ED-1 positive cells, increased terminal deoxynucleotidyl transferase nick end labelling and downregulation of pro-inflammatory cytokines.

3.4 How Does ROS Affect the Regenerative Potential of MSCs?

Few studies have explored the effect of ROS on stem cells. It is crucial to understand and prevent ROS-induced effects on stem cells as MSCs are now being widely used for therapeutic purposes. MSCs are known to export their healthy mitochondria to oxidatively stressed cells, thus decreasing their oxidative stress and stabilizing their membrane potential. The mitophagy of such rescued cells is also restored. Antioxidants have been shown to enhance mitochondrial rescue transfer mechanism. It has been seen that MSCs, like other cells, undergo a decline in their regenerative capacity and other biological functions – accumulating cellular senescence and damage with age. There are various cellular and molecular changes in their self-renewal, proliferation and differentiation capacities. MSCs are continually exposed to endogenous or exogenous oxidants. Whereas a low level of ROS is necessary to maintain normal cellular homeostasis and serves as intracellular signalling molecule, high levels can interact with a wide range of cellular molecules and cause harm. ROS can cause DNA damage by triggering specific DNA damage response, leading to cell cycle arrest via activation of p53/p21 and/or p16/Rb pathways. High levels of ROS block the activation of telomerase, thus leading to immortalization of MSCs and cancer development [11].

Oxidative stress-induced DNA damage has been found to bring about post-translational modifications. Aged mice with higher accumulated ROS have been seen to undergo extensive loss of alveolar type I and II cells and delayed regeneration of alveolar type II cells compared to young mice. Low levels of ROS in mouse airway basal stem cells exhibited higher proliferative capacity, compared to cells with higher ROS. Lung mesenchymal stem cells have been isolated from nasal mucosa and lung compartments. The isolated cells expressed MSC cell surface proteins like CD73, CD105, CD166 and CD90. These have also been shown to differentiate into multiple lineages like adipocytic, osteocytic and chondrocytic. Understanding the mechanisms of senescence of MSCs will help explore novel strategies to improve MSCs' beneficial effects in recipients with age-related diseases where oxidative stress plays a crucial role.

3.5 Strategies to Improve MSC Therapy in Lungs

MSC treatment in research settings has been shown to be effective in repair of lung damage. MSC administration is safe in COPD patients, but the effects in clinical trials have not been very robust in terms of mortality reduction or improvement in

lung function. Thus, combining MSC therapies with specific modulators that can potentiate the beneficial effects of MSC are being tested. Based on the mechanisms of anti-inflammatory, anti-fibrotic, antimicrobial and anti-apoptotic activities, MSCs have been used for lung disease therapy like acute respiratory distress syndrome (ARDS), allergic asthma, silicosis and emphysema. Preclinical studies have shown a good safety profile, thus encouraging the further clinical trials. However, clinical trials have shown limited results – small inoculation cell number, late administration during advanced disease stage, low survival of MSCs in vivo and impaired MSC biological activity/potency could be various reasons. Another issue could be poor engraftment of MSCs in the lungs, given that cells are cleared from lung tissue within 24 hours.

(i) A study overexpressing CXCR-4 surface receptor which interacts with stromal cell-derived factor-1 showed improved MSC homing to injured sites. This strategy can be used to home a greater number of MSCs into lungs in acute lung injury models.
(ii) Low-level laser (LLL) has been suggested as an interesting new therapy to boost the MSC response in emphysema. It is non-invasive, economical and safe. Under in vitro conditions, LLL has been shown to promote stem cell proliferation. It also increases cyclic AMP synthesis in alveolar macrophages, leading to reduction in NFKB activation and IL-1 and IL-6 secretion and consequently lung inflammation [6]. In another study, human tubal cells and LLL were combined to treat a CS-induced emphysema model successfully. Reduction of inflammatory cellular infiltration, collagen deposition in the lungs and mucus secretion were potentiated with this combination therapy.
(iii) Another strategy that has produced beneficial results is optimization of culture conditions. MSCs are classically cultured in tissue culture plates as 2D monolayers. However, in 2D cultures, MSCs may lose their stemness properties, which is not the case in vivo. 3D spheroid cultures of MSCs have been found to prevent apoptosis and maintain self-replicative potential of MSCs due to conservation of cell–cell interactions that are important for survival and colony formation. Aggregated AD-MSCs were found to exhibit better therapeutic performance compared to dissociated AD-MSCs in a mouse model of elastase-induced emphysema [6].
(iv) To increase longevity/potency of MSCs in vivo, two techniques have been tried. One is preconditioning of MSCs through brief exposure of cells to low doses of a sublethal/toxic agent to increase stress tolerance. It has been seen that freshly isolated MSCs have better potency in vivo than frozen and thawed MSCs. Besides in terms of preconditioning, hypoxia, heat- and nutrient-depleted microenvironments have been tested to prepare them for in vivo survival.
(v) MSC potency was shown to be improved by preconditioning the MSCs in an inflammatory milieu. Pooled sera from patients with severe ARDS were used as inflammatory preconditioning media to activate MSCs. These sera contain high levels of IL-10, IL-8 and IL-6 and low levels of IL-1β, TNFα and IFNγ.

(vi) Chemical substances like pioglitazone, *N*-acetylcysteine (NAC) and tetrandrine have been used to increase MSC potency. Pioglitazone binds to peroxisome proliferator-activated receptor. This modulates transcription of genes involved in glucose and lipid metabolism. NAC-preconditioned MSCs reduced lung inflammation and collagen content in lung tissue of bleomycin-induced PF model. Thus, treatment with primed MSCs significantly reduced mortality of bleomycin-administered animals after 28 days, compared to naive MSCs. Although tetrandrine has not yet been tested in vivo, cell culture results (5 and 10μM for 24 hours) showed that PGE2 expression was increased in MSCs post treatment.

(vii) Genetic manipulation: Genetically engineered MSCs to overexpress hepatocyte growth factor (HGF) have been seen to have better survival in vivo, and the effects have been tested in lung injury models. MSCs engineered to conditionally express HSP70-VEGF-MSCs exhibited better survival and therapeutic efficacy than control MSCs in a CS extract and emphysema model. MSCs overexpressing developmental endothelial locus (Del-1), ST2 receptor gene, angiotensin-converting enzyme (ACE-2) and manganese superoxide dismutase (MnSOD) dramatically improved the lung injury index, levels of pro-inflammatory cytokines like TNFα, IL-1β and IL-6 and neutrophil count in mouse models of ARDS. For pulmonary arterial hypertension, MSCs overexpressing heme oxygenase-1 (HO-1) gene have been found to be more effective in the reduction of RV systolic pressure and RV hypertrophy in recipient mice. Genetically modified cells are currently in early-phase clinical trials for pulmonary hypertension patients in the Pulmonary Hypertension and Angiogenic Cell Therapy (PHACeT) trial [8].

3.6 Mesenchymal Stem Cells in Clinical Trials

Lung disorders are one of the leading causes of morbidity worldwide, besides cardiovascular diseases and cancer. Considering the levels of airborne microbes, toxins and microbial by-products that lungs are exposed to, healthy lungs are able to maintain the homeostasis, plasticity and integrity of the tissue [19]. However, no definitive cure or treatment regime is available for the variety of lung disorders. Stem cells-based therapies are the upcoming treatment regimens in various lung diseases due to their characteristic properties of immune-modulation, being immunologically naive and being anti-inflammatory and anti-fibrotic in action. Of all the types of stem cells, mesenchymal stem cells (MSCs) are the easiest to obtain and transplant. They have also shown great in vitro expansion and in vivo regeneration potential.

Keeping the diverse characteristic features of MSCs into consideration, a lot of clinical trials have been conducted, and several are ongoing. According to the registered clinical trials on *www.clinitrials.gov*, 13 clinical trials have been completed, 10 are recruiting patients currently, 4 are ongoing but not recruiting patients now, 11 are not yet recruiting, 1 has been withdrawn, and status of 12 clinical trials is not

known. This data depicts those clinical trials which are using mesenchymal stem cells obtained from various tissue sources for the treatment of lung diseases. The figure given below gives a brief idea of the overall scenario in this direction (Fig. 3.1).

Also according to the clinical registry data, umbilical cord-derived MSCs are the most highly used MSC candidates in the clinical trials, followed by bone marrow-derived MSCs (29%) and adipose tissue-derived MSCs (10%). The remaining other sources of MSCs contributed 12% of the clinical trials (Fig. 3.2).

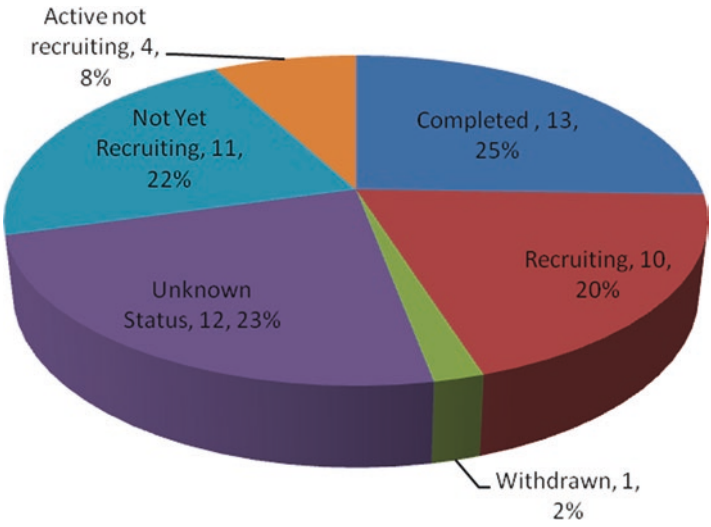

Fig. 3.1 Current status of various clinical trials using mesenchymal stem cells as the cell source. The data is based on the latest information available on www.clinicaltrials.gov

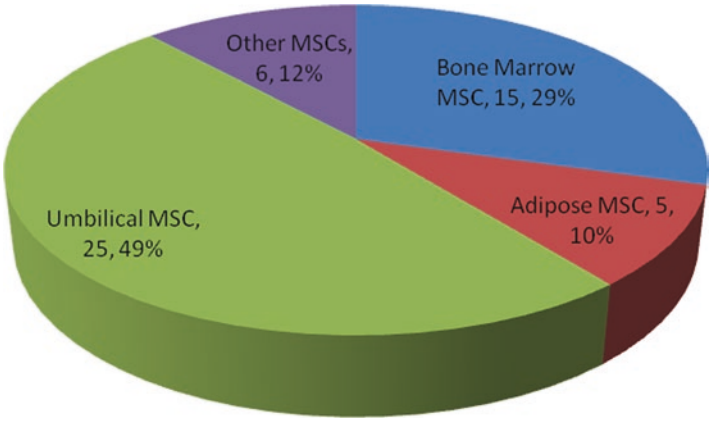

Fig. 3.2 Pie-chart showing use of mesenchymal stem cells obtained from various tissue sources used in the registered clinical trials of lung diseases

Table 3.1 Clinical trials of MSCs in lung diseases

S.No	Title of study	Number of patient/age	Cell therapy-based dose	Duration	Conclusion	Reference/ location
1	Predictive value of within-breath respiratory input impedance in the early diagnosis of Obliterative bronchiolitis after allogeneic hematopoietic stem cell transplantation	26 18–70 years	HSCT	2010–2012	Airway calibre and distensibility with lung inflation are increased after HSCT. This effect seems to be related to an increase in lung stiffness and must be taken into account when interpreting lung function changes after HSCT	Barisione et al [21]/ Italy
2	Autologous stem cell treatment for chronic lung disease study	207 16–70 years	Autologous stem cell	2016–2017	Therapy is about safety and minimization of adverse events, a perceived improvement in the patient's lung condition (to be determined by their perceived quality of life), an improvement in the FEV1 among COPD patients	Rubio et al. [22]/USA
3	A pilot study to evaluate the safety and feasibility of mesenchymal stem cells to induce remission in lung transplant patients experiencing treatment refractory moderate lung rejection	9 18–75 years	2–four million MSC per kg will be infused intravenously	2014–2017	To assess for their capacity to tolerate IV infusion of MSC without acute clinical or physiological deterioration	Zubair et al. [23]/Mayo Clinic/USA
4	An open-label, non-randomized, multicentre study to assess the safety and effects of intravenous implantation of liposuction-derived autologous adipose-derived stem cells in subjects with chronic obstructive pulmonary disease (COPD)	26 18–85 years	Adipose-derived stem cell (ADSC)	2013–2017	FEV1 decline of approximately or less than 30 ml at 12 month follow-up. Decrease in six minute walking distance (6MWD) of less than 5% over one year	Propis et al. [24]/USA

5	A pilot study of mesenchymal stem cells for treatment of acute respiratory distress syndrome in patients with malignancies	20 18–older age	Mesenchymal stem cells (MSCs)	2017–2020	NA	Olson et al. [25]/USA
6	A phase I trial to evaluate the safety, tolerability, and potential efficacy of allogeneic human mesenchymal stem cell infusion in patients with mild asthma	6 18–65 years	hMSCs	2017–2021	NA	Glassberg et al. [26]/ USA
7	Open-labeled trial to evaluate the therapeutic effects of inhaled budesonide/ Formoterol in bronchiolitis obliterans after allogeneic stem cell transplantation	32 16–older age	Allogeneic stem cell	2008–2012	Adult allogeneic stem cell transplant recipients with clinical respiratory signs assumed to be secondary to BO, without extra-thoracic extensive graft-versus-host disease	Anne et al. [27]/Paris, France
8	A phase I study to evaluate the potential role of mesenchymal stem cells in the treatment of idiopathic pulmonary fibrosis	8 40–80 years	Placental mesenchymal stem cells (MSCs)	2010–2013		Chambers et al. [28]/ USA

There have been quite a few studies reported for the use of MSCs in treating various lung diseases [20]. But most of the clinical trials were conducted to establish the safety and efficacy of use of MSCs in treating these diseases. Most of the studies have taken up intravenous as the mode of infusion of MSCs. However, there are still several ongoing clinical trials in various lung diseases. A brief background of the recent clinical trials in this area is given in the Table 3.1.

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4

Role of NADPH Oxidase-Induced Oxidative Stress in Matrix Metalloprotease-Mediated Lung Diseases

Jaganmay Sarkar, Tapati Chakraborti, and Sajal Chakraborti

Abstract

Activation of proteases is known to dysregulate the homeostasis of lung metabolomics and thereby triggers a variety of lung diseases such as chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS) and pulmonary hypertension (PH). Among proteases, matrix metalloprotease (MMP) plays a critical role in regulating the turnover (degradation and synthesis) of extracellular matrix (ECM). MMPs facilitate cell migration by modulating production of cytokines and other signaling molecules, which are involved in the pathogenesis of lung diseases. Under normal condition, proteases are controlled by endogenous antiproteases. For example, MMPs are regulated endogenously by their inhibitors, TIMPs. Agonists induced imbalance of MMP-TIMP results in MMP activation. Oxidative stress by modulating inflammatory signaling targets triggers activation of MMPs and thereby initiates the progression of lung diseases. This suggests that MMP inhibition is an attractive therapeutic strategy to ameliorate oxidant-induced lung diseases.

Keywords

NADPH oxidase · Superoxide · Metalloproteases · Antiproteases · Metabolomics

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4.1 Introduction

Normal function of healthy lungs require alveolar support by the extracellular matrix (ECM). Lung diseases are predominantly associated with the abnormal remodeling or destruction of ECM that alters normal lung function and, if extensive, cause death. Proliferation and migration of cells are regulated by cell matrix interactions and matrix turnover [1, 2]. MMP family members act on lung matrix, thereby causing a variety of pathological consequences of the lung. MMPs can proteolyse and remodel ECM proteins throughout the body [3, 4]. MMPs are classified considering their structure and functions (Table 4.1) [5, 6]. MMP-mediated alteration in tissue integrity appears to be important for normal physiology. MMP-induced activation of some signaling cascades and modification of integrins has been observed to regulate platelet function. A discernible alteration of MMP expression may produce a variety of pathological conditions in the lung such as PH, asthma, ARDS and COPD. An extensive alteration of ECM is important for a variety of lung diseases, which include emphysema and asthma due to intra-alveolar fibrosis by MMPs [7].

NADPH oxidase (NOX), a multisubunit enzyme complex, is the main source of oxygen-derived free radicals in cells and tissues. NOX has seven isoforms, and their action depends on tissue and cell types. This enzyme yields superoxide anion ($O_2^{\cdot -}$), which can further react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$). Thus, ROS-mediated stress plays a pivotal role in malfunctions of the lung that eventually cause different types of lung diseases [8, 9].

4.2 NADPH Oxidase and MMPs

NADPH oxidase (NOX)-derived $O_2^{\cdot -}$ is an important component of stress-induced cellular signal network. NOX-induced oxidative stress-mediated signaling could impinge upon normal cellular mechanism and could dictate cells either to adapt to the stress or to go for apoptosis or cancer based on anti-oxidant status. Activation of NADPH oxidase in the lung has been observed to be altered by several chemical, physical, environmental and biological factors and also by the different stimuli, which could further increase in NOX activity [10,11].

Lipopolysaccharide (LPS), a bacterial endotoxin, increases leukocyte NADPH oxidase and also nonphagocytic NOX in human epithelial cells by activating isoform-specific protein kinase C [12]. Different proinflammatory cytokines such as TNF-α, IL-1β and IFN-γ play a vital role in activating NOX [13–19]. In pulmonary smooth muscle cells, Ang II stimulates NOX activity through the involvement of c-Src, EGF receptor transactivation, phosphatidylinisitol-3-kinase and Rac [20]. Other vasoactive agents like thromboxane A_2 and endothelin-1 (ET-1) stimulate PLD activity that subsequently activates NOX [21, 22] in pulmonary vasculature. Additionally, agents such as silica, asbestos, bleomycin, cigarette smoke and automobile exhaust components have been observed to increase NOX activity in airway epithelial cells (AECs) and also in the pulmonary endothelial and smooth muscle cells [23–26].

Table 4.1 List of major matrix matalloproteases with their substrates and chromosomal localization

MMPs	Others name	Molecular weight latent/ active	Chromosome location	Substrates
MMP-1	Collagenase-1; interstitial collagenase	55,000/45,000	11q22-q23	Collagen type I, II, III,VII, VIII, X
MMP-2	Gelatinase A; 72kDa gelatinase; type IV; collagenase; MMP-5	72,000/66,000	16q13	Collagen type I, II, III, IV, V, VII, X, XI, XIV; gelatin; aggrecan; laminin; fibronectin; elastin; MMP-9; MMP-13
MMP-3	Stromelysin-1; pro-collagenase activator; transin-1; MMP-6	57,000/45,000	11q23	Collagen type II, III, IV, IX, X, XI; gelatin; aggrecan; laminin; fibronectin; elastin; MMP-1, -7, -8, -9, and -13
MMP-7	Matrilysin-1; matrin; PUMP-1; uterine metalloendopeptidase	28,000/19,000	11q21-q22	Collagen type IV, X; gelatin; aggrecan; laminin; fibronectin; elastin; MMP-1, -2, and -9
MMP-8	Collagenase-2; neutrophil collagenase	75,000/58,000	11q21-q22	Collagen type I, II, III, V, VII, VIII, X; gelatin; aggrecan; laminin; fibronectin; elastin
MMP-9	Gelatinase B; 92kDa gelatinase; type IV collagenase	92,000/86,000	20q11.2-q13.1	Collagen type IV, V, VII, X, XIV; gelatin; aggrecan; fibronectin; elastin
MMP-10	Stromelysin-2; transin-2	57,000/44,000	11q22.3-q23	Collagen type III, IV, V; gelatin; aggrecan; laminin; fibronectin; elastin; MMP-1; MMP-8
MMP-11	Stromelysin-3	51,000/44,000	22q11.2	Aggrecan; fibronectin; laminin; α -1 antitrypsin
MMP-12	Macrophage metalloelastase	54,000/22,000	11q22.2-q22.3	Collagen IV; elastin; gelatin; laminin; fibronectin; vitronectin
MMP-13	Collagenase-3	60,000/48,000	11q22.3	Collagen type I, II, III, IV; gelatin; aggrecan; MMP-9
MMP-14	MT1-MMP (membrane-type-1 MMP)	66,000/56,000	14q11-q12	Collagen type I, II, III; gelatin; aggrecan; laminin; fibronectin; elastin; MMP-2; MMP-13

(continued)

Table 4.1 (continued)

MMPs	Others name	Molecular weight latent/ active	Chromosome location	Substrates
MMP-15	MT2-MMP	72,000/60,000	16q13-q21	Gelatin; laminin; fibronectin; MMP-2
MMP-16	MT3-MMP; ovary metalloproteinase	64,000/52,000	8q21	MMP-2
MMP-17	MT4-MMP; stromelysin A; stromelysin B	57,000/53,000	12q24.3	Fibrin; fibrinogen; TNF precursor
MMP-18	*Xenopus* collagenase-4	55,000/42,000	Not applicable	Unknown
MMP-19	RASI-1; RASI-6	54,000/45,000	12q14	Gelatin; aggrecan; COMP; collagen type IV; laminin; nidogen; large tenas
MMP-20	Enamelysin	54,000/22,000	11q22.3	Amelogenin; aggrecan; COMP
MMP-21	*Xenopus* MMP; XMMP	70,000/53,000	10	Unknown
MMP-22	*Gallus domesticus*	52,000/43,000	Not applicable	Gelatin; casein
MMP-27	MMP; CMMP			
MMP-23	CA-MMP	56,000/?	1p36	McaPLGLDpaARNh2 (synthetic MMP substrate)
MMP-24	MT5-MMP	63,000/45,000	20q11.2-q12	MMP-2
MMP-25	MT6-MMP; leukolysin	63,000/?	16p13.3	Gelatin
MMP-26	Matrilysin-2; endometase	28,000/19,000	11p15	Collagen type IV; gelatin; α_1-PI; fibronectin; fibrinogen; pro-MMP-9
MMP-28	Epilysin	59,000/45,000	17q11.2	Casein

NADPH oxidase-derived oxidants target the inflammatory proteases, for example, matrix metalloproteinases, which leads to lung diseases including PH, COPD, ARDS, asthma, cystic fibrosis and cancer [27–32]. Production of ROS by activated phagocytic cells occurs through activation of NOX complex, which comprises cytosolic (p47phox, p67phox and p40phox and Rac1) and membrane (gp91phox and p22phox) components. Upon agonist-induced translocation of these cellular components to the cell membrane, NOX becomes activated and subsequently induces NADPH oxidase activity to generate superoxide anion ($O_{2^{\cdot}}^{-}$) [33]. Since lung diseases are predominantly associated with the ECM remodeling, therefore, MMPs and their endogenous inhibitors (TIMPs) play a critical role in ECM homeostasis. In pulmonary fibrosis (PF), cysteine oxidation in the enzyme's active site causes activation of pro-MMP. Importantly, ROS not only activate MMPs but also increase its mRNA expression (Fig. 4.1).

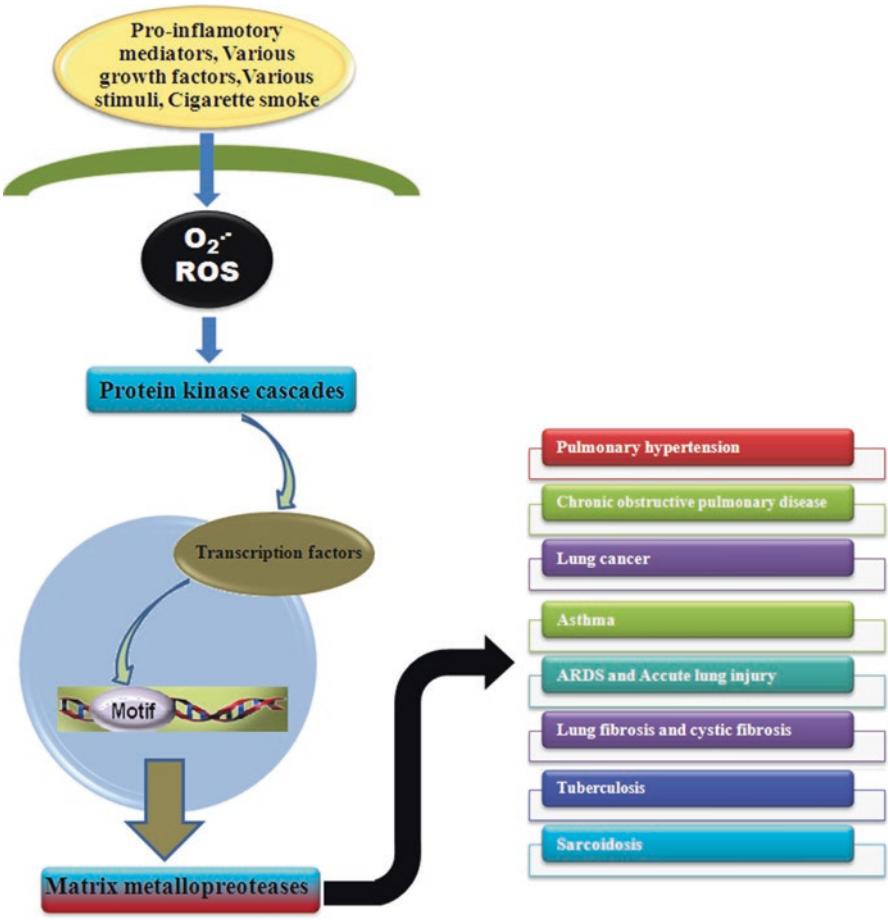

Fig. 4.1 Role of proinflammatory mediators and various stimuli generated by environmental and occupational agents, and also from metabolic pathways on ROS production in the lung and subsequent activation of MMPs leading to a variety of lung diseases

Oxidative stress has been shown to contribute to TGF-β-induced pulmonary fibrosis [34]. Several stimuli like ET-1, Ang-II and TxA_2 have been shown to induce pro-MMP-2 activation. Activation of pro-MMP-2 primarily occurs through the involvement of NOX-derived $O_2^{\cdot-}$ and subsequently by other reactive species derived from $O_2^{\cdot-}$ such as $OH^{\cdot}$, $OOH^{\cdot}$ and $ONOO^{-}$ [35, 36]. Stimulants like ET-1 and TxA_2 in pulmonary smooth muscle cells generate active MMP-2 from inactive pro-MMP-2, which occurs via MT1-MMP upon regulating NOX-PKCα-p38MAPK-NFκB pathway. This active MMP-2 may be involved in the pathogenesis of many lung disease like PH, ARDS, asthma, COPD and lung cancer (Fig. 4.1) [36].

4.3 MMPs in Pulmonary Hypertension and Right Ventricular Hypertrophy

Pulmonary arterial hypertension (PH) is distinguished primarily by remodeling of pulmonary artery, which leads to hypertrophy of the right ventricle [37, 38]. This mainly occurs primarily due to a noticeable increase in proliferation of pulmonary artery smooth muscle cells [37, 39]. In addition, the progressive phase of PH includes an increase in ECM turnover: its synthesis and degradation. ECM upon degradation could organize different types of proteases in which MMPs gained special importance [40, 41]. Several studies have shown that an increase in the level of MMPs appear to be important in the progression of PH and associated lung diseases [42, 43].

Lepetit et al. [44] have identified a marked increase in MMP-2 activity in PASMCs of patients with PH. MMP-2 is known to be produced upon activation of pro-MMP-2. Membrane-type matrix metalloproteinase-1 (MT1-MMP) interacts with tissue inhibitor of metalloproteinase-2 (TIMP-2) in the cell membrane. The bimolecular MT1-MMP-TIMP-2 complex associates with pro-MMP-2 by producing a complex (trimolecular complex) and thereby initiates the activation cascade and subsequently releases active MMP-2 into the extracellular medium [45]. Cytokines like IL-1 and TNF-α are present in significantly higher levels in serum of patients with PH [46]. Roy et al. [47] have demonstrated the role of proinflammatory cytokines: TNF-α, IL-1β and TGF-β in inducing activation of pro-MMPs (such as pro-MMP-9 and pro-MMP-2) and to study the mechanistic pathway(s) for their activation in the SMCs of pulmonary artery (Fig. 4.1).

Lungs treated with monocrotaline (MCT) for producing PH have been shown to be associated with numerous genes involved in the regulation of ECM and subsequently cell adhesion, which was confirmed by gene expression analyses. A discernible increase in MMPs like MMP-2 and MMP-9 have been shown to increase MMP-8, MMP-10, MMP-11, MMP-12 and MMP-20, which play a critical role in PH. Additionally, MMPs can increase in migration of SMCs in monocrotaline (MCT)-treated PH artery [48]. Additionally, increase in the activity of MMP-1, MMP-2, MMP-9 and MMP-3 were observed during normoxic recovery from hypoxia-induced PH [49]. In a study using rat hypoxic pulmonary artery, Herget et al. [50] demonstrated collagen breakdown by a marked increase in MMP-13 during exposure with hypoxia [50].

Failure of the right ventricle (RV) is a deadly disease with no effective treatment. In a mouse model, bleomycin-induced failure of the right ventricle (RV), stimulation of α1-adrenergic receptor (α1A-subtype) by A61603 was shown to improve PH and RV hypertrophy and failure. ROS is known to directly reduce the function of contractile proteins [51]. Thus, A61603-mediated decrease in oxidative stress could improve contraction of the myofilament by decreasing its damage by ROS. ROS also stimulates mRNA and protein expression. The resulting activation of MMP-2 affects negatively [52–55]. An increase in MMP-2 activity has been shown to alter Ca^{2+} handling characteristics of the pulmonary vasculature [56, 57]. Thus, A61603 reduces MMP-2 expression during A61603 treatment, thereby protecting RV

function due to proper Ca^{2+} handling, which results in a marked decrease in myofilament injury [58]. This indicates that A61603 protects against RV hypertrophy induced by ROS. A61603 treatment appears to be beneficial in this pathology conceivably due to its role to increase SOD and also to reduce NOX-4 activity, which in combination decreases ROS level and subsequently minimizes reductions of myofilament contractility. Additionally, chronic A61603 treatment has been shown to induce inotropic effect to augment RV pump function.

4.4 MMPs and Chronic Obstructive Pulmonary Disease

COPD is a chronic lung disease, which is ascribed by irreversible decrease in alveolar airway flow [59, 60]. Emphysema and chronic bronchitis elicit pathogenesis of the COPD. Emphysema occurs due to damage of the alveoli with a marked reduction in the plasticity of the lung. This results in lowering of gas trapping and that subsequently decreases pulmonary oxygenation. The major cause of COPD is due to inhalation of silica, asbestos, cigarette smoke and smoke associated with automobile exhaustion [61, 62]. MMPs are known to be involved in emphysema, which results in the pathogenesis of COPD [63].

Immunohistochemical studies of COPD patients lung tissue exhibit an increase in the expression of MMPs, e.g. MMP-1, MMP-2 (present predominantly in alveolar macrophages and epithelial cells), MMP-8 and MMP-9 (primarily secreted by neutrophils) [64]. COPD patients sputum elicit a marked increase in MMP-2 and MMP-9 activities [65, 66]. In bronchoalveolar lavage fluid (BALF), MMP-8 and MMP-9 levels were considerably higher in smokers having emphysema in comparison to the smokers without emphysema [67, 68]. The inflammatory cell migration to the airway cells due to ECM destruction augments MMP-9 expression [69]. As a consequence, TIMP-1 inhibits MMP-9 activity by 1:1 stoichiometric binding [70]. COPD patients sputum elicit active MMP-9, which is absent in normal subjects. Importantly, COPD patients have about 25% pro-MMP-2, while only 5% was observed in the controls. This indicates that pro-MMP-2 level also increases in COPD patients. This is in agreement with a study of Beeh et al. [71], who have shown an enhancement in MMP-9/TIMP-1 ratio in sputum of patients with COPD. Alveolar macrophages isolated from BALF of COPD patients elicited a marked increase in MMP-9 secretion with augmented enzymatic activity in comparison to the smokers and non-smokers, who are apparently healthy [72].

Cigarette smoke extracts also induce MMP-2 gene expression and MMP-2 gelatinase activity in normal lung fibroblasts [73]. Cigarette smoke extracts treatment to lung fibroblasts stimulates EGR-1 mRNA expression. Imai et al. [74] have shown that emphysema patient's lung parenchyma elicits higher MMP-1 expression. MMP-3 and MMP-7 also release cytokines and growth factors such as TNF-α, TGF-β, FGF-1 and IGF-1 that, in turn, promote cleavage of adherence junction proteins from its binding proteins, which results in damage to the cells [75, 76].

MMP-12 is another subfamily of MMPs, mostly secreted by macrophages, and is known to be associated with COPD and airway remodeling [62]. Studies with

different animal models illustrate the involvement of MMP-12 in pathogenesis of cigarette smoke-mediated emphysema in a mice model. In this context, it has been observed that MMP-12 gene knockout mice elicited complete protection from emphysema that occurs upon exposure of cigarette smoke. Cigarette smoke also stimulates the macrophage staffing in association with elastolytic properties of MMP-12 [77].

ADAMs (*a d*isintegrin *a*nd *m*etalloproteases) are another subfamily of proteases, which belongs to the metzincins family and is structurally related to MMPs [78–81]. ADAM-33 has been observed to be involved in airway inflammation along with hyperresponsiveness in the general population with COPD [82]. Rat exposed to cigarette smoke in a COPD model have higher ADAM-17 level in lung tissues compared to respective control, indicating involvement of ADAM in the obstructive lung pathology [83]. Human airway epithelial cells (NCI-H292) treated with ADAM-17 siRNA develops protection against smoke-induced mucin overproduction [84].

4.5 MMPs and Lung Cancer

Lung cancer (LC) is one of the major causes of cancer-related death in human, and annually above 1.1 million deaths occur all over the world [85]. LC may be categorized as (i) small-cell lung cancer (SCLC) and (ii) non-small-cell lung cancer (NSCLC) [86]. NSCLC is found in approximately 85% of all lung cancer cases, which include squamous cell carcinoma (SQ), adenocarcinoma (AD) and carcinoid [87]. Tobacco stimulates inflammation and oxidative stress in lung tissue [88, 89], modulates the transcription and activation of proteases and thereby elicits protease-antiprotease imbalance towards protease in the lung parenchyma leading to damage to the lung tissue, which could have influence on the progression of lung cancer [90, 91].

MMPs play a critical role in cancer pathogenesis that has been suggested to be initiated by proteolytic degradation of several ECM components and basement membranes. LC expresses high level of MMPs. Upregulation of MMPs may cause genetic alterations in addition to transcriptional changes and that subsequently activates a relatively large number of oncogenes like β-catenin or lymphoid enhancer factor-1 (LEF-1); however, a marked decrease in tumour suppressors, for example, p53, have also been observed [91, 92].

Serum collected from SCLC and NSCLC patients elicited a marked increase in MMP-9 and TIMP-1 levels with respect to healthy subjects [93]. Patients with NSCLC have shown an increase in MMP-9 level in plasma, but the exact role of MMP-9 in lung cancer is currently unknown. MMP-9 is known to play an important role for metastasis development in NSCLC. Itoh et al. [94] have demonstrated that in MMP-9-deficient mice, metastasis development was vulnerable in comparison to control mice in regard to cancer dissemination. In NSCLC, MMP-1, MMP-2 and MMP-9 have been suggested to be reliable markers as they contribute to metastasis and tumour invasion [95–97]. In stromal fibroblasts, elevated MMP-2 expression

was found in squamous lung cell carcinoma compared with adenocarcinoma. MMP-2 expression in tumour of fibroblast stroma has been suggested to be an excellent angiogenic marker for NSCLC [98]. The involvement of MMP-2 in cancer development was demonstrated in a mice model [99]. It has been demonstrated that MMP-2 inhibition could decrease production of vascular endothelial growth factor (VEGF) and subsequently inhibit angiogenesis and apoptosis of endothelial cells [100]. In patients with NSCLC, MMP-2 and MMP-9 expressions were demonstrated by immunohistochemical studies [101–103]. Additionally, the expression of endogenous MMP inhibitors (TIMPs) was found to be correlated with different stages of cancer [104]; and their levels could be considered as prognostic markers to ascertain the progression of lung adenocarcinoma [105].

MMP expression has been shown to vary with cancer subtypes [106]. Lack of MMP-13 did not show a doscernible effect on tumour growth, vascularization and lung metastasis, though MMP-13 mRNA has been shown to be significantly increased with cancer metastasis [99]. Absence of MMP-7 did not show any alteration in the progression of lung metastases, whereas MMP-9 deficiency or treatment with MMP-9 inhibitor decreases lung tumour burden in mice [94, 107, 108]. In different models of tumour metastasis, abrogation of MMP-12 significantly increases lung carcinoma metastasis w.r.t normal animals, indicating that MMP-12 could play a tumour-suppressive role [108]. By activating MMPs with FGF, VEGF and TGF-β, MMPs promote tumour angiogenesis [109, 110]. MMP-14 cleaves the hyaluronan receptor, CD44, and the released extracellular domain of CD44 subsequently binds with MMP-9 in malignant cells surface. This complex subsequently activates TGF-β and thereby triggers angiogenesis [111].

CH1104I treatment to mice has been shown to significantly inhibit metastasis of lung carcinoma cells, which indicates that abrogation of MMP-2 and MMP-9 could decrease metastasis of lung cancer cells [112]. The MMP inhibitor, MMI270, was shown to markedly decrease colony numbers in the lung following treatment with B16-F10 mouse [113]. The MMP inhibitor, BMS-275291, also showed therapeutic potentiality because of its role to ameliorate symptoms of advanced lung cancer [114]. However, more detailed studies are needed before recommending BMS-275291 for chemotherapy of advanced NSCLC [115]. The broad-spectrum MMP inhibitor, BAY 12-9566 N has been shown to counteract the neoplastic growth induced by genotoxic carcinogen [116]. GM6001 is also a potent inhibitor and displays its role in the MMTVPyMT cancer model [117].

4.6 MMPs and Asthma

Asthma is characterized as a chronic inflammatory lung disease associated with airway hyperresponsiveness, infiltration of inflammatory cells in bronchi and significant morphological alterations of airway structure (bronchial remodeling). As ageing progresses, a marked decrease in lung function has been observed [118]. In asthmatic patients, remodeling occurs through (a) thickening of epithelium due to fibrosis-associated deposition of collagen and fibronectin, (b) hyperplasia of smooth

muscle cells, (c) enhancement of blood vessels in airways and (d) hyperplasia Goblet mucus-producing cells [119–122]. In asthma, secretion of proteases in airways has also been documented.

MMPs and TIMPs are known to play a critical role in the pathogenesis asthma [123, 124]. To establish MMPs role in the pathogenesis of asthma, Suzuki et al. [125] observed an alteration in the MMP-2 and MMP-9 and TIMPs levels in sputum isolated from patients with asthma. They also found that compared to control (healthy subjects), MMP-2 and TIMP-1 levels were significantly elevated in asthmatic patients, whereas MMP-9 level was markedly greater in asthmatics. Vignola et al. [126] have observed stimulation of both MMP-9 and its inhibitor, TIMP-1, in asthma patients in comparison to control subjects. In asthma patients, MMP-9 has been observed to increase the level of proinflammatory cytokines [127, 128], which induces inflammation of the airways [129]. TNF-α and IL-1β have been shown to induce MMP-9 expression in macrophages [130–132]. Cataldo et al. [133] have shown that MMP-9 knockout mice were unable to elicit airway hyperresponsiveness [133]. Cigarette smoke treatment to asthmatic human respiratory epithelia has been observed to be liable to MMP-9-induced airway remodeling [134–137], indicating that MMP-9 inhibition could have protective role towards asthma.

Other classes of MMPs such as MMP-1, MMP-3, MMP-7, MMP-8, MMP-12 and MMP-19 were shown to be involved in asthma pathogenesis. In airway smooth muscle cells of asthmatics, immunoreactive MMP-1 expression was found to be predominantly higher, indicating that MMP-1 plays an important role in inducing the mass of airway smooth muscle cells, which is characteristically present in patients with asthma [138]. BALF collected from asthma patients elicit higher MMP-8 expression, which seems to be critical determinant in this type of lung disease [139, 140]. This idea was hypothesized from an observation of a mice model of asthma by Gueders et al. [141], which was observed in MMP-8-deficient mice. Bronchial fibroblasts MMP-3 along with MMP-2 stimulate procollagen synthesis and showed a marked increase in hyperreactivity resulting in reduced lung function [142]. However, in severe asthma patients, MMP-7 level is higher in basal epithelial cells, which induces cleavage of Fas ligand (FasL), and thereby damages the airway epithelial cells [143]. Guiders et al. [144] have shown that MMP-9 deficiency in mice decreases tenascin-C accumulation in Th2-mediated airway reactivity, indicating involvement of MMP-19 in asthma. Chiba et al. [145] demonstrated upregulation of MMP-12 in airways of bronchial asthma in a rat model system. MMP-12 was found to be enhanced in monocytes and macrophages that migrates to the site of inflammation and thereby causes remodeling. This remodeling occurs through degradation of ECM components by cytokines-mediated induction of MMPs, which eventually cause airway inflammation [146]. Mitogen-activated protein kinases like ERK1/2, c-Jun N-terminal kinase (JNK) and phosphatidylinositol 3-kinase (PI3-K) are known to be involved in IL-1β-mediated activation of MMP-12 [147, 148]. These evidences suggest that MMPs are the major class of proteases that could prove useful as therapeutic measures for asthma patients.

R-94138 and marimastat are the two broad-spectrum MMP inhibitors that reduce the progression of allergic inflammation in airways and hyperresponsiveness to

allergens in different model systems [149, 150]. However, GM6001 administration did not elicit any alteration in ovalbumin (OVA) caused asthma in mice, albeit inhibits inflammatory cells accumulation in lung parenchyma [151]. In a murine model of toluene-mediated asthma (TDI-OA), MMPI-I (matrix metalloproteinase inhibitor I) and MMPI-II (matrix metalloproteinase 2/9 inhibitor II) have been shown to decrease the number of inflammatory cells in BALF [152, 153]. Several drugs with anti-inflammatory effects, for example, corticosteroids, have been shown to inhibit LPS-induced activation of MMP-12 in macrophages [147, 154]. In patients with severe asthma, corticosteroid treatment is the most effective anti-inflammatory medication and exhibits its anti-inflammatory effect by reversing the imbalance of MMP-9/TIMP-1 [155, 156]. In this context, dexamethasone has been shown to significantly attenuate IL-1β-mediated stimulation of MMP-12 activity [157].

4.7 MMPs in ARDS and Acute Lung Injury

Acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) are the foremost life-threatening diseases. ARDS and ALI have been suggested to occur due to injury to epithelial and endothelial capillary membrane of alveoli during inflammation with the involvement of MMPs. MMP-induced ECM-degrading components are involved in the initiation and progression of different types of lung diseases [158–160].

MMPs are predominantly secreted by activated macrophages and neutrophils [161, 162]. It has previously been suggested that these cells are the only MMPs source; however, it has now been known that fibroblasts, endothelial cells and type II epithelial cells of the lung are also the source of MMPs [162, 163]. Kong et al. have shown that lungs of paediatric ALI patients produce higher level of MMP-8, MMP-9, MMP-2, MMP-3, MMP-11 and MMP-12 in comparison to controls [158, 163–165]. In brochoalveolar lavage (BAL) fluids of ALI/ARDS patients, a marked increase in MMP-2, MMP-8 and MMP-9 levels have been observed [163]. The value of the ratio of MMP-9/TIMP-1 may be considered as prognostic marker of ARDS [158, 165].

Several investigators have suggested that MMPs and its inhibitors for therapeutic potentials in ALI [166]. In a mice model, ischemia-reperfusion injury to the lung, bleomycin, hyperoxia, LPS and acid aspiration were shown to trigger ALI [167]. LPS or bleomycin treatment was shown to induce ALI in mice deficient in MMP-8. This occurs due to a marked increase in the deposition of PMNs. The accumulated PMNs cause a marked decrease in MMP-8-mediated macrophage inflammatory protein-1α (MIP-1α) inactivation and result in a marked increase in mortality [168, 169]. Induction of LPS in mice with MMP-9 deficiency has been shown to develop emphysema [170]. In mouse lung, inhibition of MMP-9 by chlomethylase-3 (CMT-3) was shown to attenuate inflammation of neutrophils [171]. BALF collected from MMP-9-deficient mice upon ozone exposure showed greater protein content and a discernible increase in epithelial cells and neutrophils with respect to the wild-type cells. This suggests protective effect of MMP-9 in the inflammation during ozone

exposure [172]. However, this kind of protective effect was not found in MMP-7-deficient mice [172]. Additionally, ALI induced by hyperoxia showed a marked increase in inflammation in MMP-13-deficient mice due to its absence in the cleavage of monocyte chemotactic protein-1 [173]. It has been observed that MMP-3 also contributes to lung injury during inflammation [174]. Thus, MMPs are involved in lung injury in ARDS, while its inhibition prevents ALI. MMP-3 and neutrophil elastase inhibition by tetracycline was shown to reduce lung injury in an animal model system [175]. Similar protective effects by inhibiting MMP-3 inhibition have also been shown to give similar protection in animal models of sepsis [176, 177]. Therefore, inhibition of MMP-3 activity could prevent acute lung injury. Importantly, treatment with MMP-3 inhibitors needs to start early in the initiation and progression of the disease in order to prevent the pathogenic mechanism.

4.8 MMPs and Lung Fibrosis

Pulmonary fibrosis (PF) is a fatal disease where interstitial myofibroblasts replaced the loss of lung epithelial cells and deposition of ECM component in the interstitial space of the lung, which causes remodeling of pulmonary vessels [178]. PF in addition elicits damage to the alveoli leading to decrease in oxygen transfer and subsequently collapse of alveoli [179]. Intraluminal fibrosis occurs due to the recruitment of inflammatory cells especially macrophages, lymphocytes and neutrophils into airways, which leads to dysregulation of the turnover of ECM components [180, 181].

In PF chronological variations in the localization and the expression of MMPs and TIMPs have been observed [182, 183]. MMP-2 and MMP-9 activities have been observed to be stimulated in BALF from patients with PF [184]. MMP-9 activity has been shown to be notably increased in the early stage of PF, whereas MMP-2 activity is predominant in the latter stage of the disease. Alveolar macrophages of patients with PF revealed greater MMP-9 expression with respect to normal [185]. MMP-2 and MMP-9 overexpression have been shown to damage alveolar epithelial cells, thereby increasing invasion of fibroblasts into the alveoli [184].

Besides the involvement of gelatinases, MMP-7 gene has also been observed to be overexpressed in patients with PF [186]. Zuo et al. [187] in a mouse model have shown that MMP-7-deficient mice inhibits PF [187]. Studies of the transcriptional behaviour of the lung genes of the patients with PF confirm the involvement of MMP-1 [188]. Moreover, collagen showed a prominent role in the progression of PF by cleaving the native helix of fibrillar collagens. Gene expression of other classes of MMPs such as MMP-3, MMP-8, MMP-12, MMP-13 and MMP-28 was also found to be stimulated in PF induced by bleomycin [189–195].

Overexpression of TIMPs causes dysregulation in MMP-TIMP balance, which leads to a marked increase in MMP activity [181]. TIMPs are expressed in different types of lung cells that have different roles in the regulation of the activities of MMPs. TIMP-2 and TIMP-3 genes were expressed during fibrosis. Thus, MMPs could be considered an important prognostic marker in lung fibrosis. In this context,

Corbel et al. [196] have demonstrated that the synthetic inhibitor of MMPs, batimastat, markedly inhibits the bleomycin-induced PF.

4.9 MMPs and Cystic Fibrosis

Cystic fibrosis (CF) is a type of lung disease where protease-antiprotease imbalance increases protease activities which may contribute to progressive bronchiectasis due to damage of alveoli [197]. It has now become clear that MMPs are involved in CF [197]. In the sputum of CF patients, a marked increase in MMPs levels have been observed [198–200].

MMP-9, one of the mostly studied MMPs, is expressed mainly in PMNs, macrophages and epithelial and endothelial cells of the lung. MMP-9 exerts proinflammatory effects by generating a novel neutrophil chemokine, proline-glycine-proline (PGP) [201], and increasing the chemokine potency of IL-8 [202]. Gaggar et al. [201] have shown that MMPs regulate the immune response during CF by generating proline-glycine-proline (PGP), which is an extracellular matrix-derived neutrophil chemoattractant [201]. A marked increase in MMP-9 level was detected in BALF and serum of patients with CF [199, 200, 203]. Geraghty et al. [204] have demonstrated that neutrophil elastase (NE) augments MMP-2 expression in epithelial cells and thereby alters remodeling and inflammatory responses in CF [205].

Other class of MMPs, for example, MMP-7, has also been shown to be associated with CF. Dunsmore et al. [206] have shown the elevation in MMP-7 expression in the AES of patients with CF. Research in the recent past provided evidence supporting involvement of MMP-2 in CF [207]. A previous study indicated that amiloride-sensitive epithelial sodium ion channels play an important role in airway surface lipid depletion, which activates MMP-12-dependent emphysema [207]. Additional studies are required to determine the role of MMP-12 on leukocytes and BALF in patients with CF [208].

In vivo dysregulation of TIMP/MMP status may be correlated to the secretion of CF. It is known that tetracyclines, for example, doxycycline, have intrinsic anti-inflammatory properties and inhibit production of MMPs in endothelial cells [209], which have been regarded as targets for CF [201].

4.10 MMPs and Tuberculosis

In global health crisis, *Mycobacterium tuberculosis* is known to be one of the most harmful human pathogens. Primarily, *M. tuberculosis* causes ECM destruction. It causes and thereby creates sites for its proliferation and transmission to another host [210]. This kind of tissue damage due to inflammation is mainly responsible for morbidity and mortality of patients with tuberculosis (TB). Notably, the mechanism by which lung matrix destruction in TB occurs is poorly understood. However, it has been suggested that MMPs are the key molecules in the pathogenesis of TB, due to its novel capacity to damage ECM, for example, collagen.

Chang et al. [211] reported that a marked increase in MMPs levels were found in human TB. They collected BALF from the patients and found an increase in MMP-9 expression in comparison to controls [211]. In TB patients, MMPs are activated and play an important role in degrading the lung matrix, while endogenous tissue inhibitors of metalloproteinases (TIMPs) failed to balance the activated MMPs [212, 213]. Elkington et al. [214] demonstrated that *M. tuberculosis* upregulate MMP-1, MMP-3, MMP-7 and MMP-10. Coussens et al. [215] have also observed an increase in the level of MMP-1, MMP-7 and MMP-10 in macrophages infected with *M. tuberculosis*. Immunohistochemical studies have identified the localization of MMP in TB granulomas. MMP-1 and MMP-7 are expressed in Langhans giant cells and macrophages in granulomas of the lung [214], whereas MMP-1 and MMP-9 are localized in epithelial cells of the lung [216, 217]. Thus, targeting MMP activity seems to be the useful intervention in drug-resistant TB [218]. Dexamethasone has been shown to ameliorate early effects in TB meningitis [219] and subsequently decreases MMP-9 level [220]. Another globally used drug to inhibit the activity of MMPs in TB patients is doxycycline. Importantly, multiple MMP inhibitors were developed as remedy for cancer [221], and these are currently under reassessment as adjunctive drugs to diminish immunopathological aspects of TB.

4.11 MMPs and Sarcoidosis

Sarcoidosis is an inflammatory lung disease affecting multiple organs including the lung and lymph nodes with unknown aetiology [222, 223]. Granuloma formation seems to be important for manifestation of a variety of lung diseases that are associated with remodeling and proteolysis of the ECM [224–226]. Proteases in macrophage especially MMPs have been suggested to play a critical role in sarcoidosis [227]. A marked increase in collagenase activity has been detected in BALF from sarcoidosis patients, where MMP-8 appears to be predominant MMPs [228]. MMP-9 levels are also elevated in BALF and sputum of patients with sarcoidosis without a discernible increase in TIMP-1 level [224, 228]. Immunohistochemical studies demonstrated that a cellular component of sarcoid granulomas in the lung has a high degree of immunoreactivity for MMP-1, MMP-2 and MMP-9, whereas the role of MMP-3 and MMP-7 has been observed to be less [229]. The cells of the sarcoid granulomas have low levels of TIMP-1 and TIMP-2, and that maintains latency in the activity of MMPs. The activated proteases cause disruption of the basement membrane leading to its damage [229]. Thus, MMP activation and ECM breakdown with subsequent remodeling could disrupt the normal lung function, which may produce advanced pulmonary sarcoidosis.

4.12 Conclusion and Future Direction

Several evidences are available implying the involvement of NADPH oxidase-derived $O_2^{\cdot -}$-induced activation of MMPs for progression of lung diseases. The evidences are well studied in the MMPs knockout mice and also by the specific MMP inhibitor, which suggests that correlation exists between the NADPH oxidase and MMPs activations with lung diseases. It has now become evident that regulation of lung health depends on MMP-TIMP balance. Dysregulation of the balance towards proteases affects lung heath. Therefore, MMP inhibitors have a great potency as useful therapeutic agent to ameliorate lung disease such as COPD, asthma, ARDS and associated PH and also lung cancer.

Recent evidence indicates that the recombinant signal regulatory protein for immunoglobulin kappa J region (RBPJ) and mastermind like 3 (MAML3) are now considered as the novel therapeutic targets for small-cell lung cancer (SCLC); however, its chemosensitivity may be reduced for long-term treatment [230]. Therefore, future research will determine appropriate combinational therapy with other known drugs, which may be of use for treating SCLC patients.

The marine actinomycetes product, 1-hydroxy-1-norresistomycin (HNM), has been observed to increase lncRNAs expression via transcriptional regulation of p53, thereby increasing apoptosis in non-small-cell lung carcinoma (NSCLC) [231]. In view of this, HNM is currently considered to be a novel therapy for NSCLC [231]. However, further research is needed to develop more potent HNM derivative that could be useful for long-term treatment of the disease.

Considering that elastin damage occurs in emphysema, a novel biodegradable polymeric nanoparticle (NP) has been developed using doxycycline loaded BSA (BSA.NPs). This opens up a promising way of controlling MMPs and, therefore, emphysema, thereby stopping further lung damage [232]. However, long-term therapeutic potentiality of BSA.NPs needs to be clearly ascertained by future research.

Anti-muscarinic agents are used for therapy of COPD and to some extent in the treatment of asthma because of their broncho-dilatory effects. Recent research showed that they also regulate remodeling of small airways by modulating MMPs and thereby COPD pathogenesis [233]. Further research is important to clearly determine the role of anti-muscarinic agents in ameliorating MMPs and subsequently COPD in animal model systems.

Acute pulmonary embolism is a critical condition that occurs due to prolonged pulmonary hypertension during abnormal activation of MMPs in pulmonary vasculature. Although doxycycline is being used to partly ameliorate pulmonary embolism and PH, yet more potent agents need to be discovered for clinical use of pulmonary embolism and associated PH [234].

Sepsis is a disease of relentless mortality due to non-availability of a potent drug. Research in the recent past indicated involvement of systemic inflammatory response associated with MMP activation in septicemia [235]. Chemically modified tetracycline, CMT-3 is currently used for sepsis, albeit it has limited effect in this scenario [235]. Further research is needed to explore the shortcomings of

therapeutic use of CMT-3 during different stages of septicemia and its association with MMPs.

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5 Oxidative Stress Mechanisms in the Pathogenesis of Environmental Lung Diseases

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Abstract

Globally, respiratory diseases are major cause of disability and mortality, and more alarmingly, it disproportionately affects developing countries, which is largely attributed to poor quality of air. Tobacco smoke and emissions from combustion of fossil fuel and biomass fuel are the major airborne pollutants affecting human lung health. Oxidative stress is the dominant driving force by which the airborne pollutants exert their toxicity in lungs and cause respiratory diseases. Most airborne pollutants are associated with intrinsic oxidative potential and, additionally, stimulate endogenous production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Elevated ROS and RNS in lungs modulate redox signals and cause irreversible damage to critical biomolecules (lipids, proteins and DNA) and initiate various pathogenic cellular process. This chapter provides an insight into oxidative stress-linked pathogenic cellular process such as lipid peroxidation, inflammation, cell death, mitochondrial dysfunction, endoplasmic reticulum stress, epigenetic changes, profibrotic signals and mucus hypersecretion, which drive the development and progression of lung diseases. Lungs are associated with robust enzymatic and non-enzymatic (GSH, ascorbic acid, uric acid, vitamin E) antioxidant defences. However, sustained production of free radicals due to continuous exposures to airborne pollutants overwhelms lung antioxidant defences and causes oxidative injury. Preclinical studies have

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demonstrated the critical roles and therapeutic potential of upregulating lung antioxidants for intervention of respiratory diseases; however, so far clinical benefits in antioxidant supplementation trials have been minimal and conflicting. Antioxidants alone may not be effective in treatment of respiratory diseases; however it could be a promising adjunctive therapy.

Keywords

Respiratory diseases · Oxidative stress · Lipid peroxidation · Inflammation · Cell death · Mitochondrial dysfunction · Endoplasmic reticulum stress · Epigenetic changes · Profibrotic signals · Mucus hypersecretion · Lung antioxidants

5.1 Introduction

With growing industrialization and rapid urbanization, the global burden of respiratory diseases is rising at an alarming rate and is a major source of disability and death following cardiovascular diseases [1, 2]. Globally, around 10% of all disability-adjusted life years (DALYs) lost is attributed to respiratory illnesses mainly chronic obstructive pulmonary disease (COPD), asthma, tuberculosis, lower respiratory tract infection and lung cancer [3]. Respiratory diseases disproportionately affect developing countries [1, 2], which is largely attributed to poor quality of air, although socioeconomic and genetic factors contribute significantly. Despite being so widespread, environmental respiratory illnesses are highly preventable.

Lungs are at the direct interface between body and the environment, and therefore, the major threat to lung health is the air we breathe. An adult individual inhales on average 10,000 to 15,000 litres of air every day. Inhaled oxygen along with airborne pollutants dissolves in respiratory epithelial lining fluid. The common pollutants that contaminate air include tobacco smoke, particulate matter, biomass fuel smoke, industrial emissions, ozone, oxides of nitrogen, oxides of sulphur and carbon monoxide. Most of these airborne pollutants are associated with intrinsic oxidative potential, and hence, oxidative stress is thought to be a dominant mechanism by which the air pollutants exert their toxicity in lungs. Several studies have reported elevated oxidatively damaged biomolecules in the exhaled breath condensate, nasal lavage, bronchoalveolar lavage and blood of human subjects following acute exposures to airborne pollutants such as cigarette smoke, ozone or particulate matter. Prolonged exposures to these airborne toxicants also stimulate endogenous cellular production of free radicals by diverse mechanisms including mitochondrial, NADPH oxidase, xanthine oxidase and myeloperoxidase activity. To defend against oxidative insult, lungs are endowed with powerful extracellular antioxidants in respiratory tract lining fluid as well as intracellular antioxidant defences. Measures to improve the air quality that we breathe and understanding the underlying mechanisms of disease development caused by exposures to airborne toxicants are pivotal to reduce the global burden

of respiratory disease. This chapter chronicles the key mechanisms by which oxidative stress mediates development and progression of respiratory diseases. The chapter begins with an overview and sources of free radicals in the lungs and a brief description of key environmental respiratory diseases mainly COPD, asthma, acute respiratory distress syndrome and idiopathic pulmonary fibrosis. Finally, the chapter reviews the antioxidants associated with lungs and clinical studies testing antioxidant therapy for treatment of respiratory diseases.

5.2 Sources of Free Radicals in Lungs

5.2.1 Free Radicals: ROS and RNS

Free radical is a highly reactive chemical species, which interacts and damages cellular biomolecules (proteins, lipids, DNA and carbohydrates). Free radicals are molecules associated with an unpaired electron in their valency shell, which renders them intrinsically unstable and reactive. In biological systems, free radicals are oxygen-centred radicals and nitrogen-containing radicals collectively called as reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively. ROS comprises of radical species (such as superoxide, hydroxyl radicals, lipid-derived hydroperoxides) and non-radical species (singlet oxygen, hydrogen peroxide and hypochlorous acid and ozone) (Table 5.1) [4, 5]. RNS includes nitric oxide, nitrogen dioxide, nitroxyl anion and peroxynitrite [6]. Elevated cellular levels of ROS and RNS with reduced capacity to detoxify or neutralize them or its derivatives result in a state of oxidative and nitrative stress, respectively.

Low levels of ROS and RNS function as secondary messengers and play a pivotal role in redox cell signalling and regulate diverse beneficial normal physiological processes such as bacterial killing during phagocytosis, vasodilation, tissue repair and regeneration [4, 7, 8]. In contrast, higher levels of ROS/RNS inflict irreversible damage to biomolecules resulting in cell and tissue injury, and if unchecked, it results in onset of inflammatory diseases including respiratory disease [9–11].

Table 5.1 List of ROS and RNS

ROS	RNS
Superoxide ($O_2^{\cdot-}$)	Nitric oxide ($^{\cdot}NO$)
Hydrogen peroxide (H_2O_2)	Nitrogen dioxide ($^{\cdot}NO_2$)
Hydroxyl radical ($HO^{\cdot}$)	Nitrous acid (HNO_2)
Peroxyl radical ($RO^{\cdot}{}_2$)	Dinitrogen tetroxide (N_2O_4)
Alkoxyl radical ($RO^{\cdot}$)	Dinitrogen trioxide (N_2O_3)
Hydroperoxyl radical ($HO^{\cdot}{}_2$)	Peroxynitrite ($ONOO^-$)
Singlet oxygen ($^1O_2$)	Peroxynitrous acid (ONOOH)
Ozone (O_3)	Alkyl peroxynitrites (ROONO)

5.2.2 Sources of ROS and RNS in Lungs

5.2.2.1 Endogenous Sources of ROS/RNS in Lungs

The endogenous source refers to free radicals produced by cells. Adult lung comprises around 40 different cell types [12], which can be broadly classified as epithelial, endothelial and neuroendocrine cells, smooth muscles, fibroblasts and immune cells (macrophages, neutrophils and T cells). All these cells have varied intrinsic capacity to generate ROS upon stimulation. In general, ROS/RNS are produced as intermediates or byproducts of cellular metabolism catalysed by enzymes localized in different organelles primarily plasma membrane, cytosol, mitochondria, peroxisomes and endoplasmic reticulum. The key enzymes that produce ROS/RNS in lungs include cytochrome c oxidase, NADPH oxidase, myeloperoxidase, xanthine oxidase and nitric oxidase synthase. In most of the lung disorders, the mitochondria are primary source of excess ROS [4, 9, 13]. Superoxide produced by mitochondria is a result of incomplete reduction of oxygen to water due to leakage of electrons by mitochondrial respiratory chain [4, 13]. NADPH oxidase (NOX) is a multicomponent transmembrane enzyme complex that generates superoxide as end product via one electron reduction of oxygen [8–11]. In humans, there are seven isoforms of *NOX – NOX1, NOX2, NOX3, NOX4, NOX5,* DUOX1 and *DUOX2*. Nox2 is localized in phagosome of phagocytes (macrophages and neutrophils) and produces massive ROS to kill the phagocytized bacteria. *NOX1, NOX2, NOX4,* DUOX1 and *DOUX2* are distributed in various cell types in the human lung [10, 14]. Myeloperoxidase (MPO), a heme-containing enzyme, is localized in lysosomes of neutrophils and macrophages [11] and catalyses chlorination of H_2O_2 to HOCl, a highly reactive radical [11]. Additionally, MPO catalyses oxidation of thiocyanate to generate hypothiocyanite. Xanthine oxidase, localized in the plasma membrane and cytosol, catalyses oxidation of hypoxanthine to xanthine and to uric acid, during which superoxide is generated [6, 15]. The enzyme nitric oxide synthase catalyses oxidation of L-arginine to citrulline and nitric oxide [7, 16]. Nitric oxide produced reacts with superoxide to form very potent RNS intermediate, peroxynitrite ($OONO^-$). There are three isoforms of NOS, namely, *NOS1* (neuronal NOS, nNOS), *NOS2* (endothelial NOS, eNOS) and *NOS3* (inducible NOS, iNOS). Pulmonary cells constitutively express NOS1 and NOS2, while NOS3 is elevated in the lung during inflammation [7, 16, 17].

5.2.3 ROS and RNS in Lungs: Exogenous Sources

Inhalation of airborne toxicants such as tobacco smoke, biomass fuel smoke, particulate matter and gaseous emissions (nitrogen oxides and sulphur oxides), ozone, chemical toxins, pesticides and pollen grains largely constitutes the exogenous sources of oxidants in lungs [18]. Among the airborne toxicants, ambient air pollution particulate matter and cigarette smoke are the main contributors of free radicals

in human lungs [1, 3, 19, 20]. Depending on the size, the ambient particulate matter (PM) exhibits different physicochemical properties [21, 22]. Coarse particles larger than 10 microns are composed of natural materials (mineral and silicates) and are trapped and cleared by the nose and upper respiratory tract [23, 24]. Fine PM of size 2.5 microns ($PM_{2.5}$) and lesser and ultrafine particles (<0.1 microns) are produced by the combustion process from anthropogenic activities and are composed of polycyclic aromatic hydrocarbons (PAH), metals, minerals, sulphates and nitrates [23, 25]. $PM_{2.5}$ and ultrafine particles could reach deep into distal lungs and cause injury to the alveoli [24]. A single puff of cigarette smoke comprises of 10^{15} free radicals [20, 26] and over 4000 chemicals including epoxides, peroxides, semiquinones, quinones and PAH. The cigarette smoke free radicals are classified into two phases: tar (particle) and gas. Tar phase has 10^{17} relatively long-lived radical molecules per gram such as quinone/hydroquinone (Q/QH2) radicals, which produce $O_2^{\bullet -}$ leading to the generation of H_2O_2 and •OH [27]. Gas phase of cigarette smoke comprises of 10^{15} organic and inorganic radicals per puff and includes NO•, NOx and ONOO– [28]. Ozone is another powerful exogenous oxidant in the lungs [29]. Plant pollens are associated with NADPH oxidase enzyme and are shown to generate superoxide in the airways following inhalation. Besides possessing intrinsic oxidative potential, the airborne toxicants also stimulate production of ROS by activating NADPH oxidase, damaging mitochondria and by recruitment of inflammatory cells (macrophages and neutrophils).

5.3 Oxidative Stress-Associated Pulmonary Disorders

5.3.1 Chronic Obstructive Pulmonary Disease (COPD)

As per the recent estimates by a global burden of disease study [1], COPD kills over three million people and is the third leading cause of death. COPD is characterized by progressive, irreversible limitation in expiratory airflow and abnormal lung inflammation. The disease process involves airway inflammation and remodeling, mucus hypersecretion, loss of the terminal bronchioles and destruction of the lung parenchyma [30, 31]. Tobacco smoking is the primary causal agent of COPD; however, recent epidemiological evidence suggests that exposures to indoor and outdoor air pollutants are also a major etiological factor, especially in developing countries. Genetic polymorphisms in genes coding for alpha-1 antitrypsin, metalloproteinase 33, superoxide dismutase-3 (SOD3) and tumour necrosis factor-α (TNFα) are additional risk factors in 10%–20% of the smokers for developing COPD [32–37]. Although the underlying pathophysiological mechanisms are complex, the lungs of patient with COPD show persistent oxidative stress, increased levels of proinflammatory cytokines, increased CD4 and CD8 cells, elevated levels of proteases and increased apoptosis and senescence [38–44]. Oxidative stress-driven mechanisms are strongly implicated in the initiation and progression of COPD [45].

5.3.2 Asthma

Asthma is a very common airway disorder affecting both children and adults worldwide. Clinically, asthmatics show airway hyperresponsiveness, reversible airflow obstruction and abnormal airway inflammation [46]. Genetic factors in the combination of environmental exposures increase the risk of asthma. The common environmental agents that trigger asthma include aeroallergens (indoor and outdoor), tobacco smoke, dust, air pollutants, cold air and viruses. The hallmark pathological features of asthmatic airways include airway remodeling, epithelial desquamation, goblet cell hyperplasia and inflammation and are associated with eosinophils, mast cells, neutrophils, macrophages and T helper type 2 cells (Th2 cells) [47]. In asthmatics lungs, allergen exposure triggers immunoglobulin E (IgE) production from B cells, degranulation of mast cells and infiltration of eosinophil into airways. These events are mediated by Th2 cytokines, namely, IL-13, IL-4, IL-5 and IL-9. Th1 cytokines (IFN-g, IL-12) and Th17 cytokines (IL-23) are implicated in severe or steroid resistance asthma, which is characterized by high neutrophilic inflammation. ROS through direct injury to airway epithelial cells and via redox signalling mechanism are shown to enhance sensitivity to allergens and augment Th2/Th1 cytokine secretion and, therefore, intricately involved in the pathogenesis of asthma [48].

5.3.3 Acute Respiratory Distress Syndrome (ARDS)

Acute lung injury and its severe form ARDS are common complications in patients admitted to intensive care unit. ARDS results from direct or indirect injury to lungs. Direct injury may be caused by gastric aspiration, pneumonia, inhalation of injuries gases and pulmonary contusion. Indirect injury includes sepsis, pancreatitis and trauma. ARDS is characterized by alveolar flooding with protein-rich oedema followed by a progressive fibrotic phase [49]. Death among the patients with ARDS is mainly due to respiratory failure and/or multiorgan failure. Pathogenesis of ARDS involves an early injury to alveolar epithelium and capillary endothelium, which results in leakage and flooding of alveolar and interstitial spaces with protein-rich oedema. This is also accompanied by a massive influx of neutrophils into alveolar and interstitial spaces. Neutrophils secrete proteolytic enzymes (elastase and metalloprotease), ROS, proinflammatory mediators and further lung injury [50]. The early inflammatory exudative phase is followed by a fibroproliferative phase in which fibroblast and myofibroblast infiltrate and proliferate within the alveolar and interstitial spaces leading to lung fibrosis [51]. Oxidative stress is shown to mediate epithelial-endothelial barrier dysfunction and perpetuate inflammation in ARDS patients [52, 53].

5.3.4 Idiopathic Pulmonary Fibrosis (IPF)

IPF is a progressive interstitial pulmonary fibrosis disorder with no known causal etiological factor. The lungs of patients with IPF show excessive deposition of matrix proteins such as fibronectin and collagen in the alveoli and lung parenchyma, which destroys the gas exchange surface leading to respiratory failure [54]. It is more prevalent in the USA and Europe than South America and South Asia. IPF disproportionately affects individuals above age 65 years, and often it is referred to as age-related disorder. Pathogenesis of IPF involves chronic insult to alveolar epithelial cells (AEC), senescence of AEC and fibroblast, increased differentiation of fibroblast to myofibroblast [54] and increased accumulation of myofibroblast, which is mediated by oxidative injury, mitochondrial dysfunction, proteotoxicity and endoplasmic reticulum stress [14, 55]. Emerging evidences implicate ROS by Nox4 as a key player in the pathogenesis of IPF [55].

5.4 Oxidative Stress-Driven Mechanisms in Lung Diseases

5.4.1 Oxidative Stress and Lipid Peroxidation

Lipid peroxidation (LPO) in biological systems refers to the oxidation of cellular membrane lipids; and uncontrolled LPO is the most significant early biological process induced by oxidative stress state. Excess LPO results in defective or dead cell, inactivation of critical proteins and activation of proinflammatory responses. Together, these events not only initiate but also ensue disease progression. Numerous studies have overwhelmingly showed that LPO is a universal pathogenic event in all the respiratory diseases including COPD, IPF, ARDS and asthma [56].

Membrane lipids mainly glycerophospholipids (PL) esterified with polyunsaturated fatty acid (PUFA) and to a lesser extent free PUFA are the targets for oxidation. Free PUFAs released by action of phospholipases inside the cells are substrates for enzymes such as cyclooxygenase, lipoxygenase and cytochrome P450s, and hence, free PUFA undergoes enzyme-dependent peroxidation. PL-PUFAs are predominantly oxidized by non-enzymatic process and highly depend on the radical species. Both radical species ($^{\bullet}OH$ and $O^{-}_{2}{}^{\bullet}$) and non-radical species (H_2O_2, HOCl, ozone and singlet oxygen) may oxidize PL-PUFA in selective or non-selective manner. The chemical reactions mediating the oxidation of PL-PUFA or free PUFA are similar; however, the products generated may vary. LPO process involves three phases – initiation, propagation and termination. During the initiation phase, non-radical lipid molecule becomes a lipid radical. The radical species abstract hydrogen from bisallylic methylene and produce a carbon-centred radical ($L^{\bullet}$) within PUFA, which subsequently reacts with molecular oxygen and forms lipid peroxyl ($LOO^{\bullet}$) radical. During the propagation phase, the $LOO^{\bullet}$ radical abstracts hydrogen from bisallylic methylene of another PUFA molecule and transforms itself into a lipid hydroperoxide and, concomitantly, generates a new $L^{\bullet}$ radical, and in this manner, the peroxidation chain reaction sustains. During termination process,

antioxidant molecule, such as vitamin E, donates hydrogen, reduces lipid radicals without transforming itself into radical and thus terminates the LPO chain reaction. Lipid hydroperoxides may further participate in additional oxidative reactions such as Fenton reaction catalysed by Fe or Cu, intra- and intermolecular oxidative modification and oxidative fragmentation. Finally, LPO produces diverse reactive aldehyde byproducts including widely studied malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4HNE). The oxidation of PL-PUFA also yields diverse species of oxidized phospholipids (Ox-PLs) (aldehyde, alkene, peroxyl and alkane derivatives), which exhibit varying carbon chain length, hydrophobicity, reactivity, physical stability and biological activity [57]. The oxidized PUFA chain of glycerophospholipid may be released by the action of enzymes such as phospholipase A2 and PAF-acetyl hydrolases [57]. Since the cell membranes are composed of 40–50% of phosphocholine (PC) phospholipids, PC-derived Ox-PLs species are the most abundant Ox-PLs detected in injured lungs.

LPO products such as MDA, 4HNE and Ox-PLs produced in the lungs are not bystanders; rather they actively take part in the pathogenesis of lung disease by inducing cell death, epithelial-endothelial barrier dysfunction, inflammation and immune responses [58]. Ox-PLs and 4HNE are shown to mediate cytotoxicity through disruption of membrane integrity and activating cell death signalling programs such as apoptosis [59, 60] and ferroptosis [61, 62]. Ox-PLs generated following particulate matter exposure caused disruption of the endothelial barrier [62]. Ox-PLs are demonstrated to be dominant mediators of acute lung injury following gastric aspiration and viral infection [63].

A large body of evidence has reported elevated levels of MDA, 4HNE or Ox-PLs or their corresponding protein adducts in the bronchoalveolar lavage fluid, lung tissue and/or serum of patients with various lung diseases such as asthma, COPD, ARDS and IPF [43, 64–66]. Immunohistochemical analysis revealed greater accumulation of 4HNE in the airways, alveolar epithelium and inflammatory cells of the lungs of COPD patient when compared to smoker non-COPD patient with similar smoking history [67]. The bronchoalveolar lavage fluid from COPD patients shows higher levels of Ox-PLs when compared to healthy subjects [43, 64, 65]. The lung parenchyma of IPF patients showed greater accumulation of LPO products [54, 55, 68]. In most respiratory diseases, the levels of LPO byproducts increased with the severity of the pulmonary diseases, which suggest that the LPO is the central pathological event.

5.4.2 Oxidative Stress in Activating Inflammatory Response

It is proven beyond doubt that oxidative stress is involved in the initiation, promotion and augmentation of inflammation by affecting multiple redox-sensitive signal transduction pathways, including Toll-like receptor (TLR) signalling, MAPK kinase signalling and inflammasome which ultimately leads to activation of proinflammatory transcription factors particularly nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and AP-1 [32, 69].

TLR signalling is central in activating pulmonary inflammatory responses following infectious stress and oxidative stress [70]. During infection, TLRs recognize highly conserved microbial motifs referred to as pathogen-associated molecular pattern (PAMP) and activate downstream inflammatory signals [71]. In mammals, there are 13 TLRs, which are present either on the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) or in endosome compartment (TLR3, TLR7, TLR8, TLR9) inside the cell. Upon activation by PAMPs, TLRs undergo hetero- or homodimer and trigger a downstream signal transduction by recruiting adaptor molecules, myeloid differentiation factor 88 (MYD88) or Toll-receptor-associated activator of interferon (TRIF). Except TLR3, a majority of TLRs recruit MYD88 that interacts with IRAK4 and phosphorylate IRAK1. Phosphorylated IRAK1 activates TNFR-associated factor 6 (TRAF6) through phosphorylation, which subsequently stimulates protein kinase C and transforming growth factor (TGF)-β-activated kinase 1 (TAK1). Activated TAK1 activates IκK complex and MAPK kinase family members (ERK1/2, p38 MAPK and c-Jun terminal kinase (JNK)) by phosphorylation mechanisms. TLR3 recruits adaptor molecule TRIF and activate TRAF3, which then activates TANK-binding kinase 1 (TBK1). Activated TBK1 initiates interferon regulatory factor 3 to transcribe IFN-beta cytokine which by autocrine or paracrine mechanism elicits interferon signalling pathway activation.

Transcription factor NF-κB is a central node in regulating inflammation leading to the pathogenesis of COPD, asthma, ARDS and IPF. NF-κB family is constituted of five members – NF-κB1 (P50) (its precursor p105), NF-κB2 (P52) (its precursor p100), p65/RelA, RelB and C-Rel – and exists as homo- or heterodimer. Only p65, Rel-B and C-Rel members have a transactivating domain. In an unstimulated cell, the NF-κB dimmer is sequestered in the cytoplasm by one of the three members of IκB protein complex consisting of IκBα, IκBβ and IκBε. Activation of NF-κB may occur through canonical or noncanonical pathways. In canonical pathway, signals elicited by TLR(s) ligands, TNFα or IL-1β converge at IκK complex constituted of IκKα, IκKβ and IκKγ (NEMO). Upon activation IκK complex phosphorylates IκB on serine 32 and serine 36, which results in its proteasomal degradation and subsequently allows NF-κB to translocate into the nucleus. NF-κB binds to κB element in the promoter/enhancer regions and activates transcriptional expression of cytokines, chemokines and adhesion molecules, which are involved in coordinating innate and adaptive immunity [70–72].

Oxidative stress regulates activation of TLR signalling by multiple mechanisms [70]. NADPH oxidase-dependent ROS production is shown to enhance surface trafficking of TLR4 to lipid rafts, thereby augmenting downstream signals leading to hyperactivation of NF-κB [72–75]. Suppression of ROS generation by pharmacological NADPH oxidase inhibitor, genetic ablation of NADPH oxidase or exogenous antioxidants mitigated lipopolysaccharide (LPS)-induced TLR4 trafficking to lipid rafts and diminished downstream inflammatory responses [74, 75]. Genetic ablation of NADPH oxidase dampened lung inflammation and injury in mice exposed to gram-negative bacteria, LPS, TNFα or bleomycin, suggesting a crucial impact of NADPH oxidase-elicited ROS in producing inflammation and associated tissue injury [55, 74, 76–78]. On the other hand, ROS derived from NADPH oxidase

have also been involved in the resolution of lung inflammation [79]. Mitochondria-derived ROS also play a crucial role in enhancing TLR1, TLR2 and TLR4 signalling [80] and production of proinflammatory cytokines [81]. ROS is shown to augment TLR3 signalling partly by increasing the expression of TLR3 [82]. Oxidatively damaged biomolecules also act as danger-associated molecular patterns (DAMPs) and engage TLR4 to activate inflammatory responses. For instance, oxidized phospholipids generated in lungs following exposures to chemicals, bacteria or virus act as DAMPs and activate TLR4 directed inflammatory responses [83], which is shown to play an essential role in the initiation of acute lung injury. In contrast, excess oxidized phospholipids may also dampen TLR4 signalling by directly interacting with LPS-binding protein, CD14 and/or MD-2 and inhibit inflammation and protect from sepsis [84]. Often oxidative or nitrosative modification of proteins gives rise to modified proteins, which act as DAMPs and perpetuate inflammation. For example, protein adducts of 4HNE or MDA are shown to elicit inflammatory and immune responses [85, 86] in the lungs. 4HNE has also been shown to activate NF-κB and p38 MAPK and promote inflammation [56]. *S*-nitrosylation of surfactant-D protein switches pulmonary surfactant protein-D from antioxidant to a proinflammatory mediator [87].

ROS may regulate NF-κB activation by modulating upstream signal kinases (IκK complex) through oxidative modification of signal transducers and/or its binding partners [88]. In early phases of oxidative stress, ROS may enhance NF-κB activation following exogenous stimuli; however sustained oxidative stress may repress NF-κB activation [88]. IκK complex, mainly IκKβ, is highly susceptible for redox modification. Exposures to H_2O_2 induced oxidative inactivation of IκKβ, which prevented phosphorylation of IκB protein and thus blocked TNFα-induced NF-κB activation [89]. In another study, H_2O_2 posttreatment augmented IκK kinase activity in response to TNFα and leads to higher NF-κB activation [90]. H_2O_2 treatment was also shown to enhance NF-κB activation in response to IL-1 cytokine by increasing NF-kappa B-inducing kinase activity [91]. IκKβ is also susceptible for S-nitrosylation, which inactivates IκKβ resulting in inhibition of NF-κB activation [92]. ROS and RNS are shown to directly modify NF-κB or its associated proteins and alter its transcriptional activity. ROS-dependent phosphorylation of serine-276 on REL-A enhanced transcriptional activity of NF-κB [93]. S-nitrosylation of p65 subunit inhibited NF-κB activity. Kelleher et al. [94] demonstrated that p65 subunit is S-nitrosylated in unstimulated lung cells and LPS challenge stimulates denitrosylation of p65 resulting in activation of NF-κB. Excess ROS may also inactivate proteasome, which impedes IkB degradation and thus inhibits NF-κB activation [95]. Certain cellular redox proteins play an important role in modulating upstream pathways leading to NF-κB activation. In the nucleus, thioredoxin binds and protects oxidation of p65 subunit and enhances its DNA-binding activity. It is also shown that thioredoxin mediates denitrosylation of p65 following LPS exposure and facilitates NF-κB activation [94] in the lungs of mice. Pretreatment with antioxidants such as N-acetylcysteine [96], GSH [97, 98] or increased expression of

antioxidant [72, 97, 98] attenuated lipopolysaccharide (LPS)-promoted NF-κB activation emphasizing the role of oxidative stress in regulating NF-κB activity.

Inflammasome is an intracellular multiprotein complex assembled in the cytoplasm, which recognizes microbial or environmental toxins and DAMPs (e.g. ATP) and activates inflammatory responses. Several airborne environmental pollutants such as silica, ozone, particulate matter and tobacco smoke are shown to activate inflammasome, and therefore, inflammasome signalling is implicated in the pathogenesis of several lung disorders such as acute lung injury, pulmonary fibrosis, COPD and asthma [99, 100]. Activation of inflammasome produces active caspase-1 via autoproteolytic cleavage, which then mediates proteolytic cleavage of precursor pro-IL1β and pro-IL18 into biologically active cytokines. Among the inflammasome members, NLRP3 is redox sensitive, and therefore, intracellular ROS generated by NADPH oxidase or mitochondria have been shown to alter the activation of NLRP3 inflammasome [101]. Ablation of NADPH oxidase or depletion of mitochondrial ROS inhibited ATP-induced caspase-1 activation and IL-1β secretion in macrophages [102, 103]. ROS may also alter NLRP3 inflammasome activation by oxidizing redox-sensitive binding partners such as thioredoxin interaction protein (TXNIP) and mitochondrial antiviral sensing (MAVS) protein to NLRP3. TXNIP is a negative regulator of thioredoxin. It is shown that ROS generated in response to a wide range of environmental stimuli oxidizes thioredoxin that liberates TXNIP. The liberated TXNIP interacts with NLRP3 and promotes NLRP3 activation [104]. MAVS regulates type 1 interferon and NF-kB signalling following virus infection. It has been shown that ROS may induce MAVS aggregation [105] on the outer membrane of mitochondria, which enables interaction with NLRP3 and promotes activation.

MAPK kinases, namely, ERK1/2, JNK and p38 MAPK, represent key effectors of signal transduction to activate inflammatory responses in the lungs following exposure to environmental toxicants [106]. In macrophages and bronchial epithelial cells, LPS stimulation induces phosphorylation of p38 MAPK and mediates the generation of numerous proinflammatory cytokines such as TNFα, IL-6, IL-1β [107] and also T cell (Th1 and Th17)-polarizing cytokines such as IFNγ, IL-12 and IL-23 [108]. In cigarette smoke-exposed mouse models, specific activation of p38 MAPK is shown to be a determinant of susceptibility to emphysema [106]. Several investigations suggest that ROS plays a pivotal function in activating and/or perpetuating MAPK kinase signalling. Exposure of cells to H_2O_2 induces phosphorylation of p38, ERK and JNK [109]. Although precisely how ROS activates MAPK kinase is less understood, it is postulated that ROS mediates oxidative inactivation of protein tyrosine phosphatases and MAPK phosphatases, which inactivate MAPK kinase by dephosphorylating [109, 110]. Because MAPK kinase plays a pivotal role in regulating inflammation, kinase inhibitors, particularly p38 MAPK inhibitors, are shown to be promising drug for treatment of airway disorders such as COPD and asthma [111].

5.4.3 Oxidative Stress in Programmed Cell Death

Apoptosis (a programmed cell death) is involved in removing damaged, infected and potentially neoplastic cells, and increased apoptotic cell death is involved in the pathogenesis of several lung disorders. Apoptosis can be activated by several factors including receptor-mediated signals and DNA damage; however, in most cases, ROS functions as an upstream activator of apoptosis. Apoptosis is mediated by extrinsic and intrinsic pathways [40, 112, 113]. The extrinsic pathway is mediated through interaction of death ligands of the tumour necrosis factor (TNF) family (FasL/FasR and TNFũFC;/TNFR1) with their appropriate cell surface death receptors, while non-receptor-mediated stimuli are involved in intrinsic signalling pathways that initiate apoptosis. Oxidative stress may induce these processes by activating several signalling pathways, including MAPK (ERK, JNK and p38), cell-cycle regulators, protein kinase B and caspases [114]. For instance, 4HNE is reported to enhance the mRNA and protein expression of pro-apoptotic regulators/adaptors such as BAX and caspases [59, 115]. Further, 4HNE can directly interact with death ligand (Fas) on the cell membrane and activate apoptotic process [114]. Finally, 4HNE alters cytosolic calcium homoeostasis and mitochondrial calcium uptake, resulting in apoptosis [116]. Similarly, cigarette smoke exposure is shown to activate apoptosis via ROS by activating MAPK/STAT1 pathway [117]. Several studies have reported oxidative stress-dependent apoptosis in pulmonary fibrosis, obstructive airway diseases and ARDS [12, 40, 41, 44, 113, 118].

5.4.4 Oxidative Stress in Mitochondrial Dysfunction

Besides the ‘powerhouse’ (ATP production by oxidative phosphorylation) of the cell, mitochondria physically interact and communicate with other organelles to maintain the metabolic homeostasis and many synthetic processes for normal function and survival of cell [13]. Mitochondria may also sense external stressors and alter its function to mount a protective adaptive stress response program [119]. However, prolonged exposures to environmental toxicants induce mitochondrial dysfunction mainly via oxidative stress mechanisms [120]. Mitochondrial dysfunction may present in the form of increased mitochondrial ROS, diminished oxidative phosphorylation, increased mitochondrial mass, secretion of mitochondrial DAMPs, mitochondrial DNA damage, decreased mitochondrial biogenesis and increased accumulation of defective mitochondria [13, 119, 121]. Several studies suggest that mitochondrial dysfunction is a predominant pathological feature in all lung diseases [13, 120, 121].

In lungs, owing to their dynamic function, alveolar type II epithelial cells, bronchial ciliated epithelial cells, vascular smooth muscle cells and macrophages are richer in mitochondria than other lung cell types. In normal conditions, lung cells preferentially use glucose end product, pyruvate, for oxidative phosphorylation. However, during stressful physiological or pathological conditions (such as increased surfactant production), alveolar type II epithelial cells rely on fatty acids

for energy demand. Under chronic stress conditions, mitochondrial bioenergetic metabolic function may get altered in lung cells. For example, cigarette smoke exposure is shown to damage mitochondrial structure and affect oxidative phosphorylation in lung cells [122]. Likewise, primary bronchial epithelial cells from severe COPD patients showed accumulation of abnormal mitochondria [122]. Airway smooth muscles and diaphragmatic and external intercoastal muscle of patients with COPD are associated with altered mitochondrial oxidative phosphorylation [123]. Bronchial epithelium in asthmatics is associated with reduced mitochondrial oxidative phosphorylation and decreased expression and activity of cytochrome *c* oxidase [120, 124, 125]. To meet the energy demand and mount stress response, chronic stress may also induce mitochondrial biogenesis in lung cells. Alveolar type II epithelial cells showed increased mitochondrial biogenesis during acute lung injury, pneumonia and hyperoxia-induced lung injury [126]. Bronchial smooth muscles of asthmatic airways are associated with increased mitochondrial biogenesis, and this was linked to higher expression of nuclear respiratory factor 1, peroxisome proliferator-activated receptor γ coactivator (PGC)-1α and mitochondrial transcription factor A [127]. Abnormal or defective mitochondria in the cells are constantly removed by a process called mitophagy, which is regulated by PTEN-induced kinase 1 (PINK1). Expression of PINK1 is negligible in healthy mitochondria; however its levels increase on the outer mitochondrial membrane of defective mitochondria, which recruits parkin and autophagy proteins and facilitates mitophagy. Impaired mitophagy leads to accumulation of damaged mitochondria in the cells, which promotes cellular senescence [128, 129]. Increased cellular senescence has been observed in the lungs of COPD and IPF patients [128–130]. Exposures to cigarette smoke in lung cells are shown to inhibit mitophagy, increase accumulation of damaged mitochondria [128, 129, 131] and induce cellular senescence. Alveolar type II cells of IPF patients are associated with abnormal mitochondria due to diminished PINK1 expression [132]. In mouse models, PINK1 knockdown impaired mitophagy and increased accumulation of defective mitochondria and promoted fibrosis in aging lungs [132]. On contrary, increased mitophagy may also contribute to pathogenesis of lung diseases. For example, *Staphylococcus aureus* infection increased mitochondrial expression of PINK1 and mediated acute lung injury, which was ablated in PINK1 knockout mice [133]. Mitochondrial dysfunction may also lead to leakage of cytochrome c, which triggers programmed cell death [116, 134]. Mitochondria have been shown to regulate various forms of cell death such as extrinsic apoptosis, intrinsic apoptosis, necroptosis and pyroptosis [134], and all these forms of cell death have been reported in various lung diseases including COPD, asthma and IPF.

At physiological concentrations, many mitochondrial-derived molecules including ROS help in normal cellular signalling. However, when secreted in excess, mitochondrial-derived molecules act as mitochondrial DAMPs (mtDAMPs) and contribute to lung injury. Mitochondrial DNA (mtDNA), a well-studied mtDAMP, is released by damaged mitochondria, which is shown to engage TLR9 and inflammasome to initiate inflammatory responses in lung cells [135, 136]. Circulatory levels of mtDNA correlate well with severity and mortality in sepsis and ARDS

patients [137]. Excess ATP released in the lungs by dead or damaged cells also acts as mtDAMPs and activates inflammatory response via NLRP3 inflammasome [119, 138]. Elevated levels of ATP are reported in bronchoalveolar lavage fluid of patients with COPD [139] and asthma as well as in mouse models of asthma [140] and pulmonary fibrosis [141]. Other mtDAMPs such as TFAM and N-formyl peptide are also implicated in driving inflammatory responses in lungs [119]. Cardiolipin, a predominant lipid located in mitochondrial inner membrane, is released by damaged mitochondria and acts as mtDAMP. Levels of cardiolipin increase during lung injury and are shown to mediate cell death and activate inflammasome signals [133, 142]. Additionally, cardiolipin in lung fluid was shown to inhibit surfactant activity and worsen lung function in mouse models of pneumonia [143]. Finally, mitochondrial-derived ROS plays diverse roles in the pathogenesis of lung diseases including perpetuating oxidative stress, augmenting TLR-NF-κB signalling and cell death [13, 81, 119, 125, 134, 135, 144].

5.4.5 Oxidative Stress in Promoting Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) is involved in protein biosynthesis and post-translational modifications and perturbation of ER homeostasis results in ER stress which affects both these process. To overcome the ER stress, cells initiate an evolutionarily conserved mechanism called unfolded protein response (UPR). Activation of UPR leads to decrease in protein synthesis by selectively inhibiting translation, increases protein folding machinery and removes misfolded proteins through endoplasmic reticulum-associated degradation (ERAD) pathway [145]. If UPR fails to alleviate ER stress, it activates apoptotic signalling mechanism [146] and, thus, helps in removal of damaged or stressed cells. Chronic ER stress is pathological and is associated in the pathogenesis of many lung disorders. Markers of ER stress are elevated in neutrophil-associated steroid-resistant asthma [147]. In COPD model, cigarette smoke exposure elicited ER stress and apoptosis [148, 149]. ER stress was found to be elevated in lungs of human IPF and murine models of pulmonary fibrosis [146]. ER stress is also reported to be involved in the hyperoxia-induced acute lung injury in neonates [150]. Sustained oxidative stress milieu may promote ER stress by increasing cellular stress and decreasing the efficiency of protein folding pathways [151]. A relationship has been established between ROS generation and activation of ER stress response [152]. NADPH oxidase(s) and mitochondria are reported as a probable ROS source during ER stress.

5.4.6 Oxidative Stress in Epigenetic Alterations

Chronic oxidative stress state may disturb the epigenetic state of the cell by multiple mechanisms. For example, superoxide radicals can directly mediate transfer of a methyl group from SAM to a cytosine residue without the need of DNMT by

deprotonating C5 [153]. ROS may increase DNMT expression and indirectly affect DNA methylation [154]. ROS may also directly or indirectly modify acetylation of histones by modifying activity or the expression of histone methyltransferases (HMTs) and/or histone acetyltransferases (HATs) [155]. On the other side, ROS and RNS are reported to modulate the activity of HDACs (histone deacetylase) that may influence the expression of target genes by removing acetyl groups [156–159] on histones. Recently, various ncRNAs, in particular microRNAs (miRNAs), are regulated by ROS. Interestingly, some miRNAs such as miR-9, miR-21, miR-200 and miR-210 are shown to control cellular ROS levels and are termed as redoximiRs [160]. ROS are also shown to interfere with miRNA biogenesis process as well as miRNA maturation by modulating Dicer and argonaute RISC catalytic component [161]. In the context of the lung, several lines of evidence support that ROS-dependent changes in the epigenetic background play an important role in the pathogenesis of respiratory diseases. For instance, cigarette smoke exposures inhibit HDAC2 enzyme activity through oxidative and nitrosative modification, which leads to enhanced inflammatory responses, senescence and steroid resistance in COPD [158, 159]. Another study shows that Sirtuin 1 promotes lung epithelial cell death following hyperoxia by selectively deacetylating the transcription factor nuclear factor (erythroid-derived 2)-like 2 (NRF2), accompanied by reduced levels of antioxidant enzymes [162]. In case of asthma, one study reported that exposure to environmental particulate matter could lead to demethylation of iNOS gene; subsequently this may lead to increased expression of proinflammatory iNOS, leading to lung inflammation [163]. The involvement of many factors including ROS in epigenetics of IPF has been reviewed [164].

5.4.7 Oxidative Stress in Profibrotic Mechanisms

Fibrotic lungs are associated with increased oxidative stress, as indicated by the elevated levels of biomarkers of lipid, protein and DNA damage, and several reports have implicated ROS in profibrotic processes. Activation and proliferation of fibroblasts/myofibroblasts are thought to be responsible for the excessive synthesis and accumulation of extracellular matrix (ECM) proteins, resulting in fibrosis. During the inflammatory phase of fibrosis, ROS along with growth factors (TGF-β, PDGF and CTGF) and cytokines (IL-6 and IL-13) stimulate fibroblast to produce ECM. Among these, TGF-β is the most dynamic pro-fibrogenic cytokine, which regulates important biological processes such as EMT, fibroblast activation and differentiation and ECM production [165]. ROS may influence the transformation of latent TGF-β complex into its active form, which then binds to its receptors and activates signalling pathways such as SMAD-dependent or SMAD-independent (e.g. MAPK and PI3K) pathways and enhances the transcriptional activity of various profibrotic genes such as α-SMA and COL1 [166]. On the other hand, elevated TGF-β itself reciprocally induces the production of NOX4-dependent ROS [167]. NOX4 is selectively upregulated in the lungs of IPF patients and is associated with the endothelial cell dysfunction and hypoxia [14, 55]. Elevated NOX4-generated

ROS triggers DNA oxidation and activates other ROS-dependent signalling pathways such as JNK and NF-κB [168]. Silencing of NOX4 by siRNA inhibited TGF-β-mediated profibrotic responses in the lungs of mice [55]. Further, NOX4 knockout mice and use of NOX4 inhibitor in mice protected against bleomycin-induced acute lung injury and the onset of fibrosis [76, 169]. In fibroblasts, mitochondrial ROS has been shown to induce the expression of profibrotic genes during fibroblast differentiation [144]. ROS may also modulate integrins, transmembrane receptors that activate FAK, which in turn activate rac1 protein and initiate production of collagen and other profibrotic actors (CTGF and α-SMA) [170]. ROS and RNS may modulate activity of matrix metalloproteinases (MMPs) through the inhibition of cysteine switch and thus influence ECM degradation. ROS are also shown to induce epithelial cell senescence that may result in a diminished capacity for regeneration of epithelium [171]. In IPF, epithelial damage and epithelial cell senescence in the lung are interconnected with increased mitochondrial ROS production. Similarly, in IPF fibroblasts, ROS generation is reported to require for the maintenance and differentiation [172]. Furthermore, oxidative stress may cause ER stress, which facilitates fibrogenesis through activation of EMT, pro-apoptotic pathways and inflammatory responses [146]. In summary, oxidative stress can alter different cellular processes that amplify fibrotic responses.

5.4.8 Oxidative Stress in Airway Mucus Hypersecretion

A thin layer of gelatinous mucus covers the apical epithelial surfaces of mammalian respiratory tract, which forms a protective barrier against airborne microbes and toxins, but conversely, excessive mucus production becomes pathologic in muco-obstructive airway diseases [173]. Mucus is secreted by goblet cells in the airway epithelium and is mainly composed of mucin, which is a large filamentous glycoprotein [174]. Mucus is also rich in antioxidant scavengers such as glutathione, uric acid and ascorbic acid. Chronic airway inflammatory diseases such as chronic bronchitis and asthma are characterized by mucus hypersecretion [175], and ROS (hydroxyl radicals, superoxide anions and hydrogen peroxides) are key regulators of mucus production in goblet cells via transcriptional regulation of mucin genes [176]. Of 12 mucin genes, MUC5AC is a major inducible mucin gene in airways and reported to be highly expressed in muco-obstructive airway disorders [177, 178]. Increased intracellular ROS and exposure to hydrogen peroxide stimulate EGFR tyrosine phosphorylation and subsequent activation of ERK1/2, resulting in increased expression of MUC5AC in lung epithelial cells [176, 179]. Similarly in nasal epithelium, exogenous hydrogen peroxides exposure induces MUC5AC expression through activation of EGFR-ERK1/2 signalling [178]. *Yu* et al. [180] reported that ROS depolymerizes hyaluronan into fragments and these hyaluronan fragments interact with CD44 receptor to activate tissue kallikrein, which cleaves precursors of EGF into mature EGF. Subsequently, mature EGF binds and activates EGFR leading to activation of ERK1/2. In addition, ROS is reported to contribute to goblet cell metaplasia, a major player in mucin overproduction through JAK/STAT

pathway [181]. Likewise, activation of other pathways, such as NF-κB, is also linked to ROS-mediated MUC5AC production in airways. However, the majority of the studies suggest that EGFR is involved in ROS-mediated mucus hypersecretion [173, 177, 178].

5.5 Antioxidant Responses in Lungs

The lungs are exceptionally exposed to greater oxidative environment than other organs. The inhaled toxicants are by themselves oxidants or may induce oxidative stress inside lung cells. To protect from the inhaled environmental oxidants, lungs are endowed with efficient antioxidant defences that includes both non-enzymatic and enzymatic antioxidant defences.

5.5.1 Antioxidant System in Respiratory Tract Lining Fluid

The airways are covered with respiratory tract epithelial lining fluid (RTLF) which forms a physical barrier between the external environment and underlying respiratory tract epithelial cell layer. The respiratory tract lining fluid traps most of the inhaled toxicants, and by the help of mucociliary action, these trapped toxicants are cleared from the lungs. The respiratory tract lining fluid is rich in many non-enzymatic low-molecular-weight antioxidant scavengers, which directly interact and detoxify the inhaled oxidants and thereby prevent the direct contact of inhaled toxicants with the underlying epithelium. The major antioxidant molecules in the RTLF are GSH, ascorbic acid, uric acid and vitamin E (Fig. 5.1). Additionally, airway epithelial cells secrete certain antioxidant proteins into RTLF, which also function as antioxidant scavengers.

5.5.1.1 Glutathione (GSH)

Glutathione, a thiol-tripeptide comprised of glutamate, cysteine and glycine, is the most important antioxidant in RTLF, and its levels in RTLF are close to 100 times more than in the plasma [182]. GSH scavenges a number of ROS products including hydroxyl, H_2O_2, hypochlorous acid and lipid peroxyl radical generated during exposures to inhaled oxidants such as cigarette smoke, ozone and allergens, and therefore, reduced bronchoalveolar lavage GSH levels has been a hallmark feature of many pulmonary diseases including COPD, asthma, ARDS and IPF [183]. Besides scavenging ROS, GSH is a co-substrate for the enzyme glutathione peroxidase and glutathione S-transferase which mediate detoxification of lipid hydroperoxides and xenobiotics, respectively. GSH in RTLF also protects secretory antiproteases such as alpha-1-antitrypsin, alpha-2-macroglobulin and secretory leukoprotease inhibitor from oxidative inactivation [184]. Therefore, a lower level of tissue GSH intensifies oxidant-induced lung inflammatory injury. GSH also maintains thiol status of extra- and intracellular proteins and facilitates post-translational modification of proteins such as S-glutathionylation. Protein S-glutathionylation may alter the function of

many intracellular proteins. For example, S-glutathionylation inhibits DNA-binding activity of p65 or p50 subunits [185], and S-glutathionylation inactivates IκKβ [186] resulting in diminished NF-κB activity.

5.5.1.2 Ascorbic Acid

Ascorbic acid is another major antioxidant in RTLF. It directly reduces the oxidative potential of oxidants present in tobacco smoke [187] or particulate matter [188] as well as inhibits ROS generation by NADPH oxidase activity [189]. Besides reducing the inhaled oxidants, ascorbic acid also reduces oxidized antioxidants in RTLF such as vitamin E, thereby maintaining the total antioxidant capacity of the lungs during oxidative insult. Ascorbic acid is rapidly used in RTLF fluid upon exposures to environmental oxidants including ozone, nitrogen dioxide, particulate matter and tobacco smoke [190–192] as indicated by the depletion of ascorbic acid levels. Supplementation of ascorbic acid protected from cigarette smoke-induced emphysema by inhibiting protein oxidation in mouse models [192] highlighting the antioxidant potential of ascorbic acid in the lungs. Asthmatics are associated with lower levels of ascorbic acid [193, 194], and the beneficial effect of supplementation of ascorbic acid in asthmatics has been mixed and inconclusive [195]. Ascorbic acid has been shown to attenuate acute lung injury caused by inhalation of oxidant chlorine gas [196]. Ascorbic acid may also take part in pro-oxidant activity in the presence of free iron by taking part in Fenton reaction.

5.5.1.3 Uric Acid

Uric acid formed due to purine metabolism is one of the major water-soluble scavengers of singlet oxygen, ozone and peroxylnitrite (ONOO) in RTLF [190] . Uric acid has been shown to be a major antioxidant in nasal secretion [197] and RTLF and helps in the removal of inhaled ozone and neutralizes the oxidative potential of inhaled particulate matter in humans [198]. Uric acid also reacts and neutralizes gaseous free radical nitrogen dioxide [190, 199]. The antioxidant scavenging activity of uric acid greatly depends on ascorbic acid and hydrophilic environment. Uric acid reacts with radical species and forms urate free radical which is then quenched by ascorbic acid. In lipophilic environment, uric acid fails to stop the self-propagating lipid peroxidation reaction. Subnormal levels of serum uric acid were associated with greater risk for COPD and greater morbidity, including reduced 6-minute walk test and greater burden of exacerbations [200, 201].

5.5.1.4 Vitamin E

Vitamin E (tocopherol) is a lipophilic antioxidant scavenger in RTLF which neutralizes ROS and attenuates self-propagating lipid peroxidation reactions in the airways. Patients with asthma and COPD are associated with lower serum levels of vitamin E [193] as compared to healthy subjects, and this formed the basis for vitamin E supplementation trials to prevent respiratory diseases. Vitamin E trials reduced levels of markers of oxidative damage in smokers [202]. Dietary intake of vitamin E improved lung function in healthy aging population [203]. Vitamin E supplements reduced endotoxin-induced sputum eosinophilia in asthma patients

[204]. In experimental mouse models, administration of vitamin E isoform γ-tocotrienol protected from cigarette smoke-induced emphysema [205] and dust mite-induced asthma. However, supplementation of vitamin E showed no benefits in the management or treatment of asthmatics [206].

5.5.2 Lung-Specific Secretory Proteins in RTLF as Antioxidants

Pulmonary surfactant which lines the alveoli surface is composed of a mixture of 90% phospholipids and 10% surfactant proteins. The surfactant proteins included high-molecular-weight hydrophilic surfactant proteins A and D and low-molecular-weight hydrophobic surfactant proteins B and C. Unsaturated phospholipids and surfactant protein are prone for oxidative inactivation following exposures to environmental oxidants such as ozone. Both surfactant proteins A and D exhibited direct antioxidant activity and protected phospholipids and LDL from copper or ferric chloride-induced oxidation [207]. Surfactant proteins A and D also protected macrophages from hydroperoxide-induced cell death [207]. Clara cell-16 (CC16) protein secreted by clara cells also exhibits antioxidant and anti-inflammatory activity [208]. Mice with genetic disruption of CC16 showed elevated oxidative damage and structural injury following exposure to cigarette smoke [209]. Low circulating levels of CC16 are shown to be associated with poor lung function growth in children [210] and smoking-dependent lung function decline in adults [211] as well as patients with COPD and asthma [208].

5.5.3 Enzymatic Antioxidant System in Lungs

Lungs are endowed with robust antioxidant protein defences to minimize oxidative stress caused by airborne environmental toxicants. Major pulmonary antioxidant enzymes include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, hemeoxygenase-1, peroxiredoxin-1, thioredoxin and thioredoxin reductase [40, 41, 212, 213]. Exposures to environmental oxidants such as cigarette smoke lead to the coordinated activation of all these antioxidant proteins [39–41] (as illustrated in Fig. 5.1) and help in efficient detoxification of ROS and lipid peroxides generated in the lungs. The importance of individual antioxidant enzyme has been exemplified using knockout and transgenic mouse models.

All the three isoforms of superoxide dismutase, extracellular-SOD (EC-SOD), copper/zinc-SOD (Cu/Zn-SOD) and manganese-SOD (Mn-SOD), are present in lungs and provide first line of defence against superoxide radicals. The importance of each of the SOD isoforms in protecting lungs from oxidants has been well studied. EC-SOD prevented fibrosis in lungs by inhibiting oxidative degradation of the matrix proteins, type I and type IV collagen [214]. Cigarette smoke exposure and elastase instillation caused greater emphysema in EC-SOD-deficient mice; however transgenic EC-SOD mice were protected from emphysema [215]. Similarly, overexpression of human Cu/Zn-SOD in lungs protected from cigarette smoke-induced

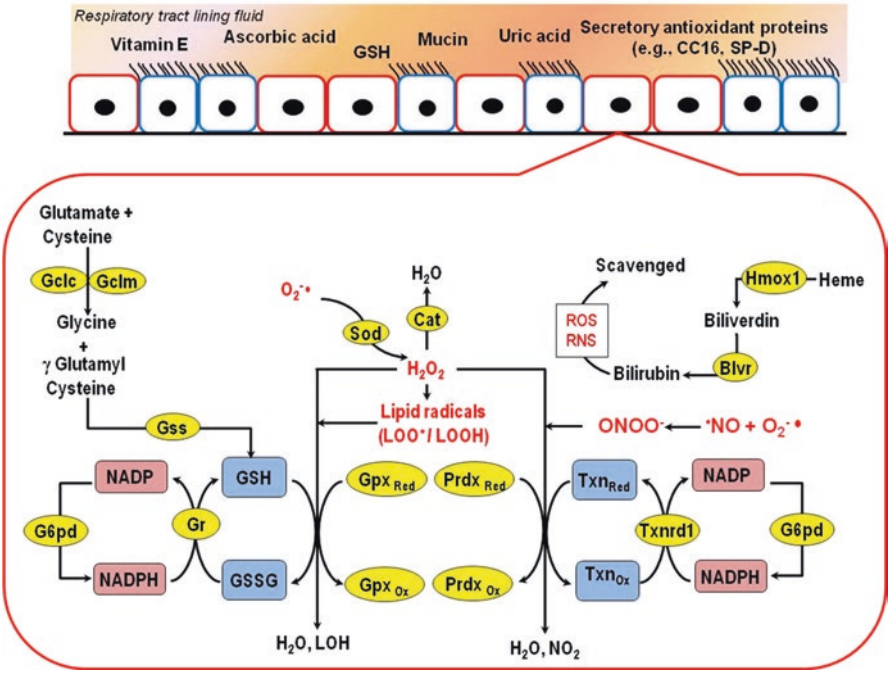

Fig. 5.1 Antioxidant defenses in lungs

emphysema in mouse model [216]. Overexpression of Mn-SOD has also been reported in alveolar macrophages during sarcoidosis and in the lung tissue of IPF patients [213]. SOD is highly sensitive for oxidative inactivation, and therefore, SOD levels are depleted in disease lungs. In IPF, there was no expression of EC-SOD in fibrotic areas where there is an enhancement of oxidative burst [214]. Enzyme activity of SOD was significantly low in asthmatics as compared to healthy subjects, which is further depleted during asthma attack [217, 218]. Oxidative and nitrosative modification of Mn-SOD was observed in the airways of asthmatic, which correlated with asthma severity [217]. Therefore, increasing SOD levels in the lungs by pharmacological approaches including SOD mimetics are thought to be a promising approach for mitigating pathogenesis of pulmonary disorders.

Glutathione peroxidase (GPx) detoxifies hydrogen peroxide and reactive lipid hydroperoxides using GSH as an electron donor. In mammalian lungs, four major selenium-containing GPx isoforms are expressed – *GPX1* (classical GPx), *GPX2* (gastrointestinal GPx), *GPX3* (extracellular GPx) and *GPX4* (phospholipid GPx). Bronchial epithelial cells and alveolar macrophages produce Gpx3 in epithelial lining fluid, which detoxify lipid hydroperoxides generated in RTLF. GPx2 is a predominant glutathione peroxidase expressed in the lungs following cigarette smoke exposure and silencing GPx2 by RNA interference enhanced cytotoxicity in bronchial epithelial cells following treatment with cigarette smoke extract [42]. In comparison with wild type, GPx2-deficient mice showed greater levels of oxidative

damage, airway inflammation and airway hyperresponsiveness in ovalbumin-induced asthma mouse model [219]. Basal levels of GPx in lungs were shown to be a key determinant of severity of pulmonary fibrosis in mouse models [220]. Peroxiredoxins are the family of peroxidase enzymes, which play a dominant role in detoxification of hydrogen peroxide within the cells. Human lung expresses all the six members of a peroxiredoxin family [221]. Thioredoxin reductase is a selenium-containing flavoprotein oxidoreductase enzyme expressed in mammalian lungs, which primarily catalyses NADPH-dependent reduction of thioredoxin, an important redox protein involved in transcriptional regulation of NF-κB [94]. Heme oxygenase-1 (HO-1) is a highly inducible protein in the lungs which exhibits anti-apoptotic, anti-inflammatory and antioxidant activities. HO-1 catalyses heme to carbon monoxide and biliverdin and the latter is converted to bilirubin. Although the mechanism by which HO-1 mediates antioxidant and anti-inflammatory activity is less understood, the end byproducts of HO-1 enzyme activity, CO, bilirubin and Fe are shown to mediate the beneficial effects [222]. HO-1 knockout mice display greater inflammation, apoptosis and tissue injury following an ischemic reperfusion injury [223], while lung-specific expression mitigated LPS- and hyperoxia-induced lung inflammation [224, 225].

5.5.4 Regulation of Antioxidant Enzymes in Lungs

Many lines of evidence show that transcription factor Nrf2 is a central regulator of nearly all cellular antioxidant proteins in the lungs and other organs [226]. In a normal cell, Nrf2 is held in the cytoplasm by a cysteine-rich, redox sensor Keap1 protein, which functions as an adaptor molecule and bridges Nrf2 with Cul3-based E3 ubiquitin ligase [227, 228]. Under normal condition, Keap1-Cul3-based E3 ubiquitin ligase ubiquitinates Nrf2 and directs it to proteasomal degradation. However, upon exposure to ROS and electrophiles, Keap1 protein undergoes conformational change due to oxidative modification of its cysteine residues, which disrupts the interaction of Nrf2 with Cul3-dependent E3 ligase and prevents Nrf2 ubiquitination. Stabilized Nrf2 moves into the nucleus and mediates transcriptional activation of its target genes by binding to cis-element called ‘antioxidant response element’ in the promoter region. Genetic disruption of Nrf2 ablates transcriptional induction of antioxidant genes in the lungs and sensitizes the mice to several environmental lung diseases such as cigarette smoke-induced emphysema [41], allergen-induced asthma [229], LPS-induced acute lung injury [97] and bleomycin-induced pulmonary fibrosis [230] and sepsis [72, 74, 231]. In contrast, activation of Nrf2 by pharmacological activators and genetic disruption of Keap1 protected mice from development of these pulmonary diseases [226]. Nrf2-regulated antioxidant has been shown to be downregulated in lungs of patients with COPD [232] and IPF [233], underscoring the importance of Nrf2 pathway in protecting the lungs from oxidative stress. Besides Nrf2, NF-κB and AP-1 also regulate transcriptional expression of certain antioxidant genes in the lungs [88, 234].

5.5.5 Antioxidant Therapy for Lung Diseases

Despite the compelling evidence from preclinical and clinical studies that pulmonary antioxidants play a pivotal role in protecting from environmental pulmonary diseases, clinical trials testing antioxidant therapy have shown modest to no significant beneficial effects. Clinical trials with N-acetyl-L-cysteine (NAC) supplementation have shown mixed results. Meta-analysis of all clinical trials using oral NAC concluded that long-term intake of NAC may reduce the acute exacerbations of chronic bronchitis [235]. However, more recent randomized double-blinded multicentre clinical trial of oral NAC reported no beneficial effect in the prevention of lung function decline and COPD exacerbation [236]. Vitamin C and E clinical trials showed no improvement on lung function decline in COPD patients [237]. Supplementation of Nrf2 activator, sulforaphane, in the form of broccoli sprout homogenates reduced bronchoconstrictor hyperresponsiveness in asthmatics [238]. In a randomized clinical trial, supplementation of sulforaphane showed no significant upregulation of Nrf2-regulated antioxidants in lungs of COPD patients [239]. In another study, consumption of broccoli sprout showed no effect on eosinophilic inflammation as well as markers of oxidative stress in atopic asthmatic patients [240]. In smokers, consumption of broccoli sprout homogenates reduced influenza virus-induced inflammation [241]. Consumption of broccoli tea has been reported to promote rapid and sustain detoxification of air pollutants in a randomized clinical trial in China [242]. The reasons for lack of consistent benefits of antioxidant trials for pulmonary disease are still puzzling. Perhaps it could be combination of poor efficacy of a single antioxidant agent as well as limited bioavailability.

5.6 Conclusions

Oxidative stress is a central hallmark pathological feature of all the respiratory disease. Oxidative stress elicits both reversible and irreversible macromolecule damage (oxidative modification of lipids, proteins and DNA). As illustrated in Fig. 5.2, besides inducing macromolecular damage, oxidative stress propagates the disease by augmenting other pathological processes such as inflammatory responses, mitochondrial dysfunction, ER stress, profibrotic signalling, cell death and epigenetic changes. Experimental evidences suggest that antioxidant therapy may prevent or mitigate oxidative stress-mediated macromolecular damage and abnormal signal transductions and, thereby, protect from development and progression of disease. However so far, most antioxidant clinical trials have shown poor efficacy to mitigate disease progression, which may be attributed to insufficient bioavailability of antioxidant agent in the lungs and also inability to reverse pathogenic events such as epigenetic changes, macromolecule damage and mitochondrial dysfunction. Antioxidant alone may not be effective in treatment of diseases; however it could be a promising adjunctive therapy.

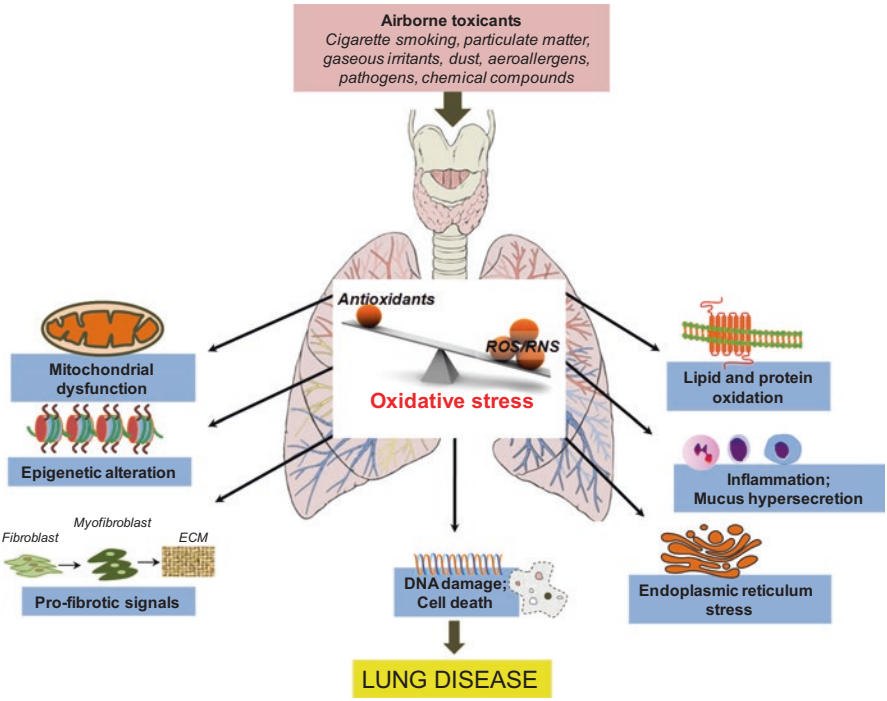

Fig. 5.2 Oxidative stress-mediated pathological events in driving lung diseases

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Part II

Chronic Lung Diseases

Oxidative Stress-Induced Mitochondrial Dysfunction and Asthma

6

Samarpana Chakraborty, Kritika Khanna, and Anurag Agrawal

Abstract

Asthma is a well-recognized global concern with ever-increasing prevalence and economic burden worldwide. Genetic susceptibility and exposure to environmental triggers such as allergens, pollutants, infectious agents and even lifestyle choices are well-established modulators of the disease. Recent studies show that irrespective of the nature of causal trigger (allergic or nonallergic), mitochondria and its dysfunction is a central player in asthma pathogenesis. This chapter discusses the studies and mechanisms through which mitochondria plays its role in causing asthma pathogenesis. Under allergic asthma conditions, immune response and epithelial barrier functions are the key players modulating the function of mitochondria. Other mechanism that leads to the development of obese-asthma phenotype involves disruption of cellular bioenergetics via modulating nitric oxide levels, calcium homeostasis, etc. Repair, reprogramming and/or replacement of the dysfunctional mitochondria are some of the possible therapeutic strategies for better management of asthma.

Keywords

Allergic asthma · Obese-asthma · Mitochondrial dysfunction · Oxidative stress · Immune responses · Epithelial barrier function · Nitric oxide metabolism · Lipoxygenase pathway · Obesity · Metabolic syndrome · ADMA

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Abbreviations

12/15-LOX	12/15-lipoxygenase
12-S-HETE	12-S-hydroxyeicosatetraenoic acid
13-S-HODE	13-S-hydroxyoctadecadienoic acid
ADMA	Asymmetric dimethylarginine
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CLR	C-type lectin receptors
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DRP1	Dynamin-related protein 1
eNOS	Endothelial nitric oxide synthase
ETC	Electron transport chain
FADH2	2-Dihydro flavin adenine dinucleotide
HDM	House dust mite
IgE	Immunoglobulin E
IL	Interleukins
LPS	Lipopolysaccharide
MCU	Mitochondrial calcium uniporter
MetS	Metabolic syndrome
MHC II	Major histocompatibility complex class II
MSC	Mesenchymal stem cell
mtDNA	Mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
nDNA	Nuclear DNA
NF-kβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFP	N-Formyl peptides
NLR	Nucleotide-binding domain/leucine-rich repeat receptor
NLRP3	Nod-like receptor family pyrin domain containing 3
NO	Nitric oxide
NRF-1	Nuclear respiratory factor-1
$OONO^-$	Peroxynitrite
OPA1	Optic atrophy protein 1
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PC	Phosphatidylcholine
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator
PKC-δ	Protein kinase C delta type
PQQ	Pyrroloquinoline quinone
PRR	Pathogen recognition receptor
RLR	Retinoic acid-inducible gene (RIG-1)-like receptors

ROS	Reactive oxygen species
siRNA	Small interfering RNA
TCR	T-cell receptor
TFAM	Mitochondrial transcription factor A
Th2	Type 2 T helper cells
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TRPV1	Transient receptor potential cation channel subfamily V member 1
UQCRC2	Ubiquinol-cytochrome c reductase core protein II

6.1 Introduction

Asthma is a chronic disorder presenting features such as airway inflammation, reversible airflow obstruction and enhanced bronchial reactivity. With as many as 339 million people affected with asthma worldwide, its global disease burden is increasing substantially. Poor management of the disease has been associated with reduced quality of life and even premature death in people of all age groups around the world. Asthma has multifactorial disease aetiology with both genetic and environmental factors playing significant roles [1]. It has now also been recognized as a complex, heterogeneous disease with multiple clinical presentations and physiological characteristics. Depending on consistent clinical features, a common natural history (like age at onset) and predictable responsiveness to therapies, asthma has been classified into various subgroups or phenotypes [2]. Due to such underlying heterogeneity, some asthma patients do not respond well to the classical interventions of bronchodilators and corticosteroids. However, detailed asthma research is now unravelling newer mechanisms of asthma pathogenesis that can provide novel handles for treatment of various sub-phenotypes of the disease.

Interestingly, recent studies, in both allergic and nonallergic obese-asthma, have shown mitochondrial dysfunction to be central to disease pathogenesis [3–5]. Mitochondria are no longer thought to be only involved in cellular bioenergetics but have been shown to have significant roles in multiple cellular processes like biosynthesis, signal transduction, danger sensor and cell death pathways [6]. With such multifaceted roles, any oxidative stress-induced mitochondrial dysfunction can perturb the cellular homeostasis at various levels. Thus, mitochondrial function impairment is now a prominent signature of various inflammatory, respiratory, cardiovascular, metabolic, neurodegenerative and infectious diseases [6–8].

In this chapter, we look into the experimental studies connecting mitochondrial dysfunction with allergic or nonallergic obese-asthma. In allergic asthma, there seems to be a bidirectional interplay, wherein allergens have been shown to induce mitochondrial dysfunction, and pre-existing defects in mitochondria have aggravated the allergic asthma phenotype [5]. Further, delving deeper into the underlying mechanisms of mitochondrial dysfunction and allergic asthma, we explore the association with immune response modulation and epithelial barrier function. As for the

obese-asthma phenotype, we see that mitochondria act as a common thread connecting asthma, obesity and metabolic syndrome [9]. Common molecular mechanisms like elevated expression of 12/15-lipoxygenase and perturbed nitric oxide metabolism lead to mitochondrial dysfunction in both obesity and asthma, thus placing mitochondria at the hub of obese-asthma pathophysiology [3]. Finally, we look into the various strategies to restore the function of mitochondria, mainly by repairing, reprogramming and replacing damaged mitochondria, as novel tools in asthma therapeutics.

6.2 Asthma

Asthma is a chronic disorder of the airways characterized by four major hallmarks, namely, increased airway inflammation, reversible airway obstruction, enhanced airway responsiveness and mucus hypersecretion. It is also accompanied by airway remodelling and epithelial barrier dysfunction. Currently, more than 300 million individuals are affected with asthma worldwide [10], and it is estimated that the numbers will increase up to 400 million by 2025.

Genetic predisposition along with environmental insults, like exposure to smoke, pollution, lifestyle, obesity and house dust mites, are the main causal factors for asthma. It is broadly classified into two types: atopic and nonatopic. Atopic asthma, triggered by allergic reaction against a plethora of reactants like house dust mites, animal dander, pollens and fur, leads to an increase in the level of serum IgE. In allergic asthma, there is strong involvement of the immune system. Allergens are recognized by membrane-bound pathogen recognition receptors (PRRs) such as C-type lectin receptors (CLR), toll-like receptors (TLRs) and nucleotide-binding domain/leucine-rich repeat receptors (NLRs). Interaction of these PRRs with allergen induces immune tolerance in healthy individuals, but in asthmatics, they activate cascade of pathways causing inflammatory immune responses [11].

Antigen-presenting cells especially dendritic cells (DCs) act as a connective link between the innate and adaptive immune responses. They pick the external allergens which are then transported to the local lymph node. After processing, the major histocompatibility complex class II (MHC II) molecules present them to the naïve T cells. Upon binding of allergens, DCs drive the differentiation of naïve T cells into T helper cell type 2 (Th2) cells that secrete pro-inflammatory cytokines IL-4 and IL-13 that recruits inflammatory cells leading to an inflammatory cascade. The primed Th2 cells also activate B cells and convert them to plasma cells causing secretion of allergen-specific IgE antibodies [12]. The IgE antibodies then bind to their receptors present on mast cell surface causing allergen sensitization. Re-exposure of the body to that allergen mediates cross-linking of these IgE receptors, leading to mast cell degranulation and subsequent release of histamine and other mediators that lead to vasodilation, smooth muscle contraction and increased capillary permeability. This process termed as immune hypersensitivity plays a key role in eliciting immune response in allergic/atopic asthma.

Nonatopic asthma, on the other hand, is caused by factors such as occupation, extreme weather conditions, heightened emotions, lifestyle choices and obesity. Neither allergen-specific serum IgE nor total IgE levels are seen to increase in patients with nonatopic asthma. Therefore, they show negative skin test result against common allergens. In addition, clinical or family history of allergy is not observed. Initially, it was hypothesized that development of nonatopic asthma involves mechanisms other than inflammation or immune response. However, studies now demonstrate involvement of different immune modulators in nonatopic asthma as compared to atopic asthma. Increase in the numbers mast cells, eosinophils and T lymphocytes characterize the airway inflammation in atopic asthmatics, whereas nonatopic asthmatics mainly display high numbers of neutrophils and mast cells, possibly via non-IgE-mediated hypersensitivity responses in airways [13]. However, the pathophysiological mechanisms guiding nonatopic asthma are poorly defined.

6.3 Mitochondria

Human mitochondria are essential organelles of the cells that have been thought to have a bacterial descent. According to the endosymbiotic theory, alpha-proteobacterium was endocytosed by host cells approximately 2 billion years ago. The resultant double-membrane organelle was semi-autonomous in nature, having its own genetic material and the ability to code for some proteins using its own transcription and translational machinery [14].

Mitochondria have classically been thought to be the powerhouses of the cell as they generate energy through oxidative phosphorylation. Citric acid cycle in the mitochondrial matrix leads to the formation of metabolites and reducing equivalents like NADH and $FADH_2$. Thereafter, in the inner mitochondrial membrane, electrons move from these energy carriers through a series of complexes (complex I–IV), to the final electron acceptor – molecular oxygen. The proton motive force generated by this electron flow powers the production of ATP from ADP and P_i [6]. Apart from their central function in bioenergetics, mitochondria are also essential to maintaining cellular homeostasis via biosynthesis. With important roles in synthesis of heme molecules and assembling of the iron–sulphur clusters, mitochondria are critical to regulation of iron metabolism [15]. Moreover, the metabolites produced during citric acid cycle also serve as precursors for synthesis of macromolecules like carbohydrates, lipids and proteins. In recent studies, mitochondria have also come up as essential signalling organelles [16]. The highly oxidative environment in mitochondria leads to the generation of reactive oxygen species like superoxide ($O^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). While, under pathological conditions, excessive production of ROS causes damaging effects in the cell, under physiological conditions, mitochondrial ROS (mROS) act as signalling molecules. mROS relay signals between the mitochondria and rest of the cell and are essential for regulating innate and adaptive immune responses, oxygen sensing, stem cell proliferation and hormone signalling [17]. Thus, this delicate balance of the mROS levels is critical to cellular homeostasis and is termed as mitochondrial hormesis or

mitohormesis [18]. Mitochondria also play a central role in apoptosis by involvement via disruption of electron transport chain, release of caspase-activating proteins in the cytoplasm and alteration of cellular redox potential [19].

Since mitochondria are at the nexus of important cellular processes, there exist multiple quality control measures to ensure higher mitochondrial fitness. One of the key pathways in place is mitophagy whereby the damaged, depolarized mitochondria are autophagocytosed, enabling mitigation of mitochondrial stress [20]. The dynamic nature of mitochondria – with continuous fission and fusion – also facilitates stress attenuation. While mitochondrial fusion is regulated by proteins, optic atrophy 1 (OPA1) and mitofusin 1 and 2 (Mfn 1/2), mitochondrial fission is controlled by dynamin-1-like (DRP1) and fission 1 (Fis1). Fusion of mitochondria reduces the heterogeneity of mitochondria and helps in diluting the mtDNA mutations and oxidized proteins. Mitochondrial fission also supports quality control. DRP1-mediated asymmetric fission enables segregation of healthy, polarized mitochondria from the abnormal, depolarized portion. The healthy part can reintegrate into the mitochondrial network, whereas the depolarized part is removed through mitophagy [21]. Another important quality control pathway is the mitochondrial unfolded protein response which enables communication between the nuclear and mitochondrial genomes and maintains the mitochondrial proteome [22].

Even with such quality control measures in place, when the mitochondrial stress exceeds a critical point, mitochondrial function gets affected leading to disruption of energy production, alteration of cellular metabolic profile, dysregulation of signal transduction pathways and eventual setting in of apoptosis. Any cellular injury or stress also leads to release of mitochondrial components like mtDNA, ATP, mitochondrial N-formyl peptides and mitochondria-specific phospholipid cardiolipin in the cytosol or the extracellular milieu. Due to microbial origin of mitochondria, these components have features akin to their bacterial counterparts and thus act as damage-associated molecular patterns (DAMPs) [23–25]. These mito-DAMPs are recognized by pathogen recognition receptors (PRRs) like NLRP3 inflammasome in the cytosol or toll-like receptors (TLRs) outside the cell, leading to activation of inflammatory signalling and triggering of immune responses [7]. Overall, mitochondrial dysfunction can disrupt critical cellular pathways and is thus central to the pathogenesis of various diseases like respiratory diseases, cardiovascular diseases, neurodegenerative diseases, autoimmune diseases, inflammatory disorders, metabolic disorders and cancer.

6.4 Mitochondrial Dysfunction in Asthma

6.4.1 Mitochondrial Dysfunction in Atopic/Allergic Asthma: Studies and Mechanisms

Asthma has been shown to have a strong hereditary component. Maternal history of atopy and asthma is considered to be a significant risk factor for development of asthma and other allergic diseases. With maternal transmission of mitochondria, an association of mitochondrial genome with asthma and atopic diseases is indicative.

A significant association has been observed between mitochondrial haplogroup U and elevated serum IgE levels in European population [26]. Moreover, mutations in mitochondrial genes encoding mitochondrial t-RNAs have also been shown to play a role in increasing the risk to asthma [27]. Also, ATP synthase mitochondrial F1 complex assembly factor 1 (ATPAF1) has been identified as a candidate gene for asthma in Caucasian European children [28]. Along with genetic links, functional studies have also shown mitochondrial dysfunction to be associated with asthma and allergies. Structural changes in the mitochondria of bronchial epithelia of asthmatic patients have been reported [29]. In addition, in the murine model of allergic inflammation, swollen and fragmented mitochondria have been observed in the airway epithelium, associated with activation of apoptotic pathways in the lungs and reduction in lung ATP levels [30]. In another study, exposure of airway epithelial cells to ragweed pollen extract was shown to cause oxidative damage to an important structural protein of complex III of the mitochondrial electron transport chain, ubiquinol-cytochrome c reductase core protein II (UQCRC2), which was in turn implicated in mtROS generation. Mitochondrial dysfunction induced by downregulation of this core protein prior to pollen extract exposure in mice led to aggravated allergic response as seen by increased bronchial hyper-responsiveness and elevated mucin secretion [31]. Thus, the study highlights how pre-existing mitochondrial dysfunction can increase the allergic phenotype. Environmental oxidative stress caused by diesel exhaust particles or tobacco smoke can induce mitochondrial dysfunction, further exacerbating the allergic features in asthmatic and atopic individuals.

The above-mentioned studies demonstrate experimental links between mitochondrial dysfunction and asthma. Multiple other studies have delved into the mechanistic connections that make mitochondrial dysfunction central to pathogenesis of allergic asthma. Broadly, defects in mitochondria translate to epithelial barrier disruption and immune response modulations which play an essential role in allergic asthma pathogenesis.

6.4.1.1 Mitochondrial Dysfunction and Epithelial Barrier Disruption

Recent studies have highlighted the crucial role of epithelial cells in asthma pathogenesis. Epithelial cells in the lungs serve as the first line of defence against noxious agents like allergens, cigarette smoke and invading pathogens. These cells act not just as a structural barrier against allergen sensitization but are also actively involved in activating signal transduction cascades and in mounting immune responses [32]. Mitochondria, through their control of apoptosis, have been shown to maintain the epithelial integrity. Mitochondrial calcium uniporter (MCU) is involved in uptake of calcium in the mitochondrial matrix. Calcium overload in mitochondria leads to mtROS generation, membrane depolarization and, ultimately, release of cytochrome c in the cytosol and induction of apoptosis. In a recent study, Sebag et al. demonstrated protective response against mitochondrial dysfunction and cellular apoptosis caused by IL-13 upon downregulation of MCU in the airway epithelial cells. In concert with in vitro studies, in murine model of ovalbumin-mediated allergic inflammation as well, MCU knockout was associated with preserved expression of

tight junction proteins and reduced epithelial cell apoptosis [33]. Another study highlighted the role of a phospholipid transfer protein (Stard7), which enables uptake of phosphatidylcholine in mitochondria, in maintaining mitochondrial homeostasis and epithelial barrier function. In vitro downregulation of Stard7 in bronchial epithelial cells and lung epithelial cell-specific knockout of the protein in mice was shown to alter mitochondrial morphology, change mitochondrial membrane potential and induce mtDNA damage in the cells. This was concomitantly associated with impaired epithelial barrier permeability which was restored by treatment with mitochondria-targeted antioxidant – MitoTEMPO [34].

6.4.1.2 Mitochondrial Dysfunction and Immune Response Modulation

Mitochondrial dysfunction has been shown to activate both innate and adaptive immune responses. Association of mtROS and NLRP3 inflammasome has been reported in allergic asthma [35]. Inflammasome, a part of the innate immune response, is a multi-protein complex that intercepts damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) and causes release of pro-inflammatory cytokines like IL-1β and IL-18 [36]. In ovalbumin (OVA), lipopolysaccharide (LPS) and house dust mite (HDM) induced murine model of allergic inflammation, the mitochondrial ROS levels were found to be elevated in the airway inflammatory and epithelial cells. This was also associated with damage-associated mtDNA in the lungs of these mice. While mtROS has been known to activate inflammasome cascade, mtDNA also acts as a DAMP and coactivates the cascade [37, 38]. In these allergic models, increased mtROS and damage-associated mtDNA have been shown to activate the NLRP3 inflammasome. This was associated with activation and nuclear translocation of NF-κB as well. Treatment of these mice with a mitochondria ROS inhibitor – NecroX5 – led to restoration of these changes and inhibition of the pathophysiological features of allergic inflammation. Blockade of IL-1β also alleviated the airway hyper-responsiveness and inflammation [35]. These observations suggest a central role of inflammasome in the mtROS-driven allergic asthma pathogenesis. On similar lines, another study using OVA- and *Aspergillus*-exposed murine model of allergic inflammation has demonstrated activation of oxidant Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in the mitochondria and increase in mtROS generation. High mtROS was further shown to activate NLRP3 inflammasome and nuclear translocation of NF-κB. There was a concomitant induction of Th2 cytokines and consequent airway inflammation and hyperactivity [39].

Mitochondrial dysfunction can also directly cause mast cell degranulation and enhance Th2 responses. Exposure of the mucosal mast cells to ragweed pollen extract was found to induce generation of mtROS from the mitochondrial respiratory complex III. This oxidative stress further induced secretion of the biogenic amines – histamine and serotonin from mast cells – independent of any IgE-mediated trigger. Concomitantly, the mtROS also led to increased release of IL-4 from the sensitized mast cells [40].

6.4.1.3 Other Studies in Mitochondrial Dysfunction and Allergic Asthma

Exposure of bronchial epithelial cells to allergens like house dust mite has been shown to hamper the mitochondrial dynamics by increasing ER-mitochondrial contacts. Such changes were demonstrated to induce recruitment of mitochondrial fission protein DRP1 and initiate fragmentation of mitochondria. This was associated with release of pro-inflammatory cytokines like IL-8 and IL-1β [41]. In asthmatic lungs, along with epithelial barrier disruption, hypertrophy and hyperplasia of smooth muscle cells are also key pathophysiological features. In airway smooth muscle cells (ASMs) of severe persistent asthma patients, increased number of mitochondria has been observed due to increased expression of proteins involved in mitochondrial biogenesis. This increased mitochondrial biogenesis has been associated with hypertrophy of the ASMs causing airway remodelling. However, in fatal, young asthmatics, such an increase was not observed in the ASMs [42]. Another study in cultured ASMs demonstrates the alteration in mitochondrial calcium flux leading to increase in cytoplasmic calcium levels and eventually promoting ASM contractility [43]. Thus, mitochondrial function, inflammation and ASM contraction are interlinked; however, they have different patterns in different asthma phenotypes.

6.4.2 Obese-Asthma Phenotype: An Overlap Between Metabolic Syndrome and Asthma

The increasing trend of high-calorie diet consumption and lack of physical activities is a mark of the modern lifestyle. Such lifestyle modifications over a prolonged period of time negatively impact the overall health of an individual leading to development of diseases. Metabolic syndrome represents the abnormal metabolic factors that increase the frequency of cardiovascular diseases, such as heart failure, thrombosis and cardiac arrhythmias, along with increase in incidence of type II diabetes. Of the factors that lead to metabolic syndrome (MetS), obesity is a strong risk factor for development of asthma and represents one of the members of the triad, with dyslipidaemia and hyperglycaemia being the other two [44, 45]. A higher intake versus lower consumption of energy leads to an imbalance, causing excessive adipose tissue accumulation and, therefore, obesity. Mitochondrial dysfunction, inflammation and perturbed antioxidant defence functions are the hallmarks of obesity.

Epidemiological studies have found a strong link between asthma and metabolic syndrome. It has been observed that body mass index shows a positive correlation with asthma severity, with abdominal obesity showing stronger association to asthma as compared to general body mass, leading to development of a subset of asthma phenotype termed as obese-asthma [46, 47]. Subjects of this phenotype are of particular concern to physicians since most of them are unresponsive to corticosteroids and conventional anti-inflammatory therapies. Thus, obese people are not

only more prone to asthma, they often experience symptoms of severe asthma with poor disease management. In addition to this, neutrophilic airway inflammation, low-grade systemic inflammation and increased morbidity are commonly observed in these individuals. Asthma and obesity are in fact termed as twin epidemics of the developing world [44, 48, 49]. In two studies in Korean population, one with 10,000 participants and a recent one with 4000 aged subjects, metabolic syndrome was found to be significantly associated with asthma [50, 51].

Obese mice show increased airway hyper-responsiveness even without allergen immunization and develop severe asthma phenotype in response to asthma predisposing factors [52]. Furthermore, obese patients show improvement in asthma on weight loss [53]. This suggests a possible relation between the two diseases. However, the mechanism by which they work is hitherto unknown. While obese-asthma subjects show altered lung and chest wall mechanics, these do not fully explain the development of the phenotype. This is because (1) not all obese subjects develop asthma, (2) subjects with metabolic dysregulation with normal body mass also frequently develop asthma and (3) maternal obesity is observed to increase risk of asthma incidence in offspring, independent of child's own obesity. This suggests the possible involvement of other mechanisms that may lead to either of the two diseases.

6.4.2.1 Mitochondrial Dysfunction in Obese-Asthma (Nonatopic): Studies and Mechanisms

Optimal cellular bioenergetics, the key to good health, is largely dependent on mitochondria and its function. Chronic high nutrient intake overloads mitochondria, increasing ROS levels and therefore oxidative damage that affects mitochondrial integrity and its function. This also leads to activation of stress response pathways and ultimately loss of healthy mitochondria. In fact, mitochondria from obese subjects were found to be smaller, with diminished biogenetic capacity as compared to lean subjects [54]. In genetically obese, as well as diet-induced obese mice, increase in mitochondrial ROS and dysfunctional oxidative respiration have been observed [55, 56]. Also, mitochondrial biogenesis is compromised in obese individuals due to decrease in levels of peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α), the master regulator of mitochondrial biogenesis and also of important transcription factors such as mitochondrial transcription factor A (TFAM) and nuclear respiratory factor-1 (NRF-1). All these provide supporting evidence that mitochondrial dysfunction is a key pathological mechanism in obesity.

Multiple studies have shown how mitochondria and its bioenergetics play a central role in asthma. In 1985, Konradova et al. showed swollen mitochondria in bronchial epithelial cells of three subjects with asthma. Murine models of allergic asthma confirmed the observation and found abnormalities in mitochondrial structure as an integral part of the asthma phenotype [30]. The key inflammatory mediators of asthma, Th2 cytokines like IL-4 and IL-13, were also found to induce mitochondrial dysfunction. Emerging evidence therefore suggests mitochondrial dysfunction to be the common player in development of these seemingly disparate diseases: obesity and asthma.

Many studies have shown alterations in mitochondrial genome to be associated to metabolic syndrome. Although the ratio of mitochondrial DNA to nuclear DNA (mtDNA/nDNA) is shown to be markedly reduced, any major genomic deletions are not observed. Few polymorphisms in mtDNA that are also risk factors for metabolic syndrome have been reported, for example T16189C in both Caucasian and Turkish patients and A10398G in Chinese population [57, 58]. These variants may be responsible for increased damage to mitochondria, their accelerated clearance and reduced bioenergetics.

6.4.2.2 Molecular Mechanisms of Mitochondrial Dysfunctions in Obese-Asthma

Molecular mechanisms through which asthma, obesity and metabolic syndrome are connected involve proteins and metabolites involved in dysregulation of mitochondrial functions. These are discussed below:

(A) Increase in expression of a non-heme iron dioxygenase, 12/15-LOX, that catalyses hydroperoxidation of polyunsaturated fatty acids in adipocytes is observed in mice fed on high-fat or western diet. Protection from obesity-related complications was observed on fat-specific deletion of 12/15-LOX, and its deficiency reduced macrophage infiltration in adipocytes, decreasing tissue inflammation [59–62]. Also, asthmatic features were seen to be alleviated on genetic ablation of the enzyme, 12/15-LOX. Also, the enzyme 12/15-LOX and its metabolites such as 13-S-hydroxyoctadecadienoic acid (13-S-HODE) and 12-hydroxyeicosatetraenoic acid (12-S-HETE) may cause mitochondrial degradation and dysfunction in airway epithelium of asthmatics by activating TRPV1 channels, thereby modulating calcium homeostasis [63]. These suggest mitochondrial dysfunction via change in expression of 12/15-LOX to play a role in development of the obese-asthma phenotype.

The increase in 12/15-LOX in obese mice also causes ER stress and unfolded protein response (UPR) which further leads to macrophage infiltration causing activation of pro-inflammatory cytokines such as TNF-α and IL-1β in adipocytes. These mediators reduce the expression of endothelial nitric oxide synthase (eNOS), thereby causing decrease in nitric oxide (NO) production. This in turn compromises mitochondrial biogenesis by inhibition of PGC-1α. The reduction in mitochondrial biogenesis hampers β-oxidation of fatty acids, causing their accumulation in adipocytes. The resultant adiposity and release of free fatty acids cause mitochondrial dysfunction, impaired oxidative phosphorylation and elevated level of ROS. Further, the lipid overload increases ER stress, decreasing eNOS, thereby leading to a vicious cycle ([64] Fig. 6.1).

(B) Perturbation of the nitric oxide metabolism is also an important pathophysiological mechanism connecting asthma and obesity. Using L-arginine as a substrate, NO is synthesized by enzymes called nitric oxide synthase (NOS) which are of three types: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS).

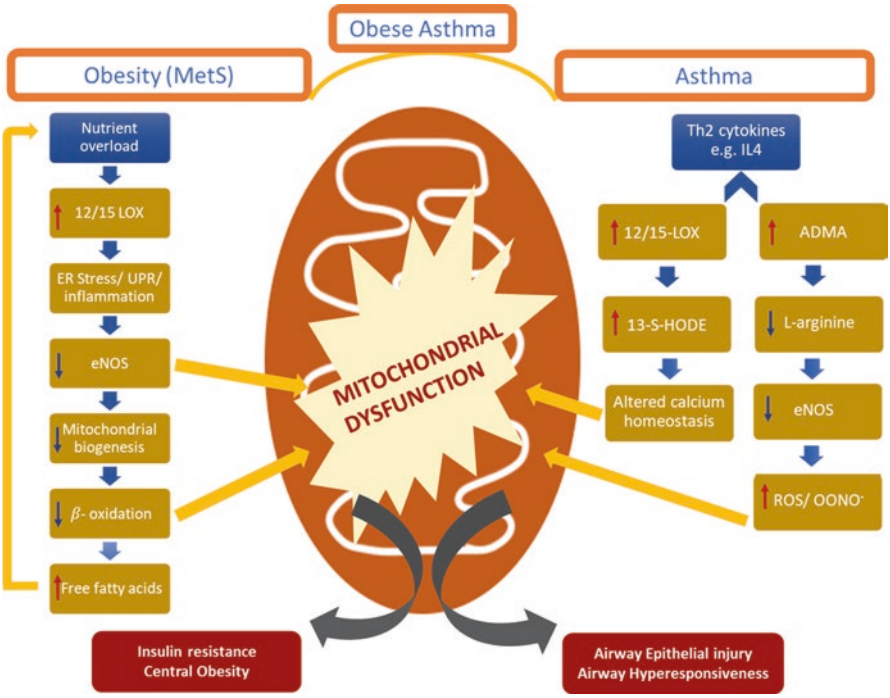

Fig. 6.1 Obese-asthma at the interface of obesity and asthma: schematic showing possible link with mitochondrial dysfunction

Nitric oxide plays an important role in maintaining the function of the airway epithelium such as bronchodilation, ciliary movements and mitochondrial biogenesis [65]. eNOS shows a protective role in both asthma and metabolic syndrome. As explained before, mitochondrial biogenesis is inhibited and fatty acid oxidation decreases on reduction in bioavailability of NO. This leads to lipid accumulation in adipocytes causing increase in formation of oxidative free radicals and mitochondrial dysfunction. Furthermore, decrease in bioavailability of L-arginine is a common pathophysiological feature observed in both diseases.

(C) In addition to that, asthmatic airways show an increase in ADMA level, which is an endogenous NO inhibitor that uncouples eNOS to generate reactive oxygen species (ROS) and peroxinitrites causing oxidative stress and mitochondrial dysfunction. Interestingly, increase in protein turnover causes ADMA levels to increase in obesity. In experimental model of obese-asthma, ADMA levels are seen to be high in plasma. Interestingly, features of metabolic syndrome are observed in eNOS-deficient mice, while its overexpression in bronchial epithelial alleviates asthma features [66]. Independent studies show that high dosage of L-arginine supplementation also alleviates features of asthma and metabolic syndrome [67, 68].

An important pro-inflammatory Th2 cytokine involved in allergic asthma, IL-4, also promotes intracellular accumulation of ADMA leading to oxo-nitrative stress, hypoxic response and ultimately loss of mitochondria. Thus, elevated levels of ADMA in obesity along with high IL-4 levels can potentiate each other and show a bidirectional relationship between the two diseases causing mitochondrial dysfunction.

In obesity (MetS), excess nutrient leads to ER stress via increased expression of 12/15-LOX in adipocytes causing decrease in eNOS and β-oxidation leading to mitochondrial dysfunction. Decreased β-oxidation leads to accumulation of free fatty acid, which in turn ends up as a signal for nutrient overload resulting in formation of a vicious cycle. The subsequent insulin resistance and central obesity are the key features of metabolic syndrome. In asthma, on the other hand, Th2 cytokines such as IL-4 increase levels of ADMA and 12/15-LOX, which leads to mitochondrial dysfunction via increase in oxo-nitrative stress and altered calcium homeostasis via activation of TRPV1 channels, respectively, leading to airway hyper-responsiveness and epithelial cell injury in asthma. Thus, mitochondrial dysfunction is the common player in MetS and asthma that leads to development of obese-asthma phenotype.

6.5 Mitochondria-Based Therapeutics

Since mitochondrial dysfunction has emerged central to asthma pathogenesis, improving mitochondrial function through targeted therapeutics offers a novel handle to tackle the disease. Towards this aim, three basic strategies have been worked upon, namely, **r**eprogramming, **r**epair and **r**eplacement of defective mitochondria.

6.5.1 Reprogramme

The reprogramming strategy entails modulating the regulatory pathways of mitochondria to improve their function. Since the obese-asthma subjects do not respond well to the traditional line of anti-inflammatory therapy, such a therapy targeting mitochondria dysfunction could have greater benefits in disease management. Calorie restriction and physical exercise enhance the production of natural antioxidants, decrease mitochondrial ROS and promote mitochondrial biogenesis [69]. While lifestyle modification and weight loss are the first lines of recommendation in treatment of obese-asthma owing to obvious health benefits, targeted therapies are also required. Using chemicals to mimic calorie restriction such as metformin is one of the potential approaches. Metformin acts via AMP-sensitive protein kinases promoting mitochondrial metabolism. Attenuation of allergen-induced inflammation on treatment with metformin is observed in mice with high-fat-diet-induced obesity [70]. Further, resolution of inflammation is faster in animals treated with metformin. While intrinsic airway hyperresponsiveness in genetically obese mice does not show much improvement, other allergen-induced models of asthma show antiasthmatic effect on treatment with metformin. This suggests shared metabolic processes between allergic asthma and dietary obesity play an important role [71].

Supplementation of L-arginine on experimental models with mitochondrial dysfunction caused by either asthma or metabolic syndrome shows benefits. Mice with arginase 2 (Arg2) knockouts show metabolic dysfunction and increased predisposition to asthma. Also, as per genome-wide association studies, Arg2 genetic variants are associated with increased risk to development of severe asthma [72]. Inhibition of L-arginine degradation using arginase or restoration of eNOS levels using statins and degradation of ADMA has similar effect on metabolism of nitric oxide and asthma [67, 73]. A shared interface between obesity and asthma is also suggested since metformin also shows important effects on nitric oxide metabolism.

Biomolecules that attenuate asthmatic features in experimental models include the following:

(a) 12/15-LOX inhibitors such as baicalein and aesculetin along with exogenous antioxidants that scavenge ROS can reduce mitotoxicity [74, 75].
(b) Sirtuin activators like resveratrol can stimulate mitochondrial biogenesis [76].

While these pathways are common in both obesity and asthma, their potential benefits in allergic as well as obese-asthma patients are yet to be ascertained.

Another novel method of restoring the mitochondrial biogenesis is through a microbial derivative – pyrroloquinoline quinone (PQQ). Bacterial origin of human mitochondria enables a cross-talk between human mitochondria and the microbiome [77]. PQQ is a microbial metabolite that has been reported to stimulate the human mitochondrial biogenesis through PGC-1α [78]. The physiological potential of PQQ as a strong antioxidant (1000 times better at tolerating oxidation than vitamin C) makes it a promising candidate for reprograming mitochondrial dysfunction [79].

6.5.2 Repair

The repair therapy works towards restoring mitohormesis and ensuring that the level of mtROS is finely balanced. General antioxidant therapy, involving α-tocopherol and vitamin C, has not been as effective as projected [80]. However, antioxidants targeted to the mitochondria have shown potential benefits. A combination of mitochondria-targeted antioxidants such as coenzyme Q10 (CoQ10) along with α-tocopherol and vitamin C was shown to reduce steroid usage [81]. This may be particularly important in obese-asthma subjects who are ineffective to glucocorticoid treatments. Potent mitochondrial antioxidants such as MitoQ, a modified form of CoQ10, MitoTEMPO and tiron are effective in preventing and reversing mitochondrial oxidative damage and therefore seem to be promising [8]. As mentioned in previous sections, MitoTEMPO has been shown to restore the paracellular leak in airway epithelial cells in both in vitro and in vivo models [34]. In experimental models of allergic inflammation, another mitochondria-targeted antioxidant – NecroX5 – has been shown to reduce mtROS and alleviate the allergic phenotype [35]. Although these repair strategies hold much promise, it must be ensured that the oxidant–antioxidant balance is optimized so that mitochondrial hormesis prevails.

6.5.3 Replacement

An emerging strategy in mitochondrial medicine is the replacement of defective mitochondria with functional ones. This can be achieved by multiple methods, namely, microinjection of isolated mitochondria, incubation with purified mitochondria, gap junction-driven transfer or direct transfer from donor cells [82]. Amongst the candidate donor cells, mesenchymal stem cells (MSCs) have come up as effective donors of mitochondria whereby they transfer the organelles through tunnelling nanotubes or extracellular vesicles. MSCs also have an added advantage of having homing properties that direct them to the site of injury for tissue repair [83]. In a recent study using murine models of allergic inflammation, mitochondrial donation from MSCs to injured epithelial cells was shown to alleviate the allergic phenotype. The donation of mitochondria was shown through TNTs, and the mitochondrial movement was mediated by Rho GTPase, Miro1 [84]. While such a stem cell-based therapy seems to be an attractive option, work needs to be carried out for optimization of essential parameters like the source of MSCs, the dose of MSCs and their donation potential.

6.6 Conclusion

Asthma is a complex disease originating from a wide spectrum of triggers and with multiple phenotypes, but there appears to be a common point of integration at level of mitochondria. In allergic asthma, multiple studies demonstrate the involvement of mitochondria, which appears to be bi-directional in nature. Also, in obesity, the role of mitochondrial dysfunction in the adipose tissue, liver and skeletal muscle has been well established by multiple studies. In addition to this, dietary obesity that leads to the obese-asthma phenotype seems to involve dysfunctional mitochondria. Therefore, irrespective of atopic or nonatopic trigger, mitochondrial dysfunction seems to be the unifying thread playing a key role in asthma pathophysiological mechanisms. While available clinical data is insufficient, mitochondria-targeted therapies are shown to be promising in experimental models. In subjects with severe form of asthma who are resistant to conventional clinical interventions, such strategies may improve asthma management.

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Regulation of Antioxidant Nrf2 Signaling: An Important Pathway in COPD

7

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Abstract

Oxidative stress plays a role in multiple disorders that include lung diseases like chronic obstructive pulmonary disease (COPD) and asthma. The Nrf2 signaling pathway is the principal regulator of the oxidative stress response and protects against various oxidative stress-related diseases. Nrf2, a bZIP transcription factor, regulates the expression of a battery of antioxidant and detoxification genes in response to oxidative stress. Moreover, Nrf2 signaling responds to multiple environmental and physiological inputs such as endoplasmic reticulum (ER) stress and insulin signaling. The effects of these inputs are often mediated through the molecular regulators of Nrf2 such as kinases, acetylases, and other Nrf2-interacting proteins. Additionally, they can also serve as drug targets for therapeutic modulation of Nrf2 signaling. Therefore, a comprehensive understanding of the molecular mechanism of Nrf2 regulation is important to analyze its role in different physiological and pathological conditions and to develop new drugs that modulate Nrf2 activity. Development of new Nrf2-inducing drugs that can complement current therapeutics is of particular importance for improving the treatment of diseases like COPD where Nrf2 activity is suppressed.

Keywords

Oxidative stress · Nrf2 signaling · COPD · Inflammation · BET proteins

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7.1 Introduction

7.1.1 Oxidative Stress Is Implicated in Multiple Diseases

"Oxidative stress" is characterized by an excessive amount of intracellular reactive oxygen species (ROS), which are extremely reactive ions including but not limited to the hydroxyl radical, the superoxide anion, and hydrogen peroxide. These are generated by oxygen metabolism in mitochondria and peroxisomes and also by cytochrome C oxidase, membrane-associated NAD(P)H oxidase, and xanthine oxidase [1]. Intracellular production of ROS is counteracted by antioxidant processes in the cell that include enzymes like catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) and non-enzymatic ROS scavengers such as glutathione, ascorbate, and carotenoids [1]. Generation of ROS is an essential biological mechanism in many cellular functions such as the "oxidative burst" in phagocytic immune response and signal transduction during growth factor-mediated proliferation [2]. However, excess ROS can damage nuclear and mitochondrial DNA and can modify proteins and lipids. Such damages disrupt cellular functions and can result in cellular senescence or apoptosis [3]. In addition to the internal biological sources, various environmental sources of ROS including ultraviolet rays, ionizing radiations, hyperthermia, toxins, and chemicals can generate oxidative stress [2]. Oxidative stress causes extensive damage to multiple tissues and is implicated in a plethora of diseases such as lung diseases, cancer, diabetes, neurodegenerative diseases, and cardiovascular diseases [4].

7.1.2 Nrf2 Signaling Protects Against Oxidative Stress

A host of signaling pathways like NF-κB, JNK, ERK, p38 MAPK, PI(3)K/Akt, p53, HSF-1, FOXO, and Nrf2 are activated after exposure to oxidative stress, and they mediate the oxidative stress response [5–10]. A typical cellular response depends on the nature of the affected tissue and can range from pro-survival mechanisms such as increased production of antioxidants, repair and replacement of the damaged macromolecules to cellular senescence, and apoptosis to remove malfunctioning cells and thereby preserve tissue integrity and organismal homeostasis. Additionally, the abovementioned pathways often cross-talk among themselves to bring about an integrated response to oxidative stress [2].

Nrf2, a major regulator of the oxidative stress response, plays a central role in the antioxidant defense system of the body [11]. Under basal conditions, Nrf2, a bZIP transcription factor, binds to its cytoplasmic inhibitor Keap1 [12, 13]. Keap1 sequesters Nrf2 in the cytoplasm targeting it for proteasomal degradation and thereby preventing its nuclear localization and binding to the antioxidant response elements (AREs) in the regulatory sections of its target genes [14]. In the absence of nuclear Nrf2 under basal conditions, the AREs are occupied by homodimers of a small Maf protein [15, 16]. Oxidative stress causes sulfhydryl modification of Keap1, and that prevents Nrf2 degradation (Fig. 7.1). Inside the nucleus, Nrf2 binds to AREs as

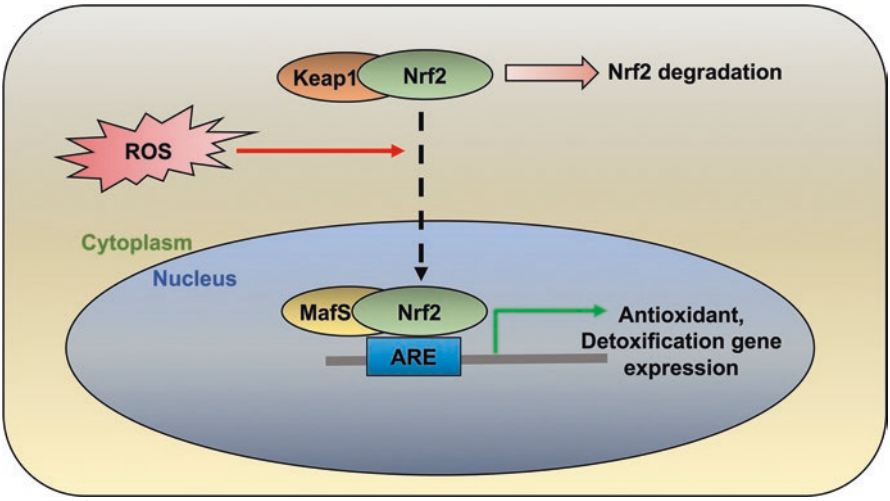

Fig. 7.1 Nrf2 signaling pathway. In the absence of oxidative stress, Keap1 binds to Nrf2, and that leads to ubiquitination and degradation of Nrf2 by proteasomes. Under oxidative stress, the suppression of Keap1-directed degradation of Nrf2 results in increased nuclear localization of Nrf2. In the nucleus, small Maf (MafS) dimerizes with proteins Nrf2; the heterodimer binds to antioxidant response elements (AREs) and promotes the expression of a variety of antioxidant and detoxification genes

heterodimers with small Maf protein to induce a group of antioxidant enzymes and detoxifying proteins such as thioredoxins and glutathione-synthesizing enzymes, glutathione S-transferases, molecular chaperones, and proteasomal subunits [17–19].

The Nrf2-Keap1 signaling pathway is conserved in invertebrates including *Drosophila* and *C. elegans*. In *Drosophila*, CncC is the functional and structural homolog of Nrf2. Like mammalian Nrf2, *Drosophila* CncC is activated by oxidative stress leading to the induction of an array of antioxidant and detoxification genes, thereby protecting against oxidative stress [20]. Similarly, Skn-1 is the ortholog Nrf2 in *Caenorhabditis elegans* and also plays a significant role in the antioxidant defenses of the nematode [21].

Nrf2 induces a group of antioxidant and detoxification genes to protect the organism against oxidative stress. An absence of Nrf2 in mice ($nrf2^{-/-}$) makes them highly susceptible to damages induced by oxidative stress [22]. Various studies have established the protective role of Nrf2 against multiple diseases such as cancer, neurodegenerative diseases, pulmonary diseases, and inflammation where oxidative stress plays a major role [11, 13, 22]. Interestingly, many of these are age-associated dysfunctions, and therefore, it can be surmised that Nrf2 protects different organs from age-related diseases. In addition to oxidative stress response, recent studies have implicated Nrf2 signaling in the regulation of lipid and glucose metabolism and in stem cell maintenance [23–26]. Moreover, Nrf2 signaling also shows

anti-inflammatory effects in different inflammatory disease models related to lung inflammation, inflammatory bowel syndrome, and inflammation associated with multiple neurodegenerative diseases [27–30].

7.1.3 Nrf2 Signaling Is Suppressed in COPD

Chronic obstructive pulmonary disease (COPD) is marked by gradual constriction of peripheral airways and depletion of the lung parenchyma and is caused by inflammation in the parenchyma of lung and in the respiratory airways [31]. A recent study found COPD to be the third leading cause of death globally [32]. Different pollutants including cigarette smoke that cause oxidative stress play a significant role in the progression of COPD [33, 34]. It has been demonstrated in COPD patients that Nrf2 expression is attenuated in the pulmonary macrophages [35]. As a result of which, the expression of different Nrf2 target genes such as glutathione peroxidase 2 (GPx2), heme oxygenase-1 (HO-1), and NAD(P)H quinone dehydrogenase 1 (NQO1) was decreased [36]. In the absence of robust antioxidant defense mechanisms, the damage caused by oxidative agents like cigarette smoke, pollutants, and hyperoxia [31] is increased. On the other hand, activation of Nrf2 by pharmacological compounds is beneficial in COPD [37, 38]. However, the molecular mechanism involved in the suppression of Nrf2 signaling in COPD patients is not well known.

7.2 Regulation of Nrf2 Signaling

7.2.1 Regulation of Nrf2 by Oxidative Stress and Keap1

The Nrf2 protein contains seven functional "Nrf2-erythroid cell-derived protein with CNC homology (ECH)" (Neh) domains, namely, Neh1–Neh7 [39]. The Neh1 domain carries a bZIP DNA-binding and dimerization motif that is required for the binding of Nrf2 to antioxidant response elements (AREs) in DNA and dimerize with other transcription factors including Maf [40]. The Neh2 domain, located near the N-terminus of Nrf2, contains DLG and ETGE motifs that interact with Keap1. In addition, the Neh2 domain carries seven lysine residues that are the sites of ubiquitination by a Cullin-3-dependent E3 ligase that directs proteasomal degradation of Nrf2 [41–44], and Keap1 is an adaptor for this ligase. The Neh3 domain of Nrf2 binds to the chromodomain-helicase-DNA-binding protein 6 (CHD6), which functions as an Nrf2 transcriptional coactivator [45]. Transcriptional coactivator CBP that acetylates Nrf2 interacts with the Neh4 and Neh5 domains and induces transactivation of Nrf2 target genes [46, 47]. The Neh6 domain interacts with the β-TrCP that promotes Cullin-1-dependent Nrf2 degradation [48, 49]. Finally, the Neh7 domain interacts with the retinoic X receptor, which represses Nrf2 target gene expression (Fig. 7.2) [50].

Oxidative stress modifies the cytoplasmic inhibitory protein Keap1 and thereby activates Nrf2 signaling. The Keap1 protein contains five different domains, namely,

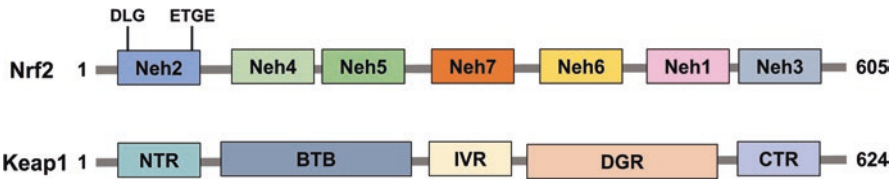

Fig. 7.2 Peptide domains involved in Keap1-mediated Nrf2 regulation. Keap1 binds to the DLG and ETGE motifs in the Neh2 domain of Nrf2 through the DGR domain containing the Kelch repeats. The interaction between the BTB domain and the Cullin-3 E3 ubiquitin ligase complex leads to ubiquitination and proteasomal degradation of Nrf2 protein. Oxidative stress causes sulfhydryl modification of the cysteine residues in Keap1, and that leads to the nuclear localization of Nrf2. In the nucleus, Nrf2 forms heterodimers with MafS proteins and binds to the ARE sequences through the Neh1 domain and, finally, promotes transcriptional activation of the target genes through the Neh4 and Neh5 domains

NTR, BTB, IVR, DGR, and CTR. The DGR domain includes Kelch motifs that interact with Nrf2 [51]. The interaction between the BTB domain and the Cullin-3 E3 ubiquitin ligase complex leads to ubiquitination and proteolytic degradation of Nrf2 protein [42, 44]. Furthermore, Keap1 contains several cysteine residues, and sulfhydryl modification of these residues by oxidative stress causes a conformational change in Keap1, which ultimately leads to Nrf2 activation [13, 52]. There are four highly reactive cysteine residues in the IVR domain, and two cysteine residues out of these four are crucial for Keap1-dependent Nrf2 repression [53, 54].

Independent of the effects of oxidative stress on Nrf2, it is also regulated by environmental and physiological signals including calorie restriction, endoplasmic reticulum stress, and insulin signaling. Moreover, different molecular modifications of Nrf2, like phosphorylation, acetylation, and other proteins that interact with Nrf2 or Keap1, modify Nrf2 activity. Therefore, multiple environmental and physiological inputs are integrated through Nrf2.

7.2.2 Regulation Nrf2 Signaling by Kinases

Nrf2 has been shown to be regulated by different kinases [55, 56]. PKC-δ-mediated phosphorylation of Ser40 in mammalian Nrf2 prevents its degradation and facilitates nuclear entry [57–59]. In contrast, GSK-3β increases the degradation of Nrf2 in a Keap1-independent manner via F-box protein β-TrCP and Cullin-1 [49]. Akt phosphorylates and inhibits GSK-3β and thereby activates Nrf2 signaling [60]. The Tyr568 residue of Nrf2 can be phosphorylated by the Src-family tyrosine kinase Fyn leading to the export of Nrf2 out of the nucleus and its degradation [61]. Moreover, phosphorylation of Fyn by GSK-3β can also increase its nuclear accumulation and thus inhibit Nrf2 signaling [62]. p38 has been shown to phosphorylate Nrf2 to promote its interaction with Keap1, thereby preventing nuclear translocation of Nrf2 and suppressing target gene expression [63]. PERK, which is activated

by ER stress, has also been shown to phosphorylate and activate Nrf2 [64]. However, it should be noted that the substrate sites of phosphorylation for some of the aforesaid kinases are not known. Furthermore, coactivators of Nrf2 such as CBP instead of Nrf2 itself might be the actual targets of some of the protein kinases [65].

7.2.3 Regulation Nrf2 Signaling by Acetylation

p300/CBP are transcriptional coactivators that acetylate histones to promote chromatin decondensation and recruitment of the RNA polymerase machinery [66, 67]. These coactivators have been shown to be associated with Nrf2 protein [46, 68]. Under oxidative stress conditions, p300/CBP binds to Nrf2 at its Neh4 and Neh5 domains acetylating a number of lysine residues within the Neh1 DNA-binding region of Nrf2. It has been demonstrated through mutational analysis that acetylation of multiple residues is required for full Nrf2 inducibility. CBP-mediated acetylation of Nrf2 promotes its binding to the ARE sites and its transactivation potential but does not affect its stability [68]. Recent studies have shown that acetylation of lysine residues in other domains of Nrf2 such as Neh2 and Neh3 also modulates Nrf2 activity [69].

7.2.4 Regulation Nrf2 Signaling by Nuclear Receptors

Nrf2 signaling is regulated by various nuclear receptors – the retinoid receptor is one of them. There are two distinct classes of retinoid receptors, namely, retinoic acid receptor (RAR) and retinoid X receptor (RXR) [70]. All-trans retinoic acid (ATRA) can suppress Nrf2-driven transcription by promoting the formation of a protein complex of RARα receptor with Nrf2 [71]. A vitamin A-deficient diet induced the expression of genes regulated by Nrf2 in the small intestine of mice. Wang et al. reported that RXRα interacts with Nrf2 at its Neh7 domain and inhibits expression of Nrf2 target genes as well [50].

Estradiol suppresses the expression of Nrf2 independently of Keap1-mediated degradation [72, 73]. Yao et al. reported that ERα localizes to the promoter region of Nrf2 target gene NQO1 and inhibits its expression in breast cancer cells [74]. On the other hand, antiestrogen drug shikonin suppressed this ERα-mediated inhibition. Dexamethasone, a synthetic glucocorticoid, suppresses the induction of Nrf2 target gene GSTA2 [75]. In addition, Nrf2-mediated antioxidant response and resistance to H_2O_2 are suppressed by 11β-HSD1 that activates glucocorticoid receptor (GR) through glucocorticoids [76].

7.2.5 Regulation Nrf2 Signaling by BET Proteins

BET proteins have recently been shown to inhibit Nrf2 signaling both in mammals and in invertebrate *Drosophila* [77–79]. BET proteins that contain two

bromodomains and an extra-terminal domain interact with acetylated lysine residues in both histone and nonhistone proteins through their bromodomains. Four mammalian BET protein-coding genes (*Brd2*, *Brd3*, *Brd4*, and *BrdT*) are involved in chromosome organization and in the regulation of gene expression [80–83]. It has recently been reported that *fs(1)h*, the only BET protein-coding gene in *Drosophila*, can suppress Nrf2 signaling and consequently regulates oxidative stress responses [14, 56, 79]. Likewise, BET proteins were shown to suppress Nrf2 signaling in multiple mammalian cell lines, and their inhibition imparts resistance to oxidative stress [77, 78]. Interestingly, BET proteins have been reported to inhibit Nrf2 signaling in a Keap1-independent fashion, and as a consequence, a combination of a Keap1 inhibitor with a BET inhibitor synergistically activates Nrf2 signaling [79].

7.2.6 The Effect of Calorie Restriction on Nrf2 Signaling

Calorie restriction (CR) is a well-established process for extending lifespan in different species. CR also improves several physiological parameters. It increases insulin sensitivity, lowers cancer risk, lowers blood pressure, and improves neuronal function [84]. There is no report demonstrating the importance of Nrf2 signaling in CR-mediated lifespan extension in mammals. However, CR has been documented to activate antioxidant genes that are regulated by Nrf2 in mice, and this induction was impaired in *nrf2*$^{-/-}$ mice. In addition, it was shown that CR mediated protection against cancer by activating Nrf2 [85, 86]. In contrast, in *C. elegans*, the role of Nrf2-like signaling as the mediator of the longevity effects of CR is well established. The activity of Skn-1, the Nrf2 ortholog in worms, in ASI neurons is required for CR-mediated lifespan extension [87]. This indicates that the cell nonautonomous effect of Nrf2 signaling is involved in CR-mediated lifespan extension in worms.

7.2.7 Regulation of Nrf2 by IIS/Akt Signaling

Insulin and its downstream signaling mediated through PI3K/Akt are principal anabolic signals for metabolism and growth. Studies across species have shown that loss-of-function mutations in the insulin signaling pathway (IIS) can extend lifespan [88–90]. Daf-16/Foxo, which is phosphorylated and suppressed by IIS/Akt signaling, has been characterized as the primary mediator of longevity in response to a loss of IIS [91–93]. However, Tullet et al. showed that Skn-1 is also phosphorylated and inhibited by Akt [94]. Moreover, IIS signaling affects Daf-16 and Skn-1 function independently of each other. In contrast, PI3K/Akt signaling activates mammalian Nrf2 signaling pathway [95, 96]. Rizvi et al. reported that the suppression of PI3K/Akt signaling causes decreased nuclear retention of Nrf2 and increased Nrf2 ubiquitination. PI3K/Akt suppression, thereby, suppressed Nrf2 target genes leading to an increase in oxidative stress-mediated cytotoxicity [60]. Interestingly, Akt is phosphorylated in response to oxidative stress. Phosphorylated Akt, in turn, phosphorylates and inhibits GSK-3β. As discussed earlier, GSK-3β represses Nrf2

signaling by promoting the degradation of Nrf2 protein. In addition, it also activates Fyn kinase, which phosphorylates Nrf2 to increase its nuclear export and thereby suppresses Nrf2 signaling. However, the physiological and evolutionary significance of these differential effects of IIS/Akt signaling on Nrf2 and Skn-1 in mammals and worms, respectively, is not clear.

7.2.8 ER Stress and Nrf2 Signaling

An excess of unfolded or misfolded proteins is detrimental to the cellular milieu – this gives rise to ER stress. The unfolded protein response (UPR) operates to either refold or remove these defective proteins. PERK, which is activated during UPR, activates Nrf2 by destabilizing Nrf2-Keap1 interaction [64]. Furthermore, the stress signaling pathway JNK, activated by IRE1, activates Nrf2 signaling by promoting Nrf2-Keap1 dissociation [97–99]. The activation of Nrf2 signaling by UPR ultimately leads to the induction of detoxification, chaperone, and proteolytic genes that help the cell combat ER stress [99]. Interestingly, Glover-Cutter et al. recently reported that ER stress in *C. elegans* can activate Skn-1, which in turn binds to common downstream targets like XBP-1 and ATF6 to orchestrate the transcriptional ER stress response [100]. Therefore, it can be concluded that ER stress-mediated activation of Nrf2-like signaling is conserved across different species.

7.2.9 Regulation Nrf2 Signaling by Nuclear Lamin

Oxidative stress is implicated in the process of aging, and loss of Nrf2 activity is a characteristic of multiple age-related diseases. A recent study by Kubben et al. [101] linked loss of Nrf2 signaling to Hutchinson-Gilford progeria syndrome (HGPS) that is characterized by accelerated aging. HGPS is caused by mutations in lamin A gene that leads to the expression of a truncated lamin A protein (also known as progerin). The accumulation of dominant-negative progerin at the nuclear membrane affects nuclear architecture and genome stability. Cardiovascular diseases emanating from severe atherosclerosis is the principal cause of death in these patients [102]. It was found that sequestering of Nrf2 by progerin causes subnuclear mislocalization of Nrf2 protein and, thereby, suppression of Nrf2 signaling that leads to heightened chronic oxidative stress. Consistent with this observation, genetic or therapeutic activation Nrf2 rescued the phenotypes of cells expressing progerin [101].

7.2.10 Regulation Nrf2 Signaling by Pgk1

Phosphoglycerate kinase (Pgk1) is a glycolytic enzyme that has very recently been shown to regulate Nrf2 signaling [103]. Bollong et al. reported that inhibition Pgk1 by a small-molecule inhibitor CBR-470-1 results in Nrf2 protein accumulation and subsequent induction of Nrf2 target genes. Inhibition of Pgk1 leads to the

accumulation of methylglyoxal, a reactive metabolite. Interestingly, post-translational modification of Keap1 by methylglyoxal leads to the formation of a methylimidazole cross-link between cysteine and arginine residues (MICA) in Keap1. This results in the accumulation of Nrf2 and the transcriptional induction of Nrf2 target genes. This study establishes a direct connection between glucose metabolism and Nrf2 signaling.

7.3 Conclusion and Future Outlook

Nrf2 signaling is affected in multiple disease conditions, including COPD, diabetes, neurodegenerative disorders, and cancer, and consequently is an attractive drug target [11]. Nrf2 signaling is induced by various types of oxidative stressors such as paraquat which affects the mitochondrial electron transport chain or hydrogen peroxide (a reactive oxygen species) [104]. Additionally, different categories of small molecules such as Michael acceptors (bardoxolone methyl, dimethyl fumarate), quinones (tBHQ), isothiocyanates (sulforaphane), dithiolethiones (oltipraz), selenium-based compounds (Ebselen), and heavy metals (sodium arsenite, cadmium chloride) have been identified as inducers of Nrf2 signaling [105, 106]. Some of these compounds including sulforaphane, bardoxolone methyl, oltipraz, dimethyl fumarate (DMF), and Ebselen are being studied in clinical trials for the treatment of different diseases like COPD, asthma, multiple sclerosis, breast and prostate cancer, cystic fibrosis, chronic kidney disease in type 2 diabetes, and nonalcoholic fatty liver disease [105, 107]. Interestingly, a number of the known Nrf2 inducers form covalent adducts with the sulfhydryl groups of cysteines in Keap1 and thereby affect Keap1-Nrf2 interaction. Therefore, they can also react with other cysteine-containing proteins and that might result in "off-target" toxic effects [108, 109].

In addition to these compounds, BET protein inhibitors can be used to activate Nrf2 signaling and thereby may present a separate therapeutic approach for the treatment of these diseases. A recent study by Liang et al. reported that the suppression of Nrf2 activity in the hippocampus caused by hyperglycemia can be reversed by treatment with JQ1 [110]. Moreover, a strong and specific induction of Nrf2 signaling without the toxic "off-target" effects can be achieved by exploiting the synergistic activation of Nrf2 signaling by simultaneous application of BET protein inhibitors and Keap1 inhibitors (Fig. 7.3) [111].

Inflammation plays a major role in the etiology of COPD. However, COPD patients are resistant to widely used corticosteroid treatment because they cannot recruit histone deacetylase 2 (HDAC2) to suppress active inflammatory genes [112, 113]. PI3K/Akt-mediated phosphorylation and degradation play a role in the decrease in the levels of HDAC2. Incidentally, inhibition of HDAC2 enhances Nrf2 acetylation and thereby can suppress Nrf2-mediated antioxidant gene expression in COPD [114, 115]. Furthermore, the suppression of CncC, the ortholog of mammalian Nrf2 in *Drosophila*, by BET protein Fs(1)h relies on the acetylation of CncC [79]. Therefore, it will be interesting to investigate whether a decrease in HDAC2 activity in COPD patients leads to an increase in Nrf2 acetylation and thereby makes

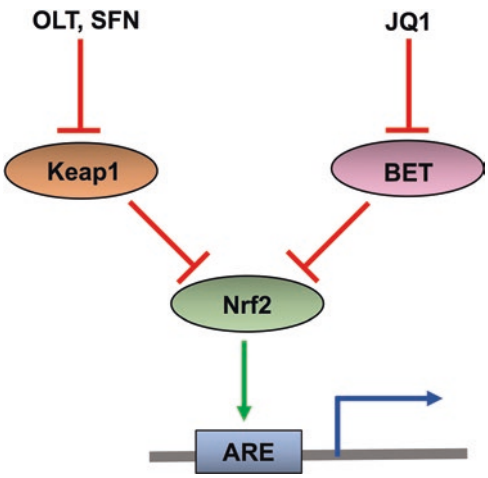

Fig. 7.3 Proposed model for cooperative activation of Nrf2 signaling by Keap1 inhibitors and BET protein inhibitors. Nrf2 is regulated independently by Keap1 and BET proteins. Treatments with the Keap1 inhibitor such as oltipraz (OLT) or sulforaphane (SFN) and BET protein inhibitors like JQ1 relieve different mechanisms of Nrf2 inhibition. Therefore, synergistic activation of Nrf2 target genes can be achieved by combining those treatments

it more susceptible to inhibition by BET proteins. Moreover, it can also be tested whether a combinatorial treatment with a BET inhibitor like JQ1 and a Keap1 inhibitor like sulforaphane yields better results in COPD resulting from a robust induction of antioxidant genes and a better suppression of inflammation through a stronger activation of Nrf2 signaling [111]. Thus, a comprehensive understanding of the molecular regulation of Nrf2 signaling will lead to new therapeutic approaches to induce Nrf2 signaling in the treatment of oxidative stress-associated diseases.

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Role of Oxidative Stress Induced by Cigarette Smoke in the Pathogenicity of Chronic Obstructive Pulmonary Disease

8

Anuradha Ratna, Shyamali Mukherjee, and Salil K. Das

Abstract

Cigarette smoke (CS) exposes lungs to oxidative stress and inflammation and is a major risk factor for the development of chronic obstructive pulmonary disease (COPD). COPD is a complex lung disease characterized by chronic inflammation with limited airflow and chronic bronchitis associated with mucus hypersecretion, thickened small airway walls, and emphysema. CS-induced oxidative stress is responsible for altered cellular metabolism, including increased infiltrating immune cells, pro-inflammatory cytokine production, protease–antiprotease imbalance, lipid peroxidation, apoptosis, upregulation of unfolded protein response (UPR), and protein misfolding. This chapter reviews the current knowledge on different mechanisms through which both direct and secondhand CS-induced oxidative stress in lungs plays a significant role in the pathogenesis of COPD. Despite the presence of considerable reports recognizing the harmful effects of CS-generated oxidative stress, effective treatment for COPD is lacking. Extensive research on the immune and pathogenetic mechanisms of COPD will help in developing new treatment strategies. Clinical trials leveraging multiple antioxidants, anti-inflammatory processes, and UPR inhibitors are urgently needed to advance COPD therapies.

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8.1 Introduction

Chronic obstructive pulmonary disease (COPD) is a leading cause of chronic morbidity and mortality and is becoming a global public health concern. According to the World Health Organization (WHO) in "The Global Burden of Disease Study," there were 251 million cases of COPD globally in 2016 [1] and is likely to be the third leading cause of death worldwide by 2020 [2]. The two conditions that lead to COPD are chronic bronchitis and emphysema. Around 12 million adults are diagnosed with COPD, 120,000 deaths are reported each year, and about 12 million adults go undiagnosed for COPD [3]. It currently affects about 10–12% of the population over 45 years of age, rising to 50% in heavy smokers [4]. COPD is a complex disease associated with chronic airflow limitation along with an inflammatory pulmonary response to smoke and toxic particles. COPD is characterized by lung emphysema, with degradation of alveolar structural protein, loss of alveolar tissue and loss of lung elastic recoil, and chronic bronchitis with increased deposition of structural proteins, narrowing of airway lumen, and mucus hypersecretion [5, 6]. Secondary to the structural changes in the airways, reduced airflow in COPD presents irreversible conditions such as peribronchiolar fibrosis and increased collapsibility due to destruction of elastase in lung tissues [7]. Exposure to both direct and secondhand cigarette smoke (CS) has been widely accepted as a major risk factor in the pathogenesis of COPD [8, 9]. Although smoking is considered the most important risk factor for COPD, not all smokers develop COPD. This implies other contributors including air pollution, exposure to occupational dust, fumes and chemicals, and genetic predisposition [10–12]. The most common genetic factor associated with COPD is the deficiency of α1-antitrypsin [13]. Individual with α1-antitrypsin deficiency has reduced levels of circulating proteinase inhibitor, a protective factor against proteolytic attack that leads to emphysema. Although much research has focused on the protease and antiprotease theory of pathogenesis of COPD, less attention has been paid to the role of the oxidant–antioxidant imbalance in COPD. Besides the detrimental effects of tobacco smoking, secondhand smoke exposure from burning tobacco products such as cigarettes, cigars, pipes, and beedi or biri also has significant adverse effects on health [14]. Homa et al. [15] reported that 2,500,000 nonsmokers have died from different types of health problems caused by exposure to secondhand smoke. In this book chapter, we focus mainly on the both direct and secondhand CS-induced oxidative stress and its role in the pathogenesis of COPD.

8.2 COPD: Pathogenesis and Risk Factors

Although the pathogenesis of COPD remains poorly understood, multiple studies have reported that oxidative stress, inflammation, protease–antiprotease imbalance, and apoptosis of lung cells are involved [16–18]. All these pathogenic mechanisms lead to a series of physiological abnormalities including airflow obstruction, airway hyperresponsiveness, hyperinflation of the lungs, dysfunction of cilia, loss of lung elasticity, and abnormalities in gas exchange due to destruction of alveoli [19]. Figure 8.1 illustrates the pathogenesis of COPD and changes in the lungs due to exposure to both direct and secondhand CS. The pathology of COPD can be represented as a persistent inflammatory immune response and oxidative and chemical injury by noxious gases. Next to chronic inflammation of the lungs, systemic alterations have been seen in COPD patients. Systemic inflammation decrease in body weight and loss of skeletal muscles are observed as other manifestations of the disease. COPD patients frequently develop skeletal muscle dysfunction. Oxidative stress has been reported to cause posttranslational modifications of specific muscle proteins, for example, creatine kinase, rendering them susceptible to increased protein breakdown leading to muscle loss in smokers and COPD patients [20].

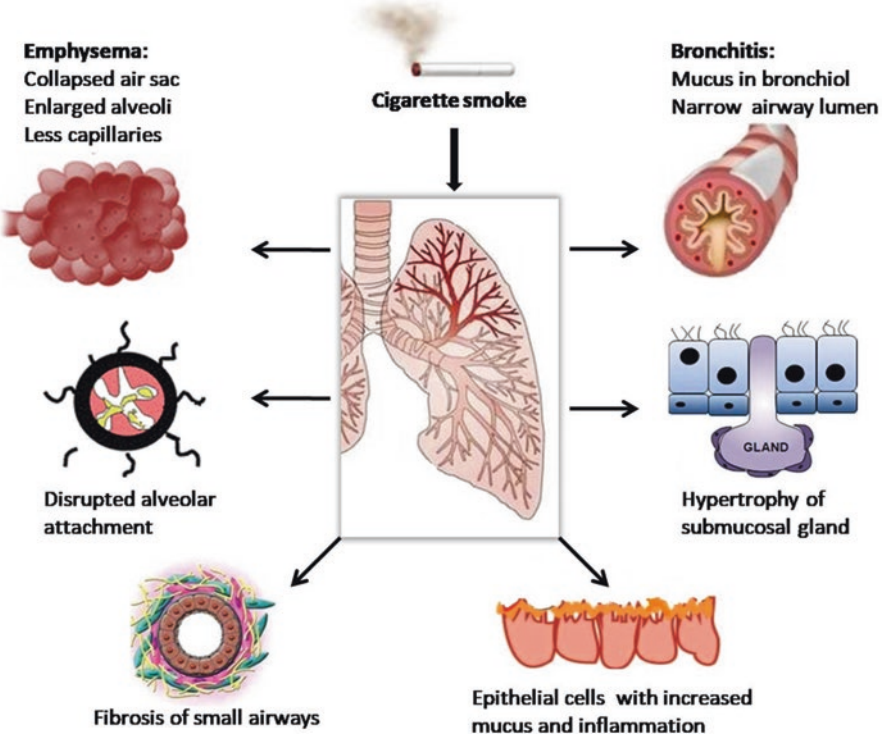

Fig. 8.1 Pathogenesis of COPD induced by both direct and secondhand cigarette smoking

The most prominent risk factors for COPD are cigarette smoking, passive CS exposure, and exposure to other noxious particles including tobacco smoke, fuels, and wood smoke [21]. Smoking harms nearly every organ of the body leading to different diseases such as cancer, heart diseases, lung diseases, and COPD. The WHO reports that cigarette smoking causes more than 480,000 deaths per year in the United States, including more than 41,000 deaths resulting from secondhand smoke exposure [22]. Many oxidants contained in CS induce severe adverse effects through oxidative damage of key biological structures. Inhaled CS induces an aggravated inflammatory response and abnormal tissue damage in the lungs of COPD patients. The gaseous and particulate phases of CS contain 4000 to 1×10^{15} different chemicals and carcinogens and also produce highly toxic reactive oxygen species (ROS) through various processes such as the Haber–Weiss reaction [23]. ROS are able to initiate inflammatory responses by altering biological molecules, signaling pathways, and antioxidant molecule function predominantly in epithelial cells and inflammatory cells in the lung [24]. Chronic inflammatory response in the airways of COPD patients is characterized by infiltrating inflammatory cells (neutrophils, eosinophils, macrophages, and $CD8^+$T cells) and enhanced oxidant production in tissues such as lipid peroxidation [25], protein and thiol oxidation [26], and DNA oxidation [27]. The inflammatory cells release a variety of proteases, known to be responsible for the degradation of elastin and thus leading to emphysema development [19]. Inhaled CS damages lung epithelial cells and releases various inflammatory mediators such as tumor necrosis factor-α (TNF-α), C-reactive proteins, and interleukins (IL-6, IL-8) resulting in structural damage to lung parenchyma [28].

8.3 Smoke-Induced Oxidative Stress in COPD

Recent experimental studies and clinical research have established that CS-induced oxidative stress is a major predisposing factor for COPD. The increased oxidative stress in COPD patients is attributed to the increased burden of inhaled oxidants, along with increased amount of ROS produced by several inflammatory, immune, and epithelial cells in the lungs [29], and cessation of cigarette smoking does not stop progression of this disease. Louhelainen et al. [30] reported that inflammatory cells (particularly neutrophils) are continuously recruited to the lungs and released more inflammatory mediators such as leukotriene B_4 (LTB_4) and IL-8 and cause continuous oxidative stress after smoking cessation, indicating that endogenous ROS/reactive nitrogen species (RNS) resulting from the successive inflammatory response might also contribute to the progression of oxidative stress-induced COPD. Oxidative stress interferes with multiple events of lung physiology, thus contributing to COPD pathogenesis. Oxidative stress is responsible for inactivation of surfactants and antiproteases, hypersecretion of mucus, lipid peroxidation and formation of malondialdehyde (MDA), alveolar epithelial damage, and a loss of lung elasticity and remodeling of the extracellular matrix (ECM) (Fig. 8.2). MDA causes disruption of the membrane lipid bilayer that may lead to the inactivation of membrane-bound receptors and increases tissue permeability. As a result, there is

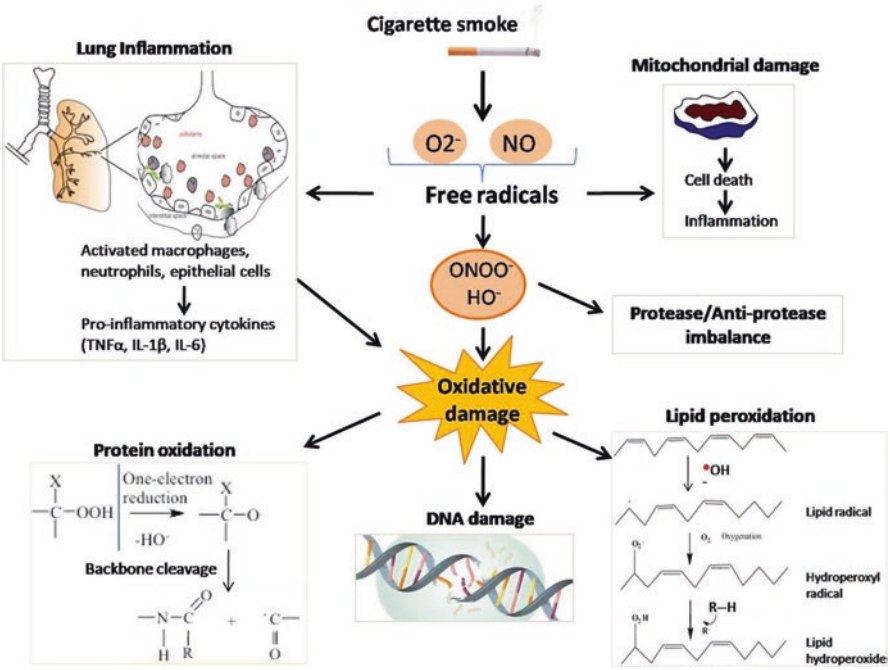

Fig. 8.2 Oxidative stress originated by cigarette smoke causes alterations of multiple pathways and induces cellular damage and inflammation

induction of pulmonary inflammation, as evident from the observation that the level of MDA is increased in peripheral circulation of COPD patients [24, 31]. Birben et al. [32] reported that two of the indirect biomarkers of oxidative stress, isoprostanes and thiobarbituric acid, were increased in the bronchoalveolar lavage and exhaled breath condensate of COPD patients or smokers. Further, CS-induced ROS/RNS upregulate redox-sensitive transcription factors and activate their downstream signaling pathways in lung epithelial and inflammatory cells [24, 33]. In order to sustain a pro-inflammatory state, ROS also initiate small G proteins and pro-inflammatory transcription factors such as NF-κB [34].

Cigarette smoke has been found to contain several reactive species, such as oxides of nitrogen, organic peroxides, and hydroperoxides [35]. Muller et al. [36] reported that the peroxynitrite ($ONOO^-$) is formed in aqueous CS fractions as a reaction product of superoxide and nitric oxide (NO) which are released by CS. Peroxynitrite plays a critical role in the oxidative inactivation of α_1-proteinase inhibitor. Furthermore, peroxynitrite also inhibits both half-life of CS-induced c-fos activity and expression [37]. It has been also reported that CS exposure causes increased blood counts of neutrophils and monocytes, which are the main endogenous sources of myeloperoxidase (MPO), which catalyzes H_2O_2, producing highly oxidizing hypochlorous acid (HOCl). The heme enzyme MPO is a potent endogenous scavenger for peroxynitrite and acts as a protective mechanism from oxidative/

nitrosative stress. Martins et al. [38] reported that serum MPO activity is significantly higher in smoker than nonsmoker and thus responsible for imbalance of the lung protease–antiprotease activity. Moreover, CS exposure also increases production and secretion of metalloprotease by macrophages [39]. It has also been reported that the high levels of matrix metallopeptidase 9 (MMP-9) are present in the airways and serum and persist after smoking cessation [40]. Hence, both increase activity of MPO, and metalloproteases emerge as a critical contributor for the development and progression of COPD in smokers.

8.4 Oxidative Stress and Inflammation in COPD

Airway and parenchymal inflammation in patients with chronic airflow limitation is the hallmark of COPD. Multiple experimental studies and clinical investigations performed by bronchoscopy, sputum analysis, and lung lavage have suggested that inflammation plays a critical role in pathobiology of COPD [41–43]. Besides airflow limitation and bronchiolar constriction, inflammation may also be responsible for fibrosis, mucus gland hypertrophy, increased connective tissue deposition, and deforming the airway lumen [44]. CS triggers a cascade of pro-inflammatory response at the site of inflammation caused mainly due to the infiltration of leukocytes mediated by pro-inflammatory cytokine signaling (IL-1β and TNF-α) [45], upregulation of matrix metalloproteases (MMP-1, MMP-9, etc.), and adhesion of monocytes to the endothelium [46]. The activated endothelial cells express higher levels of selectins, VCAM-1 and ICAM-1, and promote monocyte adherence [47]. The most important cellular players for inflammation in COPD are alveolar macrophages, neutrophils, lymphocytes, and epithelial cells [48, 49]. Increased levels of monocytes and neutrophils secreting free radicals, elastase, and collagenase have been reported in smokers [50, 51]. Many mechanisms could be responsible for increased number of neutrophils in the lungs of cigarette smokers with COPD. For example, oxidants decrease neutrophil deformability and enhance their sequestration in small blood vessels [52]. Upregulated expression of E-selectin causes increased neutrophil adherence in lung vessels of chronic bronchitis patients [53]. CS was found to elicit adhesion of neutrophils to hamster endothelium in vitro by inhibiting $Cu^{2+}Zn^{2+}$-SOD (superoxide dismutase) [54].

Several in vitro studies done on macrophage (U937), alveolar (A549), and bronchial epithelial cells (BEAS-2B) reported that ROS induces the expression of cytokines, such as IL-1β and TNF-α that cause inflammation [55–57]. Alveolar macrophages and airway epithelium, under direct or indirect oxidant stress, also produce TNF-α, which in turn activates epithelial cells to induce pro-inflammatory genes, such as IL-8, IL-1, IL-6, inducible nitric oxide synthase (iNOS), ICAM-1, MIP-1α, heat shock proteins, and antioxidant enzymes [29, 55]. These genes are regulated by redox-sensitive transcription factors NF-κB and AP-1 (activator protein-1). Oxidative stress activates the enzyme I-κB kinase, which degrades κB protein inhibitor, and promotes the release of NF-κB. Intracellular ROS has been also reported to inactivate histone deacetylase 2 that leads to increased acetylation of

proteins. As a result, there occur chromatin decompaction and greater accessibility of transcription factors to the genes [58, 59]. Studies have demonstrated that acetylation of histones is associated with the transcription of inflammatory mediators such asIL-8 [60], eotaxin and GM-CSF [61], MIP-2 [62], and IL-6 [63].

8.5 Oxidative Stress-Induced Mitochondrial Dysfunction in COPD

Mitochondrial dysfunction hampers a multitude of cellular functions, giving rise to the onset of various diseases. The major purpose of mitochondria, the "power house" of the cell, is production of adenosine triphosphate (ATP). Mitochondria perform several other essential functions such as cellular signaling, redox homeostasis, and cell survival and proliferation [64]. They are major endogenous source of ROS. Alteration of mitochondrial function is associated with a variety of disorders in human health [65]. Oxidative stress occurs when exposure of both exogenous and endogenous ROS/RNS is enough to overwhelm antioxidant defenses of our body (Fig. 8.3). Superoxide anion (O_2^-) is generated mainly by mitochondrial metabolism,

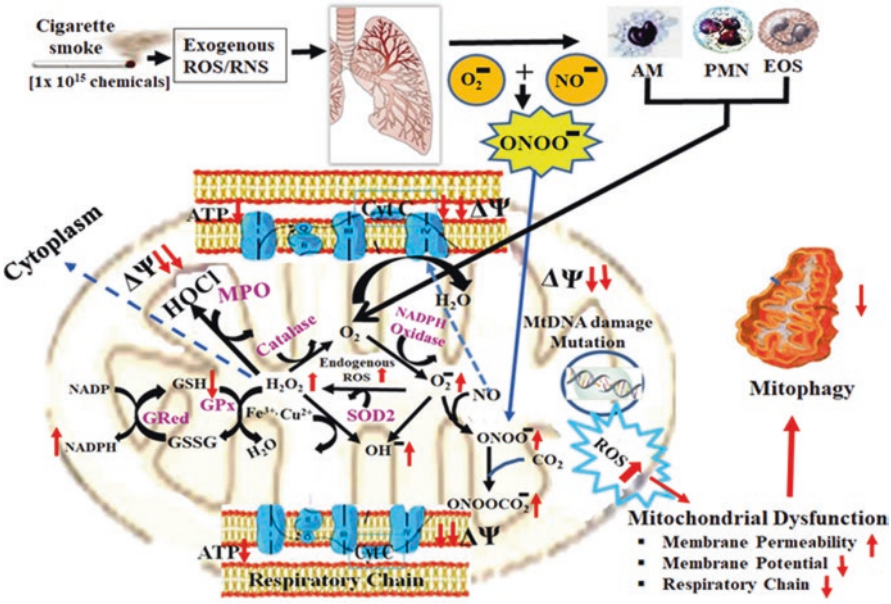

Fig. 8.3 Proposed mechanism of mitochondrial-centered pathogenesis by ROS/RNS of both direct and secondhand cigarette-smoke-induced mitochondrial dysfunction in development and progression of COPD. Abbreviations: *AM* Alveolar microphages, *PMN* polymorphonucleocytes, *EOS* eosinophils, *CAT* catalase, *GSH* glutathione, *GSSG* glutathione disulfide, *GPx* glutathione peroxidase, *GRed* glutathione reductase, *NADP* nicotinamide adenine dinucleotide phosphate, *NADPH* reduced NADP, *MPO* myeloperoxidase, *ONOO*$^-$ peroxynitrite molecule, *ONOOCO*$_2^-$ nitrosoperoxycarbonate, *cyt c* cytochrome c

molybdenum hydroxylase reactions, arachidonic acid metabolism, heme peroxidase system, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent processes in phagocytic cells [66]. Although superoxide anion is unstable, it is considered to contribute little to oxidative stress, because of rapid dismutation of superoxide anion into hydrogen peroxide (H_2O_2) by enzyme cytosolic superoxide dismutase (SOD1) and mitochondrial SOD2. NO is produced by iNOS which in the presence of O_2^- forms an extremely reactive ROS, peroxynitrite anion ($ONOO^-$) [67]. Peroxynitrite causes oxidation of multiple targets such as lipid, proteins, and DNA [25–27]. Nitrosative stress from an upregulated iNOS system and its downstream oxidative pathways is involved in different stages of proteolytic pulmonary emphysema related to cigarette smoking [68]. CS-induced NO acts as inhibitor of electron transport chain (ETC) which increases mitochondrial oxidant (O_2^-) production and impairs the flow of electron at cytochrome c (cyt c) oxidase (Complex IV). Peroxynitrite also inactivates mitochondrial protein SOD2 and mediates a variety of biological effects. Furthermore, in the presence of CO_2, it produces nitrosoperoxycarbonate ($ONOOCO_2^-$), which diminishes oxidation and increases nitration reaction. ROS and RNS are generated by both direct and secondhand cigarette smoke. The superoxide anion radical (O_2^-) is generated from CS and mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and can be converted to H_2O_2 by superoxide dismutase (SOD), which can diffuse from mitochondria and act as a signaling molecule, or is reduced by GSH to form H_2O. In the presence of (Fe_2^+), H_2O_2 can produce hydroxyl radicals (OH^-) via Fenton–Haber–Weiss reaction or can produce highly reactive HOCL by mitochondrial MPO.

CS induces mitochondrial dysfunction by the following mechanisms:

1. Overproduction of ROS/RNS and overutilizing GSH in mitochondria cause defective ETC, which leads to mitochondrial membrane potential loss and increased membrane permeability.
2. Modification of mitochondrial DNA (mtDNA)-associated immune response, which will increase mtDNA fragmentation.
3. Impairment of mitophagy leads to accumulation of damaged mitochondria and increased cellular senescence via suborganellar signaling [69–71].

Hence, CS exposure generates excessive oxidation by inducing tremendous mitochondrial ROS/RNS burden in the cell, which induces an oxidant–antioxidant imbalance in CS-induced COPD [72]. Thus, understanding the underlying mechanisms on oxidant–antioxidant imbalance and involvement of signaling pathway may provide important information for therapeutic treatment of pathogenesis and progression of CS-induced COPD.

While the role of mitochondria in COPD has not been adequately elucidated, several studies have demonstrated that CS disrupts mitochondrial membrane potential ($\Delta\Psi m$), reduces ATP content, and lowers complex protein expression [73]. Prolonged exposure to CS was found to significantly increase the expression of fission/fusion markers, oxidative phosphorylation proteins, and oxidative stress markers [74]. Prohibitin complexes, an essential component of mitochondrial fusion

machinery, were found to be downregulated in COPD lung tissue. Moreover, prohibitin levels were associated with the degree of airway destruction [75]. The peroxisome proliferator-activated receptors (PPARs) and PPAR-γ coactivator (PGC)-1α are key regulators of mitochondrial formation and skeletal muscle oxidative ability [76]. Several studies have reported reduced expression of PPARs and PGC-1α in peripheral skeletal muscle of patients with moderate-to-severe COPD and muscle weakness, suggesting these molecules as potential targets for pharmacological management of COPD [77, 78]. The genes driving iron regulatory proteins (IRPs), known to regulate cellular iron homeostasis, have been identified as major COPD susceptibility candidates [79]. Later studies showed that IRP2 is associated with mitochondrial dysfunction in experimental COPD, where mice treated with a mitochondrial iron chelator showed protection from CS-induced COPD [80].

Mitochondria orchestrate signaling pathways that regulate both innate and adaptive immunity. Two mitochondrial proteins, nucleotide-binding domain and leucine-rich repeat-containing protein X1 (NLRX1) and mitochondrial antiviral signaling (MAVS), have been previously reported to control cytoplasmic, nucleic acid-mediated innate immune pathway [81]. Later studies demonstrated that these two proteins were implicated in COPD pathogenesis [82]. CS-induced dysregulation of NLRX1/MAVS signaling pathway was responsible for enhanced inflammasome activation, increased cytokine response, disturbed ROS production, intra-alveolar inflammation, and alveolar cell death. The role of NLRX1 in the pathogenesis of COPD is also evident from clinical studies. In human COPD cohorts, the suppressed expression of NLRX1 was strongly correlated with the degree of airflow limitation [82]. Some other mitochondrial regulatory pathways, including mitochondrial Tu translation elongation factor and ubiquinol–cytochrome c reductase core protein 2 (UQCRC2), a subunit of electron transport chain complex III, have been found to be relevant in COPD [83, 84]. Mitochondrial signaling is also known to control adaptive immunity, including differentiation of $CD4^+$ T cells and formation of $CD8^+$ memory T cells. Despite all this, the role of mitochondria in COPD immunology is still not very clear. Further studies confirming the participation of other mitochondrial molecules and signaling in COPD pathogenesis will help in understanding the immunologic aspects and in developing disease-modifying therapeutics.

8.6 Oxidative Stress and Antioxidant Defenses in COPD

Oxidative stress may result from increased exposure to oxidants and/or decreased antioxidant capacities, which are central features of COPD. Since oxidants play a pronounced role in CS-induced pulmonary damage, the status of pulmonary antioxidant defense mechanisms holds paramount importance. Pulmonary antioxidant defenses include both enzymatic and nonenzymatic systems. The major enzymatic antioxidants are superoxide dismutase (SOD) and glutathione (GSH) redox system [85]. SOD degrades superoxide (O_2^-) and catalase, whereas GSH inactivates hydrogen peroxide (H_2O_2) and hydroperoxidases. CS compromises the antioxidant machinery of the lung. Acute exposure of airway epithelial cells to CS depletes

airway cell GSH [86]. From a cellular perspective, CS irreversibly modifies redox-sensitive transcription factors, such as nuclear factor erythroid 2-related factor 2 (Nrf2) in epithelial and alveolar macrophages which activates antioxidant response elements and cytoprotective genes [87, 88]. One study reported that erythrocytes from smokers had decreased glutathione peroxidase (GPX) and glucose-6-phosphate dehydrogenase (G6PD) activity and were more susceptible to lipid peroxidation than erythrocytes from nonsmokers [89]. In contrast, there are various studies showing increased antioxidants in cigarette smokers. Certain cigarette smokers had increased GSH and GPX activities in their epithelial lining fluids compared with nonsmokers [90]. On a related note, CS increased lung SOD and catalase activities, but these responses were unable to protect rats against CS [91]. This imbalance between the smoke-induced oxidative stress and antioxidant defense machinery in the lung is believed to be a major step in the progression of COPD.

Mukherjee et al. [92] have evaluated the effect of direct and passive CS exposure on the activity of antioxidant enzymes and lipid peroxidation in guinea pig erythrocytes. Smoke-exposed groups showed increased activity of SOD and decreased activities of GPX and NADPH enzymes [92]. A significant increase in the lipid peroxidation potential of erythrocytes was also observed in vitro, thus indicating the free radical-mediated lung injury. An association between increased peroxidation of erythrocyte lipids and incapability of erythrocytes to quench free radicals in smoke-exposed animals may be due to a deficiency of antioxidants. Lower levels of vitamin C and carotene in plasma of smokers have been reported in some of the earlier studies [93]. Vitamin A and its metabolites play important role in the respiratory system by influencing differentiation and the integrity of the epithelial cells [94, 95]. Several studies [95] have been carried out to understand the relationship between level of vitamin A, lung function, and lung diseases such as vitamin A deficiency leading to type I brittle asthma, degree of bronchopulmonary dysplasia in the neonate, and cellular defense against lung infection due to widespread reduction in the number of ciliated cells throughout the trachea, bronchi, and bronchiolar epithelium [94]. Earlier study from our laboratory suggests that vitamin A deficiency decreases SOD, GSPx, and GSH level in guinea pig lung and simultaneously causes a marked increase in microsomal oxidation [96]. Vitamin A deficiency in smokers increases the risk of developing COPD [97, 98]. Mukherjee et al. [99] reported in a CS-exposed guinea pig model that there is an accumulation of lung retinol and decrease in all-*trans* retinoic acid (ATRA) in both mainstream (MS) and sidestream (SS), and these are still significantly higher in both groups than sham and room control after cessation of smoking, suggesting disturbed retinoid metabolism and signaling. Furthermore, electron microscopic study also revealed that mainstream CS exposure led to massive accumulation of dense amorphous granulated materials and enlarged type II cells in the alveolar space. In secondhand CS-exposed guinea pigs, there was no accumulation of dense, amorphous granulated materials in the alveolar space. However, abnormally enlarged type II cells protruded into the lung alveolar space similar to mainstream CS-exposed group indicated a rapid turnover of type II cells in guinea pigs exposed to both mainstream and secondhand CS. Furthermore, cessation of CS did not improve morphology of the lung of both groups. Above studies suggest

that vitamin A plays a crucial role in pulmonary defense system through cellular removal of toxic radicals [95–100]. Pinnock et al. [101] reported that a lower serum retinol concentration in patients with moderate-to-severe COPD and treatment with retinol improves the FEV1 in these individuals.

Several recent evidences suggest that dietary antioxidants vitamin C, vitamin E, and β-carotene are positively associated with respiratory function [102, 103]. Lutein, a fat-soluble carotenoid without vitamin A capacities, is present in eggs and green leafy vegetable, for example, kale, spinach, and collards [104]. It has also been reported that lutein plays an important role in ocular health, where it protects the eye from inflammation and oxidative stress and in the prevention of stroke, cardiovascular disorders, and lung cancer. It has been reported that higher lutein blood levels were associated with mortality [104]. Grievink et al. [105] reported that α-carotene, β-carotene, and lycopene were positively associated with lung function. Recently, we reported vitamin A, tocopherol, β-carotene, and lutein status in the lung of mild-to-moderate-to-severe COPD patients as judged by their forced expiratory volume (FEV1%) [107]. Figure 8.4 showed retinol (A), retinyl stearate (B), and retinyl palmitate (C) levels in different stages of COPD lung.

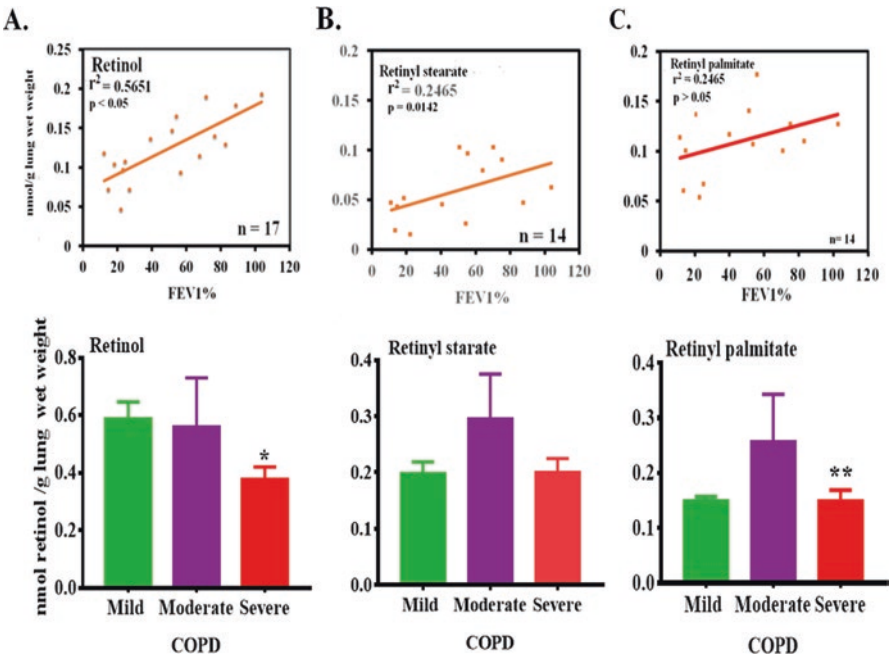

Fig. 8.4 The level of vitamin A in different stages of COPD lung. Upper panel is demonstration of an inverse linear correlation between the degree of emphysema among group of COPD patients, expressed in FEV1% values, and calculated amount of specific vitamin A expressed in μg/g lung wet weight. Lower panel represents the mean ± SEM values in each group (mild, moderate, and severe). A = Retinol, B = retinyl stearate, C = retinyl palmitate

The resulting graph demonstrates that there is a significant inverse relationship between the degree of emphysema among COPD patients, expressed in FEV1% values, and content of specific vitamins in subgroup of vitamin A content (Fig. 8.4—upper panel), whereas Fig. 8.4—lower panel showed the difference between vitamin A metabolites from mild, to moderate, to severe COPD patients. Unpaired t-test indicates that difference in total retinol levels (ROH, Fig. 8.4A) between the mild and the severe group was significant ($^{*}p > 0.05$), whereas total retinyl palmitate (RP) levels (Fig. 8.4C) between moderate and severe were highly significant ($^{**}p > 0.05$), but there is no significant change in total retinyl stearate (RS) level between moderate and severe group among COPD patients (Fig. 8.4B, $p = 0.0142$). Our study indicates that the level of lung vitamin A reduces with the disease progression. Increased local vitamin A deficiency may occur due to high demand for its active form *all-trans* retinoic acid (ATRA), followed by depletion of intracellular retinyl ester stores. Previous studies reported that the COPD risk increases with the decreasing level of serum vitamin A, and ATRA-inhalation therapy showed improved sign and reduction in inflammatory markers in the serum of patient with severe emphysema [108, 109]. In our study, significant reduction in RP level in the severe versus moderate COPD lung tissue indicates the preference of retinyl ester hydrolase toward palmitate ester than stearate ester as observed by others [110]. It has been reported that inhalation of RP aerosol improved the vitamin A in preschool children, and reversal of metaplasia/dysplasia and partial remission in smokers [111].

Figure 8.5 shows an inverse linear correlation (upper panel) and level of different subtypes of tocopherol (lower panel) in difference of emphysema among groups of COPD patients. Our data indicates that only δ-tocopherol levels (Fig. 8.5C) were significantly (**, $p > 0.05$) changed between moderate and severe group of COPD. Although not significant, a decrease of 25.8% in α-tocopherol levels was observed in the severe group compared to samples in the mild group of COPD (Fig. 8.5A), whereas level of β/γ-tocopherol showed a nonsignificant increase with the increasing severity (Fig. 8.5B). It should be noted that α-tocopherol levels are to be found to be higher than β/γ-tocopherol, which are in turn higher than δ-tocopherol. α-Tocopherol is the most abundant tocopherol in our body. As reported in our study, 25.8% reduction of α-tocopherol levels in the lung of the mild-to-severe groups reduced availability of this antioxidant, and increased oxidative stress burden in CS-induced COPD patients may be a critical risk factor for the development of lung cancer in COPD patients [104, 105]. It has been reported that α-tocopherol inhibits retinyl ester hydrolase (REH) activity [106]. The decrease of α-tocopherol levels may impair its ability to inhibit REH, resulting in reductions in RP in our study COPD patients.

Figure 8.6 shows an inverse linear correlation (upper panel) and total concentration of β-carotene and lutein (lower panel) in difference of emphysema among groups of COPD patients. Our result indicates that level of β-carotenes did not change between mild, moderate, and severe COPD patients (Fig. 8.6A). In contrast, lutein level increases between the mild and moderate (Fig. 8.6B), whereas it decreases significantly (**, $p < 0.05$) between moderate and severe COPD. A population-based prospective study reported a strong association between lutein/

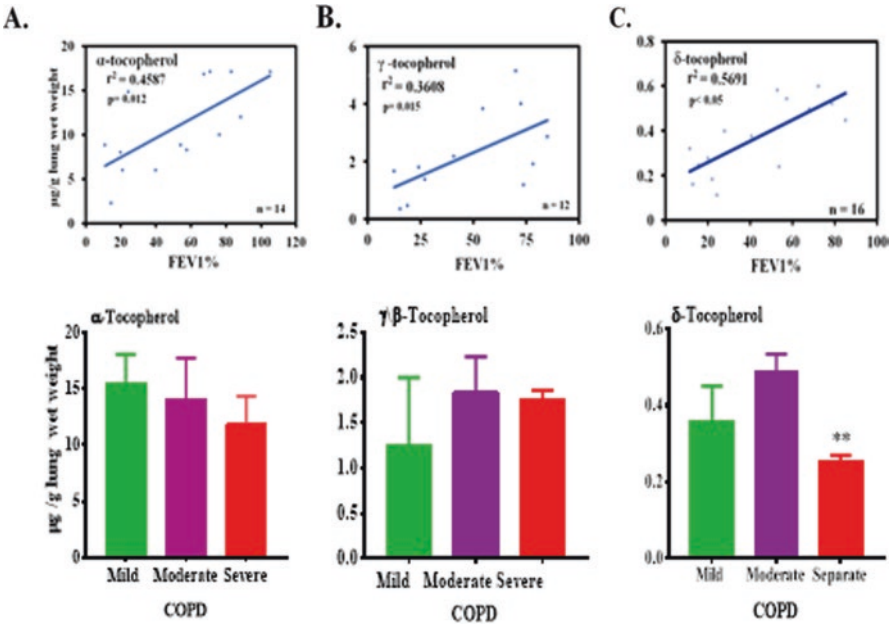

Fig. 8.5 The level of tocopherol in different stages of COPD lung. Upper panel is demonstration of an inverse linear correlation between the degree of emphysema among group of COPD patients, expressed in FEV1% values, and calculated amount of specific tocopherol expressed in µg/g lung wet weight. Lower panel represents the mean ± SEM values in each group (mild, moderate, and severe). A = α-tocopherol, B = β/γ-tocopherol, C = δ-tocopherol

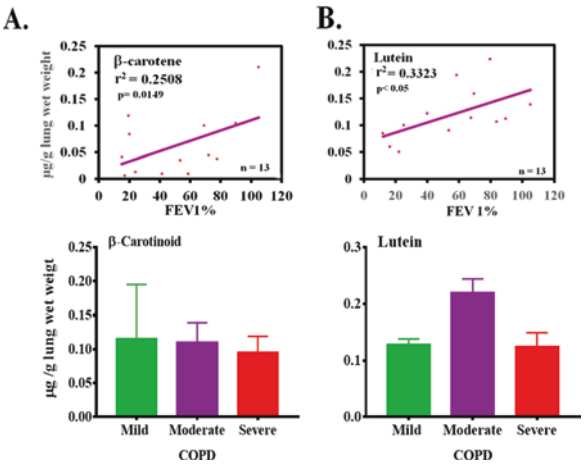

Fig. 8.6 The level of carotenoid in different stages of COPD lung. Upper panel is demonstration of an inverse linear correlation between the degree of emphysema among group of COPD patients, expressed in FEV1% values, and calculated amount of specific carotenoid expressed in µg/g lung wet weight. Lower panel represents the mean ± SEM values in each group (mild, moderate, and severe). A = β-carotene; B = Lutein

zeaxanthin and FEV1, FVC, and FEV1/FVC% in adults with chronic airflow limitation. However, this study also found that lutein intake was significantly associated with lower FEV1/FVC% in current smokers (−1·69 (95% CI −2·93, −0·45) % per SD increase of lutein) independent of other carotenoids [107].

Hence, our overall results suggested that the lung levels of retinol, vitamin A, and other critical components of antioxidative defense, such as lutein and α- and δ-tocopherol, were reduced with the increasing severity of emphysema in COPD patients [107]. Recent emerging evidence suggests that tocopherols modulate the activity of several signal transduction enzymes such as protein kinase C; protein kinase B; protein tyrosine kinases; 5-, 12-, and 15-lipoxygenases; cyclooxygenase-2 (COX-2); phospholipase A2 (PLA2); protein phosphatase 2A; protein tyrosine phosphatase; and diacylglycerol kinase, with consequent alterations of gene expression [108]. It has been suggested that excessive activity of COX-2 is associated with the development of COPD and bronchial tumors of the patients with chronic COPD and a limited activation in idiopathic pulmonary fibrosis [109]. It has also demonstrated that γ-tocopherol has better antioxidative efficacy and protection against emphysema and lung function in COPD than other forms, α, β, and δ [110]. Recently, it has been reported that the expression of P27, Bcl2, α-TTP, CYP3A, tropomyosin, Il-2, PPAR-γ, and CTGF appears to be upregulated by α-tocopherol, whereas cyclin D1, collagen-α1, MMP-1, MMP-19, E-selectin, ICAM-1, integrins, glycoprotein IIb, as well as Il-2, IL-4, and IL-β genes are downregulated [111]. This heterogeneity of mediators of tocopherol action suggests that tocopherols may play critical role in the development of CS-induced COPD. Our study and a recent population-based cross-sectional study indicate that there is a stronger association between antioxidant vitamin levels and the CS-induced COPD [107, 112]. We have reported previously the presence of high-density translocator protein (TSPO) formerly known as peripheral benzodiazepine receptors (PBRs), in lung [113] and alveolar type II cells [114], which are involved in surfactant synthesis and secretion [115]. We and other laboratories also reported that TSPO is predominantly located in the mitochondria, playing an important role in steroidogenesis, inflammation, oxidative stress, cell survival and proliferation, and mitochondrial permeability transition pore (mPTP) formation [113, 116]. Mark and Barnes [117] reported that TSPO is also located in submucosal glands in intrapulmonary bronchi of human and airway epithelium and alveolar walls of both human and guinea pig [118]. Our laboratory also reported that vitamin A deficiency caused a decrease in binding capacity of TSPO in both nuclear and mitochondrial fraction of the guinea pig lungs [118]. These reports suggest a close functional relationship between vitamin A and TSPO in the lung.

Das et al. [119] reported a group of proteins, called annexins or Ca^{++}-dependent phospholipid-binding proteins (PLBP), was identified in alveolar type II cells, which play a role in surfactant biogenesis and lung development. The secretion of surfactant is mediated by the action of catecholamines on β-adrenergic receptors in the lungs [120]. Das and Mukherjee [121] also reported that the treatment with isoproterenol (IP) Ro5–4864 (TSPO agonist) increases the secretion of surfactant 24%, 52%, and 171%, respectively. Our laboratory also reported that MS and SS CS exposure causes a significant increase in levels of PLBP in alveolar type II in

comparison with that in sham control guinea pig lung, which indicates that rapid turnover of PLBP due to CS-induced oxidative stress [122]. This finding explained our previous observation of a massive accumulation of dense, amorphous granulated materials in the alveolar space and irregular expansion of type II cells in both mainstream and sidestream CS-exposed guinea pig [78]. In a guinea pig model of CS exposure, we also observed desensitized β-adrenoreceptors (AR) in guinea pig type II cells [123]. Wang et al. [124] demonstrated that chronic CS exposure triggered lung inflammation by increasing TNFα and IL-1β in both bronchoalveolar lavage fluid (BALF) and lung tissue and downregulated β_2-AR in the rat lung. Moreover, CS exposure inhibits both β_2-agonist-mediated epithelial permeability and transforming growth factor-β1 signaling, which may provide a possible explanation to why CS lowers surfactant level in bronchial lavage. Gavish et al. [125] reported that CS exposure causes significant increases of 72 KDa TSPO degradation and redox metal-ion-induced oxidative stress. CS-induced progression of COPD is associated with small airway obstruction of mucous exudates. It is reported that β_2-AR-β-arrestin2-ERK1/2 signaling was involved in CS-induced mucus hypersecretion in rat and chronic administration of propranolol significantly attenuated the CS-induced airway goblet cell metaplasia and ameliorated airway mucus hypersecretion [126]. Taken together, all these studies indicate that CS causes a multifactorial damage to the lungs by interfering with antioxidant machinery, surfactant biogenesis, and proper lung regeneration.

8.7 Oxidative Stress and Protease/Antiprotease Imbalance

Proteases are responsible for mucin hypersecretion and mucociliary clearance [127]. The deficiency and/or decreased activity of antiproteases contributes to mucus hypersecretion and emphysema [128, 129]. There is strong evidence that neutrophil infiltration and oxidative stress contribute to the imbalance between proteases and antiproteases by activating various proteases, which further leads to COPD pathogenesis [130, 131]. Studies show an acute increase in airway proteases in COPD, degradation of airway mucin proteins, and mucus obstruction [132]. The three classes of proteases contributing to the etiopathogenesis of COPD are serine protease, cysteine proteases, and MMPs. Among serine proteases, neutrophil elastase, cathepsin G, and proteinase-3 degrade elastin and collagen, subsequently destroying alveolar tissue [133]. In cysteine proteases, caspase-3, caspase-8, and caspase-9 are responsible for ECM degradation and controlling apoptosis. MMPs, which act on collagen, laminin, and gelatin [7], play an influential role in severity of COPD. The protease activity is regulated by different inhibitors such as α1-antitrypsin, neutrophil elastase inhibitor, and leukocyte protease inhibitor (PI).

One probable mechanism causing protease imbalance is the oxidation of methionine residues at active sites of α1-antitrypsin under oxidative stress conditions. This decreases α1-antitrypsin's in vitro inhibitory ability dramatically [130, 134]. Mutation in the α1-antitrypsin gene (*SERPINA1*) is the best example of genetically induced emphysema. α1-Antitrypsin possesses a pleiotropic effect known to inhibit neutrophil

chemotaxis and has anti-inflammatory effect independent of neutrophil elastase inhibition [135, 136]. Deficiency of this protein leads to the release of neutrophil elastase creating a pro-inflammatory state within the lungs, accelerating tissue damage and emphysema, which is exacerbated by smoking. Neutrophil elastase which is produced by activated neutrophils and macrophages causes airway and epithelial cell apoptosis via caspase-3 [137] and is a potent inducer of mucus gland hyperplasia [138]. The protease–antiprotease balance may be disturbed by insufficient production of α1-antitrypsin due to genetic defects or smoking-induced oxidants. Deficiency of α1-antitrypsin occurs primarily from the Z allele [Glu342Lys; denoted as protease inhibitor (PI) ZZ in homozygote)] which causes polymerization of newly synthesized protein [139]. Longitudinal and meta-analysis studies show that even a single allele of Z α1-antitrypsin increases the risk for COPD [140, 141]. Besides genetic deficiency, smoking also causes protease–antiprotease imbalance by disturbing the functional activity of α1-antitrypsin in alveolar lining fluid and lung interstitium [142]. Literature suggests that cysteine proteases play a critical role in COPD and pulmonary emphysema by controlling apoptosis [128, 142]. An increase in apoptotic alveolar epithelium, lung endothelial cells, and mRNA expression of caspase-3, caspase-8, and caspase-9 has been reported in COPD cells [143]. Mutation in MMPs is often associated with COPD pathogenesis. Single nucleotide polymorphisms (SNPs) in MMP-9, MMP-1, and MMP-12 have been associated with COPD in different population [144–146]. One study showed that rs652438 SNP, which creates a hyperactive A allele that altered MMP-12 activity, increased macrophage infiltration and emphysema in the lungs of COPD patients [147]. MMP-9 plays an important role in cell migration and airway inflammatory response, affecting the severity of COPD [148]. Since expression of proteases and their inhibitors plays a significant role in COPD, they make obvious targets for research and treatment of COPD.

8.8 Oxidative Stress and Unfolded Protein Response in COPD

Accumulation of potentially cytotoxic misfolded proteins in the ER is known as ER stress. While the effect of CS-associated oxidative stress in COPD has been extensively investigated, the profound effects of oxidative stress on the function of endoplasmic reticulum (ER) through disturbed oxidative protein folding, eliciting an ER stress, have gained interest in recent years. A triad of ER sensor proteins, namely, RNA-dependent protein kinase (PKR)-like ER kinase (PERK), inositol-requiring protein 1 (IRE1), and activating transcription factor 6 (ATF6), induces a cascade of downstream signaling pathways called the unfolded protein response (UPR) [149] (Fig. 8.7). The UPR includes a series of transcriptional, translational, and posttranslational modifications that decrease protein synthesis, enhances protein folding capacity, and eliminates misfolded proteins. Diverse functions of the UPR potentially play a role in the pathogenesis of COPD. Multiple studies have reported the presence of damaged proteins and impairment of their elimination from COPD lungs [150–153]. Elevated levels of UPR targets such as binding immunoglobulin

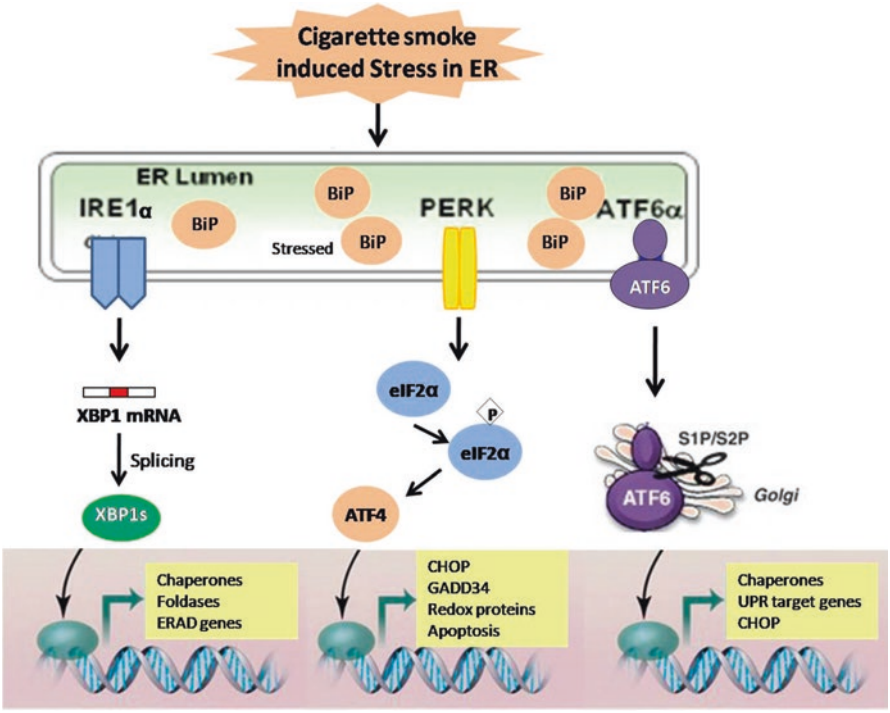

Fig. 8.7 Schematic illustration of endoplasmic reticulum (ER) stress and unfolded protein response (UPR) signaling pathways activated by three sensors, inositol-requiring protein 1 (IRE1), RNA-dependent protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6). *BiP* binding immunoglobulin protein, *XBP1* X-box binding protein 1, *ERAD* ER-associated degradation, *eIF2α* eukaryotic initiation factor 2α, *CHOP* C/enhancer-binding protein homologous protein, *GADD34* growth arrest and DNA damage-inducible protein 34, *S1P/S2P* site 1 and site 2 proteases, *P* phosphorylation

protein (BiP), X-box binding protein 1 (XBP1), and glucose-regulated protein 94 KDa (GRP94) have been obtained in a variety of lung cancer and extrapulmonary cell lines when exposed to CS and aqueous smoke extracts [154, 155]. Cigarette smoking is linked with activation of UPR as evidenced by increased protein level of chaperones (GRP78, calnexin, calreticulin) and protein disulfide isomerase (PDI) [156]. Multiple in vivo studies done in mouse demonstrate increased expression of p-eIF2α, CHOP, p50 ATF6N, and ATF4 proteins in lung lysates after cigarette smoke exposure at different time points [6, 153]. CS inhibits XBP1 splicing, ameliorating IRE1α-XBP-1 axis even when A549 cell line was treated with ER stress inducers such as tunicamycin [157].

Oxidant stress due to CS irreversibly damages several lung proteins, thereby requiring ubiquitin–proteasome degradation or autophagic degradation. The ubiquitin–proteasome system is required for both the normal turnover of cytoplasmic and nuclear proteins and oxidatively modified proteins. CS directly impairs the proteasome activity in alveolar and lung epithelial cells [158]. The study also reported that

CS induced the accumulation of carbonylated and polyubiquitinated proteins in epithelial cell lines and alveolar macrophages. CS has been reported to cause oxidation and misfolding of PDI, an ER-resident foldase, both in vitro and in vivo [6]. Different constituents of smoke, namely, acrolein, hydroxyquinones, or peroxynitrites, cause nitrosylation of PDI and impair its enzymatic activity [154].

CS has been also linked with the autophagy dysfunction. Autophagy is a complementary degradative pathway that captures and recycles protein aggregates and removes defective mitochondria and internalized pathogens [159]. Studies have reported increased autophagosomes but with functional immune deficits in the lungs of smokers and in epithelial cells exposed to CS extract in vitro [160, 161]. The decrease in functional autophagy was attributed to the protein aggregate accumulation, mitochondrial damage, and impaired lysosomal delivery. In summary, oxidative stress in the form of CS exposure induces protein misfolding and UPR in the lungs and the isolated cells. Due to the major role played by UPR in protein metabolism, autophagy, and antioxidant defense, it is potentially of great importance in the pathogenesis of COPD.

8.9 Oxidative Stress and Antioxidant and/or Anti-inflammatory Therapeutic Strategies for COPD

There is considerable evidence that an increased oxidative burden occurs in the lungs of patients with CS-induced COPD, and this results in an imbalance between pro-inflammatory–anti-inflammatory, oxidants–antioxidants, or oxidative stress, which may play a role in many of the processes involved in CS-induced pathogenesis of COPD. Hence, it becomes important to target systemic and local oxidative stress with therapeutic administration of a variety of antioxidants from diet or drugs in the treatment of pathogenesis of COPD and control different signaling molecules (such as nuclear factor-kappa B, nuclear erythroid 2-related factor 2 (Nrf2) signal transduction) and hence inflammatory gene expression [162–170].

Dietary antioxidant supplementation of vitamin A, vitamin E, vitamin C, β-carotene, lutein, and lycopene is the modest approach to modulate antioxidant redox system or to improve the endogenous levels of antioxidants to manage CS-induced COPD. Table 8.1 describes the mode of action of dietary supplementation of vitamin and/or carotenoids in pathogenesis of COPD.

In order to counterbalance the lethal effects of ROS/RNS, the normal lung has various endogenous antioxidants, which play very important role in both enzymatic and nonenzymatic mechanisms. The antioxidant glutathione (GSH) is the most abundant cellular thiol, and the glutathione redox cycle is the fundamental component of the cellular antioxidant defense system. GSH present in higher concentrations in the epithelial lining fluid than plasma plays a protective role in the airspaces and epithelial cells against oxidative stress and in detoxifying and maintaining the integrity of the lung airspace epithelial barrier. A reduced level of GSH has been reported in the airways of smokers compared with nonsmokers, signifying that CS predisposes lung cells to ongoing oxidant stress [179]. It has been observed that

Table 8.1 Effect of dietary vitamins and carotenoid supplement in pathogenesis of COPD

Antioxidant	Mode of action	References
Vitamin A[a]	Reduces the annual FEV1 and exacerbation symptoms by improving oxidant and antioxidant levels	Refs. [171, 172]
Vitamin E[a]	Acts as a strong free radical scavenger and inhibits progressive inflammatory response in COPD	Ref. [173]
Vitamin C[a]	Improves antioxidant status by increasing plasma GSH level in Male COPD patients	Ref. [174]
All-trans retinoic acid (ATRA)[a]	Regulates various gene expressions and induces production of various proteins in rats with elastase-induced emphysema	Ref. [95]
β-carotene[a]	Increases plasma antioxidant level and inhibits inflammation	Refs. [175, 176]
Lycopene[a]	Modulates various biochemical pathways of COPD	Ref. [177]
Lutein[a]	Inhibits DNA damage and lipid peroxidation in COPD patients	Ref. [156]
Diet (fruits and vegetables)[a]	Modulates various biochemical pathways and reduced COPD incidences in both current and ex-smoker but not in never smokers	Ref. [178]

[a]Clinical trial has been done in COPD patient

direct administration of GSH leads to undesirable effects, which suggests that direct therapy with GSH will not be an appropriate way in increasing GSH levels in lung airspace fluid and epithelial cells of the CS-induced COPD patients [180]. Table 8.2 describes the mode of action of thiol-based drugs in pathogenesis of COPD.

Nrf2 is an important transcription factor that interacts with Kep1 and ubiquitinated by Cullin 3 in the cytoplasm. It has been reported that COPD patient lungs showed (*1*) marked decline in NRF2-dependent antioxidants and glutathione levels, (*2*) increased oxidative stress markers, (*3*) significant decrease in NRF2 protein, and (*4*) significantly decreased DJ-1 levels (a protein that stabilizes NRF2 protein by impairing KEAP1-dependent proteasomal degradation of NRF2) than nonsmokers [192]. Table 8.3 describes the effect of agonists or modulators of nuclear factor erythroid 2-related factor 2 (Nef2) in pathogenesis of COPD.

Enough scientific evidences exist supporting the fact that oxidative stress resulting from CS exposure is involved directly in the pathogenesis of COPD. It has been reported that the activities of Cu^{+}, Zn^{+}superoxide dismutase (Cu^{+}, Zn^{+}SOD), glutathione peroxidase (GPx), and glutathione-S-transferase are decreased in alveolar macrophages of elderly smokers when compared with that of nonsmoker [200, 201] as we observed in erythrocytes of guinea pig when compared that with room control [92]. NADPH oxidase (NOX) has been suggested as a potential mediator of oxidative stress. Cheng et al. [202] reported that ROS generation from CS particle extract (CSPE) is mediated via a c-Src/NADPH oxidase/MAPK pathway which activates Nrf2 and finally induces HO-1 expression in human tracheal smooth muscle. MPO released after neutrophil activation has a beneficial role in terms of the immune response to invading pathogens, but there is considerable evidence that inappropriate stimulation of oxidant formation can cause host tissue damage. It has been reported

Table 8.2 Effect of thiol-based drugs in pathogenesis of COPD

Drugs	Mode of action	References
N-acetylcysteine (NAC)[a]	Increases intracellular GSH by reducing cysteine to cystine disulfide and modulates chemotaxis and NF-κB production of c-reactive marker (CRP)	Refs. [181, 182]
Nacystelyn (NAL)	Increases intracellular GSH	Ref. [183]
N-isobutyrylcysteine (NIC)[a]	Increases intracellular GSH	Ref. [184]
Carbocisteine[a] (mucolytic agent)	Attenuates tumor necrosis factor-α (TNF-α)-induced inflammation via suppressing nuclear factor erythroid 2-related factor 2 (NF-κB) and ERK1/2MAPK signaling pathways and reduces exacerbation associated with COPD	Refs. [185, 186]
Erdosteine[a]	Decreases number of respiratory exacerbation and reduces bacterial adhesiveness by modulating pro-inflammatory cytokines in current smokers with mild COPD	Refs. [187, 188]
Ebselen	Reduces the exacerbated BALF inflammation and pro-inflammatory cytokine, chemokine, and protease expression by modulating glutathione peroxidation in COPD mice	Ref. [189]
Fudosteine	Reduces airway hyperresponsiveness, inflammation, remodeling, goblet cell hyperplasia, subepithelial collagenization, and basement membrane thickening by decreasing level of MMP-2, MMP 9, eotaxin, IL-4, and TGFβ in a murine model of COPD	Ref. [190]
Procysteine	Improves macrophage function in CS-induced COPD by increasing efferocytosis and availability of GSH	Ref. [191]

[a]Clinical trial has been done in COPD patient

that MPO levels in sputum of stable COPD patients were higher than non-COPD patients, and this increase was especially pronounced during exacerbations as compared to the stable state [203]. Table 8.4 describes mode of action of antioxidant enzyme mimetics, spin traps, and other enzyme inhibitors in pathogenesis of COPD.

The hallmark of COPD is an increase or abnormal inflammatory response of the lungs to inhaled CS, which is characterized by increased numbers of neutrophils, macrophages, and T lymphocytes. The suppression of this inflammatory response for treatment of CS-induced COPD improves symptoms such as mucus secretion and cough and reduces exacerbations and disease progression [209]. Table 8.5 describes mode of action of anti-inflammatory modulators in pathogenesis of COPD.

The most important source of antioxidants is diet not drugs or supplements. The disease-preventing ability of variety of dietary plants, fruits, tea, and wine has been attributed to polyphenols and natural antioxidants present in these natural sources. Certain micronutrients (vitamins and minerals) and phytochemicals (carotenoids and phenols) show protective effect on several diseases such as cancer, COPD, and cardiovascular disorders. Natural products contain dietary polyphenols and other active compounds such as curcumin, green tea catechins, quercetin, resveratrol, and lycopene. Tocotrients, acai, ginkgo biloba, tocotrienols, α-lipoic acid, omega-3 fatty acid,

Table 8.3 Effect of nuclear factor erythroid 2-related factor 2 (Nrf2) agonists or modulators in pathogenesis of COPD

Drugs	Mode of action	References
Crocin	Attenuates CS-induced lung injury by modulating Nrf2 pathway	Ref. [193]
Ursolic acid	Downregulates PERK pathway and upregulates Nrf2 pathway	Ref. [170]
Sulforaphane (SFN)[a]	Protects alveolar epithelial cell injury, attenuates G1-phase cycle arrest, and abrogates apoptosis by upregulating Nrf2 expression	Refs. [194, 195]
Oroxylin A	Attenuated oxidative stress and CS-induced lung inflammation upregulate antioxidant response element (ARE) via activations of Nrf2 signaling	Ref. [196]
Platycodin D	Inhibits CS-induced malonaldehyde (MDA) and NO production by suppressing NF-κB and activating Nrf2 signaling pathway	Ref. [197]
15-Deoxy-prostaglandin J2 (15d-PGJ2)	Plays a protective role by activating Nrf2 in both rat COPD model and human bronchial epithelial cells	Ref. [198]
CDDO-Imidazole	Protects against smoke-induced COPD via Nrf2/HO-1 pathway in mice	Ref. [199]

[a]Clinical trial has been done in COPD patient

Table 8.4 Effect of antioxidant enzyme mimetics, spin traps, and other enzyme inhibitors in pathogenesis of COPD

Drugs	Mode of action	References
SOD mimetics, e.g., M40419 and AEOL-10113	Inhibits CS-induced inflammatory response by mimicking extracellular SOD and significantly reduces lung markers of oxidative stress	Refs. [204, 205]
GPx mimetic ebselen	Reduces the exacerbated BALF inflammation and pro-inflammatory cytokine, chemokine, and protease expression by modulating glutathione peroxidation in COPD mice	Ref. [189]
Thioredoxin	Modification of oxidoreductase and ameliorates smoke-induced inflammation	Ref. [206]
α-phenyl-N-tert-butyl nitrone, STANZ, NXY-059, L-NL, and L-NAME	Attenuates emphysema by quenching free radicals in animal model	Ref. [177]
Celastrol	Decreases free radical formation by inhibiting NADPH oxidase (NOX) and stimulates Nrf2	Ref. [207]
2-Thioxanthine (AZ1)	Protects morphological change in CS-induced emphysema by inhibiting myeloperoxidase in animals	Ref. [208]

Table 8.5 Effect of anti-inflammatory modulators in pathogenesis of COPD

Drugs	Mode of action	References
Ginsenoside Rg1	Attenuates CS-induced pulmonary epithelial–mesenchymal transition via inhibition of transforming growth factor-β1 (TGF-β1)/Smad pathway	Ref. [210]
Salidroside	Ameliorates the progression of CS-induced COPD by inhibiting the generation of pro-inflammatory cytokines (e.g., TNFα, interleukin (IL)-1β, IL-6, and MAPK/NF-κB)	Ref. [211]
Isoliquiritigenin (ILG)	Attenuates inflammation and oxidative stress via upregulating Nrf2 and downregulating NF-κB in CS-induced COPD	Ref. [212]
Sodium tanshinone IIA sulfonate (STS)	Inhibits CS-induced inflammation and oxidative stress via blocking the activation of MAPK/HIF-1α signaling	Ref. [213]

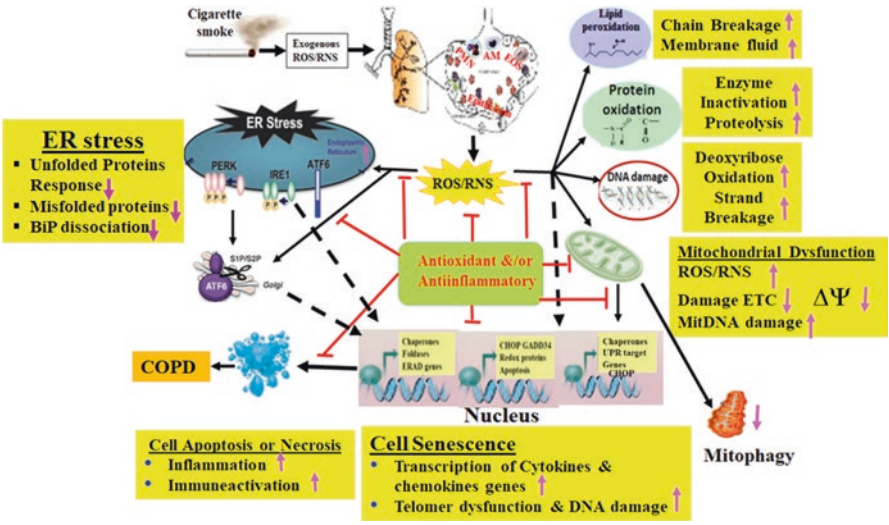

Fig. 8.8 Schematic illustration of targets of antioxidant and/or anti-inflammatory agents on pathogenesis of COPD induced by both direct and secondhand cigarette smoking

and apocynin play a protective role against lung function loss by improving average lung function and reducing declined rate [214, 215]. Several studies reported that curcumins and phenols have significant positive dose–response relationship between their intake and FEV1 and FEV1/FVC in adults [216]. A special benefit of curcumins for smokers came into light when the pulmonary function in smokers with curcumin-rich curry intake was almost equal to that in nonsmokers [216]. Recent studies showed the intake of catechins, green tea polyphenols, epigallocatechin gallate, flavonols (e.g., quercetin and kaempferol), and flavones (e.g., apigenin and luteolin) was positively associated with FEV1 [217]. Dietary polyphenols have beneficial effects due to their antioxidant and anti-inflammatory effects [218, 219]. Figure 8.8

shows the possible mechanism of action of role of antioxidants and/or anti-inflammatory agents in the treatment of CS-induced pathogenesis of COPD.

Although antioxidant treatments have shown promising effects in targeting ROS/RNS and oxidant-mediated cellular alterations of CS-induced COPD, few antioxidant agents have undergone formal clinical trials to assess the clinical benefit in COPD. Development of novel wide-spectrum small molecule antioxidants with a good bioavailability and potency is needed in clinical trials of COPD. Furthermore, the effects of combination of various antioxidants along with enzyme inhibitors and/or blockers are needed for management of COPD.

8.10 Conclusion and Future Perspective

Epidemiological studies have demonstrated that CS is the primary causative agent of various respiratory system diseases, including COPD whose pathobiology is mainly due to oxidative stress induced by CS. We have endeavored in this chapter to highlight the various pathways through which CS-induced oxidative stress and chronic inflammation contribute to COPD. It is evident from several studies that lung oxidant–antioxidant balance is disturbed in cigarette smokers. However, it remains unexplained why only some cigarette smokers develop COPD. Due to this variability among smokers and difficulties in measuring oxidative status, the treatment and prevention of COPD remain challenging. Understanding the oxidant–antioxidant balance, genetic factors, and other intrinsic factors that vary among individual smokers is highly necessary. Targeting ER stress proteins and UPR pathways in COPD will gain practical advancement only after detailed evaluation of their mechanisms in various and robust in vivo models. As COPD is a major inflammation-related lung disease, the role of mitochondria cannot be neglected. While no mitochondria-targeted therapies are available for COPD, several mitochondria-based antioxidants and oxidative phosphorylation inhibitors are undergoing clinical trials in lung diseases. Additional investigation of the COPD targets discussed in this chapter is warranted and could pave the way for mitochondria-based biomarkers and targeted therapies. Due to complexity of COPD, a multi-targeted therapeutic approach is highly required.

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Oxidative Stress in Obstructive and Restrictive Lung Diseases

9

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Abstract

The lung has ample and vascularized surface area constantly exposed to endogenous and environmental oxidants (in particular cigarette smoke). Thus, the imbalance between oxidants and antioxidant defenses has a pathologically important role in several lung disorders. This chapter describes the sources of free radical generation, ROS-induced signaling pathways, and mechanisms of oxidative stress damages in the pathogenesis of obstructive pulmonary diseases, idiopathic pulmonary fibrosis, and asthma. ROS are regulatory factors in different molecular pathways involved in miscellaneous lung diseases and might represent potential suggestions for therapeutic approaches. Given the limited effectiveness of current strategies, novel experimental approaches to develop improved antioxidant therapies are discussed.

Keywords

Oxidative stress · Damage mechanisms · Lung diseases · COPD · IPF · Asthma · Antioxidant therapies

9.1 Introduction

Oxidative stress is an insalubrious condition occurring when a variety of free oxygen radicals, collectively termed reactive oxygen species (ROS), prevail on antioxidant systems and lead to cellular damages [1, 2]. The lung is particularly susceptible to

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this imbalance being the organ designed for gas exchange, continuously exposed with a large surface area and blood supply to high oxygen tensions and exogenous oxidants such as ozone (O_3) and sulfur dioxide (SO_2) [3, 4]. Although some environmental pollutants (e.g., particulate matter, silica, or asbestos) are not oxidants, they may promote oxidative stress in the lung through recruitment and activation of ROS-producing cells and by triggering oxidative chemistry, as Haber-Weiss or Fenton's reactions [5–8]. The endogenous defense against oxidative stress induced by free radicals stress involves several preventive, repair, enzymatic, and non-enzymatic mechanisms [9]. Molecular antioxidant systems in the lung comprise scavengers (glutathione, ascorbic acid, tocopherol, uric acid, β-carotene, or thiol-containing proteins), detoxifying enzymes (superoxide dismutases, catalase, GSH-peroxidase, GSH S-transferase, peroxiredoxin-thioredoxin, glutaredoxions, or hemeoxygenase), and metal-binding proteins (transferrin or metallothioneins), and mucins [9, 10]. Except in some unusual exposures such as those to UV light and ionizing radiations, reactive oxygen and nitrogen species (RONS) are naturally generated by the cellular metabolism through enzymatic or non-enzymatic electron transfer reactions. These reactions are involved in a plethora of cellular processes, including cell signaling, microbial activity, cell fate, differentiation, proliferation, vasodilation, inflammation, neurotransmission, cell migration/adhesion, and hormone synthesis [11–13]. Mitochondrial electron transport chain, NADP oxidases, peroxidases, nitric oxide synthase, and xanthine oxidase are only some of the main RONS-generating pathways occurring in alveolar macrophage (AMs), fibroblasts, neutrophils, eosinophils, bronchiolar epithelial cells, alveolar epithelial cells (AECs), and endothelial cells [4, 14]. The expression of antioxidant enzymes is finely regulated and often induced in response to RONS exposure through transcription factors such as Nrf2 and FoxO3 in the bronchial and alveolar epithelium [15, 16].

As the oxidant/antioxidant imbalance is embroiled in the pathogenesis of miscellaneous diseases affecting the lung and pulmonary vasculature [3, 15, 17], this review will highlight its involvement especially in the chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) and will provide an overview of new therapeutic strategies.

9.2 Mechanisms of Oxidative Stress Damage

Reactive radical species can cause protein, DNA, and lipid oxidation and, through the generation of secondary metabolic RONS, can induce a variety of cellular responses [7, 10, 18]. The higher reactivity of RONS with different macromolecules (due to presence of unpaired electrons) leads to tissue damage, cellular dysfunction, and activation of different signaling pathways [11]. Proteins are the main target, and the oxidative/nitrosative stress through their oxidation, glycation, carbonylation, sulfonation, sulfenylation, or nitration affects their catalytic activity, conformation, and interactions and induces crosslinking [19]. Protein modifications, together with lipid peroxidation, impact on the cellular homeostasis; promote catabolite accumulation, cytotoxicity, and

apoptosis; and activate immune and inflammatory cytokines and chemokines (through TLR, NF-kB, p38-MAPK, or inflammasome pathway), such as IL-1, IL-6, IL-18, and TNF-α [e.g., 1, 14, 18]. Moreover the oxidative stress enhances the production of advanced glycation end products (AGE, heterogeneous compounds formed by glycated proteins or lipids), which contribute to pro-inflammatory and apoptotic response [1]. Positive feedback among the abovementioned inflammatory mediators and RONS is well-known, and it might sustain chronic inflammation and lung injury [1]. The RONS-induced protein modifications cause Endoplasmic Reticulum (ER) stress and the subsequent activation of Unfolded Protein Response (UPR), a protein folding restoration pathway [20]. Unresolved accumulation of misfolded proteins overcoming the UPR induces apoptosis (or senescence) and inflammation and strengthens the oxidative stress [21, 22]. There is substantial evidence that many respiratory diseases such as cystic fibrosis, COPD, IPF, and asthma are associated with excessive ER stress [21, 22]. RONS (or hypoxia) and ER stress can also regulate autophagy, an homeostatic catabolic process involving lysosomal degradation of damaged intracellular structures, that seems to influence cell differentiation [23, 24].

Interestingly, oxidative modifications of proteins and lipids are a potential source of autoantigens, and a role of oxidative stress in autoimmunity was postulated [2]. RONS damage DNA, and through chromatin remodeling and methylation inhibition they affect epigenetics, leading to instability, mutagenesis, and telomere shortening. Being close to endogenous ROS sources and unprotected by histones, the mitochondrial DNA (mtDNA) is more sensitive to oxidative stress damages than nuclear DNA. Thus, together with the well-know induction of intrinsic apoptosis or aging acceleration, oxidative damage altered the normal mitochondrial function, influencing the electron transport chain and promoting aerobic glycolysis (the Warburg effect) [25]. The structure and integrity of mitochondria are also compromised with swollen and elongated shape, fusion, and reduced cristae definition [26]. The leaks of cardiolipin or mtDNA are additional inflammatory and apoptotic signals [27]. The physiological response to mitochondrial dysfunction involves sensor proteins such as AMPK and sirtuin that further activate antioxidant gene regulators, such as Nrf2 and FoxO3 [28]. As a rule, alterations of mitochondrial function are observed in several pulmonary diseases and cancers [29].

Oxidative stress triggers the premature aging, through the abovementioned DNA instability and by inhibiting sirtuin-1, a regulatory protein of DNA repair system [15, 18]. Changes in cell morphology and physiology and their permanent proliferative arrest (senescence) further increase DNA damage, ER stress, insufficient autophagy, mitochondrial dysfunction, and ROS production [19, 28, 30, 31]. Furthermore, senescent cells express a peculiar secretory pattern, defined as Senescence-Associated Secretory Phenotype (SASP), consisting of cytokines, chemokines, and growth factors such as TNF-α, IL-β, IL-1, -6, CCL2, CXCL1, CXCL8, and TGF-β [32–34]. The SASP molecular microenvironment is linked with a persistent low level of inflammation and with immunosenescence (i.e., the immune cells, although chronically activate, show reduced functioning) [9, 15]. Premature aging markers and telomere shortening are often associated with IPF and COPD [31, 35].

9.3 Oxidative Stress in Chronic Obstructive Pulmonary Disease (COPD)

Exogenous oxidative stress and cigarette smoking are recognized as the principal pivotal factors in COPD etiology, prompting tissue injury, chronic inflammation, and mitochondrial dysfunction, accelerating aging, and altering the protease-antiprotease balance [36]. Increased levels of different markers of oxidative stress (such as 8-oxo-2′-deoxyguanosine, nitrotyrosine, isoprostanes, and AGEs) and pro-inflammatory molecules (TNF-α, IL-6, -8, CCL2, CCL3, ICAM-1, and leukotriene B4), as well as low levels of antioxidants, characterize biofluids and lung tissue of COPD patients [36–38]. The cellular mechanisms promoting the oxidative stress induced by smoking are complex and poorly understood; however, the gas and tar phases of cigarette smoke contain short-lived oxidants and long-lived radicals, respectively, and these compounds can react to form highly reactive molecules such as peroxynitrite or hydrogen peroxide [39]. Inhaled particles in ambient air have also the ability to generate free radicals and to activate cellular oxidative stress-response signaling pathways [40]. Thus, the inhalation of cigarette smoke and airborne particles depletes antioxidants (as glutathione), recruits macrophages, encourages AECs and inflammatory cells (especially AMs and neutrophils) to produce ROS and to release pro-inflammatory cytokines (through inflammasome, NF-kB, IRAK1, JNK, ERK, or TLR signaling), and, in parallel, induces apoptosis/cytotoxicity through ER stress [38, 41]. In fact, higher amounts of ROS and inflammatory proteins (as TNF-α, IL-1β, IL-6, IL-8, and CXCL1) as well as an increased number and altered functions of AMs and neutrophils are reported in COPD [15, 36, 38]. In particular, AMs from COPD patients show reduced phagocytic and antigen-presenting activity contributing to inflammation, apoptosis induction, reduced T-cell activation, and susceptibility to infections [36, 38]. The up-regulation of TNF-α signaling may have a pathogenic role in COPD by supporting further recruitment of inflammatory cells and tissue remodeling through induction of extracellular matrix (ECM)-degrading enzymes by neutrophils and AMs [42]. Indeed, BAL and sputum samples from COPD patients with exacerbations have higher TNFRII levels than those from healthy controls, and TNFRII concentrations are suggested as a prognostic biomarker of COPD [42]. Furthermore, ROS foster the breakdown of several ECM components (collagen, elastin, hyaluronic acid, fibronectin, and proteoglycans) and the inactivation of anti-proteases (α1-antitrypsin and other serine protease inhibitors) and, in parallel, induce the transcription and the proteolytic activation of proteases (as MMPs, cathepsins, or neutrophil elastase), triggering lung tissue destruction [26, 38, 43]. Independently of the smoking history of COPD patients, the increase of senescence markers and SASP proteins in their fibroblasts, endothelial cells, and AECs compared to controls shows that ROS influence the premature aging of the lung [15, 19].

ROS-induced mitochondrial abnormalities are reported in airway smooth muscle, bronchial epithelial cells, and AECs of COPD patients, and through accelerated cell senescence, apoptosis, and inflammation, they contribute to COPD pathogenesis and progression [7, 28]. Several protective mechanisms against DNA damage and mitochondrial stress are dysregulated by ROS in COPD lung, including reduced levels of parkin (a regulator of mitochondrial autophagy), sirtuin1, and FoxO3 [28, 38].

Nitrogen metabolism, in particular nitric oxide (NO), is hypothesized to impact on COPD pathogenesis [41]. It is well-known that oxidative stress can reduce the activity of nitric oxide synthase (NOS) positively regulating the metabolism of asymmetric dimethylarginine (ADMA), a potent inhibitor of this enzyme, and negatively influencing the production of arginine, a substrate of NOS [44]. Serum from COPD patients shows low NO concentration, high ADMA levels, and high ADMA/arginine ratio as compared to that in controls and correlates with disease severity. Interestingly, ADMA is hypothesized to be a comorbidity risk factor, as well as a prognosis and mortality biomarker, being increased in serum from non-survivors COPD and in patients with pulmonary hypertension or with acute exacerbation as compared to COPD stable patients [44]. Another possible mechanism of NOS down-regulation in COPD might involve PAR-1 (a ROS-induced protein involved in DNA repair and modulating NOS transcription) which is observed to be up-regulated in PBMCs from COPD patients [37].

9.4 Oxidative Stress and Idiopathic Pulmonary Fibrosis (IPF)

Several pieces of evidence show that oxidative and nitrosative stresses give a substantial contribution to IPF pathogenesis and progression, although they are not the main causative factor [26, 45]. Hence, insufficient concentration of antioxidants and high levels of oxidative/nitrosative markers (such as isoprostane, hydroperoxides, nitrogen oxides, nitrosotyrosine, uric acid, and etane), oxidized lipids, oxidized, nitrated, and carbonyl proteins have been found in biofluids or lung of IPF patients as compared to healthy subjects [45, 46]. Correlating with progressive worsening of dyspnea, acute exacerbation incidence, and BAL neutrophil content, some of these molecules may constitute potential prognostic biomarkers in serum or BAL samples from IPF patients [3, 4]. Positive and intricate interactions between the transforming growth factor β (TGF-β; the most well-known fibrogenic cytokine) and RONS signaling represent another important aspect in IPF pathogenesis [26]. TGF-β induces mitochondrial oxidant radical formation in lung fibroblasts by enhancing NADP oxidase, inhibiting sirtuin 3 expression and inactivating Nrf2; on the other hand, ROS support the profibrotic TGF-β downstream signaling at different levels [28, 47]. In general, ROS amplify the TGF-β-mediated pathway through oxidation of redox-sensitive proteins such as thioredoxin, which has inhibitory effects in physiological conditions [13]. In particular, the induction of NADPH oxidase-4 (NOX4) expression by TGF-β is postulated to have a central role in driving fibrotic response in IPF through ROS generation [13, 48]. NOX4 is highly expressed in fibroblast foci of IPF by myofibroblasts and AECs with opposite effects: it promotes differentiation (increasing expression of α-smooth muscle actin, fibronectin and procollagen I) and apoptotic resistance in fibroblasts/myofibroblast as well as apoptosis, mitochondrial stress, and epithelial-to-mesenchymal transition (EMT) in alveolar epithelial cells [25, 29]. The presence of myofibroblasts additionally boosts the ROS-TGF-β positive loop because these cells generate high levels of ROS which support myofibroblasts survival, differentiation, and contractility through ROCK pathway [49, 50].

Among the products released by damaged AECs in active sites of fibrosis, tenascin-C and sonic hedgehog (SHH) represent an interesting integration between ROS and TGF-β signaling in IPF patients [51, 52]. In AECs, TGF-β promotes tenascin-C secretion and inhibits SHH release; vice versa, oxidative stress stimulates the release of SHH and the transcription of tenascin-C. Tenascin-C is a profibrotic factor associated with EMT and tissue remodeling, while SHH is an AEC proliferative factor possibly related to re-epithelialization [51].

Furthermore, increased activity of NOS is observed in IPF lung and NO seems also to promote TGF-β and ECM-degrading enzymes in fibroblasts, at least in murine models [26, 43].

As abovementioned, the ECM degradation may occur through not only enzymatic but also oxidative mechanisms leading to remodeling and fibrosis. In IPF lung, there are increased levels of several ECM-degrading enzymes (in particular MMPs), whereas antioxidant enzymes (as extracellular superoxide dismutase) and pathways (as Nrf2) are barely present in fibroblast foci region [3, 4]. Thus, ROS critically contribute to the shedding and activation of latent form of TGF-β that is physiologically stored in intact ECM. In addition, low-molecular fragments of ECM components (as syndecan and hyaluronic acid) are observed in IPF and have been suggested to promote fibrosis and inflammation by facilitating the neutrophil recruitment [43, 48].

9.5 Oxidative Stress in Asthma

Oxidative stress may also affect asthma pathology, influencing several aspects associated with the disease, including alterations in airway smooth muscle contraction, mucus secretion/clearance, vascular permeability, and airway hyper-responsiveness [26].

In asthma, the observed high levels of oxidative stress markers in biofluids, as well as the low levels of NO and decreased activity of pulmonary antioxidant enzymes, are associated with disease severity [9, 15, 44]. The boost of oxidative stress seems to be related to the altered response to inhaled allergens or inflammation and is suggested to play a driving role in exacerbations [29]. Alterations in ROS production are observed in asthma in different cells. In particular, histamine and Th2 cytokines induce secretion of ROS by alveolar and bronchial epithelial cells [9, 29]. The latter exhibit elevated levels of NADPH oxidases (such as DUOX1 and DUOX2), and the activities of inflammasome, NOX4, and TNF-α signaling are increased in neutrophils and macrophages as compared to healthy controls [29, 42].

The enhanced inflammation and hyper-responsiveness in asthma airways through ROS-mediated mechanisms involve β-adrenergic receptor, DNA damage-response (especially PARP signaling), incorrect T cells maturation, and alteration of cytokines secretion by dendritic cells and epithelial cells [53, 54]. Hence, IL-8 (induced by NOX), IL-5 (induced by PARP1), and IL-33 and IL-25 (induced by DUOX1) foster inflammation, Th2 response, and leukocytes recruitment [29]. Cumulative evidence suggested the additional importance of oxidative stress in pathogenesis of neutrophilic, severe, and elderly asthma [9].

9.6 Conclusion and Future Perspectives

The oxidant/antioxidant imbalance and the accumulation of highly reactive molecules cause damage to DNA, lipids, proteins, and carbohydrates and are implicated in the pathogenesis of diseases affecting the lung and pulmonary vasculature. Redox-regulated signaling pathways are important mechanisms to regulate cellular functions, and ROS and RNS have specific targets conferring them signaling properties and determining their biologic effects [55]. For instance, GSH and NADP homeostasis is regulated by GSH peroxidases, S-transferases, and reductases, and when the mechanism is altered, the induced signaling pathway promotes airway inflammation in COPD and asthma [55]. There are several methods to measure the oxidative stress in lung pathologies such as increased lipid peroxidation products, DNA oxidation, or protein carbonyl formation in lung tissue, and several antioxidant scavengers have been tested in clinical trials to restore oxidant/antioxidant imbalance in pulmonary and cardiovascular diseases [4, 19, 36, 55]. Unfortunately, the results of these studies were conflicting or unsuccessful in IPF, COPD, and asthma treatments. Although dietary supplementation with antioxidants (vitamin A, C, E, β-carotene, glutamine, polyphenols, melatonin, and coenzyme Q10) may produce some beneficial effects, such as lower risk for COPD, asthma incidence, attenuation of inflammation, and lung deterioration, it cannot be considered a valid therapeutic strategy [55, 56].

Recently, a double-blinded, placebo-controlled crossover study on asthma patients reported that γ-tocopherol may have potential therapeutic effects reducing inflammation and eosinophils in the induced sputum [57]. N-acetyl-cysteine (NAC) is an antioxidant (acting as scavenger and restoring glutathione), mucolytic, and anti-inflammatory drug widely tested in lung diseases yielding contrasting results [52, 55]. In the context of COPD (and cystic fibrosis), NAC seems to ameliorate the pulmonary function and to reduce the risk of exacerbation, whereas it has not been shown to prevent mortality in asthma [52, 55]. Although there is evidence of an improvement of 6-min walking test distance, NAC therapy is not recommended (even when combined with antifibrotic drugs) for IPF therapy due to the lack of beneficial effects in pulmonary functional tests parameters such as DLCO and VC and in the mortality rate [52, 55].

Interestingly, pharmacogenomics seems to affect the NAC therapy in IPF, as reported by the differential response of patient with different *TOLLIP* genotypes [58]. For this reason, recent insights in personalized medicine are oriented toward implementing the efficiency of antioxidant therapy in selected stratified patients. In fact, genetic polymorphisms in antioxidant enzymes such as glutathione-S-transferase or superoxide dismutase are associated with susceptibility and symptom development in asthma and COPD patients [59–61]. Thus, for appropriate therapies it seems necessary to consider individual genetic and epigenetic factors that may influence the oxidant/antioxidant system. Moreover, the monitoring of oxidative stress markers as indicators of treatment response could be helpful in the optimization of individual dosage. The asthma management with inhaled corticosteroids based on the monitoring of fractional exhaled nitric oxide appears to reduce

exacerbations in adult patients [59]. However, the establishment of targeted and patient-specific therapies will be a difficult task without appropriate systems to directly provide a proper antioxidant, in the right concentration and in a specific tissue or cell of the lung environment, especially in the initial disease phase, before oxidative stress compromises the tissue integrity.

Conflict of Interest The authors declare no conflict of interest.

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TRP Channels, Oxidative Stress and Chronic Obstructive Pulmonary Disease

10

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Abstract

Chronic obstructive pulmonary disease (COPD) is a lung disease that is often associated with chronic bronchitis, bronchiolitis and emphysema. The disease pathology is heterogeneous in nature and usually results from several environmental factors including cigarette smoke, biomass smoke particle, diesel and automobile exhausts that can potentially expose lung tissues into severe oxidative stress condition. Some individuals, with genetic predisposition, are worse affected. The disease pathology becomes complicated and deadly when environmental and genetic factors both work in a concerted manner. In recent years, transient receptor potential (TRP) channels have been identified as key factors in COPD initiation and progression. TRP channels have been widely implicated as potential targets for genetic manipulation and pharmacological intervention to control the disease. The present chapter briefly discusses expression pattern of different TRP channel members in the lungs and airway epithelium, their physiological role in developing COPD disease pathology with special attention to oxidative stress and the pharmacological intervention and possible genetic manipulation to tackle the disease in near future.

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Lung · TRP channels · COPD · Oxidants · Airway epithelium · Inflammation · Calcium influx

Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium
4αPDD	4α-phorbol 12, 13-didecanoate
BS	Biomass smoke
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
COPD	Chronic obstructive pulmonary diseases
CRAC	Ca^{2+} release-activated Ca^{2+} (CRAC) channels
CS	Cigarette smoke
EET	5′, 6′-epoxyeicosatrienoic acid (EET)
EGTA	Ethylene gycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ER	Endoplasmic reticulum
GST	Glutathione-S-transferase
NKA	Neurokonin A
PM	Plasma membrane
SNPs	Small nuclear polymorphisms
SOD	Superoxide dismutase
SP	Tachykinins substance P
STIM	Stromal Interaction Molecule
TRP channels	Transient receptor potential channels

10.1 Introduction

Chronic obstructive pulmonary disease (COPD) is usually caused by multiple factors, and it is the fourth leading cause of death worldwide [1]. COPD disease pathology is heterogeneous in nature, and it includes chronic bronchitis, bronchiolitis and emphysema. The condition is presented by chronic airway inflammation [2], and the disease pathology is associated with obstruction of airflow into the lung [3–5]. The condition is not fully reversible at the onset of the disease and is usually progressive in nature, causing debilitating disability and finally death.

Asthmatic condition is often treated with glucocorticoids, but it is unsuccessful in treating COPD-related inflammation. Until now, no effective therapeutic and pharmacological intervention is available to reduce COPD-associated mortality [6, 7]. Extensive research performed in this specific subject area has identified

oxidative damage to lung epithelial cells is directly linked to the COPD disease pathology [8–10]. Several factors, including smoking, domestic smoke exposure, outdoor pollution, socio-economic status, and ethnicity, have been identified as major contributors towards developing COPD [11].

Reactive oxygen species (ROS) are known causative agents for cellular oxidative stress and tissue damages. Oxidative stress in pathological condition causes oxidant burden. Free dioxygen radical ($O_2^{-\cdot}$) in oxidative stress condition may function as signal transduction molecule in initiation and progression of the COPD disease state. Transient receptor potential (TRP) channels have been widely implicated in relation to COPD initiation and progression [12–15]. TRP channels are polymodal cation selective ion channels that sense and respond to environmental changes and stimuli such as pH, temperature, osmolarity and exposure to chemical agents. TRP channels also play a significant role in several cellular processes, including apoptosis and neural functions. The mechanism of calcium influx through ROS-sensitive channel and subsequent cellular signaling mechanisms are still largely unknown.

In recent past, scientists put significant effort in order to inhibit ROS-activated TRP channels by antioxidant treatment. TRPM2, one subgroup of the TRP channels, have been reported to be ROS-sensor [16]. Recent development in this specific area has identified TRPM2 channel as a potential candidate in order to modulate the antioxidant enzyme glutathione peroxidase activity [17].

TRPA1, expressed in the chemosensory C-fibers, has been reported to be activated by most of the oxidizing and electrophilic chemicals including but not limited to chlorine, acrolein, isocyanates and tear gas. The chemical stimuli exert their toxic effects by activating TRPA1 through covalent protein modification [18].

COPD disease pathology is not only restricted to lungs, but it has been established as a systemic disease that significantly affects multiple organ systems. Smoking cigarette is directly linked to developing COPD-associated morbidities, and the beneficial effect of quitting smoking has been emphasized as a first line of correcting measure to treat the disease [19].

Human body is constantly exposed not only to oxidants from exogenous sources but also to the reactive oxidant species (ROS) produced endogenously. Glutathione-S-transferase (GST) and superoxide dismutase (SOD) are two main antioxidant enzymes responsible for scavenging ROS activity and play pivotal roles in maintaining redox homeostasis. Cigarette smoke and other environmental pollutants irritate various immune cells located in the lung and cause oxidative stress. Epithelial cells being first line of defense is usually worse affected. This phenomenon ultimately leads to a disruption in redox homeostasis and cause severe damage, which in turn contributes toward developing COPD [20].

Different experimental approaches have identified TRP channels respond to several exogenous stimuli to the airway sensory neurons. The stimuli include harmful chemicals, stimuli causing pain, glandular secretion, depression, cough and other protective responses.

Till date, about 30 TRP channels have been identified. These are further subdivided into seven main subfamilies on the basis of sequence homology. These are TRPC (canonical), TRPV (vanniloid), TRPM (melastatin), TRPP (polycystein),

TRPML (mucolipin), TRPA (ankyrin) and TRPN (NOMPC-like). TRPN channels are found only in invertebrates and fish, and the expression of other six subfamilies has been confirmed in human. The subsequent effect of TRP channel activation leads to either neurogenic inflammatory and/or brain-mediated responses of the airways. If undiagnosed and left untreated, these responses mature into severe breathing problem, and eventually COPD disease pathology sets in. The exact and specific roles of individual TRP channels in specific disease conditions are still largely unknown.

10.2 TRP Channel-Mediated Chemosensation and Associated Responses

Trigeminal chemosensory nerve endings located in the nasal mucosa is the first line of defense in combating exposure of toxic chemical-induced pathological events in the airway [21]. Release of calcitonin gene-related peptide (CGRP), neurokinin A (NKA) and tachykinins substance P (SP) occurs from the nerve ending because of chemical stimulation. The associated downstream signaling events include neurogenic inflammatory vasodilation and leakage, leading to constriction and obstruction of the nasal passage [22, 23].

Oxidative stress and other noxious chemical compounds activate unmyelinated bronchopulmonary C-fibers and initiate action potentials that conduct centrally to evoke unpleasant sensations (e.g. coughing, dyspnea and chest tightness) and to stimulate/modulate reflexes (e.g. cough, bronchoconstriction, respiratory rate and inspiratory drive) [24].

Key components of this pathological event are highly sensitive to regulation of intracellular calcium concentration ($[Ca^{2+}]_i$) and play a significant role in nociception and other exogenous stimuli-induced responses. This finding actually emphasizes the importance of cellular calcium mobilization and calcium-mediated signal transduction. As COPD pathology is usually associated with cellular signaling mechanisms initiated by an increase of $[Ca^{2+}]_i$ as a result of cellular calcium influx, TRP channels draw wide attention due to its cation selective gating properties with a focused interest of calcium influx, specifically through these channels [25].

Localization of different subtypes of TRP channels was confirmed in the epithelium and smooth muscle of the lung tissue. Cigarette smoke, industrial pollutants, aldehyde, chlorine and fragrances are the known activators of TRP channels in the human lung epithelium and in the airways. During signal transduction event, calcium is released into the cytoplasm from endoplasmic reticulum (ER), and the immediate effect is store-operated calcium entry through store-operated calcium influx channels, including TRP channels [18]. TRP channels are mostly plasma membrane (PM)-bound (except nuclear membrane and mitochondrial membrane) and selectively allow influx of cations including of Ca^{2+}, Mg^{2+} and trace metal ions [26].

One of the important mechanisms of cellular calcium influx happens through Store-Operated Calcium Entry (SOCE) [27]. ER calcium store is usually

replenished by such a mechanism in a faster way after store depletion. Stromal Interaction Molecule 1 (STIM1) with PM localization has been discovered as critical communicating protein that controls SOCE [28] when the store becomes empty. Immediately after STIM1 discovery, ER Ca^{2+} sensor Orai1 has been identified as the pore-forming subunit of the Ca^{2+} release-activated Ca^{2+} (CRAC) channels [29–31]. STIM1 mediated activation of SOC channels require Orai and TRPC1 interaction [32–40]. The role of STIM1 and Orai1 variants in SOCE has been reviewed extensively in Ref. [41].

10.3 Oxidative Damage and COPD

COPD pathogenesis mainly happens due to oxidative stress in the lung tissue. Exposure of lung to inhaled exogenous oxidants along with endogenously produced oxidative stress in the lung due to ageing and various metabolic processes creates significant oxidative damage. Oxidant and COPD-associated pathology includes but not limited to cell membrane damage due to destruction of membrane lipid bilayer, proteins and nucleic acids [42].

Cigarette smoke (CS) has long been identified as a major cause of COPD due to oxidative stress produced in the lower airways [43]. CS-induced damage to the lung tissue and the development of COPD depends on the extent of inhaled cigarette smoke exposure. Hydroxyl radical ($OH^{-\cdot}$) in the inhaled CS causes lipid peroxidation of the cell membrane proteins. $OH^{-\cdot}$, upon reacting with unsaturated fatty acids of the membrane phospholipid, generates organic acid free radicals and causes membrane damage [44]. The secondary metabolite formed due to rapid degradation of the unstable intermediate oxidant molecules causes further lipid peroxidation. The intermediate oxidants molecules include alkanes (e.g. ethane/pentane) and aldehydes (e.g. malondialdehyde). The concentration of thiobarbituric acid reactive substance (TBARS) has been found in higher quantities in smoker lungs with COPD [45].

Non smoking-associated COPD development and progression of the disease has been linked to several factors. Deficiency of α1 antitrypsin, presence of chronic asthma, ROS-exposure due to polluted air, biomass smoke (BS) exposure, etc. has been found to be the major cause of nonsmoking-associated COPD [45].

Smoker lungs have been shown to have elevated high granular density alveolar macrophages, which has been identified as a major contributor for increased ROS production [46, 47]. The deadly association among H_2O_2, $O_2^{-\cdot}$ and $OH^{-\cdot}$ radicals results in bronchial hyper responsiveness in COPD patients [48].

Endogenous cell-derived ROS produced in metabolically active cells is a result of enzymatic reactions involving a group of oxidant enzymes. Three main members of such an oxidant enzymes are NADPH oxidase, eosinophil peroxidase (EPO) and myeloperoxidase (MPO) [49]. Mitochondria are the source of reactive nitrogen species (RNS), $O_2^{-\cdot}$ and H_2O_2 production [50, 51]. Sources of exogenously produced ROS are CS [52] and the lipid peroxidation in inflammation of airway epithelium due to environmental ozone exposure [53].

10.4 TRP Channels and COPD

Increased TRPC6 mRNA expression in human alveolar and lung tissue macrophages has been reported in COPD patients [54]. The pathophysiological roles of non-neuronal TRPV1/TRPA1 channels have been widely studied in infection, inflammation and immunity. The sensory input of non-neuronal TRP channel mediated signal transduction mechanisms ultimately results indirect neurogenic pain or inflammation.

TRPV1/TRPA1 activation has been positively correlated with airway neurogenic inflammation. Non-neurogenic inflammatory responses produced by non-neuronal TRPA1 results inflammatory airway diseases. Thus TRPA1 has been identified as a prominent target to treat inflammatory respiratory diseases [55]. TRP channels are also involved as active removal mechanisms of foreign toxic substances in the cell. TRPV1/TRPA1 isoforms are widely expressed in lung sensory neurons, and those specific TRP channel activation causes alteration in vagal output associated with change in respiratory pattern, blood flow and coughing behavior.

TRPV2/TRPV4 expressed in the alveolar macrophages play critical roles in immune response initiation [56]. Contribution of different TRP channel family members in relation to COPD development is summarized in Fig. 10.1.

10.4.1 TRPC6

TRPC6 and TRPC7 both gene expression has been detected in lung tissue [57]. TRPC6 being predominantly expressed in macrophages, lymphocytes and neutrophils [12] and also in the airway epithelium became a target gene for inflammation-induced lung diseases.

Increased TRPC6 gene expression has been reported in macrophages isolated from COPD patients [54]. In COPD patients phospholipase C (PLC), one of the important modulators of TRPC channels including TRPC6, has been found to be activated [58] as a result of CXC chemokine receptor activation. Thus TRPC6 activation and CXC chemokine receptor activation-mediated inflammation in COPD has been emerging as an interesting area of research.

10.4.2 TRPC4

Expression of TRPC proteins has been reported in endothelial cells, vascular smooth muscle cells and mast cells [59]. Discovery of TRPC4 knock out (KO) mouse model [60] opened up the possibility of detailed study for the role of TRPC4 in lung diseases and in COPD. Vascular endothelial cells of lungs in TRPC4 KO mice have a defective Ca^{2+} influx mechanism which has been found to be induced by thrombin [61]. Investing future research effort on TRPC4 in context to COPD and respiratory diseases certainly has potential to shed lights on COPD disease pathology.

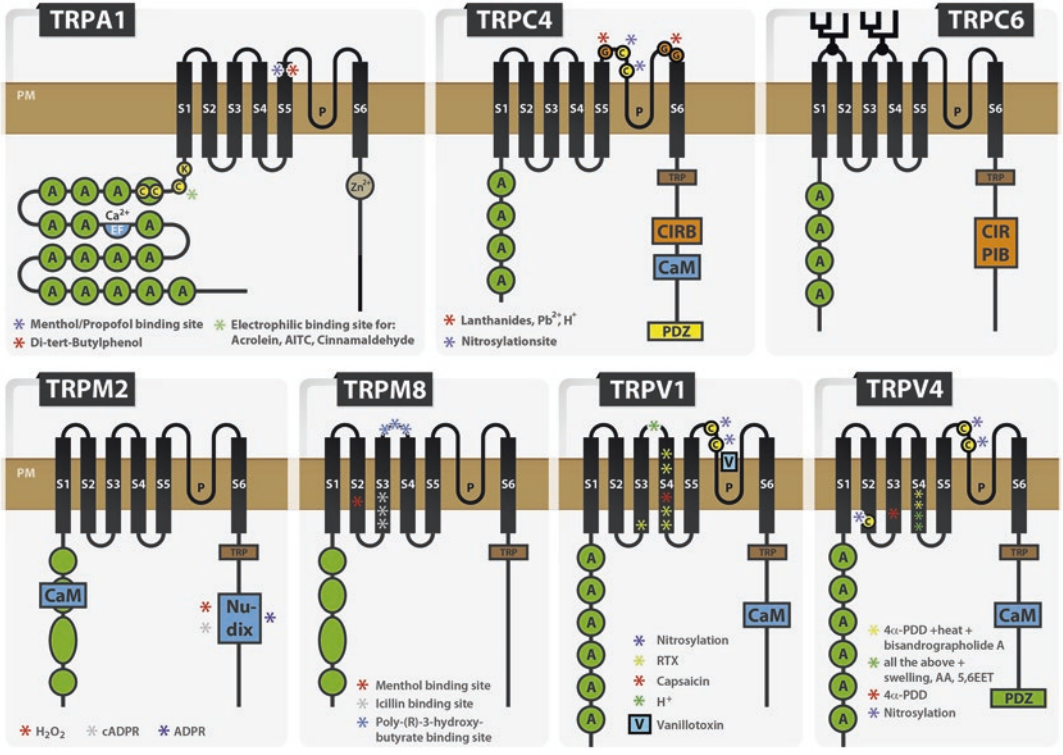

Fig. 10.1 Summary of different TRP channels expressed in lung tissues and the exogenous toxic agents they sense and respond. Respective membrane topology, functional domain and proposed binding sites with respect to the modulators of individual TRP channels are depicted in this figure. *4α-PDD* 4α -Phorbol 12,13-didecanoate, *5,6 EET* 5,6-Epoxy-eicosatrienoic acid, *AA* arachidonic acid, *A* Ankyrin repeat, *AITC* allyl isothiocyanate, *ADPR* ADP-ribose, *cADPr* cyclic ADP-ribose, *CaM* calmodulin binding site, *CIRB* calmodulin-IP3-receptor-binding site, *CIRPIB* calmodulin-IP3-receptor-phospho-inositide-bindingsite, *RTX* Resinferatoxine, *TRP* TRP box, *Nudix* nudix box, *P* pore, S1–6 transmembrane segment 1–6, TRP box, PDZ, PSD95/SAP90-Discs-large-Zonula-occludentes-1 domain. (Taken from Ref. [151] with permission)

10.4.3 TRPM2

Recently TRPM2 channel has been described as an oxidant sensor [62]. TNF-α and lipopolysaccharides (LPS) are two known potent activators of TRPM2 channels [63]. TRPM2 channel is largely expressed in inflammatory cells, including endogenous ROS-producing cells. Primary human monocytes have been shown to cause TRPM2 mRNA upregulation upon LPS or TNFα challenge [63]. Targeted gene knockdown studies of TRPM2 employing specific siRNA have been shown to reduce TNF-α, IL-6, IL-10 and $[Ca^{2+}]_i$ rise upon LPS exposure [63].

Direct activation of TRPM2 and IL-8 production by H_2O_2 and subsequent cellular calcium influx have been shown in human monocyte cell line [64]. TRPM2 has been identified as an important target in oxidative damage-induced cellular inflammatory processes. The involvement of TRPM2 channel-mediated oxidative stress-induced cellular inflammatory processes has been tested in $TRPM2^{-/-}$ mice compared to WT counterparts [65, 66]. Further research on monocytes isolated from $TRPM2^{-/-}$ mice has shown reduced Ca^{2+} influx and reduced macrophage inflammatory protein-2α (MIP-2α or CXCL-2α) production in response to oxidative stress compared to WT mice [67].

Research from Heiner group [68] has shown involvement of TRPM2 in the neutrophil chemotaxis in human. Yamammoto group [64] also has provided the evidence for the existence of similar TRPM2-mediated mechanisms in experimental mice model. All those immune cell-induced cellular inflammatory pathways have been so far characterized in the COPD disease state.

10.4.4 TRPM8

Expression of TRP channels has been confirmed in vagal afferent neurons. Cold and menthol, a TRPM8 ligand, both have been reported as TRPM8 activators [69]. So-called thermoreceptor sensory function of TRPM8 channels operating at non-physiological low temperature zone has not proved to be of beyond doubt. Cold air is known to cause airway constriction, mucus secretion, cough and plasma protein infiltration which is characteristic to processes associated with inflammatory airway diseases [70, 71]. COPD being one of the well-characterized inflammatory airway diseases certainly draws attention with a possible linkage between TRPM8 pathology and the disease presentation. Presence of a functional variant of TRPM8 protein in human epithelial cell has recently been reported that promotes ER calcium release and subsequent increase in inflammatory cytokine transcription [72, 73].

Consistent with this notion of the presence of oxidant and TRM8-mediated mechanisms in COPD has been further supported by the fact that menthol cigarette smokers in COPD patients had shown severe airway inflammation compared to non-menthol smokers with COPD individuals [74]. In the same study employing in vitro model, the degree of ROS production has been compared between non-menthol cigarette smoke extract (Non-M-CSE) and menthol cigarette smoke extract (M-CSE) groups. Initially similar degree of increased extracellular ROS production

has been reported in both groups. However, M-CSE group eventually produced a robust cytoplasmic calcium elevation, MAP Kinase (MAPK) activation, NF-κB signaling and release of IL-8. N-acetyl-cysteine (NAC), a ROS scavenger, was able to block the ROS-induced responses in both CSE treatment groups.

Additionally EGTA (an extracellular Ca^{2+} chelator) and AMTB (a TRPM8 antagonist), or both were able to completely inhibit both CSE-induced responses. Those findings strongly indicate a functional role of TRPM8 channel in oxidant-induced airway inflammation and possibility of TRPM8 being a therapeutic target to treat COPD. When menthol has been introduced into the Non-M-CSE groups, the rise in cytoplasmic calcium and release of IL-8 had been significantly increased compared to the Non-M-CSE only group. The involvement of TRPM8 in oxidative stress-induced inflammatory responses in smokers has been supported by employing either TRPM8 knocked down cells or TRPM8 knock out animal models [75].

10.4.5 TRPA1

Involvement of TRPA1 channel has been proved beyond doubt as major signaling mechanisms in COPD disease pathology [76]. Cigarette smoke extract (CSE), acrolein and crotonaldehyde have been shown to produce contraction of bronchial rings in guinea pigs which has been shown to be prevented by pretreatment with HC-030031, a specific TRPA1 antagonist and not by capsazepine, a TRPV1 antagonist or reactive oxygen scavengers [77].

Covalent modification of the N-terminus cysteine residues of TRPA1 by prostaglandins is one of the well-studied mechanisms of the channel activation [78, 79]. Another important activation mechanism is the lipid peroxidation, a mediator of cigarette smoke-induced inflammation [80, 81]. TRPA1 agonist-induced tussive responses in preclinical guinea pig model were found to be inhibited by HC-030031 [82]. Considering COPD disease etiology, the involvement of TRPA1s role in neurogenic inflammation is not well established. Recent evidence also suggests the involvement of TRPA1 in the non-neurogenic inflammatory pathways in experimental mice model [55].

TRPA1-induced neurogenic inflammation is usually associated with COPD [77]. Studies on preclinical animal models have provided evidence that TRPA1 channels play a significant role in cigarette smoke-induced bronchial inflammation [77]. Cigarette smoke is a complex mixture of several irritants known for potentially activating TRPA1 channel. Acrolein and crotonaldehyde [77, 83–86], along with nicotine [87] present in cigarette smoke, have been identified as direct TRPA1 activators. Biomass smoke (BM), mainly produced by burning wood, has been recently identified as activator of TRPA1-induced chemosensation in cultured jugular ganglia isolated from guinea pig [88]. Primary cultures of human airway fibroblasts, smooth muscle cells and epithelial cells have been reported to release IL-8 upon cigarette smoke-induced TRPA1 stimulation [55].

10.4.6 TRPV1

Neuronal TRPV1 channels are mostly expressed in C- and Aδ- fibers of primary sensory neurons. This channel has been widely described as nociceptors. TRPV1 channels are major intra and intercellular communication channels of the respiratory tract covering nose, alveoli, smooth muscle and blood vessel [13, 89].

Present TRPV1 research in relation to COPD is revolving in the areas of TRPV1's role in sensory nerves and, especially, in tussive response associated with COPD [90, 91]. Involvement of neuronal TRPV1 responses in COPD pathology in human is still questionable while the role of non-neuronal TRPV1-response is becoming more evident in recent years [92].

Heat, protons, voltage, endogenous chemicals (including lipoxygenase products) and exogenous chemicals (including capsaicin and resiniferatoxin) are the known activators of TRPV1 [57]. Protein kinase A (PKA), protein kinase C (PKC) and other kinase-induced direct phosphorylation also activate TRPV1 channel [93, 94]. Phospholipase C (PLC) also has been shown as a TRPV1 mediator [95]. TRPV1-induced release of TNF-α and downstream proinflammatory response in sensory neurons has been reported [96]. Elevated levels of endogenous TRPV1 activators such as arachidonic acid metabolites involved in PKA, PKC and PLC pathways have been found in the lungs of COPD patients.

Low pH, a known TRPV1 activator, has been found in the exhaled breath condensate of COPD sufferers [97]. Hypersensitive tussive response upon capsaicin inhalation has been noted in COPD patients, an indicator of TRPV1 signaling mechanisms [98]. In experimental rat model, hypersensitivity of capsaicin-induced airway inflammation responses in pulmonary myelinated primary afferents was reported [99]. A systematic meta-analysis also suggests a strong correlation of TRPV1 in COPD disease pathology [100].

Apoptosis caused by inhaled airborne particulate material has been found to be completely inhibited by capsazepine in human airway epithelial cells and in TRPV1$^{-/-}$ mice [101]. Parallel studies also reported TRPV1 agonist-induced ER stress and loss of cell viability in BEAS-2B and A549 airway epithelial cell lines [102]. TRPV1 stimulation also caused release of IL-6, a proinflammatory cytokine from airway bronchial epithelial cells [103]. These evidences strongly support the role of non-neurogenic TRPV1 responses in COPD. Back in 1984, it has been reported that capsaicin treatment-induced ablation of TRPV1 in neonatal rats were resistant to cigarette smoke (CS)-induced increase in vascular permeability in the airways. In recent years, TRPV1 homozygous KO (TRPV1$^{-/-}$) mice were found to be resistant to LPS-induced inflammation and bronchial hyperactivity, and that pretreatment with TRPV1 agonist SA13353 failed to produce both neutrophil influx and increase in cytokines TNFα and CXCL1 [104].

Tiotropium, a widely prescribed drug for COPD treatment as bronchodilator opened up the initial idea about possible linkage of TRPV1 in COPD disease pathology [105]. Tiotropium was found to inhibit capsaicin, a potent TRPV1 agonist-induced cough (Tussive stimulation) and single C-fiber firing in the guinea pig model [105] and in other preclinical studies [106].

Both TRPV1 and TRPV4 mRNA have been found to be upregulated in patients with COPD and were shown to be involved in CS-induced elevated ATP release in the COPD airways [107].

10.4.7 TRPV4

Known functions of TRPV4 channels include epithelial cell volume control, epithelial and endothelial permeability, bronchial smooth muscle contraction and participation in autoregulation of mucociliary transport. Those functions of TRPV4 appear important for the regulation of COPD pathogenesis, and thus TRPV4 emerges as a candidate gene for COPD. TRPV4 is widely expressed in heart, lung, kidney, CNS and skin [108]. In the lungs the highest levels of TRPV4 expression have been found in the epithelial linings of the trachea, bronchi and lower airways and the alveolar septal walls [109, 110].

TRPV1 and TRPV4 both channels are thermo- and osmo-sensitive [111]. TRPV1 has been emerging as a hyperosmotic sensor and TRPV4 as hypoosmotic [112] and mechanical sensor [113]. TRPV4 also senses and responds to chemical stimuli including, 4α-phorbol 12, 13-didecanoate (4α PDD) [111], GSK1016790A [110] and 5′, 6′-epoxyeicosatrienoic acid (EET) [114]. TRPV4 is important in controlling epithelial and endothelial barrier function, especially in response to increased vascular pressure and stretch. TRPV4 channel activation has been implicated in cellular ATP release mechanisms and subsequent downstream purinergic signaling pathways. It is important to note that increased levels of ATP have been found in bronchoalveolar lavage fluid (BALF) from COPD patients [115]. Recently association of small nuclear polymorphisms (SNPs) in TRPV4 in relation to COPD disease pathology has been confirmed [25].

10.5 ROS and RNS: Potential Activators for the TRP Channels

Infiltrating neutrophils, eosinophils and macrophages into the lung alveolar space significantly increases the pulmonary oxidant burden by generating ROS including $O_2^{-\cdot}$, H_2O_2 and hypochlorite. The NO produced by the inflamed tissue occasionally reacts with ROS and results in more damaging reactive nitrogen species (RNS) including peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2). RNS cause additional nitrative stress in airway diseases [116].

TRPV1 and TRPA1 both have been identified as a potential target for ROS/RNS-mediated cellular calcium signaling processes in both chronic and acute responses to oxidative stress into the lung. ROS-mediated activation of different TRP channels expressed in airway epithelial cells and in sensory nerves towards neurogenic inflammation is schematically shown in Fig. 10.2.

RNS damages the membrane integrity by directly attacking the unsaturated fatty acids (e.g. oleic acid) of the cell membrane and generates highly reactive nitro-oleic acid [117]. Oxidative stress can directly activate TRPA1 channels by

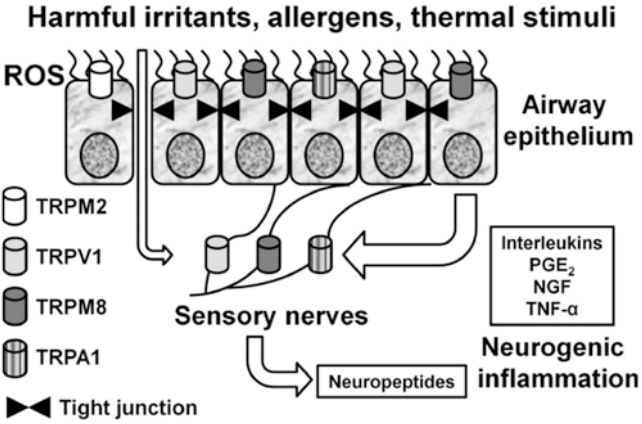

Fig. 10.2 Schematic diagram showing the ROS-induced activation and signal transduction events of TRP channels expressed in sensory nerves and in airway epithelial cells. ROS-induced TRP channel activation following release of proinflammatory mediators leads to the neurogenic inflammation. *PGE2* Prostaglandin E2, *NGF* nerve growth factor, and *TNF-α* tumor necrosis factor-α. (Taken from Ref. [152])

oxidizing cysteine residue of the cytoplasmic N Terminal [118]. TRPA1 channels are found to respond to both the electrophiles and the oxidizing agents entering in the airways. 3-Niro-tyrosine (3-NT), with high biological activity, is one of the several RNS generated by the reaction between RNS and NO and has potential to be used as a marker for NOO^{-}-mediated cellular damages in vivo. Reactive lipid aldehydes are usually formed by an autocatalytic pathway in lipid peroxidation of the cell membrane.

10.6 Biomass Smoke and TRP Channel Activation

The correlation between COPD and biomass smoke (BS) exposure is now well established. At present there is around 3 billion COPD sufferer worldwide. Burning biomass fuel such as wood and coke is common in developing countries as a cheap alternative to the conventional source of energies including electricity and gas. Burning of these materials releases several air pollutants in large quantities that includes nitrogen oxides, sulphur oxides, hydrogen chloride, polyaromatic hydrocarbons, volatile organic compounds, methane, furans, dioxins and aerosol particulates of both organic and inorganic origin [119]. In COPD patients, inhaled BS has been identified as a major contributing factor towards developing inflammatory responses. The late responses of such inflammatory processes result in tissue proliferation in small airways and severe tissue damage in lung parenchyma. Additionally, the disease state contains recruitment of immune cells to the airway compartments [120].

10.7 Diesel Exhausts Particle and TRP Channel Activation

Diesel exhausts particle (DEP) is a very common component in the city environment generated by the automobiles. DEP inhalation has been widely implicated in developing COPD and chronic asthma worldwide. The role of DEP as a direct activator of lung-specific afferent sensory nerves in relation to initiation of respiratory symptoms has been studied. The study on the effect of organic extract of diesel exhaust (DEP-OE) on human and in vitro studies and in vivo electrophysiological studies has identified a list of compounds causing TRPA1 activation. DEPs contain high amount of polyaromatic hydrocarbons (PAHs) on their surface and exert toxic and carcinogenic effects. Phenanthrene, a common PAH found in DEP has been found to cause depolarization of vagus nerve [121]. DEP exposure in human primary airway epithelia has been reported to reduce ciliary beat frequency and results in increased oxidative damage, NF-κB pathway activation and increased secretion of proinflammatory cytokines. Some of the secreted immune-responsive biomolecules also act as mediators and sensitize airway sensory neurons [122–130]. Signal transduction pathways specifically responsible for such a DEP-evoked events are not fully understood.

Electrophiles activate TRPA1 channels and involve covalent modification of the cysteine residues on the N-terminus (Cytoplasmic domain) [131, 132]. This finding possibly provides clues why endogenously produced oxidative stress causes TRPA1 activation as an integral event in intracellular oxidative stress [118, 133]. Robinson et al. [121] have shown that H_2O_2 or DEP-OE depolarizes the vagus nerve in a TRPA1-dependent manner [134, 135], and this response was inhibited by the antioxidant N-acetyl cysteine (NAC).

10.8 Genetic Contributors of COPD

Finding key genetic contributor for the chronic diseases has been a challenge for the investigators and COPD is no exception. Earlier studies [136, 137] have shown evidence that genetic factors are linked to pulmonary function and COPD. Existence of familial aggregation of COPD strongly suggests this notion [136]. Till date, it is not very clear how genetic factors are associated for COPD development and progression. Environmental pollutants have been shown to produce adverse reactions to the bronchial epithelium and recruit inflammatory cells causing pulmonary disease pathology [138]. Association of COPD with polymorphisms of genes like α1-antitrypsin, TNFα and surfactant protein B genes has been suggested in case control studies [139–141]. A study on Indian population exposed to industrial pollutants has shown evidence that microsatellite (MSI) instability is weakly associated with smoker's age and the extent of exposure to exogenous toxins, which are the known cause for developing COPD [142].

α1-antitrypsin deficiency has been previously positively correlated with COPD development in young adults [143]. Phosphorylated serine 19 residue in TRPV4 protein, a human genetic polymorphism, has been previously documented as COPD

susceptibility locus. This specific polymorphism has been directly linked to matrix metalloproteinase (MMP1) activation associated with increased calcium influx and downstream signaling pathways [25, 144, 145].

10.9 Modulators of TRP Channel Expression and Function

Significant development took place in recent years in order to find both competitive and non-competitive inhibitors for the TRP channels in order to control disease states including asthma, COPD and several other airway diseases. The development of TRP antagonists happened slowly, but in recent years, there has been a wide interest in developing TRPV1 and TRPA1 antagonists because of their potential therapeutic role in targeting neuropathic pain. Ruthenium red (RR), a non-selective calcium channel blocker, blocks several TRP channels including TRPV1. Unfavorable cytotoxicity prevented this potent molecule to be considered as potential candidate for further drug development perspective. In recent years (±) camphor has been identified as week TRPA1 antagonist [146, 147]. SB-705498, a potent TRPV1 antagonist developed by GSK cleared its Phase 1 clinical trial in 2007. SB-705498 has shown promise for further clinical trials as it has been shown to be well tolerated in Phase 1 clinical trial with no serious adverse effects. Topical applications of SB-705498 have also been tested in two Phase 2 clinical trials in relation to chronic cough and non-allergic rhinitis [148].

Competitive TRP channel antagonists are therapeutically attractive because of their direct mode of action without upregulating or activating the receptors for the respective channels and usually do not associate with unwanted drug use-related side effects [149]. So far TRPA1 and TRPV1 both appear to be potential target of therapeutic intervention in order to treat respiratory airway diseases including COPD [150].

10.10 Conclusions and Future Directions

TRP channels have now gained wide interest because they have been documented as sensors for environmental stimuli that can sense and respond to exogenous stimuli. The multifunctional roles played by different TRP channels are important in terms of understanding how the cellular sensors work to respond to exogenous stimuli in normal and pathophysiological conditions and how these channels are linked to the mechanisms of disease progression.

A wide variety of exogenous and endogenous stimuli-induced activations of different TRP channels play a significant role in COPD development and progression. Targeting TRP channels has enormous potential in treating pulmonary diseases including COPD. TRP channels appear to be a family of endogenous defense system to combat noxious stimuli-induced cellular damages and play critical immunological roles in many lung diseases including COPD. Rise in cytoplasmic calcium through TRP channel and subsequent cellular signaling pathways that lead to

hyperactivated proinflammatory and immunological responses including activation of different transcription factors, chromatin remodeling and altered gene expression have potential to shed lights on the mechanisms of the COPD for future drug development to combat the disease. Identification of specific TRP genes responsible for COPD will provide further knowledge about how a specific population of chronic COPD is predisposed to the disease and what genetic manipulation and pharmacological intervention could be done in order to prevent or slow down the disease progression.

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Paraquat-Induced Oxidative Stress and Lung Inflammation

11

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Abstract

Lung pathogenesis is associated with the oxidative stress which is one of the major causes of the lung damage. Oxidative stress is an important factor (cause) for development of chronic and degenerative diseases including cancer, aging, rheumatoid arthritis, diabetes, cataract, chronic inflammatory diseases, autoimmune disorders, cardiovascular and neurodegenerative diseases. Emerging evidences suggest that the glutathione redox couple may entail dynamic regulation of protein function by reversible disulfide bond formation on kinases, phosphatases, and transcription factors. Reactive oxygen species (ROS) enhances inflammation through the activation of transcription factors, such as nuclear factor (NF)-κB and activator protein-1 through various kinases (c-Jun-activated kinase, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinase). This results in enhanced expression of proinflammatory mediators. Many environmental pollutants play an important role in causing oxidative stress leading to lung damage. In present chapter impact of paraquat, a known herbicide has been discussed in detail for its effects on oxidative stress and lung inflammation causing injury.

Keywords

Oxidative stress · Reactive oxygen species · Inflammation · Lung damage

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11.1 Introduction

11.1.1 Oxidative Stress, Free Radicals, and Inflammation

Oxidative stress and inflammation is related to a variety of chronic diseases mainly cardiovascular diseases, neurodegenerative diseases, liver diseases, cancer, and aging. It is an imbalance between the production of free radicals and the body's defense mechanism through antioxidant system. The term free radicals is used to define molecular species which contains one or more unpaired electrons, and therefore, radicals are unstable and highly reactive because they have a tendency to donate or accept an electron from other molecules [1, 2]. Oxygen-derived radicals are collectively known as reactive oxygen species (ROS), and nitrogen-derived radicals are reactive nitrogen species (RNS) that include hydroxyl radical, oxygen singlet, superoxide anion radical, hydrogen peroxide, hypochlorite, nitric oxide, nitrogen dioxide, and peroxynitrite radical [3–5].

Development of chronic and degenerative diseases including cancer, aging, rheumatoid arthritis, diabetes, cataract, chronic inflammatory diseases, autoimmune disorders, and cardiovascular and neurodegenerative diseases are due to oxidative stress condition [6].

When any harmful stimuli, virus, bacterium, or fungus infects or affects a part of our body, there is a body's response to remove it, and this phenomenon of body's attempt of self-protection is known as inflammation. It is the protective mechanism of body which involves immune cells and blood vessels, redness, hotness, swelling and pain as signs of inflammation [7, 8]. Due to excessive secretion of cytokines and more expression of inflammatory genes, inflammation, a defensive mechanism of the body, turns into lethal process, like generation of acute lung injury (ALI) condition [9, 10]. These reactive oxygen species are produced either internally in the human body by metabolic processes or from externally exposed sources such as environmental pollutants, radiation, certain drugs, industrial chemicals and pesticides [11, 12].

11.1.2 Commonly Used Herbicides Available in India (http://www.agriinfo.in)

- 2, 4-D (2,4-dichloropnenoxy acetic acid)
- DICAMBA: (3,6-dichloro-2-methoxybenzoic acid)
- SIMAZINE: (2,chloro-4,6-bi(ethylamino)-s-triazine)
- Paraquat (1,1-diethyl-4-bipyridinium ion)
- Diquat (6,7-dihydrodipyrido (1,2:2, I-C) Pyrazinediiumaion)
- Benthiocarb or thiobencarb: (S-(4-chlorobenzyl) N,N-diethyl-thicarbamate)

Environmental toxins include exposure to paraquat (a commonly used herbicide), and chronic ethanol consumption is an established example of lung injury. Here in the present chapter, we are discussing paraquat-induced inflammation and its mechanism in detail.

11.1.3 Paraquat (PQ)

Paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) is a quaternary nitrogen-containing compound. Paraquat dichloride, Gramoxone, and methyl viologen are other common trade names of paraquat [13, 14]. Paraquat is the third best-selling pesticide in the world and is used on over 100 crops in more than 120 countries across the world [15]. It is the most widely used herbicide because it is quick-acting, non-selective contact herbicide which acts on the photosystem I of the chloroplast [16]. Paraquat is a broad-spectrum herbicide, and due to its many uses in crops, it has increased the productivity in agriculture. However, this herbicide is highly toxic and has acute toxicity; its use has been restricted in some countries due to health issues. But, it is a cheap and labor-saving herbicide, therefore popular in developing countries [15].

11.1.4 Structure of Paraquat

Chemical formula of PQ is $C_{12}H_{14}N_2\ Cl_2$. The structural formula of PQ is given below (Fig. 11.1):

IUPAC name is 1,1′-Dimethyl-4,4′-bipyridinium dichloride.

11.1.5 Properties of Paraquat (Table 11.1)

11.1.6 PQ Toxicity

PQ is toxic to human beings and animals; in humans, PQ poisoning causes Parkinson's disease (PD) and severe lung damage. Self-poisoning is a major health issue in the developing countries is associated with PQ [17, 18]. Since its introduction in agriculture, thousands of deaths occur yearly due to accidental or intentional ingestion. PQ has been reported to be a major health hazard because it can cause severe lung injury in human and experimental animals. It is a well-characterized pneumotoxicant [19].

PQ affects the lungs, heart, liver, kidneys, cornea, adrenal glands, skin, digestive system, and central nervous system, but lungs, liver, and kidneys are mainly organs from affected PQ toxicity and irreversible lung injury is the most common cause of death from PQ poisoning [20, 21].

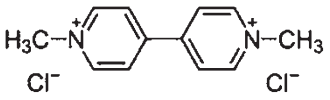

Fig. 11.1 Structure of paraquat (wssroc.agron.ntu.edu.tw)

Table 11.1 Properties of Paraquat

Molecular Formula	C12 H14 N2 (cation only)
	C12 H14 N2 Cl2(dichloride salt) (Worthing 1983)
IUPAC name	1,1′-Dimethyl-4,4′-bipyridinium dichloride
Molar mass	257.16 g·Mol^{-1}
Appearance	Yellow solid (NIOSH pocket guide to chemical hazards)
Physical state	White crystalline solid (pure salt); dark red solution (technical product). Technical product >95% pure (Worthing 1983)
Odor	Faint, ammonia-like (NIOSH pocket guide to chemical hazards)
Density	1.25 g/cm^3
Decomposition temperature	Approximately 300c (pure compound) (Weed Science Society of America 5th ed.)
Boiling point	Salts decompose at high temperatures, charring rather than melting or boiling (Weed Science Society of America 5th ed.)
Solubility in water	High
Vapor pressure	Nonvolatile. Vapor pressure of salts is very low, below 1×10^{-7} mmHg (pure compound) (Weed Science Society of America 5th ed.)
Other names	Paraquat dichloride; Methyl viologen dichloride; Crisquat; Dexuron; Esgram; Gramuron; Ortho Paraquat CL; Para-col; Pillarxone; Tota-col; Toxer Total; PP148; Cyclone; Gramixel; Gramoxone; Pathclear; AH 501

The PQ toxicity basically depends on its amount, route of exposure and amount. The most frequent routes of PQ exposure to the body are its ingestion, or direct contact with damaged skin, and it can be rapidly absorbed by inhalation [22, 23]. PQ causes damage when it comes in direct contact with the skin; normal (intact) skin is barrier to paraquat absorption, but if the skin is broken or wound is present, its absorption may lead to death in humans [24]. Paraquat ingestion (either intentional or accidental) is the most common cause of death due to paraquat toxicity. PQ is one of the common suicidal ingestions and is the major health problem in developing countries, and after ingestion, PQ can damage the inner layer of the stomach and intestine by inducing burning sensation and irritation, resulting in nausea, vomiting, abdominal pain, and diarrhea [25].

Poisoning by inhalation is the most common route of exposure for people working with paraquat in agriculture [14]. In the case of PQ poisoning, inhalation is not considered too toxic due to its low volatility and the formation of large droplets during spray in field. PQ has not been shown to cause serious systemic toxicity from inhalation because spray droplets are too large to be inhaled into small airways, but these large droplets of paraquat can deposit and may cause local irritation in the upper respiratory tract [15, 26].

11.2 Lungs: Target Organ for Paraquat Toxicity

PQ is extremely toxic, causing multiple organ failure, and its toxicity leads to lung injury. Lungs are main target organs for paraquat-induced toxicity in rats and humans [27]. The most widely accepted reason for lung specific toxicity of paraquat

is its tendency to concentrate in lung tissue than any other organ [28–30]. Many studies have suggested that the higher concentration of paraquat is retained in lungs in comparison to other organs, and its concentration has been found six to ten times more in the lungs than in the plasma [31–33]. Therefore, it is clear that lung specific toxicity by paraquat is associated with its accumulation process [31]. The explanation of more accumulation of PQ in lung cells is that alveolar epithelial cells have higher polyamine uptake system as compared to other organs and PQ uptake into lung cells occur through a polyamine uptake system because it is structurally similar to the diamines and polyamines [34–36]. After paraquat intoxication, lungs are the most affected and severely damaged organ due to its ability to accumulate and retain PQ which is completely independent of its level in plasma, where PQ level decreases with time [30, 37].

11.2.1 PQ: A Potent Inducer of Oxidative Stress and Inflammation

The toxicity of PQ is based on oxido-reduction cycle of PQ which leads to production of superoxide radicals, and these free radicals trigger inflammatory response and oxidative stress in lungs. Several studies have been conducted to determine whether the PQ-induced oxidative stress in humans or animals is related to their toxic effects. Many studies have suggested that paraquat is potent oxidative stress inducer, and its toxicity is linked with the free radical generation [38, 39]. Reactive oxygen species which are produced from redox cycling of paraquat are highly reactive compounds to degrade cellular macromolecules which induce lipid peroxidation and protein and nucleic acid degradation that leads to oxidative stress and finally cell death [40]. PQ-induced ROS generation and cellular and subcellular effects on the splenocytes were studied in albino mice. Oxidative stress and splenomegaly induced by PQ lead to the activation of the pathways responsible for inflammation, immunomodulation, and apoptosis in murine splenocytes [42]. Many experimental studies have confirmed that airway inflammation is characteristic feature of PQ-induced lung injury in which airway obstruction occurs due to infiltration of inflammatory cells [43, 44] (Figs. 11.2 and 11.3).

11.2.2 PQ-Induced Lung Inflammation

Histopathological analysis of lung tissues after PQ intoxication has shown recruitment of many inflammatory cells like neutrophils, macrophages and lymphocytes [44].

Neutrophils being one of the inflammatory cells have significant impact in the lung pathogenesis [45, 46]. Neutrophils and monocytes synthesize and secrete myeloperoxidase (MPO), which is a peroxidase enzyme that contributes to oxidative stress and inflammation by generation of reactive oxygen species [47]. After measuring the MPO activity in lung tissue and BALF, some experimental studies found that PQ intoxication caused influx of neutrophils into the lungs [48, 49]. These recruited neutrophils, after activation, release superoxide ($O^{\cdot}$) and hydrogen

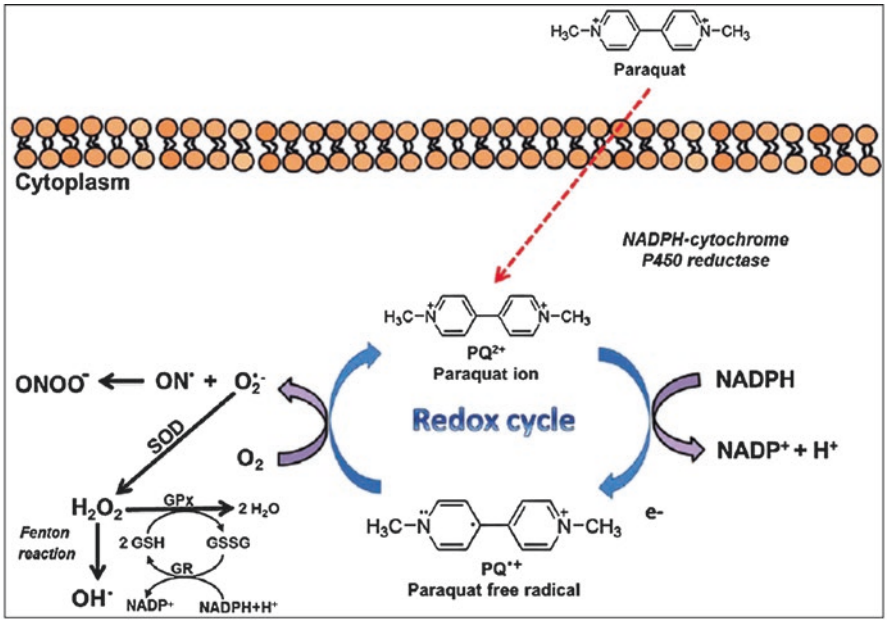

Fig. 11.2 Redox cycle of paraquat (PQ) [41]

peroxide (H_20_2) and other toxic oxidant which further cause the parenchymal cell injury [50, 51]. Nowadays, neutrophil-to-lymphocyte ratio (NLR) is being used as an inflammatory indicator in many diseases [52, 53]. A recent study evaluated the hematological parameters in PQ-poisoned patients and found significantly higher leukocyte, neutrophil counts and increased neutrophil-to-lymphocyte ratio, whereas lower lymphocyte counts were observed in non-survivors as compared to survivors with PQ poisoning [54]. Cytokines are a large family of secreted proteins released by numerous cells and act as signaling molecules that mediate and regulate the cells of the immune system, and it is accepted that cytokines are main mediators to induce inflammatory responses [55]. Previous studies confirm that proinflammatory cytokines and inflammatory mediators also play an important role in the PQ-induced lung damage. Free radicals act as inducers of cytokine secretion at the sites of inflammation and stimulate the immune responses [56]. In PQ-induced toxicity, the great amount of ROS is generated, which evokes inflammatory cell recruitment, and after activation these inflammatory cells such as macrophages, neutrophils, and lymphocytes release IL-1β, TNF-α, and other cytokines. It was reported that the level of proinflammatory cytokines like TNF-α, IL-1β, and IL-6 was induced due to PQ intoxication [44].

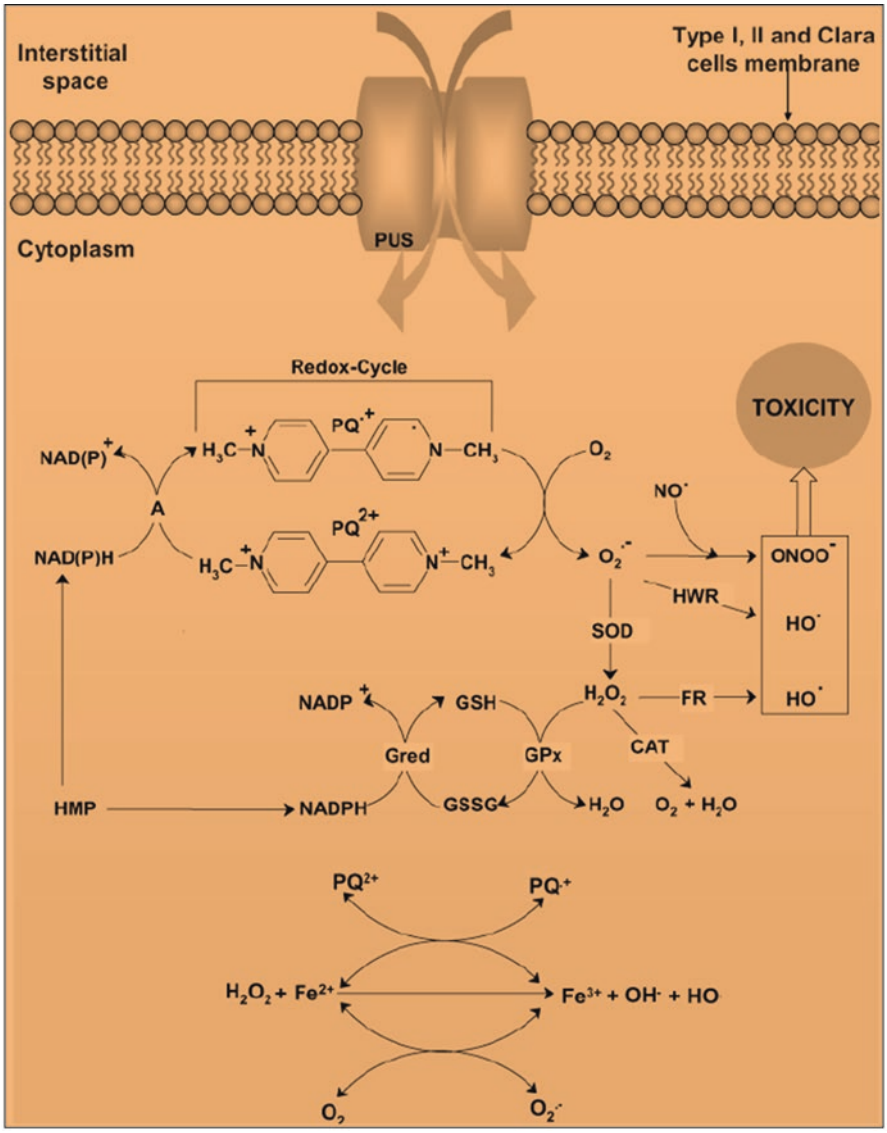

Fig. 11.3 The important intermediate species or products (*SOD* superoxide dismutase, *CAT* catalase, *GPx* glutathione peroxidase, *Gred* glutathione reductase, PQ^{2+} paraquat, PQ^{+} paraquat monocation free radical) which is involved in in vivo toxicity of PQ. The increased number of these intermediates causes imbalance between oxidant and antioxidant system of body [13]

11.3 Free Radicals-Induced Lipid Peroxidation

It has been reported in a number of in vitro and in vivo studies that paraquat has potential to damage the cell membrane by initiating the process of lipid peroxidation [57–59]. Lipid peroxides act as mediators for paraquat cytotoxicity because cell rupture and impaired cellular functioning occurs due to the peroxidation of membrane lipids [60]. The study conducted by Hara et al. (1991) to determine the mechanism of PQ-stimulated lipid peroxidation in mouse brain and pulmonary microsomes suggested that superoxide and singlet oxygen may be responsible for stimulation of PQ-induced NADPH-dependent microsomal lipid peroxidation in both the brain and lung [61]. Some other studies have also reported that singlet oxygen lead to lipid peroxidation [62, 63]. Low physiological levels of NADPH, due to depletion in redox cycling of PQ and lipid peroxidation, can lead to cell death.

11.4 PQ-Induced Structural Changes in Lungs

Environmental toxins, medications and infection may cause pulmonary fibrosis [64–66]. Pulmonary fibrosis is a chronic respiratory disease associated with irreversible fibroproliferative and wound healing cascades. Various reports have suggested that PQ intoxication leads to pulmonary fibrosis or pulmonary structural remodeling (interstitial lung disease) and is characterized by increased fibroblast mass, their migration, and excessive accumulation of matrix-associated proteins [67–69]. In this process, transition of fibroblasts into myofibroblasts in both the interstitium and the intra-alveolar space of the lungs leads to production and deposition of collagen fibers. Many in vivo and in vitro studies report that the pulmonary toxicity caused by PQ is associated with enhanced matrix metalloproteinase expression [70]. In earlier studies it has been reported that ECM modulation plays a vital role in the pathogenesis of PQ-induced pulmonary fibrosis [71–74].

On the basis of substrate specificity, domain organization and sequence homology, MMPs are classified as collagenases, gelatinases, stromelysins, matrilysins, membrane-bound and others [75]. Ouchi et al. (2008) studied the role of collagenases in bleomycin-induced pulmonary fibrosis model, and they found collagenase is strongly associated with fibrosis phase than inflammatory phase [76]. It has been reported that increased activity of MMP-2 and MMP-9 is associated with pulmonary fibrosis. MMP-2 and MMP-9 can cause disruption of the alveolar epithelial basement membrane because it is well known to damage type IV collagen [74, 77].

11.5 PQ-Induced Lung Damage: Acute Lung Injury (ALI)

Acute lung injury (ALI) is an inflammatory response of the lungs due to respiratory failure, a life-threatening condition. It may be caused by many ways.

- **Direct lung injuries** are caused by lung infections, exposure to various pesticides (chemicals) aspiration, transfusion, pulmonary contusion and pneumonia that directly affect the lungs **[**78, 79**]**
- **An indirect lung injury** is caused by different condition elsewhere in the body. These include: sepsis, trauma, severe bleeding, fractures, pancreatitis, burns and a car accident etc. **[**80, 81**].** An initial inflammatory phase is followed by disruption of both lung endothelial and alveolar epithelial cells which is followed by the destruction of epithelial basement membranes leading to severe fibrotic phase [82]

PQ induces lung injury in two phases:

(A) **Early destructive phase:** Early phase of ALI characterized by pulmonary edema, alveolitis and infiltration of inflammatory cells. There are many studies which proved through histopathological analysis that PQ intoxication caused alveolar damage with infiltration of inflammatory cells [49]. Mainly neutrophils play a critical role in the pathogenesis of PQ-induced ALI. After activation, neutrophils release certain proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL1), IL6, and IL8 and some harmful mediators like reactive oxygen species (ROS) and matrix metalloproteinases, which caused further damage [83]. Through the generation of reactive oxygen species, PQ intoxication causes oxidative damage to the cells.

(B) **Proliferative phase/fibrotic phase:** The vascular injury in acute phase is followed by fibroproliferative phase and leads to severe fibrosis phase, which evolves from early destructive phase approximately after 1–2 weeks approximately. Pulmonary fibrosis is an interstitial lung disease; during fibroproliferation, the degraded epithelial cells are replaced by myofibroblasts and fibrotic phase characterized by proliferation of fibroblasts, migration, and excessive accumulation of extracellular matrix proteins and collagen deposition resulting in airway remodeling due to PQ intoxication [67–69, 71, 72]. PQ exposure induces an alveolitis comprised of neutrophils and macrophages that play a role in the development of fibrosis. Activated alveolar macrophages release a chemotactic factor for neutrophil recruitment and also play a role in the recruitment and replication of fibroblasts by releasing fibronectin and a growth factor for fibroblasts [84].

It has been proved by many experimental studies that interstitial and intra-alveolar fibrosis is characteristic findings in PQ-induced structural remodeling of the lungs. PQ intoxication shows similar symptoms like pulmonary fibrosis (interstitial lung disease), i.e., fibroblast proliferation, migration, and excessive accumulation of extracellular matrix proteins [85, 86]. Interstitial fibrosis is characterized by thickening in the alveolar septa, whereas in intra-alveolar fibrosis, activated fibroblast migrates through the gaps of epithelial basement membrane to alveolar lumen and collapsed alveoli which undergo fusion [72, 87, 88]. The extracellular matrix also plays an important role in regulating cellular growth and migration of

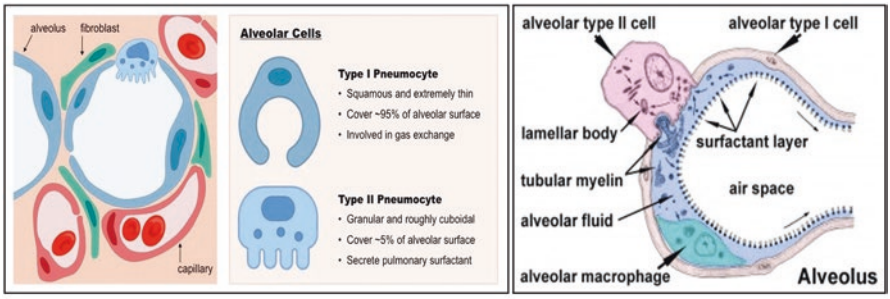

Fig. 11.4 Structure of alveolus with alveolar type I and II cells; (**a**) (http://ib.bioninja.com.au) (**b**) (https://embryology.med.unsw.edu.au)

cells during lung development, and it provides mechanical support to the epithelial cells [71, 89]. As reports suggest, higher molecular weight proteins mainly ECM like fibrous collagen and elastin are secreted by fibroblasts [89, 90]. Collagens with type I and type III are the most abundant components of matrix, and some experimental studies have demonstrated excessive deposition of type I and III collagens in lung fibrosis [91]. In PQ-induced toxicity, recruitment of inflammatory cells and excessive secretion of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β initiate the inflammatory cascade [92, 93] which further caused the structural changes in lungs. Transforming growth factor (TGF-β1) is a profibrotic cytokine responsible for the fibroblast proliferation, migration and gene expression of collagen in fibrosis phase [94, 95]. Many experimental studies have suggested that TGF-β is a strong stimulus for the induction of pulmonary fibrosis by PQ [49, 67–96]. Many evidences suggest that matrix metalloproteinases (MMPs, a family of zinc-dependent endopeptidases) have a critical role in ECM disruption and remodeling in lung injury [75]. MMPs play a major role by acting on chemokines, cytokines, growth factors, and cell surface proteins in airway inflammation [73, 97]. Reports also suggest that MMP-2 and MMP-9 are being related to pulmonary fibrosis because of their well-known ability to promote the degradation of type IV collagen [98, 99]. Studies have also shown that in ALI, proliferation phase begins much earlier than reported which contradicts the finding where fibrotic phase begins after 2 weeks of PQ intake [100]. Therefore, on the basis of above findings, we tested the hypothesis that a single toxic dose of PQ could activate the fibroproliferation after 48 h of PQ exposure (Fig. 11.4).

11.6 PQ-Induced Toxicity and Signaling Pathway

Many evidences have suggested that PQ act as a stimuli to activate a number of intracellular signaling cascades that are associated and responsible for its toxicity in cells, for example, PQ activates protein kinase B and the members of the

mitogen-activated protein kinase (MAPK) family, NF-κB, and increased ROS [82, 101, 102]. Several studies have shown that PQ is a potent inducer of reactive oxygen species, which plays a primary role in PQ-induced lung damage. More ROS production causes disruptions in cell and tissue function by inducing oxidative stress condition, protein, and DNA damage. Other than ROS-induced pathology, inflammation is the second factor which is responsible for PQ poisoning and disease pathogenesis. ROS act as intrinsic signal transduction molecules in the pathogenesis of PQ-induced toxicity. It is a well-known fact that ROS act as a second messenger and are involved in many signaling pathways such as NF-kB and MAPK pathway [103–107]. The role of MAPK signaling has been already studied in the pathogenesis of various human diseases including respiratory diseases [108, 109].

It is well known that paraquat toxicity is mediated via oxidative stress and the members of MAPK family activate the stress condition. After PQ exposure, the activation of ERK1/2 and JNK1/2 generates more oxidative stress condition. Some experimental studies have shown that by using selective inhibitor (SP 600125) of JNK1/2 pathway, PQ-induced cell death was significantly reduced [110]. Several evidences suggest that PQ stimulates the expression of p-38 MAPK, which act as mediator to regulate inflammation and apoptosis. Many studies have demonstrated that the activation of p-38 MAPK pathway is associated with over expression of proinflammatory cytokines such as TNF-α and IL-1β [111, 112].

Prostaglandins are naturally occurring compounds derived from arachidonic acid by the action of cyclooxygenase (COX) isoenzymes, and these prostaglandins are involved in various homeostatic and inflammatory processes [113]. Cyclooxygenase exists in two isoforms known as COX-1 and COX-2 where COX-1 is constitutively expressed in most tissues and COX-2 is an inducible isoform which is mainly responsible for prostaglandin formation in inflammation, and its expression was upregulated by different types of inflammation stimulus such as cytokines, mitogen, and growth factors [113]. It has been already reported that PQ intoxication increased COX-2 expression in lungs, liver and kidneys in rats [114]. Some recent studies have linked the biosynthetic pathway of prostaglandins with the activation of MAPK signaling pathway [115]. The study conducted by Pei et al. (2014) suggested that p38 MAPK signaling cascades play a vital role in regulating the IL-1β and TNF-α proinflammatory cytokine production in PQ-induced lung injury [116].

Nuclear factor kappa B (NF-κB) is considered as a major transcription factor for regulating inflammation, lung injury and repair process. It is activated during oxidative stress condition by ROS and proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α). IκB (nuclear factor of kappa light polypeptide gene enhancer in B cells) is a cellular protein that keeps NF-κB in an inactive state in the cytoplasm and blocks its nuclear localization signals, but after degradation of IκB, activated NF-κB is translocated to the nucleus where they bind to the promoters of target genes to enhance gene expression and amplification of proinflammatory genes including cytokines, chemokines, and adhesion molecules [117, 118].

It is well known that NF-κB have major induction of the expression of proinflammatory gene, but some studies have suggested its anti-inflammatory role to regulate inflammatory resolution [119]. The activation of transcription factor (NF-κB) is

important in the regulation of various cellular processes including proliferation, inflammation, also control angiogenesis and many other biological processes. The role of NF-κB in lung development and diseases was described earlier [120]. Transcription factor, NF-κB, is associated with the pulmonary diseases such as acute lung injury, cystic fibrosis, asthma, and severe sepsis, and extensively studied evidences suggest that activation of NF-κB signaling plays an important role in the pathogenesis of PQ-induced toxicity [109–111].

11.6.1 Treatment Strategy for PQ Toxicity

The major cause of high mortality rate in case of PQ poisoning is lack of antidote (antagonists) against PQ toxicity. Although there is no effective therapy for PQ intoxication, antioxidants and anti-inflammatory drugs are the drug of choice in clinical treatment regimen for PQ-intoxicated patients.

Bipyridylium herbicides, like PQ, tightly bind to the clay minerals and are easily absorbed by soil [85]. On the basis of above ability, it also has shown strong binding with montmorillonite [121]. Fuller's Earth is a calcium montmorillonite, and till now, it is one of the most common treatments for PQ toxicity. In various studies, it has been investigated the potential of fuller's Earth for treatment of PQ poisoning [121–124].

Clinicians have tried a number of treatments such as antioxidants (vitamin C or E), nitrous oxide, N-acetylcysteine, desferroxamine, some corticosteroids and cytotoxic agents [125–128]. Unfortunately, none of them helped to reduce the mortality rate due to paraquat poisoning. Generally, corticosteroids and immunosuppressive drugs are used to treat PQ-induced lung injury [129]. According to a preliminary report of Lin jl et al. (1996), cyclophosphamide and methylprednisolone by inhibiting the inflammation and fibrosis may prove useful to severe paraquat poisoning. In many cases, hemodialysis (HD) and hemoperfusion (HP) are part of the treatment for PQ poisoning, but the conclusion of some clinical studies is that HD/HP was effective to remove PQ from plasma but was ineffective in reducing paraquat lung exposure [130]. Hence, the overall outcome of these treatment methods and immunosuppressive therapies are not well effective for PQ-poisoned patients. There is urgent need of suitable antidote to treat PQ-poisoned patients with no side effects. It is clear that PQ toxicity is due to generation of reactive oxygen species which further initiates inflammatory cascades. So, the treatment strategy against PQ-induced toxicity should be to select a molecule with anti-inflammatory and antioxidant properties. The human body has a defense system to prevent oxidative stress by producing antioxidants. Antioxidant is the basic defense mechanism to inhibit oxidative damage in the human body. These antioxidants have free radical scavenging property because antioxidants are molecules which neutralize the free radicals by donating an electron to it. A number of studies suggested the role of antioxidant in case of paraquat toxicity [125, 131]. The body makes some of the antioxidant enzymes such as catalase, superoxide dismutase (SOD), and glutathione and the other principle antioxidants including vitamin A, E, C, and B carotene found in the diet. It has been reported that deficiency of vitamin C and E is directly associated with the development of acute paraquat toxicity [132–135].

11.7 Herbal Drugs

The relationship between plants and man is associated from the very beginning of human existence, and it can be said that plants play a central role in the development of human civilization around the whole world. Plant-based products have been used for centuries for various purposes. The ancient medicine system of India known as "Ayurveda" uses mainly natural plant products and its formulations to treat various human diseases. Many of these natural plant products are secondary metabolites produced by higher plants, used as a drug against infections and diseases because these products have many pharmacological or biological activities [137–140].

11.7.1 Curcumin: As an Antioxidant and Anti-Inflammatory Molecule

About two centuries ago, Curcumin was discovered by Vogel and Pelletier from the rhizomes of *Curcuma longa (turmeric*). Curcumin (diferuloylmethane) is a low molecular weight phenolic compound and a major component of the golden spice *turmeric* (*Curcuma longa*). Curcumin, a yellow pigment, is mainly attributed to the medicinal properties of the turmeric which contains 2–5% curcumin [138]. Curcumin being an immunomodulatory agent with antioxidant and anti-inflammatory properties may prove to be an effective treatment strategy for PQ-induced airway inflammation, oxidative stress, and structural changes in lungs.

11.7.2 Properties of Curcumin

Curcumin (diferuloylmethane) is (1E,6E)-1,7-bis (4-hydroxy- 3-methoxyphenyl) -1,6- heptadiene-3,5-dione. It is a polyphenolic and lipophilic molecule which is soluble in ethanol, DMSO, methanol, and acetone and insoluble in water and ether. Its molecular formula is $C_{21}H_{20}O_6$, and its molecular weight is 368.38 g/mol (Fig. 11.5). It has low aqueous solubility at both acidic and neutral pH but soluble at alkaline pH. Curcumin exhibits keto-enol tautomerism that has a predominant keto form in acidic and neutral solutions and a stable enol form in alkaline solution [141, 142].

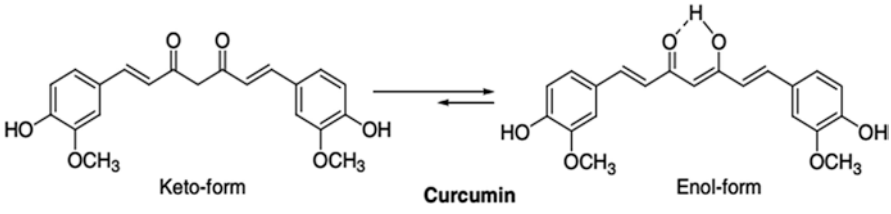

Fig. 11.5 Molecular structure of curcumin. This scheme shows the keto and enol forms of curcumin which exist in two tautomeric forms in solution (Moussawi and Patra 2016)

Majority of the studies suggested that curcumin is a potent scavenger of reactive oxygen species including hydroxyl radicals, superoxide anion radicals, and nitrogen dioxide radicals [143]. Due to the ability of curcumin to scavenge hydrogen peroxide and other free radicals, it may be used in free radical-related disease therapies. There are many evidences that curcumin has the ability to modulate the immune system and disrupt the proinflammatory cascade through a variety of mechanisms, including antioxidant effects and alterations in cell signaling pathways [144]. There are overwhelming reports from both in vitro and in vivo studies which have identified the therapeutic potential of curcumin and characterized it as a potent "drug" for treatment of diseases of diverse nature. As an alternative route of drug delivery, Intranasal administration (i.n) has a long tradition. As described earlier, intranasal drug administration has been highly investigated by the scientific community.

The drug administration by nasal route has more potential for medical purposes because mucosal surface of nasal cavity is well supplied by blood vessels which ensures rapid drug absorption [145, 146]. Curcumin administration by intranasal route avoids the first pass metabolism which is major problem of its administration via oral route. Two separate studies done in our laboratory revealed that curcumin administration via nasal route is effective against airway inflammation and in chronic asthma as it directly targets the lungs [147, 148].

Curcumin inhibits lipid peroxidation, which is one of the major reasons of PQ-induced lung injury [149]. So being an antioxidant, curcumin administration prior to PQ intoxication may attenuate the PQ toxicity. Our results demonstrated that PQ-induced intracellular ROS production and cellular inflammation were significantly suppressed by intranasal curcumin (5 mg/kg) pretreatment. We found that total inflammatory cell count (mainly neutrophils and lymphocytes) was significantly increased after 48 h of PQ exposure, whereas pretreatment with both curcumin and dexamethasone has significantly reduced infiltration of inflammatory cells [150]. These results are also consistent with histological analysis of mice lungs which revealed suppressed lung injury after curcumin pretreatment by inhibiting inflammatory cell infiltration to the lungs (Figs. 11.6 and 11.7).

Cytokines being signaling molecules are thought to play a critical role in pathogenesis of ALI. In our study where animal model of ALI is induced by PQ, we suggested that TNF-α plays a critical role in PQ-induced lung toxicity (Fig. 11.8). We found that after 48 h of PQ intoxication, TNF-α level was significantly increased as compared to control whereas curcumin pretreatment has significantly ameliorated its level [150].

Therefore, the balance of oxidant-antioxidant defense mechanism is vital for the treatment of ALI. It has been reported that polyunsaturated fatty acids undergo lipid peroxidation in presence of superoxide after PQ intoxication, and curcumin is known to inhibit lipid peroxidation [149]. Our results have also demonstrated significant increase in MDA content and decrease in SOD and catalase activities which is consistent with the previous findings (Fig. 11.9) [150–152].

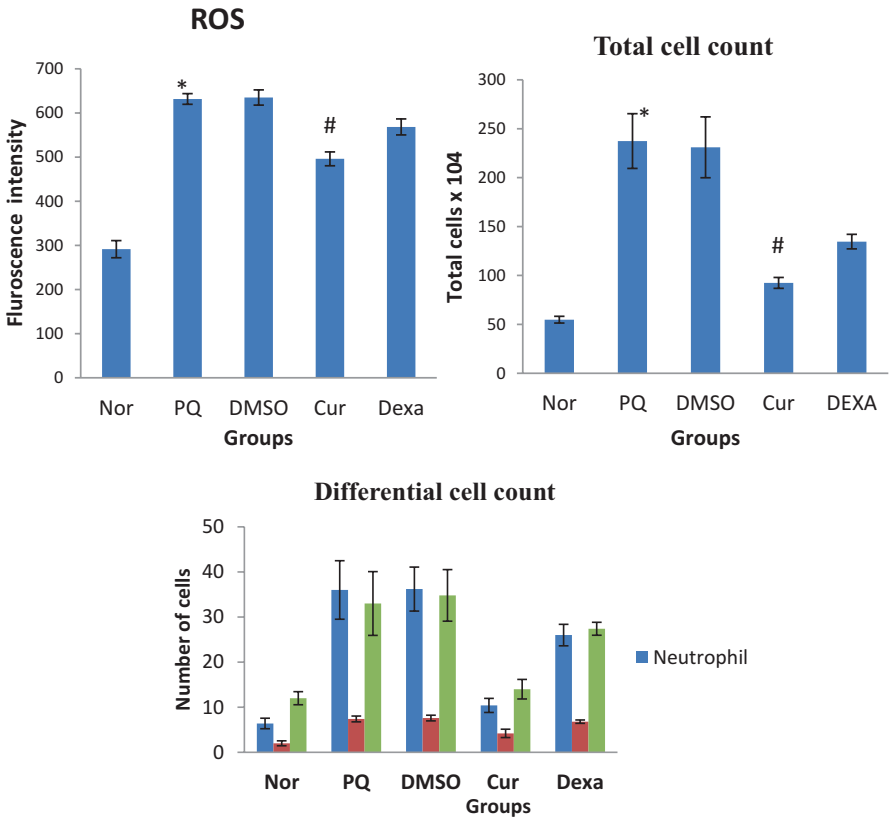

Fig. 11.6 Effect of curcumin on intracellular ROS level; intracellular ROS level was increased by PQ intoxication correlated with increased no. of inflammatory cell recruitment which was significantly decreased by curcumin pretreatment; total inflammatory cell count by trypan blue assay. PQ intoxication resulted in an increased total cell count than normal and significant reduction in curcumin pretreatment group; differential cell count in BALF. Results are shown as means ± SEM ($*p < 0.05$) compared to normal group

11.7.3 Immunomodulatory Potential

Inflammation is an uncontrolled condition of activated immune responses, and many reports have suggested that curcumin plays a vital role in the modulation of immune responses. The study conducted by **Srivastava et al. (2011)** [144] described the effectiveness and regulation of immune responses by curcumin in various diseases. In pathological conditions, curcumin have potential to affect both innate and adaptive immunity, and its anti-inflammatory action plays a vital role in the treatment of immunological disorders.

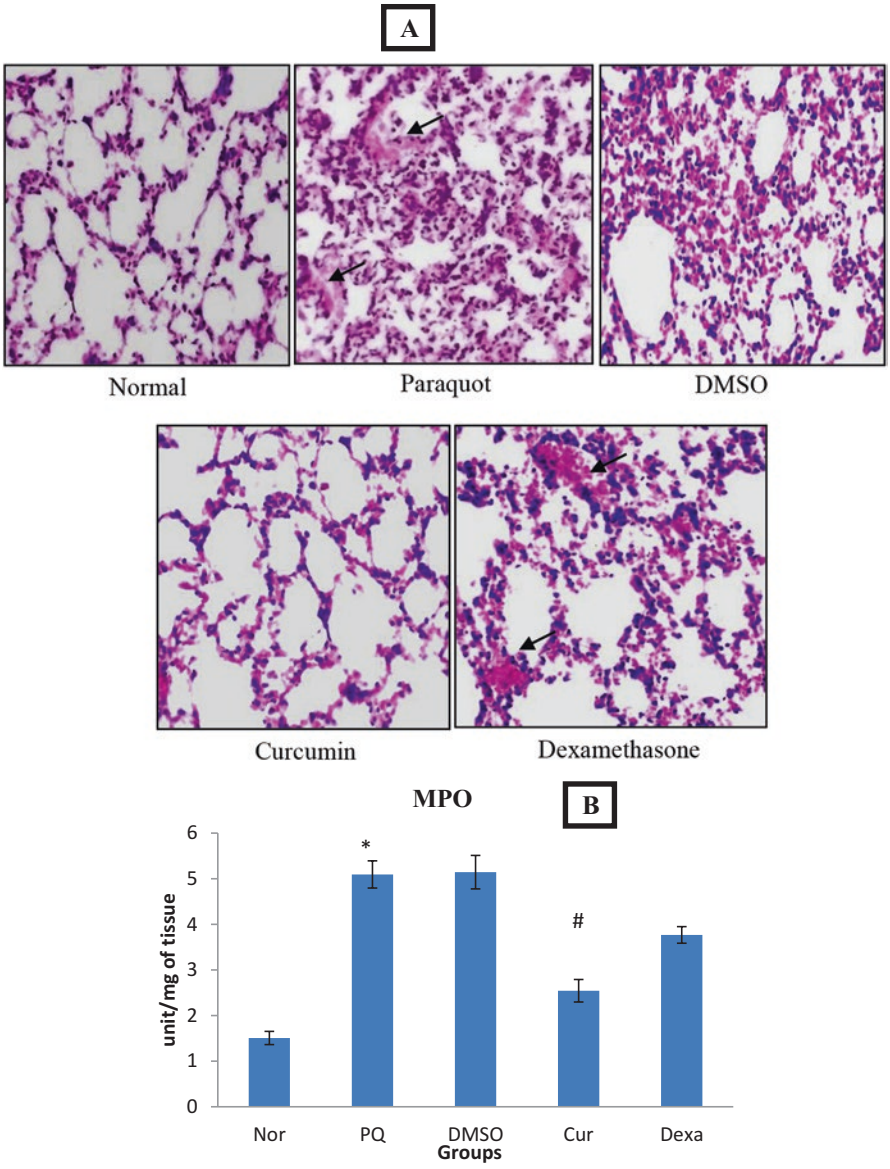

Fig. 11.7 (**a**) Effects of curcumin on PQ-induced structural changes in lungs. Histopathology of hematoxylin- and eosin-stained lung sections (magnification ×400); (**b**) myeloperoxidase (MPO) activity in lungs, which was significantly increased in PQ in comparison to control and curcumin-treated groups. The results are shown as means ± SEM ($*p < 0.05$) compared with normal group; ($\#p < 0.05$) compared with PQ group

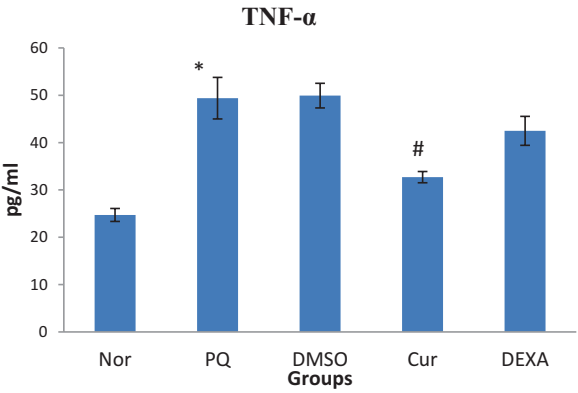

Fig. 11.8 Effects of curcumin on TNF-α level in serum. TNF-α level significantly increased in PQ group as compared to control and curcumin-treated group. Results are shown in means ± SEM. (∗$p < 0.05$) compared with normal group; (#$p < 0.05$) compared with PQ group

Strong evidences support potential of curcumin to modulate the function of neutrophils, macrophages, monocytes, B cells, T cells, and dendritic cells (DCs) [153–155]. Several studies have evaluated that curcumin inhibits the neutrophil-mediated inflammatory response by affecting its activation or infiltration [156]. An independent study [157] suggests that curcumin (40–60 mg/kg) through oral route suppressed the LPS-mediated neutrophil infiltration in the liver, and they also have shown that the reduction of neutrophil infiltration was correlated with altered levels of ICAM-1 and VCAM-1, a type of adhesion molecules. In another report [158], the impacts of curcumin on activated neutrophils both in vitro and in experimental arthritis were investigated, and they found that curcumin decreased activity of neutrophils by inhibiting the protein kinase C.

Curcumin (10 or 20 mg/kg, BW) administration through intraperitoneal route has suppressed recruitment of eosinophils and other inflammatory cells and decreased the level of IL-4 and IL-5 in bronchoalveolar lavage fluid along with expression of iNOS in lungs in a murine model of ovalbumin-induced asthma [159]. An independent study has also shown that curcumin can inhibit IgE-mediated allergic response and the degranulation of mast cells along with inhibiting secretion of IL-4 and TNF-α [160]. An in vitro study has reported that curcumin (10 μM) inhibits immunoglobulin production from rat spleen lymphocytes [161]. An independent study has suggested that curcumin inhibits mitogen-induced lymphocyte proliferation at two different concentrations (0.01 and 0.05 μg/ml) [162], and another study reported reduced proliferation of rat thymocytes by curcumin after Con-A induction [163]. Some in vivo studies conducted in a mice model to check the immunomodulatory effects of curcumin revealed that curcumin can increase $CD8^+$ T cells and NK cell populations [164, 165]. Cytotoxic T cells (CTLs) with CD8 marker play a protective role against viral-infected tumor cells; it has been found that curcumin exhibits antitumor activity by increasing the number of $CD8^+$ T cells and $CD4^+$ T cells in tumor-bearing animal models [166]. Therapeutic effects of curcumin were studied in chronic lymphocytic leukemia (CLL) patients, where a number of $CD4^+$, $CD8^+$, and NK cells were enhanced and absolute lymphocyte counts (ALC) were reduced [172].

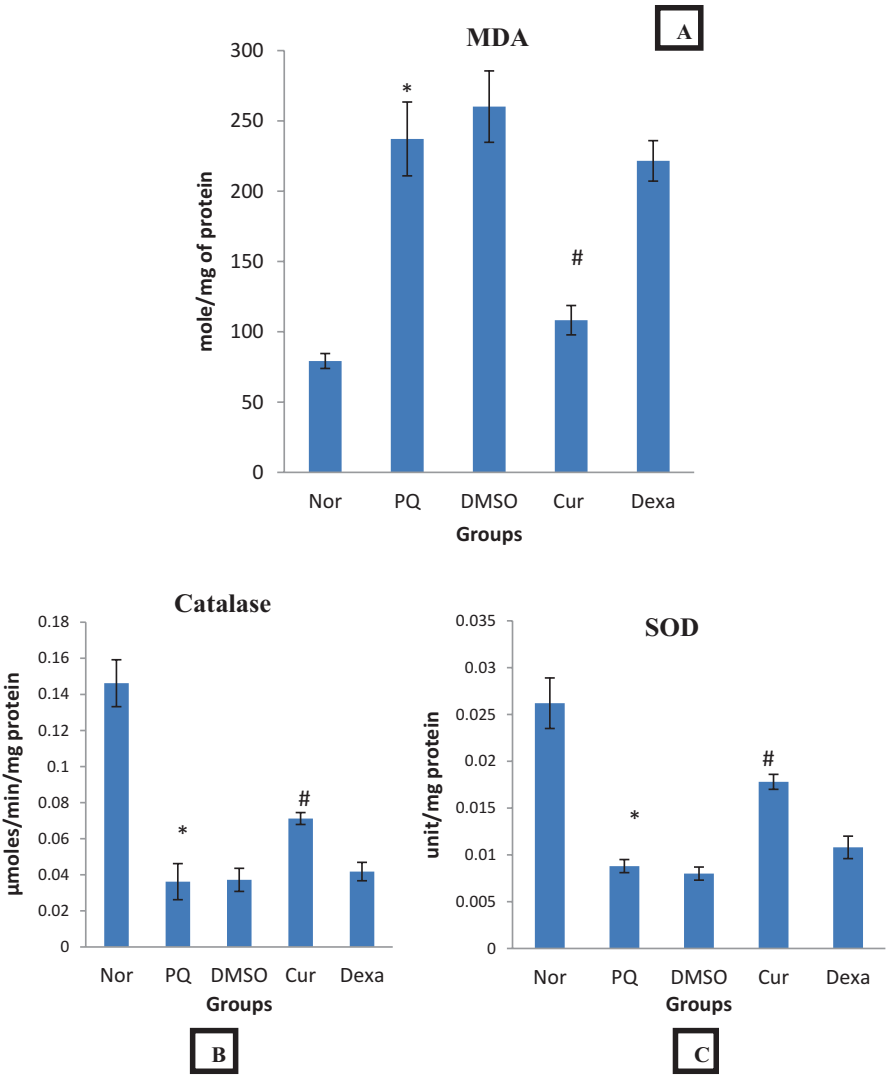

Fig. 11.9 Effects of curcumin in PQ-induced oxidative stress in mice; (**a**) malonaldehyde (MDA) level was significantly increased in PQ group in comparison to control and curcumin-treated groups; (**b**) catalase activity was significantly reduced in PQ group as compared to control and curcumin groups; (**c**) SOD activity was significantly reduced in PQ group in comparison to curcumin-treated group. The results are shown in means ± SEM ($*p < 0.05$) compared with normal group; ($\#p < 0.05$) compared with PQ group

11.8 Conclusion

Airway inflammation, pulmonary hyperpermeability and oxidative stress due to production of ROS are major characteristics of acute lung injury and damage. So an ideal mouse model of PQ-induced lung injury was developed to replicate these hallmarks of acute lung injury. After 48 h of intraperitoneal administration of PQ, mice were used to study inflammatory responses and oxidative stress leading to lung damage. Number of studies have suggested molecular mechanism of PQ toxicity based on production of superoxide anion and other oxygen free radicals. Numerous evidences suggest the roles of ROS as regulators of cell function and as a second messenger in intracellular signaling cascades. PQ intoxication increased the concentration of MDA, which suggests that this toxic dose of PQ induces lipid peroxidation. We also observed elevated level of nitric oxide (NO) after PQ intoxication which was consistent with earlier reports, where NO was shown to play a crucial role in PQ-induced lung damage. More infiltration of inflammatory cells leads to increase in alveolar and vascular capillary permeability, and we also have observed enhanced BALF protein concentration as an indicator of increased pulmonary permeability.

Intranasal curcumin has shown better efficacy over glucocorticoid (dexamethasone) as it may directly target lungs and proved much better than intraperitoneal route. Based on these evidences, we may conclude that intranasal curcumin by inhibiting the oxidative stress, infiltration of inflammatory cells, and secretion of inflammatory cytokines could inhibit PQ-induced acute lung injury.

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Environmental and Occupational agents and Cancer Drug-Induced Oxidative Stress in Pulmonary Fibrosis

12

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Abstract

Pulmonary fibrosis (PF) is a fatal lung disorder with high mortality rate. Foreign bodies can easily enter into the lung, and, therefore, the lung is armed with several defence mechanisms including antioxidants and immune systems (innate and adaptive immunity systems). Importantly, inflammasomes in the lungs have been observed to play an important role in the progression of PF. Apoptosis of alveolar epithelial cells (AEC) is crucial for inducing environmental and occupational contaminants such as silica and asbestos and some cancer drugs especially bleomycin-induced PF. In the past, PF has been believed to occur due to inflammation of AECs with subsequent proliferation of fibroblasts followed by collagen deposition that leads to fibrosis. However, recent research revealed that PF proceeds due to epithelial-fibroblast pathway in association with interstitial inflammation that results in collagen deposition. Notably, cell membrane- and mitochondria-mediated ROS generation with the involvement of inflammasomes play a crucial role in the pathogenesis of PF caused by environmental and occupational agents like asbestos, silica and also certain drugs like bleomycin.

It has been hypothesized that initial injury by oxidants during asbestos, silica or bleomycin treatment targets to AECs. Upon injury to type I AECs, the types II AECs proliferate in the exposed basement membranes. The hyperplastic type I AECs die during the repair process, and the remaining cells are differentiated into normal type I AECs. In PF pathology by TGF-β, fibroblasts differentiated into myofibroblasts that secrete collagen and other fibrotic proteins. Compounds that target fibroblast activation and the synthesis of ECM are currently under evaluation for therapies of PF.

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12.1 Introduction

Fibrosis occurs due to proliferation and accumulation of connective tissue by replacing normal parenchyma. Fibrosis leads to a marked deposition of exuberant ECM components and interstitial tissue in the epithelium and mesenchymal cells, i.e. myofibroblasts, the cells in between fibroblast and smooth muscle cells [1–5].

Pulmonary fibrosis is a pathologic condition that occurs due to a marked increase in the generation of ECM molecules in the lungs [6, 7]. To understand the mechanism of PF, agents such as bleomycin, asbestos and silica are generally used in studies of different animal model systems [6, 7]. The pathology of PF caused by environmental, occupational and cancer drugs (silica, asbestos and bleomycin, respectively) proceed initially due to damage of the alveolar walls. Fibrotic lung occurs due to thickening of alveolar upon narrowing of alveolar spaces upon infiltration of immune cells and fibroblasts into the lung interstitium resulting in the production of a marked increase in collagen and fibronectin [2–4]. Myofibroblasts are known to be responsible for the generation of ECM, which are accumulated in the lung interstitium [1]. Type I myofibroblast cells take up the alveolar space for about 90% and elicit critical effects on gas exchange by type II cells. Type II cells are observed in the surroundings of alveoli, produce surfactant and serve as progenitor for type I cells upon damage of the lung. The type II cells differentiate to type I cell in the recovery process. In the progression of PF, however, a sequence of pathological changes occur starting with the deposition of fibrin into the intra-alveolar space followed by haemorrhage and subsequently hyperplasis of type II pneumocytes [8]. The resultant deposition of excessive collagen in intra-alveolar and alveolar wall spaces causes microcyst associated with epithelial cuboidalization. This is a common feature of the fibrotic lung, which exemplifies sustained epithelial proliferation. Type II epithelial cell hyperplasia has been observed to be prominent in the pathogenesis of PF [8–10].

Alveolar macrophages have been suggested to start these inflammatory responses via NOD-like receptor (NLR family member) PYD domain-containing protein 3 (Nalp3), a component of the inflammasomes that along with the adaptor ASC (apoptosis-associated speck-like protein with a CARD) protein (PYCARD) forms a complex with caspase-1 for activation of IL-1β, a proinflammatory cytokine [1]. Nalp3 inflammasomes act as a pathogen recognition receptor (PRR), which accepts pathogen-associated molecular patterns (PAMPs) and detects products of damaged cells and subsequently triggers innate immune response [1, 11–13]. The Nalp3 inflammasomes are also activated by changes in intracellular K^+ due to its efflux via relevant ion channels present in the cell surface [14]. ROS-mediated regulation of Nalp3 is well known, albeit the underlying mechanism of such regulation is

currently unclear [15]. Stimulation of macrophages, for example, with silica causes activation of caspase-1 in a Nalp3-dependent manner [16]. Macrophages deficient in Nalp3 inflammasome components are unable to secrete the proinflammatory cytokine, interleukin-1β in response to silica, asbestos and bleomycin. Nalp3 inflammasomes activation requires both an efflux of intracellular K^+ and generation of ROS [14–16].

At low levels, ROS may activate antioxidant defence, but a discernible increase in ROS level may trigger cellular dysregulation, DNA damage, p53 activation, cell cycle blockade and associated apoptosis and/or necrosis. All of these have been suggested to be important in the manifestation of fibrotic response [17–19].

12.2 Role of Reactive Oxygen Species

Oxidative stress causes change in cellular, molecular, tissue and organ functions due to increase in the generation of reactive species, especially, ROS along with a marked decrease in antioxidant defence [20, 21]. Lung is the most prominent target of oxidant generating environmental contaminants. At low levels, ROS ameliorates cell proliferation due to triggering of antioxidant defence; however, at higher levels, ROS embraces DNA damage, stimulates transcription factors and blocks cell cycle and cell death through pathways that regulate apoptosis and/or necrosis. Collectively, all of these have been suggested to be important for producing PF [1].

Several evidence suggested that ROS is a critical determinant for the progression of PF [1, 22, 23]. These are as follows: (i) oxidized lipid and protein like 8-isoprostane and carbonylated proteins, respectively, have been observed in bronchoalveolar lavage fluid, lung tissue and exhaled air from patients with fibrotic lung diseases; (ii) PF induced by cancer drugs, for example, bleomycin has been observed to be associated with ROS-mediated production of oxidized lipid and proteins; and (iii) a marked increase in oxidative DNA damage has been identified in PF of experimental animals exposed with silica, asbestos and bleomycin [22, 24].

12.3 ROS Production by NADPH Oxidase

NADPH oxidase (NOX) is one of the most important and dedicated mechanisms for generation of ROS of different organs of human and animals. NOX, especially NOX-4, is known to play a critical role in the initiation and progression of PF [25, 26]. NOX-4 is induced by profibrotic stimulants, for example, transforming growth factor-β (TGF-β) has been shown to promote myofibroblast differentiation and subsequently impair re-epithelialization leading to PF [27].

A marked increase in NOX-4 level was found in animals, for example, rodents with bleomycin-induced PF [25, 26]. A discernible increase in the expression of NOX-4 has been shown to contribute to the disease pathology. Using NOX-4 inhibitors, attenuation of PF has been demonstrated in an animal model system [25, 26]. This inhibition was observed with a marked decrease in the components of ECM

such as fibronectin and collagen gene transcripts. Hypoxia-inducible factor (HIF), TGF-β, plasminogen activator inhibitor-1 (PAI-1) and NOX with the involvement of NOX-4 have been shown to contribute to AEC remodelling, which subsequently play an important role in PF. It has been observed that inhibition of NOX-4 activity attenuates TGF-β-mediated increase in profibrotic gene expression and subsequently inhibits fibroblast to myofibroblast differentiation and thereby decreases the progression of PF [27–29]. Thus, targeting NOX-4 could prove useful as a therapeutic measure to ameliorate PF.

NOX-4-dependent production of $O_2^{\cdot-}$ and subsequent generation of other ROS were shown to play an important role in TGF-β-induced myofibroblast differentiation and subsequently ECM production. Using siRNA or chemical inhibitors of NOX-4 was shown to attenuate bleomycin-induced PF in mice [26, 30]. NOX-4 expression has also been shown to increase the production of hyperplastic epithelium especially alveolar type II (AT2) cells [27]. Notably, mice lacking NOX-4 showed protection against pulmonary inflammation-mediated by bleomycin [28–30]. NOX-4 inhibitors have been obsered to attenuate TGF-β-induced ROS generation and AEC apoptosis [26]. Thus, NOX-4-dependent ROS production seems to be important for AEC apoptosis during the progression of PF (Fig. 12.1).

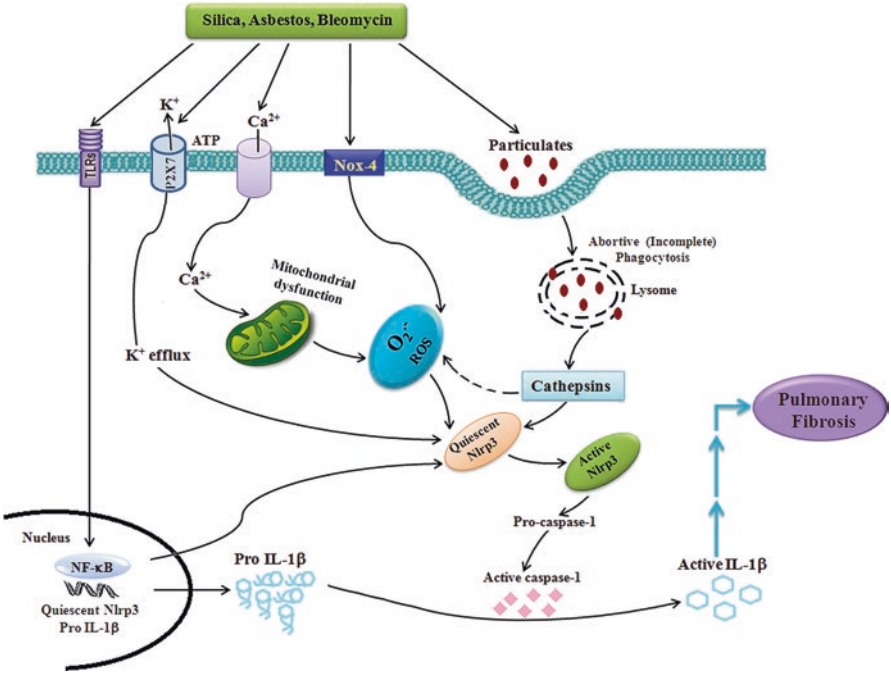

Fig. 12.1 Induction of ROS by environmental (silica), occupational (asbestos) and cancer drug (bleomycin) in TGF-β production and subsequent progression of pulmonary fibrosis

12.4 ROS Production by Mitochondria

H_2O_2 generation by alveolar macrophages (AM) mitochondria was found to be important in producing asbestos-induced PF [31–33]. Asbestos-induced H_2O_2 production in AMs has been observed to be attenuated upon knockdown of complex III, a major site of $O_2^{\cdot-}$ generation in the electron transport chain of mitochondria [31–33]. In mice AMs, an increase H_2O_2 generation by asbestos has been observed to stimulate inflammation, which causes cellular injury leading to asbestosis [31–33]. The role of H_2O_2 in mediating PF has been exemplified by the fact that catalase gives protection against asbestosis in an animal model system [34]. Environmental contaminants such as silica and asbestos fibres were shown to augment ROS production in AECs mitochondria, which in turn results in PF [34, 35].

TGF-β is known to augment production of ROS production in lung epithelial cells, which increases fibrotic gene expression, epithelial–mesenchymal transition (EMT) and myofibroblast differentiation leading to PF [36–40]. Exogenously expressed mitochondrial thioredoxin was shown to inhibit TGF-β caused mRNA expression of fibronectin and a non-histone chromosomal high mobility group AT-hook 2 (HMGA2), a central mediator of epithelial–mesenchymal transition-EMT). This has been suggested to be a crucial mechanism for TGF-β-induced gene expression in mitochondria [41]. Different mechanisms have been proposed for TGF-β-induced production of ROS by mitochondria. TGF-β was shown to induce a sustained production of mitochondrial ROS via a discernible decrease in the activity of complex IV of mitochondrial respiratory chain activity in lung epithelial cells [42]. In the recent past, Sundarson et al. [43] indicated that GSK-3α and -β subunits phosphorylation could contribute to the inhibition of the complex, which augments ROS production in mitochondria during stimulation with TGF-β. Notably, Jain et al. [44] have demonstrated that an increase in TGF-β stimulates ROS generation both in normal and fibrotic human lung fibroblasts by blocking complex III (electron transport chain of mitochondria) activity. They also observed that treatment with mitochondrially targeted antioxidants upon genetically disrupting the activity of the mitochondrial complex III attenuates the expression of profibrotic genes induced by TGF-β [44]; however, TGF-β could not produce any discernible change in Smad phosphorylation. This suggests that generation of ROS could mediate fibrogenic activity of TGF-β, which appears to be independent of Smad pathway [44]. It has also been observed that activation of mammalian target of rapamycin (mTOR) associated with TGF-β-induced mitochondria-derived ROS generation [45].

12.5 Cross-Talk between NADPH Oxidase and Mitochondria

Cross-talk between mitochondria-generated ROS and cell membrane NADPH oxidase-mediated ROS generation play a major role in lung epithelial cells. Mitochondria-derived ROS contributes to significant increase in the expression and activation of NOX-4 under agonists, for example, TGF-β triggered condition [46]. A marked increase in the expression of NOX-4 has been observed in mice

mitochondria. Abrogation of NOX-4 expression by its siRNA attenuates ROS level in mitochondria, which has been shown to be reversed by NOX-4 siRNA [46]. This indicates that a relationship exists between mitochondria-generated ROS and NOX-4-generated ROS, and that together plays an important role for augmentation of oxidative stress during ageing [47]. TGF-β caused increase in the expression of profibrogenic proteins such as α-SMA and CTGF, which have been shown to occur via stimulation of mitochondria, derived ROS. Antioxidants can be targeted to inhibit the expression of profibrotic genes and also expression of NOX-4 induced by TGF-β [48–51]. Thus, an interaction between mitochondria and NOX-4 during exposure of lungs with environmental and occupational agents, such as silica and asbestos, and some cancer drugs e.g. bleomycin plays a critical role in TGF-β-induced production of PF (Fig. 12.1).

12.6 Silocosis and Asbestosis

Airborne silica particles and asbestos fibres are implicated for PF especially for workers of different occupations such as mining, construction, manufacturing and farming. Inhalation of these particles triggers the pathogenesis associated with the chronic interstitial lung diseases, silicosis and asbestosis [52, 53]. IL-1β produced by alveolar macrophages (AMs) is known to be involved in the initiation of silica- or asbestos-induced inflammatory responses [33, 54].

Prolonged exposure to silica and asbestos induces inflammation and subsequently progression of PF, which has been exemplified by prolonged leukocyte infiltration with eventual proliferation of fibroblasts, and subsequently collagen deposition. AMs are known to respond to stimuli during initial inflammatory responses. Exposure of silica and asbestos to lung causes macrophages to undergo apoptosis due to the production of ROS, which include SOD, $OH^{\cdot}$ and $ONOO^{-}$ [55, 56]. Generation of ROS eventually causes lung damage via increase in the expression of inflammatory cytokines like IL-1β, TGF-β and TNF-α. These cytokines activate signalling pathways mediated by isoform-specific PKC, MAPK and transcription factors (e.g. NF-κB), which trigger inflammation and thereby cause proliferation of pulmonary fibroblasts with a marked increase in the production of collagen [1, 57, 58]. This eventually leads to the formation of lung granulomas. Silicosis and asbestosis reduce normal functions of the lung and are of current major global health concern [57, 58].

12.7 ROS and DNA Damage in PF

ROS dose-dependently activates the transcription factor, p53, which modulates expression of downstream target genes involved in DNA damage by increasing DNA repair thereby inhibiting the progression of PF [1].

Oxidative injury to mtDNA is important in driving ROS induced PF. Oxidative stress mediated by asbestos, silica and bleomycin predominantly activates mtOgg1

rather than nuclear Ogg1 [59] in order to attenuate mitochondrial DNA damage in lung mesothelial cells [60]. Ogg1 is a DNA glycosylase enzyme that is important for base excision repair. Ogg1 is known to be involved in the excision of the mutagenic base byproduct, 8-oxoguanine (8-Oxo-G), that results from ROS exposure [61]. The bifunctional glycosylase, Ogg1, can cleave the glycosidic bond of the mutagenic lesion and also causes strand break in the DNA backbone. The activity of Ogg1in mitochondria is threefold higher compared to the nucleus. MtOgg1 overexpression regulates AEC upon exposure of agents like silica and asbestos [62, 63].

Aconitase (ACO2), involved in the formation of isocitrate from citrate in mitochondrial TCA cycle, can be inactivated by O_{2^-} and that has been obsered to be prevented by Mn-SOD [64, 65]. Overexpression of mtOgg1 and ACO2 has been shown to inhibit mtDNA damage that occurs under oxidative stress. Several evidence suggest association between p53, ACO2 and Ogg1 in ROS-mediated DNA damage and associated signalling. Importantly, P53 regulates the transcription of the Ogg1 gene. Ogg1 decreases oxidative stress-mediated fibroblast apoptosis through p53-dependent signalling in lungs of mitochondria during exposure to asbestos and silica [66]. The significance of ACO2 transcription in maintaining the integrity of mtDNA and subsequent attenuation of PF is currently unknown and requires further investigation. Ogg1 and ACO2 along with p53 have been suggested to play a crucial role in mtDNA maintenance.

12.8 Antioxidant Defence Mechanisms

Lungs express a myriad of antioxidants to attenuate oxidative stress. Inhibition of antioxidant defence may occur due to excessive generation of ROS, which augments PF. The primary lung antioxidant defence that plays a role in ameliorating PF includes the enzymes: glutathione peroxidase, SOD and catalase in addition to the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) that regulates generation of antioxidant enzymes [1]. Intratracheal administration of catalase in asbestos-treated mice can prevent PF by attenuating H_2O_2 generation via Rac-1 in inflammatory cells [67]. In a rodent model, the antifibrotic role of the GSH precursor, N-acetylcysteine (NAC), has been observed in rodent models where NAC augments the level of GSH and inhibit bleomycin-mediated PF [68, 69]. NAC has been shown to dramatically decrease PF upon decreasing ROS formation [70]. In a mouse model, it has been observed that an increase in the activity of catalase (CAT) is associated with a marked decrease in PF [71]. Thus, increase in the expression of endogenous antioxidants, which appears to be a novel therapeutic approach to ameliorate PF [72].

Available evidence suggest that endogenous antioxidant defence is based mainly on dietary vitamins and nutraceuticals. Antioxidants, therefore, may improve ROS-induced dysfunction of alveolar type II cells [1]. Therefore, development of pharmacological agents aimed at increasing antioxidants level in the lung could prove useful in the treatment of PF.

12.9 Inflammasomes

Inflammasomes, which consist multiprotein oligomers, have been shown to act as a molecular platform and induce production of proinflammatory cytokines, for example, IL-1β in response to cellular stress to provide innate immune response. Activation of Nalp3 inflammasomes plays a critical role in many degenerative diseases [58, 73].

Recent research suggest that silica and asbestos particles activate innate immunity, which releases proinflammatory mediators and growth factors to target fibroblasts leading to initiation and progression of PF [74, 75]. Asbestos and silica exposures have been shown to cause production of proinflammatory cytokines like IL-1β, which occur in caspase-1/Nalp3 inflammasome-dependent manner [74, 75]. Nalp3 forms the inflammasome oligomer upon recruiting the adaptor protein, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and procaspase-1, which activates procaspases and cleaves pro-IL-1β into IL-1β and subsequently triggers inflammatory response leading to the pathogenesis of PF [76, 77] (Fig. 12.2).

Nalp3 inflammasome activation occurs via two-step mechanisms; the initial signal comes upon stimulation of Toll-like receptors (TLRs), which enhances Nalp3 expression and subsequently activates pro-IL-1β via NF-κB-dependent manner [14]. TLR belongs to a family of pattern recognition receptors (PRRs) that elicit a

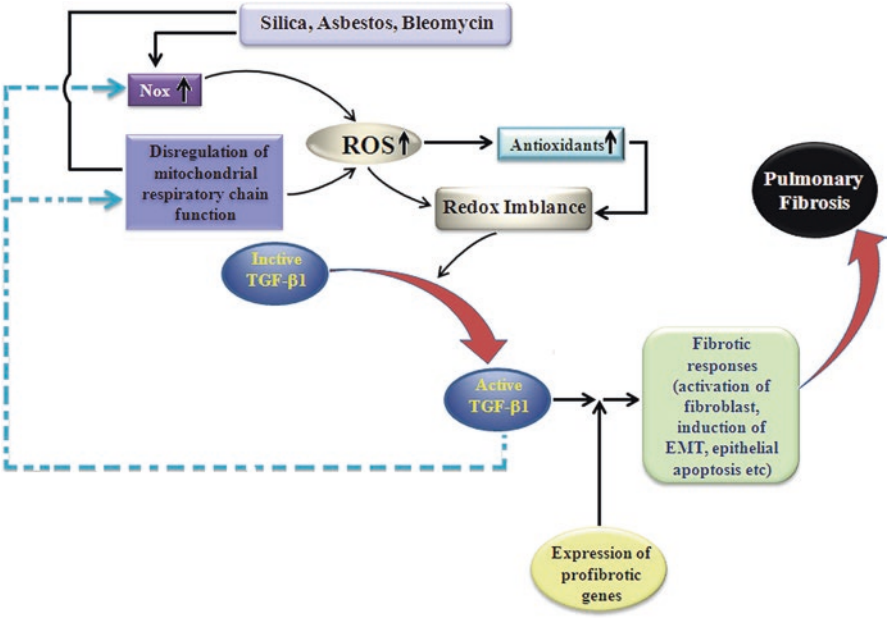

Fig. 12.2 Environmental (silica), occupational (asbestos) and cancer drug (bleomycin)-induced oxidative stress-mediated IL-1β production and subsequent progression of pulmonary fibrosis

critical role in inflammasome-mediated innate immune response to determine pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Members of the TLR family use myeloid differentiation primary response 88 (MYD88), a key adaptor molecule that recruits IL-1β receptor-associated kinases (IRKs) for downstream signalling events leading to the activation of NF-κB [78–80] (Fig. 12.2).

AMs, the predominant immune cells in the lung, enter into the lung parenchyma and mediate inflammation upon releasing IL-1β to elicit inflammatory responses [81]. Nalp3 inflammasome is known to mediate IL-1β production [82]. IL-1β binds to its receptor and recruits MyD88. MyD88 then phosphorylate the IL-1β associated kinases 1–4 (IRAK 1–4) leading to activation of IKK with consequent inactivation of IκB. This leads to stimulation of the transcription factor, NF-kB [3, 83, 84, 85], followed by upregulation of IL-1β RNA expression. The role of IL-1β has been exemplified by the fact that exogenous IL-1β augments lung inflammation followed by remodelling of lung tissues for the progression of PF [85, 86] (Fig. 12.2).

MyD88 signalling mechanism occurs in response to mobilization of silica particles in the lung for (i) granuloma formation, (ii) inflammatory response associated with Th17-mediated inflammatory processes, and (iii) neutrophil accumulation. Th lymphocytes play an important role in the progression of neutrophilic granulomatous-mediated silicosis [87]. Notably, Thlymphocytes has also been implicated in elastase –mediated cigarette smoke, bleomycin, inorganic dusts induced inflammatory lung diseases [88, 89] (Fig. 12.2).

MyD88 is associated with activation of mitogen-activated protein kinases (MAPKs) [90]. MyD88 plays a critical role in the progression of silica- and asbestos-induced inflammation and granulomas. TNF-α upon activation of innate immune cells plays a crucial role in the progression of silicosis and asbestosis. Silica- and asbestos-mediated lung inflammation and subsequent granuloma formation were reduced by administration of inhibitors of TNF-α receptors [23, 91]. Repeated triggering of innate immune response upon exposure with LPS can amplify silica-induced granulomatous response in a mice model system [92] (Fig. 12.2).

A large number of stimuli have been observed to activate the Nalp3 inflammasomes [93]. Importantly, efflux of cellular K^+ seems to be a common point where these stimuli induce Nalp3-mediated activation of caspases [94]. K^+ efflux upon P2X7 purinergic receptor activation is currently considered to be an important signalling pathway for activation of Nalp3 inflammasomes [95]. An increase in extracellular K^+ has been observed to significantly inhibit silica-induced IL-1β production from macrophage. Additionally, an increase in extracellular K^+ has also been shown to inhibit IL-1β production, which is dependent on Nalp3 inflammasome [94–96]. Notably, silica and asbestos require cellular K efflux to activate caspase-1 [93, 97]. Overall, depletion of cytosolic K leads to Nalp3 inflammasomes activation, which stimulates caspase-1 with subsequent production of IL-1β leading to PF (Fig. 12.2).

Another mechanism of Nalp3 inflammasomes activation is associated with the actions of lysosomal enzymes stimulated, for example, by silica, which is relatively big in size and difficult to get entry in to lysosomes. This causes incomplete (abortive) phagocytosis of crystals of silica and asbestos fibres leading to lysosome

swelling and rupture and subsequently releases cathepsins for activation of Nalp3 inflammasomes [98].

The implication of intracellular Ca^{2+} ($[Ca^{2+}]_i$) signalling in Nalp3 inflammasome activation has been exemplified by Brough et al. [99, 100], who observed a marked decrease in IL-1β using BAPTA-AM (Ca^{2+} chelator) in ATP triggered murine macrophages [100]. A marked increase in $[Ca^{2+}]_i$ has been shown to augment production of mitochondrial ROS (mtROS) generation, which subsequently cause damage to the mitochondria [101, 102]. Thus, mitochondrial dysfunction due to high $[Ca^{2+}]_i$ levels may trigger Nalp3 inflammasome activation [103, 104]. Importantly, blocking mitochondrial Ca^{2+} uniporter has been shown to prevent not only accumulation of mitochondrial Ca^{2+} but also IL-1β production [105, 106]. In neutrophils and macrophages, Nalp3 can be rapidly deubiquitinated under different stimulations, while pretreatment of deubiquitinase inhibitors has been observed to decrease caspase-1 activity and IL-1β production via disruption in the assembly of NAlp3 inflammasomes [107, 108] (Fig. 12.2).

Receptors for P2X subfamily of purinergic receptors play a critical role in the production of cytokines in AMs, which is induced by extracellular ATP upon stimulation of the assembly of Nalp3 inflammasomes and caspase-1-dependent generation of IL-1β and IL-18 [109] (Fig. 12.2).

12.10 Innate Immunity, Inflammation and PF

Th1- and Th2-mediated activation of cytokines was suggested to be important for inflammation and fibrosis. T cells are known to be the key player in the pathogenesis of silicosis and asbestosis [110, 111]. T cell influx into the lungs has been observed to play an important role in the immune response to silica and asbestos [110, 111]. However, T cell genetically abrogated mice elicit inflammation associated lung injury upon concomitant increase in neutrophil response to silica, asbestos and bleomycin with a marked increase in collagen deposition leading to PF [111–113]. This observation was supported by Helene et al. [114], who demonstrated that in bleomycin-induced PF, involvement of T cells is not essential, indicating that innate immunity is not the only requirement for the pathogenesis of PF. Thus, inflammation leading to PF by silica and asbestos exhibits separate pathways, which require activation of immunological responsiveness. Silica- and asbestos-mediated lung inflammatory response is associated with MyD88-dependent innate immunity. Therapeutic strategies are currently aimed at dissociating inflammation from this pathway and to link it with T cell-mediated immunosuppression, a new concept to manage PF of asbestosis and silicotic patients [87, 114].

NK cells in the lungs play an important role during silica exposure. Initial interaction between silica and phagocytic cells trigger functional alterations of NK cells [115]. However, the exact role of NK cells in PF induced by silica, asbestos and bleomycin remains to be determined.

NF-κB is known to play a pivotal role in altering the inflammatory cytokine production owing to ROS production especially in macrophages, for example, silica,

and subsequently inhibits the pathogenesis of PF [85]. A large number of researches implicated involvement of PI3K/Akt signalling components as upstream activator of NF-κB signalling cascade [116, 117]. PI3K-dependent activation of Akt may promote the transcriptional activity of NF-κB by increasing the degradation of IKK and phosphorylation of NF-κB/p65. P-AktSer473 and also the NF-κB/p65 levels were enhanced during the progression of silica- and asbestos-induced PF [118–120].

12.11 Proteases and Pulmonary Fibrosis

Uncontrolled activation of proteases has been suggested to be associated with the progression of PF. In an animal model of elastase-mediated pulmonary inflammation, it has been observed that inflammation is IL-1β and Toll/IL-1 signal transduction adaptor MyD88 dependent [121]. Elastase augments IL-1β, TNF-α, keratinocyte-mediated secretion of chemokines and subsequent recruitment of neutrophils in the lung [122]. These are markedly reduced upon inhibition of Il-1β or MyD88. The effect produced by elastase on PF depends on activation of inflammasomes, which subsequently increases production of IL-1β. IL-1β, therefore, is currently considered as an important mediator of silica-, asbestos- and bleomycin-induced PF and appears to be its potential therapeutic target.

An imbalance between MMPs and TIMPs has been implicated in a variety of lung diseases, for example, PF [123, 124]. MMPs are expressed at low levels in normal lung tissues, but their levels are increased upon inflammation leading to tissue damage [125]. MMP-2 damages alveolar basement membrane upon solubilizing several ECM proteins such as elastin, fibronectin and type IV collagen [126], and assists immune cells and fibroblasts to migrate to intra-alveolar space [127]. In order to counter the effects of MMPs, and thereby to attenuate matrix degradation and enhancement of fibroblast or myofibroblasts growth for increasing deposition of collagen in ECM [128, 129]. Dysregulation of MMP/TIMP expression may cause epithelial disruption [130]. Bleomycin-induced increase in the production of apoptotic or damaged cells cannot be cleared from the lungs. Some of these molecules play a role in activating the damage-associated molecular patterns (DAMPs) [131], which increases sustained inflammatory response. BLM groups augment mRNA and protein expression of Fas and other apoptotic proteins such as Bax and caspase 3. Inhibitors of caspase-1 or interleukin-1β activation or use of IL-1β receptor antagonist have been shown to abrogate bleomycin-induced lung inflammation [78, 132] (Fig. 12.2).

Many peptides, e.g. elastin-derived peptides, are cleaved by proteases [133–135]. Some of the peptide fragments have chemoattractive activity and were shown to trigger the chemokine, CXCR2-mediated inflammation of the lung [136]. Additionally, CXCR3 signalling seems important for PF that is triggered by inflammation-induced elastase [136, 137].

Instillation of elastase in lungs activates Nalp3/ASC inflammasomes. This activation process has been shown to be triggered mainly by two signals: The first proceeds upon activation of TLR or Nalp3 expression in inflammasomes, which leads to generation of pro-IL-1β. The second one is provided by dying cells uric acid, which stimulates the Nalp3-ASC inflammasomes leading to activation of caspase-1 followed by production of IL-1β [138] (Fig. 12.2).

12.12 Ageing and Pulmonary Fibrosis

Ageing is an established factor of PF [139, 140], albeit the pathogenic mechanisms associated with advanced ageing remain mostly unexplored. Telomeric repeat-containing RNA (TERRA) exhibits a type of long noncoding RNA, which plays a critical role in PF [141].

TERRA expression has been observed to be markedly enhanced in the peripheral blood mononuclear cells in patients with PF and that have been implicated as important physiological indicators of fibrogenesis [142]. RNA interference on TERRA expression has been shown to ameliorate the functions of mitochondria and genes that are associated with telomerase reverse transcriptase, cyclin E, cyclin D, MMPs and members of the Bcl-2 family. Notably, inhibition of TERRA expression by its siRNA was shown to improve functions of the antioxidant enzymes such as catalase and SOD [141–143].

Chronic and progressive pneumonia may produce PF, which occurs primarily in adults [144]. The incidence of PF has often been observed in aged patients. Young individuals are normally unaffected by PF. It, therefore, appears conceivable that a correlation probably exists between age and the disease. ROS abrogates oxidant-antioxidant balance in tissues and that has been suggested to play a critical role during ageing. López-Otín et al. [145] have observed a positive correlation between telomeres shortening and associated epigenetic changes that cause mitochondrial dysfunction and subsequently contribute to age-related PF. Understanding the regulatory function of TERRA may be of significance to identify therapeutic target(s) that could prove useful in the treatment of age-associated PF.

12.13 Bleomycin-Induced PF

Bleomycin is commonly used for the treatment of different types of cancers. However, bleomycin treatment causes unwanted deleterious side effects and the most prominent of which is PF. Using proteomic approach, annexin A2 (ANXA2) has been identified as a target of bleomycin-induced PF. In a mice model, genetic depletion of ANXA2 has been shown to mitigate bleomycin-induced PF [146].

Glu139 (E139) of ANXA2 has been observed to be important for the binding of bleomycin in lung epithelial cells. In lung epithelial cells, ANXA2E139A mutation

produced by a CRISPR-Cas9 technique attenuates bleomycin caused activation of transcription factor EB (TFEB), a prime regulator of autophagy, leading to a marked acceleration of autophagic flux in pulmonary epithelial cells [147, 148]. This subsequently attenuates apoptosis of the proliferative epithelial cells and eventually ameliorates PF. Importantly, a decrease in TFEB level was observed in human PF tissues compared to normal condition indicating an important role for TFEB-mediated autophagy in PF [147, 148]. ANXA2, therefore, is considered to be the target of bleomycin binding with ANXA2, which impedes TFEB-induced autophagic flux and subsequently PF [147, 148].

ROS increases expression of profibrotic genes such as α-SMA and type1 collagen [1]. In vitro silencing of Nalp3 attenuates IκBα degradation and, thereby, decreases the synthesis of type I collagen [149]. Nalp3 inflammasomes were activated by bleomycin, and this activation was relieved by an inhibitor of nuclear factor NF-κB [150]. Thus, Nalp3 inflammasome is involved in bleomycin-induced ROS-mediated type 1 collagen synthesis, which is mediated by the NF-κB signalling pathway and contributes to the development of PF.

12.14 Therapeutics

12.14.1 Sirtuin 3 as Regulator of Mitochondrial Antioxidant Response

ROS has been shown to cause alveolar epithelial cell injury mainly due to increase in fibroblast-myofibroblast differentiation (FMD), which is one of the critical steps for the pathogenesis of PF [151]. Expression of sirtuin 3 (SIRT3), a regulator of antioxidant response, has been observed to be reduced in human lung fibroblasts in response to TGF-β [152]. A marked decrease in SIRT5 (a mitochondrial deacylase) was shown to promote acetylation (inactivation) of mitochondrial stress response enzymes, for example, SOD. Decrease in SIRT3 level in fibroblast of human lung has been shown to promote FMD. However, overexpression of SIRT3 has been observed to attenuate TGF-β-induced FMD and SMAD3 expression [152, 153]. Administration of resveratrol has been shown to stimulate SIRT3 expression and also inhibits SIRT3 acetylation induced by TGF-β. Mice deficient in SIRT3 have been observed to be susceptible to mice with PF and elicit a marked increase in SMAD3 expression. Recent research demonstrated that interaction between SIRT3 with TGF-β during ageing elicits a prominent role in PF pathogenesis [153].

12.14.2 Role of Fasudil

PF is mediated by several routes under oxidative stress, which include inflammation, epithelial-mesenchymal transition and coagulation processes. The effector of RhoA, the Rho-kinase (ROCK), could imply different biochemical routes, which contribute to the initiation of PF. Fasudil, a selective ROCK inhibitor, has recently

been successfully tested in mice to ameliorate PF caused by bleomycin [154]. In the lung of bleomycin-treated mouse, hydroxyproline content decreases in response to fasudil treatment. This may be one of the mechanisms by which fasudil attenuates bleomycin induced infiltration of inflammatory cells in bronchoalveolar lavage fluid (BALF) [154]. Fasudil has also been shown to reduce bleomycin-mediated increase in mRNA and protein expression of TGF- β, α-SMA and PAI-1. [155]. Thus, fasudil could prove useful for treatment of PF.

12.14.3 Role of Salidroside

Bleomycin (BLM) application is experimental animals, e.g. rat elicited a prominent stimulation in the production of malonaldehyde (MDA) in lung tissues, which was inhibited by salidroside. In addition to MDA production, BLM-induced oxidative injury was shown to attenuate levels of antioxidants such as SOD, GSH and GSH-Px in the lung [156]. However, upon administration of salidroside, the levels of these antioxidants were restored to basal level, indicating the efficacy of salidroside for clinical use in PF [157].

12.14.4 Role of Corilagin

In bleomycin-treated mice lung, MDA, NF-κB, IKKα, TNF-α and IL-1β expression have been observed to be increased, albeit a marked decrease in the I-κB expression was noticed. These effects of bleomycin were reduced by corilagin [158]. Corilagin has also been shown to inhibit TGF-β and α-SMA expression in lung tissue, thereby attenuates BLM-induced oxidative stress-mediated lung epithelial cell injury and fibrosis of lung due to inhibition in the release of proinflammatory cytokines and concomitant signalling of NF-κB and TGF-β [159]. Corilagin has recently been considered as a potential therapeutic agent to attenuate PF [158].

12.14.5 Role of Fluofenidone

Fluofenidone (FD) has been demonstrated to abrogate asbestos- and silica-induced accumulation of ROS. It inhibits the interaction of Nalp3 inflammasome-associated molecules and decreases caspase-1 and IL-1β levels in THP-1 in lungs of mice [160]. FD was shown to inhibit NF-κB-mediated nuclear transcription instead of IKKα or IkB-α phosphorylation, the upstream signals for translocation of NF-κB to the nucleus. Importantly, FD attenuates bleomycin caused inflammation and fibrosis of lung by inhibiting the IL-1β/IL-1βR/MyD88/NF-kB signalling pathway [147, 148]. However, the exact target of FD on component(s) in the pathway is currently unknown and needs further exploration.

12.14.6 Role of Oleanolic Acid

Oleanolic acid (3β-hydroxyolean-12-en-28-oic acid; OA), a pentacyclic terpenoid, is present in vegetable oil, food and certain medicinal herbs as free acid, or an aglycone of triterpenoid saponins. OA has the ability to reverse the oxidant/antioxidant balance, and also can decrease production of cytokines and collagen in lung by modulating AKT/NF-κB signalling pathway in silica-induced PF [161].

PI3K/Akt pathway is a proximal inducer of NF-κB signalling cascade [117]. Upon activation, AKT promotes transcription of NF-κB by enhancing the degradation of IKB upon NF-κB/p65 phosphorylation. OA attenuates the stimulation and translocation of NF-κB to the nucleus, thereby inhibiting agonists such as silica- and asbestos-induced inflammatory response [162, 163]. The levels of phosphorylated Akt and NF-κB/p65 were found to be increased in silica-induced PF in rats OA treatment has been observed to attenuate the phosphorylation of AKT and decreases the level of NF-κB/p65 [163, 164]. OA inhibits pulmonary inflammation and fibrosis, conceivably by altering AKT/NF-κB signalling mechanism. This indicates that inhibition of NF-kB activation with OA decreases the severity of PF caused by agents such as silica and asbestos [164].

12.15 Conclusion and Future Direction

8-Oxoguanine DNA glycosylase (Ogg1), mitochondrial aconitase (ACO2) and the transcription factors like NF-κB were shown to play a critical role in silica-, asbestos- and BLM-induced ROS-mediated mitochondrial DNA (mtDNA) repair mechanism. The association of oxidative stress with environmental (silica and asbestos) and cancer drugs (bleomycin) induced ROS generation of TGF-β and IL-1β and subsequent initiation and progression of PF have also been delineated in this review (Figs. 12.1 and 12.2).

The role of ROS in mitochondrial dysfunction due mainly to mtDNA damage and cross-talk between AECs and AMs in the progression of PF induced by the environmental and occupational agents, and cancer drugs have also been briefly portrayed in this review.

A mere imbalance between ROS levels and antioxidant defences does not explicitly depict the multiple independent pathways involved in the pathogenesis of PF. Mitochondria- and p53-associated mechanisms and also the ER stress-mediated AEC apoptosis have been observed to play a crucial role during the early stages of the initiation of PF by asbestos, silica and bleomycin. Cross-talk among mtOgg1, ACO2 and p53 to repair mitochondrial DNA damage induced by the environmental occupational agents, and cancer drugs to modulate PF of a variety of signaling pathways require further investigation.

Although extensive researches have been performed to gain insights into the mechanisms of PF, our current understanding of its pathogenesis is limited. Several key questions remain to be addressed. Future studies need to focus on prognostic significance of common complications of PF and the risk factors (biochemical,

clinical and genetic) for the development of PF. Mechanisms of the therapies involving steroids and anticoagulants in ameliorating PF require thorough investigations. Understanding of the epidemiology of this condition seems important to design future drug trials for treatment of PF. Till date no definitive therapy for PF has, however, been available. Considering our current knowledge on ROS induced PF, this article enumerates novel insights that may be considered for the development of therapeutic strategies to ameliorate PF.

Studies on the correlation of the mechanisms associated with the pathobiology of ARDS and PF seem important because it is possible that similar biochemical mechanism underlie their pathogenic processes. Conceivably, development of therapies targeting patients with ARDS could prove useful for treatment of PF.

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Part III

Other Lung Diseases

Respiratory Syncytial Virus-Induced Oxidative Stress in Lung Pathogenesis

13

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Abstract

Respiratory viral infections remain the major cause of severe lower respiratory tract disease in both children and adults worldwide. Respiratory syncytial virus (RSV) is a negative-sense single-stranded RNA virus of the family *Pneumoviridae*, which is responsible for acute lower respiratory tract infections (LRTI) in children and is a major cause of severe respiratory morbidity and mortality in the elderly and immunocompromised. These LRTI in young children are often characterized by wheezing and are defined as "bronchiolitis." RSV bronchiolitis in infancy is strongly associated with the subsequent development of asthma and other forms of bronchial disease. Currently, there is no effective vaccine or specific therapy available for RSV infection, and natural immunity is inadequate, resulting in reinfections through adulthood. The high risk of recurrence and mortality rates of respiratory viral infections in young children and the elderly explains the importance for continuing efforts to understand the pathogenesis of respiratory virus-induced lung inflammation in order to design better therapeutic strategies. Lung epithelial cells are the major targets of RSV infection and play a central role in orchestrating the response to oxidative stress. Although the pathogenic mechanisms of RSV-induced acute airway disease and associated long-term consequences are still unclear, experimental evidence suggests that early inflammatory and immune events in the lung play a fundamental role in the outcome of the disease. Moreover, oxidative stress plays an important role in the pathogenesis of many inflammatory lung diseases including asthma and chronic obstructive pulmonary disease. Studies have shown that the oxidative stress response in the airways, which results from an imbalance between reactive oxygen species (ROS) production and lung antioxidant defenses, plays a major role

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in the pathogenesis of RSV-associated lung inflammatory disease as RSV induces excess oxidant production and inhibits antioxidant enzymes expression. Studies have also demonstrated the role of ROS as important intracellular messengers of RSV-induced cellular signaling leading to the expression of key proinflammatory mediators, such as cytokines and chemokines. This chapter reviews the various mechanisms of RSV-induced oxidative stress and associated pathogenicity. Specifically, we will focus on recent studies demonstrating the role of ROS as important regulators of respiratory virus-induced cellular signaling and inflammatory responses induced as a result of RSV-induced oxidative stress.

Keywords

RSV · Human airway epithelial cells · Oxidative stress · Reactive oxygen species · Oxidants · Antioxidants · Antioxidant enzymes · Cellular signaling · Inflammation · TLR · RAGE · NF-κB · MAPK · STAT · IRF · Monocytes · Macrophages · Eosinophils · Dendritic cells

Abbreviations

4-HNE	4-hydrynonenal
AECs	airway epithelial cells
AMs	alveolar macrophages
AOE	antioxidant enzymes
AP-1	activator protein-1
ARE	antioxidant response element
BHA	butylated hydroxyanisole
bZIP	basic-leucine-zipper
CF	cystic fibrosis
CNC	cap-n-collar
COPD	chronic obstructive pulmonary disease
DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
DCs	dendritic cells
DHE	dihydroethidium
DNA	deoxyribonucleic acid
DUOX	dual oxidase
eNOS	endothelial type nitric oxide synthase
ERK 1/2	extracellular signal-regulated kinase 1/2
GPx	glutathione peroxidase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
HIF	hypoxia-inducible factor

HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
IFN	interferon
IL	interleukins
iNOS	inducible type nitric oxide synthase
IRF	interferon-regulatory factor
Keap 1	kelch-like ECH-associated protein 1
LRTI	lower respiratory tract infections
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MDA-5	melanoma differentiation antigen-5
Mn	manganese
MSK1	mitogen- and stress-activated protein kinase 1
MΦs	macrophages
N_2O_3	dinitrogen trioxide
N_2O_4	dinitrogen tetroxide
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa B
nNOS	neuronal type nitric oxide synthase
NO.	nitric oxide radical
NO_2^-	nitrite
NO_2	nitrogen dioxide
NO_3	nitrate
NOS	nitric oxide synthase
NOX	NADPH-oxidase
NQO1	NAD(P)H:quinone oxidoreductase
Nrfs	nuclear factor (NF)-E2-related transcription factors
$O._2^-$	superoxide ion radical
OH.	hydroxyl radical
$ONOO^-$	peroxynitrite
P13K	phosphatidylinositol 3-kinases
pDCs	plasmacytoid DCs
PRR	pattern recognition receptors
RIG-I	RNA helicases, retinoic acid-inducible gene-I
RNA	ribonucleic acid
RNS	reactive nitrogen species
RO.	alkoxyl radicals
ROO.	peroxyl
ROS	reactive oxygen species
RSV	respiratory syncytial virus
SOD	superoxide dismutases
STAT	signal transducer and activator of transcription
TLR	Toll-like receptor
TNFα	tumor necrosis factor α
XO	xanthine oxidase

13.1 Introduction

Respiratory viral infections are the most common causes of wheezing illness in both children and adults and are also associated with asthma exacerbations in patients with preexisting lung inflammatory diseases ([1, 2]). Bronchiolitis is a major clinical syndrome in hospitalized infants, accounting for up to 60% of all lower respiratory tract infections (LRTI) during the first year of life and is primarily associated with infections that are caused by respiratory syncytial virus (RSV). In a screen for viral and atypical bacterial respiratory pathogens, RSV was the most prevalent (43.3%) as it was found to infect young children below 5 years of age with acute respiratory infection, as well as being an extremely common agent (44.1%) in cases of co-infection with two or more pathogens in the same cohort [3]. RSV presents a unique challenge to epidemiologists as it exhibits distinct infection and disease patterns compared to other respiratory viral pathogens. High-risk groups for severe RSV infection include: infants with a history of premature birth, children with congenital heart disease, cystic fibrosis (CF), or other chronic lung diseases; immunocompromised patients or those with congenital immunodeficiencies; and the elderly. Currently, no efficacious vaccine or specific therapy available for RSV infection and natural immunity is inadequate, resulting in repeated infections throughout life [4].

Airway epithelial cells (AECs) are the major targets of RSV infection. Initially, virus replicates in nasal epithelial cells but will spread to epithelial cells of the lower airways, where it activates and modulates several signaling cascades to control the host immune and inflammatory response, including cellular oxidant and antioxidant pathways. These pathways regulate expression of a variety of transcription factors, proinflammatory mediators, activate the adaptive immune response for virus clearance, and facilitate the clinical features of the disease. Although the molecular mechanisms of RSV-induced acute lung disease are not fully understood, experimental evidence suggests that early lung inflammatory and immune responses play a central role in the outcome of the disease.

This chapter will comprehensively discuss the impact of RSV disease, clinical aspects of the RSV, cellular responses to RSV infection, the role of ROS in RSV pathogenesis and associated cellular signaling pathways, and the oxidant/antioxidant response to RSV infection.

13.2 Respiratory Syncytial Virus (RSV): A Major Human Pathogen

13.2.1 Clinical Features of RSV Infection, Common Risk Factors, and Its Impact

RSV, also called human *Orthopneumovirus*, is an enveloped, anti-sense, single-stranded RNA virus of the *Pneumoviridae* family (*Orthopneumovirus* genus and *Mononegavirales* order). The RSV genome is about 15.2 kb and possesses 10 genes that encode for 11 proteins [5–7]. In 1956, RSV was first isolated from a

chimpanzee with common-cold-like illness and was then recovered from young children with severe lower respiratory tract disease [8]. Since its isolation, RSV has been identified as a leading cause of epidemic LRTI and is responsible for most hospital visits during infancy and childhood throughout the world [4, 9, 10]. There are two subtypes of RSV, type A and B, which differ in the envelope proteins present on the viral shell. Both subtypes are infectious, with some evidence that type A is the more common cause of severe RSV infections [11–13].

RSV accounts for ~64 million clinical infections and ~160 thousand deaths annually worldwide as estimated by the World Health Organization [14] and is responsible for the hospitalization of >200,000 individuals and the deaths of 4500 children every year in the USA alone [15]. RSV infection is most common in infants and young children, with nearly all children being infected by age three. It is the most common etiologic agent that cause severe bronchiolitis, a clinical syndrome characterized by wheezing, dyspnea, respiratory distress, and radiological evidence of hyperaeration of the lung [16]. About 30–40% of adults exposed to RSV infections will develop a spectrum of respiratory tract diseases ranging from a common cold to otitis media and to pneumonia with wheezing. RSV infections are also linked to the development and severity of asthma. Recurrent respiratory symptoms are observed in ~30% of children hospitalized for acute bronchiolitis in consistent with airway hyperreactivity in the years following infection, and RSV bronchiolitis has been proved to be the dominant risk factor for the development of asthma [17, 18]. Moreover, recurrent incidents of wheezing in asthmatic subjects are often precipitated by RSV infections. In addition to the clinical and epidemiological relationship between bronchiolitis in infancy and asthma later in life, the two diseases are also linked by the histopathologic findings of profound inflammation of the airway mucosa. Surprisingly, viral antigen has been found in only a small amount and patchy in distribution in autopsy samples from patients with RSV bronchiolitis [19]. These findings suggested that other secondary pathogenic mechanisms are responsible for the damage of the airway mucosa observed in RSV bronchiolitis. Although the pathogenic mechanisms underlying the severity of RSV bronchiolitis are unknown, studies have shown that excessive oxidative responses in the lung play a central role.

13.2.2 High-Risk Population of RSV Infections

RSV infections often manifest as a mild cold in healthy adults but can cause serious illness in the infant population. The severity of the disease is influenced by environmental and genetic modifiers, including passive smoke exposure, day care attendance, the presence of school-aged siblings, and birth within 6 months of RSV season. Young children and the elderly population are highly susceptible to RSV infection. Children with immunosuppression due to transplant are particularly at high risk for RSV related complications. Children with immature lungs, as in premature infants, are at higher risk for LRTI. Children with CF are more susceptible to RSV infection and greater incidence of hospitalization because the CF mutation

results in impaired immune function that inhibits inducible nitric oxide synthase (iNOS) expression [20]. Rates of RSV hospitalizations and significant morbidity and mortality are much higher in infants and children with congenital heart disease, bronchopulmonary dysplasia, and asthma [21–24], as well as in adults with underlying cardiopulmonary diseases [14, 25, 26], and immunocompromised adults receiving organ transplantation, including heart transplants.

13.2.3 Treatment of RSV Infections

The treatment of RSV infection is mainly symptomatic, except in high-risk infants, the elderly, and immunocompromised individuals [27]. The supplemental oxygen administration to maintain oxygen saturations of ≥93% and the replacement of fluid deficits are sufficiently effective for infants hospitalized with severe RSV infection. Treatment of RSV infection with bronchodilators such as albuterol and epinephrine, and corticosteroids including dexamethasone, prednisolone, methylprednisolone, and hydrocortisone are ineffective in reducing the rate of hospitalization when administered to outpatients and in shortening the length of hospitalization among inpatients. Apart from Synagis (palivizumab), a monoclonal antibody specific for the F protein of both subtypes of RSV, and Ribavirin, the only antiviral drug approved for the treatment of serious lung infections caused by RSV, there are no potential vaccines available to prevent RSV infections [28–30]. In children born with heart problems, Synagis was associated with low blood oxygen levels and abnormal heart rhythms. Use of Ribavirin is restricted to high-risk or severely ill infants, and its use has been limited by its cost, toxicity, variable efficacy, and tendency to generate resistant viruses [28, 31]. As shown by the studies with RSV, administration of immune globulin, humanized monoclonal antibody, or antiviral agents alone are unlikely to be of benefit in post-infectious treatment, despite their efficacy when used in prophylaxis regimens [32]. Thus, despite the fact that a specific antiviral therapy for RSV is currently not available, the current belief is that such an approach alone would not be sufficient to treat RSV bronchiolitis. The current need for additional effective combination therapies, which include anti-RSV agents and anti-inflammatory or immunomodulatory agents, is well-acknowledged [33].

Several treatment approaches are being investigated targeting different proteins associated with the replication and/or infection processes of the virus with the promise to treat RSV infections. However, they are either in preclinical development or early clinical trials, suggesting that even if successful, they will not be available for years to come. Development of vaccines for RSV infection are hindered due to the limited knowledge of the pathogenic mechanisms that determine the severity of acute LRTI caused by the virus. Studies from the formalin-inactivated vaccine that led to more severe disease in those exposed to natural RSV infections have contributed significantly to the understanding of the immune-mediated mechanisms responsible for the enhanced disease that occurred in a subset of the vaccinated infants [34, 35]. Extensive studies on RSV-mediated airway illness revealed the role of the host response and immunopathogenesis, either as an induced immune/

inflammatory response in the airways or as a failing to control or stop viral replication as a result of impaired immune response. Thus, understanding the molecular mechanisms that control the viral-induced immune/inflammatory response is critically important to identify new therapeutic strategies to treat acute LRTI caused by RSV and other respiratory viruses.

13.3 Cellular Responses to RSV Infection

13.3.1 Airway Epithelial Cell Responses to RSV Infection

The respiratory epithelium is the first protective physical barrier against injurious inhaled stimuli and pathogens, and it represents the major target site of respiratory virus infections. Specifically, airway epithelial cells (AECs) are armed with pathogen-sensing membrane and cell-surface pattern recognition receptors (PRR), such as the Toll-like receptor (TLR) family and cytosolic PRRs, which include the RNA helicases, retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation antigen-5 (MDA-5), to initiate innate immune responses upon sensing the presence of viral patterns [36, 37]. After exposure to infectious agents or environmental toxicants, AECs has been shown to secrete a variety of molecules involved in the antiviral and innate immune response and play a major role in the inflammatory and infectious processes in the lung [38–42]. The mechanism(s) of RSV-induced acute airway inflammatory disease and its associated long-term consequences are largely unknown, but the delicate balance between immunopathology and immunoprotection in the airway mucosa appears to be altered by an exuberant local inflammatory response [43]. In this regard, RSV is considered among the most potent known biological stimuli for inducing the expression and/or secretion of proinflammatory mediators in association with evidence of oxidative "stress" (i.e., lipid peroxidation) [39, 40, 42]. RSV induces intracellular signals during the process of viral replication leading to activation of a subset of transcription factors, including nuclear factor-kappa B (NF-κB), interferon-regulatory factor (IRF), signal transducer and activator of transcription (STAT), and mitogen-activated protein kinase (MAPK) signaling cascades that regulate the expression of a number of important immune/inflammatory mediators [44]. Activation of these transcription factors following RSV infection occurs through redox-sensitive pathways [44, 45]. These events have been extensively demonstrated in AECs, the primary site of RSV replication, and play a central role in orchestrating the response to oxidative stress. RSV infection elicits severe oxidative damage to AECs by significantly inducing the production of lipid peroxidation products/ROS [46, 47]. Also, RSV infection of human AECs results in a remarkable downregulation of the level of expression of several anti-oxidant gene products [47, 48]. Studies in experimental animal models and in human infants with natural infections have confirmed that many inflammatory gene products, as well as some that have not yet been described, are induced or upregulated in the airway mucosa during RSV infection and can play a significant role in the pathogenesis of lung inflammation [41, 49, 50]. Conserved structural motifs

expressed by microbial pathogens are recognized by AECs via TLRs expressed on their surface. Although TLRs are expressed at low levels under normal physiological conditions in AECs, their expression is increased under inflammatory conditions and during infection with pathogens [51–53]. Reports also show that A549 cells, a carcinoma-derived airway epithelial cell line, behave very similarly to primary AECs, such as human small alveolar epithelial (SAE) cells, in response to RSV infection and are suitable airway epithelial cell models for RSV infection [40, 54].

13.3.2 RSV Infection in Immune Cells

After the initial encounter with AECs, RSV next comes in contact with innate immune cells such as monocytes, alveolar macrophages (AMs), and dendritic cells (DCs) in the airways [55]. As blood leukocytes and respiratory tract epithelial cells actively contribute to inflammation during infection, the levels of proinflammatory mediators may be indicative of disease severity. These cells produce significant amounts of proinflammatory cytokines and chemokines in response to viral infection that are involved in controlling adaptive immunity by their interaction with T helper cells.

Monocytes, macrophages (MΦs), and DCs, which share common morphologic and functional features, are the major part of the first line of immune defense against a wide range of pathogens, including RSV, and contribute significantly to acute inflammation during infection [56, 57]. AMs are the predominant cell population at the alveolar space, and they work as major innate sentinels for pathogen recognition. AMs are required for the early immune response against RSV and promote viral clearance as well as control immunopathology through multiple mechanisms, including pathogen phagocytosis, cytokine production, direct interaction with helper and cytotoxic T cells, and antigen presentation [58]. Studies have shown that AMs and AECs respond to RSV infection distinctly in kinetics, magnitude, and TLR utilization. RSV induces IL-1β, IL-6, IL-8, IL-10, IL-12, and TNF-α in AMs [59], whereas RSV-infected AECs secrete several distinct groups of CC, CXC, and CX_3C cytokines [40].

DCs are the major antigen-presenting cells with a low phagocytic capacity and their function is to process antigens and present them to T cells to promote immunity to foreign antigens and secrete cytokines to regulate immune responses [56, 60]. A lower number of blood plasmacytoid DCs (pDCs) and increased lung pDCs as well as induced expression of inflammatory and immunomodulatory cytokines, including TNF-α, IL-6, IL-1β, IL010, and IL-12p70, have been associated with RSV bronchiolitis [61–63]. Eosinophils are leukocytes produced in the bone marrow that migrate to tissues throughout the body in order to fight against infections, and have been shown to be activated during the acute phase of RSV LRTI and contribute to antiviral immunity [64–67].

RSV infection elicits a strong systemic neutrophil response, which becomes activated during the initial pathogenesis of RSV LRTI that coincides with disease severity and viral burden [68–74]. Reduced circulating T cell counts are observed in

children with severe RSV illness compared to those with less severe infection [70, 75–78], whereas during the course of RSV disease the circulating T cell counts increase [75, 79]. Increased circulating B cells were observed in infants with RSV LRTI [76, 80, 81]. RSV infection also results in reduced total systemic natural killer cell counts, which correlates with greater severity of infection [75, 82, 83]. Studies have also shown that RSV can infect basal cells and alter human airway epithelial differentiation [84].

13.4 Oxidative Stress and Lung Inflammation

13.4.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are ubiquitous, unstable, highly reactive molecules derived from molecular oxygen produced during the normal cellular aerobic metabolism. ROS are classified into two groups: radical and non-radical. Members of the radical group, also called free radicals, include superoxide ion radical ($O._2$-), hydroxyl radical (OH.), nitric oxide radical (NO.), peroxyl radical (ROO.), and alkoxyl radical (RO.) and have at least one unpaired electron in the outer orbital and readily donate or accept an electron to attain stability [85–89]. The non-radical group includes hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), organic peroxides, and aldehydes. ROS can be generated from both endogenous and exogenous substances. ROS toxicity depends on the presence of a "fenton catalyst," such as iron ions and peroxidases, which, in the presence of O_2^- and H_2O_2 give rise to the extremely reactive (OH.) radicals. ROS and particularly (OH.) can interact with a variety of molecules, like membrane lipids, leading to lipid peroxidation, that impairs membrane functions, inactivates receptors, and increases tissue permeability [90]. Aldehydes including malondialdehyde (MDA) and 4-hydrynonenal (4-HNE) are generated during the process of lipid peroxidation and attack targets far from the site of the original free radical generation after they leaked from cells.

ROS formation occurs continuously in every cell during metabolic processes. Cellular sites for ROS generation include the mitochondria, cytochrome P-450 metabolism, peroxisomes, microsomes, inflammatory cell activation, and various enzymes like cyclooxygenase, lipoxygenase, xanthine oxidase, and membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (NOX) (Fig. 13.1) [91]. Enhanced levels of ROS can be generated by increased activation of NOX (mitochondrial and plasma membrane-associated) as well as by several other mechanisms that induce excessive ROS production due to mitochondrial dysfunction or increased activity of the above-mentioned enzymes.

Mitochondria are the most important physiological sources of free radicals in living organisms and are known to generate significant amounts of ROS. ROS are generated as byproducts by the partial reduction of oxygen during the mitochondrial electron transport of aerobic respiration or by oxidation of nutrients in order to generate energy. They are also generated during the cellular response to xenobiotics, cytokines, bacterial invasion, and infection with certain viruses [90, 92–96]. In

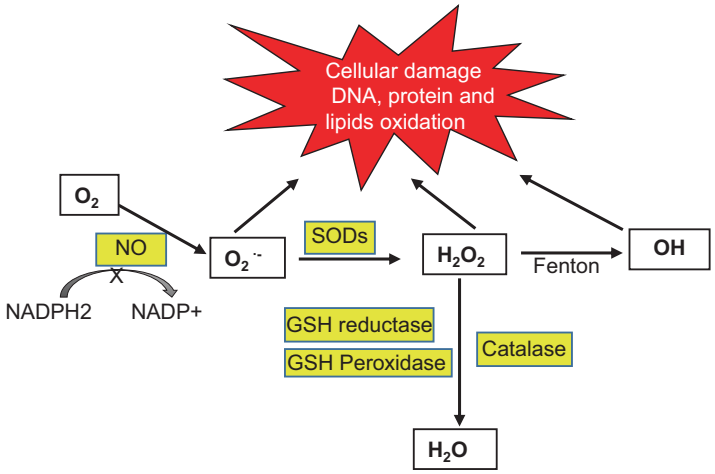

Fig. 13.1 Reactive oxygen species generation: Intracellular superoxide ($O_2^{\cdot-}$) is primarily produced from the oxidation of NADPH by oxidase enzymes (NOX) or from mitochondrial electron transport by aerobic respiration. Superoxide is rapidly converted into hydrogen peroxide (H_2O_2) by compartment-specific superoxide dismutases (SODs), which may be converted to H_2O by cellular antioxidants, such as glutathione peroxidase, glutathione reductase, and catalase. Hydoxyl radicals (OH.) accumulates when H_2O_2 levels increase uncontrollably through the reactions with metal cations and irreversibly damage cellular components

addition to endogenous ROS, exogenous compounds like air pollutants, cigarette smoke, radiation, heavy metals, etc., can generate ROS that have been shown to modulate the expression of several genes, which highlights their role as redox regulators of intracellular signaling (Fig. 13.1) [97–99]. Mitochondria are also involved in the generation of nitric oxide (NO.) free radicals via the nitric oxide synthase (NOS) reaction, which reacts with $O._2^-$ radicals to form another harmful oxidant, peroxynitrite ($ONOO^-$), a potential source for OH. radical. The endoplasmic reticulum is another source of ROS where resident cytochrome P-450 oxidizes unsaturated fatty acids and xenobiotics such as pollutants, drugs, infectious agents, and toxins to generate $O._2^-$ and H_2O_2. The xanthine oxidase (XO) is a form of xanthine oxidoreductase enzyme that catalyzes the reaction of NO with $O._2^-$ leading to the generation of highly reactive $ONOO^-$. ROS are also generated as byproducts during metabolism of arachidonic acid, which occurs in every cell. During the respiratory burst in response to xenobiotics, NOX generate ROS from activated phagosomes in neutrophils and macrophages, by reacting with the intracellular NADPH, to reduce molecular oxygen superoxide [100].

13.4.2 Reactive Oxygen Species-Mediated Cellular Signaling

Low levels of ROS in the cells act as redox regulators of several cellular signaling pathways including those involved in the regulation of the antiviral and

proinflammatory response induced by paramyxoviruses [98, 99, 101]. In contrast, oxidative stress refers to the damaging side of excessive ROS levels that can modify cellular components, induce mitochondrial permeability transition, and signal the induction of several biological processes such as autophagy, apoptosis, and necrosis [90]. ROS regulate signal transduction pathways through the cyclic oxidation/reduction of cysteine residues in kinases, phosphatases, and other regulatory factors. Signaling pathways play specific roles in different phases of the cell cycle and regulate cellular processes that are influenced by the oxidative state of the cell. NO is an intra- and extracellular messenger that transmits signals to cells in the cardiovascular, nervous, and immune system and mediates activation of several signaling cascades and has been shown to play a major role in various physiological processes [102]. Superoxide and H_2O_2 in association with antioxidant enzymes (AOE) play a role in turning enzymes on and off by redox signaling [102]. ROS-mediated mitogenic signaling activates receptor tyrosine kinases, which in turn activates MAPK cascades required for cell proliferation. Activation of these signaling cascades leads to the generation of H_2O_2 from several enzyme catalysts, including NOX, which interacts with SOS-RAS-RAF-ERK and P13K/Akt pathways to promote cell proliferation, nutrient uptake, and cell survival [103, 104]. Low-level increases in H_2O_2 result in increased reentry into the cell cycle, while sustained high levels of H_2O_2 lead to cell arrest and eventual apoptosis after prolonged arrest.

In addition to aerobic metabolism, ROS are generated physiologically through PRR signal transduction pathways. The innate and adaptive immune systems are critical for pathogen-specific defense and immunological memory and play a crucial role in tissue repair. It is evident that ROS are second messengers in innate and adaptive immune cells [105, 106], but increased ROS levels within the immune cells can result in hyperactivation of the inflammatory response that results in tissue damage and pathology [107]. Adaptive immunity involves the expansion of T cells and B cells specific for pathogens via rapid proliferative responses and has been shown to be mediated by redox signaling [108–110]. Ligation of TLRs induces ROS generation, which is critical for the activation of key transcriptional mediators of the innate immune response [111, 112]. Likewise, ligation of the TNF receptor induces ROS and oxidative DNA damage [113]. Studies have shown that PRR-generated ROS plays an important role in signal transduction by controlling phosphorylation [113–115]. Low levels of ROS in the immune system might enhance normal immune function but high levels lead to elevated levels of proinflammatory mediators and promote inflammation [116]. ROS have been shown to induce an inflammatory response through activation of NF-κB and activator protein-1 (AP-1). Interleukin (IL)-8 expression has been shown to be redox-sensitive following influenza, rhinovirus, and RSV infection, since antioxidant treatment significantly reduces its secretion from viral-infected AECs [94, 95, 117]. The likely mechanism of antioxidant-mediated modulation of viral-induced IL-8 expression is through inhibition of viral-induced NF-κB and AP-1 binding to its cognate site on the IL-8 promoter [94, 118]. NF-κB-regulated genes have been shown to play a major role in regulating the amount of ROS in the cell, and ROS have several inhibitory or stimulatory effects on NF-κB activation. ROS have been reported to stimulate NF-κB

pathways in the cytoplasm, but inhibit NF-κB activity in the nucleus, suggesting ROS involvement in both activation and repression of NF-κB signaling [119].

13.4.3 Intracellular Antioxidant Response to Oxidative Stress

Oxidative stress refers to the imbalance due to excess ROS or oxidants over the antioxidant capacity of the cell resulting in direct or indirect damage to nucleic acids, proteins, carbohydrates, and lipids that play a critical role in the preservation of essential biological functions (Fig. 13.2). Oxidative stress has been implicated in many disease states such as cancer, diabetes, aging, COPD, CF, and asthma [120–127]. Cells are protected against oxidative damage due to exogenous and endogenous ROS by well-developed enzymatic and nonenzymatic antioxidant systems. Endogenous antioxidants comprise a number of enzymes, such as SODs [three isoforms of SOD: the cytoplasmic Cu/Zn SOD or SOD1, the mitochondrial Mn SOD or SOD2, and the extracellular EC SOD or SOD3], catalase, glutathione reductase, glutathione peroxidase (GPx), and glutathione S-transferase (GST) [90]. The nonenzymatic antioxidants include transferrin, ferritin, and vitamins A and C. Each enzyme system plays a significant role in limiting intracellular and extracellular oxidative stress, and it depends on the source and type of ROS generation. Glutathione (GSH) is the major nonenzymatic oxidant defense system, which is present in large amounts in most mammalian tissue and helps to detoxify peroxides and generate a number of antioxidants [128]. Reduced GSH is generated from its oxidized form (GSSG) by the action of an NADPH dependent glutathione reductase.

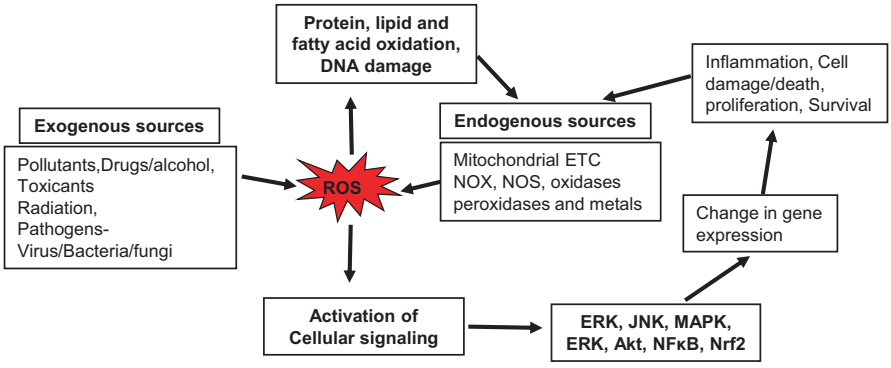

Fig. 13.2 Sources of reactive oxygen species. Reactive oxygen species (ROS) are generated by endogenous as well as exogenous sources, and leads to accumulation of oxidized proteins, lipids and DNA, which cause cellular damage. These ROS activates/modulate the cellular signaling pathways that alters gene expression by up/down regulation of antioxidant enzymes, transcription factors, cytokines, chemokines, followed by altered cellular responses such as inflammation, mitochondrial and other organelle damage, cell death, cell proliferation, and cell survival

Antioxidant enzymes (AOE) can either directly metabolize ROS (SOD and catalase) or indirectly eliminate ROS using reducing agent GSH. SOD enzymes convert $O._2^-$ anion to H_2O_2 and molecular oxygen, while catalase converts H_2O_2 to water and oxygen [129]. GPx functions in the detoxification of H_2O_2 and lipid peroxides in a glutathione and other cofactors dependent reaction. GST catalyzes the addition of GSH to endogenous and xenobiotic substrates and plays an important role in xenobiotic and endobiotic compounds detoxification and metabolism [130]. Peroxiredoxins (Prdx) are cysteine-dependent peroxidases that catalyze the reduction of H_2O_2 and alkyl hydroperoxides in the presence of thioredoxin and NADPH [131–134].

The antioxidant response element (ARE) is a *cis*-acting regulatory sequence present in the 5′ region of the genes encoding antioxidants and phase-2 detoxification enzymes/cytoprotective proteins and mediates the transcriptional activation of many oxidative stress-inducible genes [135]. Nuclear factor (NF)-E2-related transcription factors (Nrfs) are basic-leucine-zipper (bZIP) proteins belonging to the cap-n-collar (CNC) family of transcription factors that regulate the expression of antioxidant and phase 2 metabolizing enzymes in response to oxidative stress by binding to the ARE sequence. Nrf1, Nrf2, Nrf3, and p45NFE2 are closely related, and Bach1 and Bach2 are distantly related factors of the CNC family. CNC-bZIP factors interact with the small Maf family of bZIP proteins to form heterodimers and homodimers among themselves. The homodimers repress gene expression, while the small Maf-Nrf heterodimer activates it. Nrf1 and Nrf2 have been shown to be positive regulators of ARE-dependent gene transcription [135]. On the other hand, Nrf3 and Bach 1 seem to function as negative regulators of ARE-mediated gene expression [136, 137]. The key transcription factor that regulates the expression of antioxidant defense enzymes is Nrf2. During normal levels of ROS generation, Nrf2 is retained in the cytoplasm by kelch-like ECH-associated protein 1 (Keap 1), which targets Nrf2 to ubiquitin-dependent degradation. Under oxidative stress, Nrf2 dissociates from Keap 1 and translocates into the nucleus, where it binds to ARE in the promoter regions of antioxidant genes and regulates their expression.

13.4.4 Measurement of Reactive Oxygen Species

ROS are continuously produced in the cells during metabolic processes, which are counterbalanced by AOE. ROS measurement in samples is dependent on the analytic target along with the ROS in question. At the cellular level, ROS can be individually assessed from tissue culture. At the animal level, however, typically the effects of oxidative stress are measured from various biological samples including serum, plasma, and urine samples. ROS levels are measured indirectly either by using redox-sensitive cell membrane permeable compounds (2′,7′-dichlorodihydrofluorescein diacetate, DCFDA; dihydroethidium, DHE, etc.), which are trapped intracellularly after cleavage by cellular esterases and become quantifiable fluorescent products once oxidized by ROS, or via quantification of cellular oxidation

products. Measurement of GSH subcellular levels and its localization is critical in understanding the modulation of cellular redox status as well as the mechanisms of detoxification. GSH to GSSG ratio is a good indicator of oxidative stress within cells, which can be determined by HPLC [138], capillary electrophoresis [139], or biochemically [140], including a luminescent and fluorescent-based, and colorimetric assays for the detection and quantification of GSH in cells and biological samples [141, 142].

Lipid peroxidation is a key indicator of oxidative stress and is widely used as an indicator of free radical formation. Lipid peroxides are unstable and decompose to form a highly reactive carbonyl compounds such as MDA [143]. F2-isoprostanes (F2-like prostanoid derivatives of arachidonic acid) are stable and are not produced by any enzymatic pathway but are formed by lipid peroxidation and can be measured using fluorescent derivatives [144–148]. Superoxide detection is based on its interaction with other compounds to generate a measurable result [149–153]. The generation of ROS in mitochondria can be observed using fluorescence microscopy with MitoSOX Red reagent [154]. Hydrogen peroxide concentrations can be measured using either fluorogenic or colorimetric substrates [155–158]. Intracellular oxidative activity is also detected by calcein-acetoxymethylester, a fluoregenic cell permeable compound that is converted by intracellular esterases into the cell impermeant anion calcein, which is fluorescent [159]. NO. radical has a very short half-life and reacts with several different molecules to form either nitrate (NO_3) or nitrite (NO_2^-), which can be determined by either colorimetric or fluorescent assays [160–162]. Genetically encoded fluorescent protein-based biosensors have been developed for the detection of the ROS in situ in real time and can be targeted to specific cellular compartments [163–166].

13.4.5 Reactive Oxygen Species and Lung Inflammation

Oxidative stress plays an important role in the pathogenesis and development of many acute and chronic inflammatory airway diseases [123, 127, 167–169]. The lung is a highly specialized organ that is exposed to high levels of oxygen due to its unique structure that provides an enormous surface area to outside ambient air. This makes the lung vulnerable to a wide range of environmental pollutants, toxicants, oxidants, and numerous infectious agents with the potential to induce oxidative damage [170]. ROS are produced from pulmonary epithelial and endothelial cells or released from activated macrophages and leukocytes as a result of inhaled toxic air pollutants and microorganisms that cause damage to the lungs, which in turn initiate the cascades of pro-inflammatory reactions propagating pulmonary and systemic distress [171]. Several studies have either directly or indirectly demonstrated the role of ROS generated by pulmonary epithelial and inflammatory cells in the pathogenesis of acute and chronic lung diseases such as acute respiratory distress, asthma, acute lung injury, pulmonary fibrosis, lung cancer, and COPD [123, 127, 172–175].

ROS plays a major role in enhancing lung inflammation through the activation of stress kinases, redox-sensitive transcription factors, and histone modifications such as acetylation, phosphorylation, and methylation that results in increased expression of a battery of distinct proinflammatory mediators and promotes inflammation [175]. Increased nitrated proteins, lipid peroxides, and O_2^- and XO activity was observed in COPD and asthma patients, compared to normal subjects [174, 176–180]. Elevated levels of DNA (8-hydroxydeoxyguanosine), lipid (MDA, F2-isoprostane, 7-ketocholesterol, and 7-hydroxycholesterol), protein (carbonyl content), and sterol oxidation have been reported with influenza infection [181–184]. Increased ROS, inducible type NOS (iNOS), and decreased antioxidants have been observed with influenza, Sendai virus, human metapneumovirus, and rhinovirus infection [48, 95, 185–192]. In order to restore normal physiological functions, elimination of pathogenic insult and injured tissue components occurs in the tissue as a part of the inflammation process. Currently, several studies have focused on the molecular mechanisms by which oxidants exert their pathological effects on the lungs and are still the subject of debate.

13.5 RSV-Induced ROS in Lung Inflammation

13.5.1 Reactive Oxygen Species Generation During RSV Infection

ROS generation has been shown to be induced by various viruses, including influenza, hepatitis B and C virus, rhinovirus, and HIV-1 in a variety of cell types [193, 194]. Enhanced production of ROS is observed in response to respiratory viral infections like influenza and rhinovirus [187, 189]. ROS plays a major role in the pathogenesis of acute bronchiolitis caused by RSV [47, 48, 98, 195]. Severe RSV infections can promote oxidative stress in respiratory epithelial cells and induce production of lipid peroxidation products that are responsible for the magnitude of the response to stress in relation to the disease severity [47]. Elevated total ROS levels and lipid (MDA and F2-isoprostane) peroxidation products have been reported in AECs and in RSV-infected patient samples [44, 47, 48, 196]. Accumulation of lipid peroxidation products and oxidized glutathione (GSSG) as well as decreased GSH/GSSG ratio in the plasma of infants with RSV-induced acute bronchiolitis were indicative of increased oxidative stress [195]. Mitochondrial ROS, specifically $O._2^-$ formation, occurs due to the leakage of electrons from electron transport chains located on the inner mitochondrial membrane during the process of oxidative phosphorylation. RSV infection induced a dramatic increase in the formation of $O._2^-$ anion radicals as detected with MitoSOX by immunofluorescence microscopy and flow cytometry [196], indicating that the ROS generated by RSV is of mitochondrial origin. DNA damage observed in RSV infection is attributable to mitochondrial ROS, which are able to induce double-strand breaks, and antioxidant treatment alleviates symptoms of RSV infection. RSV infection of phagocytes, including monocytes, neutrophils, and eosinophils, leads to $O._2^-$ production, which becomes the substrate for myeloperoxidase that leads to the release of potent

pro-oxidative mediators in the extracellular environment [197, 198]. RSV-induced $O._2^-$ generation, chemokine secretion, and activation of IRF and STAT proteins is mediated via an NADPH oxidase-dependent pathway [45, 199, 200]. RSV-induced oxidative stress likely contributes to the preservation and augmentation of the inflammatory response [201].

13.5.2 Reactive Nitrogen Species and RSV Infection

ROS such as $O._2^-$ can rapidly react with NO to form reactive nitrogen species (RNS) via nitric oxide synthase (NOS), which in turn induces nitrosative stress by covalent modification of protein tyrosine residues and adds to the proinflammatory burden of ROS. RNS including NO, $ONOO^-$, and nitrogen dioxide (NO_2) have been shown to be involved in the pathophysiology of many inflammatory lung diseases. NO is a gaseous nitrogen-centered inorganic free radical synthesized endogenously by the oxidative deamination of L-arginine to L-citrulline by a family of three NOS, which includes a neuronal type (nNOS), an inducible type (iNOS), and an endothelial type (eNOS) [202]. Among these, iNOS gene expression is significantly induced under inflammatory conditions due to its transcriptional regulation by proinflammatory cytokines, redox-sensitive transcription factors, and viral infections [203]. NO as a mediator of tissue injury, the immunoregulatory as well as toxic effects of NO are due to its ability to react with molecular oxygen and oxygen-derived free radicals. Under aerobic conditions, NO is rapidly oxidized to RNS as NO_2, dinitrogen trioxide (N_2O_3), and dinitrogen tetroxide (N_2O_4), with N_2O_3 being the major oxidative product in biological systems.

The free radical NO has been implicated in the pathogenesis of many inflammatory lung diseases as they are produced in different cell types, including human AECs that express eNOS and iNOS, with the latter one being highly induced after exposure to proinflammatory mediators and oxidants [204, 205]. NO production has been demonstrated with several viral infections including RSV, and iNOS induction has been considered as a likely universal event in all viral infections [203, 206–211]. Although *iNOS* gene expression can be regulated through cytokine secretion [203, 212], its initial induction appears to be independent of cytokine stimulation as observed with other viruses [206, 213, 214]. Increases in iNOS protein levels with increased nitrite levels have been reported in AECs after RSV infection, which is viral replication-dependent [209]. RSV-induced NO production was significantly reduced with IL-4 pretreatment, and no change in iNOS expression was observed, indicating that Th1/Th2 affects the ability of AECs to produce NO in response to RSV infection [209]. Studies have also reported that RSV infection has no effect on NO production in human immune cells [209]. Significant induction of NO and iNOS has been reported in RSV-infected mice [215]. In contrast to the in vitro and animal studies, no change in NO generation was observed in adult volunteers who were experimentally infected with RSV compared to control subjects [216]. In addition, a reduction of exhaled NO was observed in infants with acute RSV bronchiolitis, compared to healthy controls, which returned to normal levels during the convalescence phase [217]. High levels of NO metabolites, including nitrites and

nitrates, were observed in the spinal fluid from RSV-infected infants with central nervous system symptoms [218].

NO generation in respiratory viral infections has been shown to inhibit viral replication, cause viral mutation, and play a major role in disease pathogenesis [67, 210, 215, 219]. Studies show that iNOS constitutive expression and NO production in AECs results in reversible, dose-dependent inhibition of RSV replication, whereas treatment with increasing concentrations of the chemical donor S-nitroso-N-acetylpenicillamine did not affect the viral replication. This suggests that NO derived from endogenous iNOS expression was more effective in RSV replication inhibition than exogenous NO addition via chemical donor [210]. Several signaling molecules are regulated by NO including NF-κB, AP-1, and kinases, as they contain critical cysteine residues that undergo nitrosylation [220]. Treatment of AECs with iNOS inhibitors such as L-arginine methyl ester, L-N^G-monomethyl arginine, and amino guanidine did not have a significant effect on RSV-induced chemokine secretion suggesting that NO does not affect the intracellular signaling pathways leading to RSV-induced NF-κB, AP-1, and interferon regulatory factor (IRF) activation [44, 118]. However, treatment with free 3-nitrotyrosine inhibited viral replication and chemokine secretion via formation of α-tubulin [221]. NO production in response to RSV infection has been shown to modulate ion channel activity as well as the stabilization of the transcription factor hypoxia-inducible factor-1α (HIF-1α), a master regulator of mammalian oxygen homeostasis [211, 222, 223]. Studies have also shown the association of excessive NO production with RSV severity, and prevention of its formation significantly decreased inflammatory cell recruitment and airway hyper responsiveness, suggesting that NO modulation during early events in the course of RSV infection could ameliorate the resulting lung disease in children [215].

13.5.3 ROS-Mediated Cellular Signaling in RSV Infection

Airway epithelial cells are the major targets of RSV infection and have been shown to secrete a variety of proinflammatory molecules that play a key role in inflammatory and infectious processes in the lung [38]. Several studies have characterized the transcriptional mechanisms that control gene expression in lung epithelial cells, which is mediated through redox-sensitive signaling pathways (Fig. 13.3). RSV infection results in the activation of the several cellular signaling cascades involved in the expression of early response genes, including cytokines, chemokines, and type I interferons (IFN), which is coordinated by a subset of transcription factors. Several redox-sensitive transcription factors, including nuclear factor-IL6 (NF-IL6), NF-κB, AP-1, IRF, HIF-1α, and STAT proteins, which regulate the expression of a variety of proinflammatory/immunological mediators, such as cytokines and chemokines (Fig. 13.3). RSV infection of AECs induces NF-IL6, in a time-dependent manner and is replication-dependent [224].

RSV infection induces NF-κB activation in AECs [44, 225, 226]. NF-κB is required for the transcription of several RSV-inducible inflammatory and immunoregulatory genes and/or are dependent on an intact NF-κB-signaling pathway.

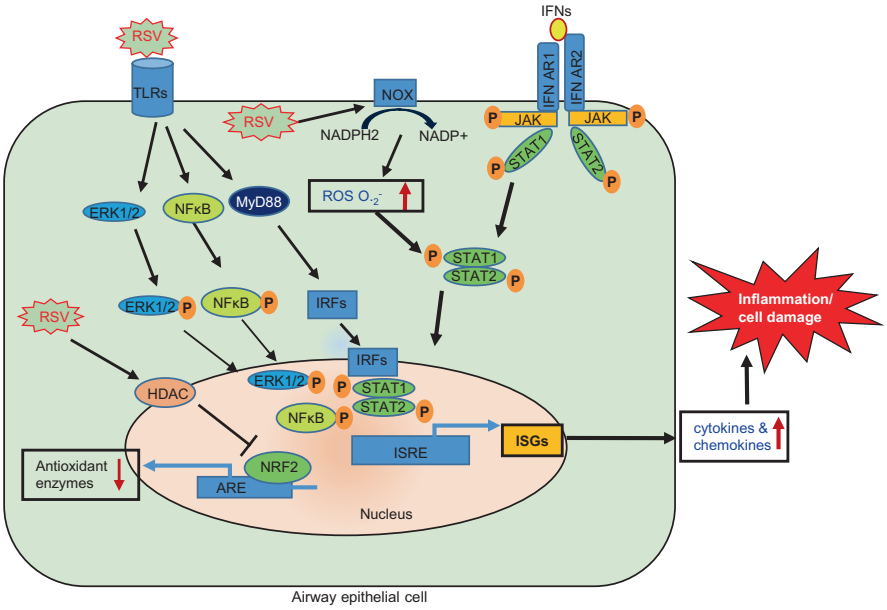

Fig. 13.3 Redox-sensitive signaling pathways in RSV infection: RSV infection in airway epithelial cells activates a multitude of redox-sensitive signaling pathways. RSV infection via Toll-like receptors (TLRs) activates NF-κB/ERK/MAPK pathways, which subsequently activates JAK/STAT signaling pathways via MyD88, and is mediated through reactive oxygen species (ROS). On the other hand RSV-induced ROS via NADPH oxidase (NOX) activates STAT pathways and promotes translocation of NF-κB, ERK1/2, STAT1/2, and interferon regulatory factors (IRFs) to the nucleus and binds to interferon sensitive response element (ISRE) and induces interferon stimulated genes (ISGs) to produce cytokines and chemokines that induces inflammation and cell damage. RSV-induced ROS promotes Nrf2 translocation to the nucleus and induces histone deacetylases (HDACs) that leads to nuclear deacetylation of Nrf2 and dissociation from antioxidant response element (ARE) that causes inhibition of antioxidant gene expression

NF-κB activation is crucial for RSV-induced expression of cytokines, chemokines, secreted proteins, and signaling molecules [227–230]. Several NF-κB family members, in particular RelA, have been shown to be phosphorylated on specific serine (ser) residues, which is required for optimal NF-κB activity. RSV induces RelA phosphorylation on ser-276 and ser-536, and this depends on activation of mitogen- and stress-activated protein kinase 1 (MSK1), a serine/threonine kinase downstream of extracellular signal-regulated kinases 1 and 2 (ERK1/2), and p38 MAPKs that play key roles in several cellular processes [115, 231]. RSV-induced NF-κB phosphorylation has been shown to be regulated by TLR3 [229, 232], and oxidative stress has been shown to play a major role in TLR3 activation [233]. Several members of the AP-1 family, including c-Jun and activating transcription factor-2, are activated by RSV-induced oxidative stress and regulate RSV-induced gene expression [234, 235]. ROS generated in response to RSV infection can activate IRF and STAT family transcription factors and regulate signal transduction cascades leading to chemokine expression [44, 45]. The Janus-activated tyrosine kinases (JAK) have

been shown to phosphorylate STAT proteins, leading to an induction of gene expression. RSV infection leads to the activation of HIF-1α via NO-stabilization, which regulates the expression of several genes, including vascular endothelial growth factor, CD 73, and cyclo-oxygenase-2 [236].

13.5.4 Antioxidants in RSV Infection and Their Use as Therapeutics

Oxidative stress is the result of an excess of oxidants and/or depletion of antioxidants. Respiratory viruses have been shown to induce ROS generation and reduce cellular antioxidant defenses. Decreased antioxidant levels, another marker of oxidative stress, were observed in RSV-infected infants, mice, and cells (Table 13.1) [47, 48, 115, 237]. Several studies suggest antioxidant activity is impaired in lung inflammation [238–240]. Upregulation of protective antioxidants has also been observed as a result of increased oxidative stress in cells along with its proinflammatory mechanisms. Studies have also shown increased levels of antioxidants in children with post-infectious bronchiolitis obliterans, which suggests that enhanced antioxidant enzyme activity preserves redox status homeostasis as well as their actuation as a defense mechanism for oxidative damage [48]. RSV infection induced increased expression of antioxidant enzymes as a protective mechanism only at early stages of infection, including SOD1, SOD3, catalase, GST, and GPx, but their expression progressively decreased as the infection progressed. Meanwhile, SOD2 levels continued to increase during the course of RSV infection, suggesting that RSV infection results in enhanced intracellular H_2O_2 production that is not detoxified by AOEs, which leads to the generation of (OH.) free radicals that can react with lipids, proteins, and DNA and cause cellular damage [47, 48]. RSV infections result in oxidation of Prdx antioxidant family members, including Prdx-1, Prdx-3, and Prdx-4, and their inhibition has been shown to enhance ROS production and protein carbonylation [48]. RSV infection leads to a decrease in nuclear levels of Nrf2, and several studies show that the Nrf2-ARE pathway plays a protective role in RSV-induced lung injury [47, 241]. Studies have shown that RSV induces RNS and NOS in the lungs of infected mice, and that inhibition of NOS expression significantly reduces RSV-induced lung inflammation [215]. These studies suggest that airway oxidant-antioxidant imbalance as a result of RSV infection could play a major role in the pathogenesis of RSV-induced inflammatory lung disease.

Several studies have explored antioxidants as novel therapeutic strategies to treat RSV infections. RSV infection induces ROS generation in AECs and pretreatment with the antioxidant such as butylated hydroxyanisole (BHA), and synthetic SOD, and catalase mimetic compounds blocks RSV-induced signal transduction cascades that lead to chemokine expression through inhibition of IRF and STAT family transcription factors [44, 45]. Treatment with the pan-NOX inhibitor, dibenziodolium chloride, significantly inhibited ROS generation induced by influenza virus, RSV, and rhinovirus [187, 189]. Several RNA viruses, including RSV, modulate cellular oxidative stress through the involvement of NOX/DUOX pathway, downregulation

Table 13.1 Antioxidants levels in respiratory syncytial virus infection

Antioxidants	Model system	Change in levels	References
GSH/GSSG	Cells Mice Human	Decreased	[47, 115, 195, 237]
SOD1	Cells Mice Human	Decreased	[47, 48, 244]
SOD2	Cells Mice Human	Increased Decreased Unchanged	[47, 48]
SOD3	Cells Mice Human	Decreased	[47, 48]
Catalase	Cells Mice Human	Decreased	[47, 48]
Glutathione peroxidase	Cells Mice Human	Decreased Decreased Increased	[47, 48, 195]
Glutathione S-transferase	Cells Mice Human	Decreased	[47, 48]
Peroxiredoxin	Cells Mice Human	Decreased	[48]
Thioredoxin	Cells Mice Human	Decreased	[48]

of AOE, increased production of ROS as well as induced antioxidant response [44, 47, 101, 115]. N-acetylcysteine treatment drastically reduced the damaged nuclei with decreased viral titer. Similarly, reduced GSH ethyl ester, a cell-permeable derivative of GSH, reduced DNA damage foci and virus titer suggesting that these antioxidants might also influence virus entry into cells [196]. Studies in transgenic mice have demonstrated that a deficiency in SOD, especially SOD 2 and 3, reduces survival and increases lung injury in response to hyperoxia, while overexpressing SODs results in improved survival [242]. This suggests that suppression of oxidative stress can be achieved by the overexpression of AOEs. Recombinant SOD, and several SOD mimetics that have been developed based around organo-manganese complexes, and their therapeutic potential have been explored in a variety of disease models. Most commonly studied SOD mimetic compounds include metalloporphyrin-based compounds (such as AEOL10113 and 10150), cyclic polyamine-based molecules (such as M40403 and 40419), and the salen-manganese compounds (such as EUK-8, EUK-134, and EUK-189 with significant catalase and peroxidase activity) [243]. Significant inhibition RSV-induced IL-8 and RANTES secretion in AECs was observed with EUK-134 treatment [47]. Although the specific mechanisms by which antioxidants protect the cells in the context of RSV infection are

unclear, attenuation of inflammation is likely playing a major role. Currently, there is no effective antioxidant therapy that has good bioavailability and potency is available. These studies suggest that RSV infection induces ROS generation that leads to the disruption of the antioxidant system. The modulation of ROS generation may enhance cellular antioxidant capacities that might attenuate RSV-induced inflammation.

13.6 Summary and Future Directions

RSV is an important human pathogen, which induces enhanced ROS/RNS generation in lung epithelial cells and leads to severe oxidative stress that is likely to play a major role in initiating and amplifying lung injury and inflammation. ROS generated in response to RSV infection can activate and modulate intracellular signal transduction cascades including NF-κB, IRF, and JAK-STAT pathways in host sentinel cells in the airways leading to induced expression of proinflammatory mediators and promoting inflammation. Modulation of ROS/RNS production and oxidative stress via antioxidant approach could represent a novel therapeutic strategy to ameliorate severe lung disease associated with RSV infection. Studies have shown that RSV infection alters antioxidants/antioxidant pathway/ARE/Nrf2 in the airways and that modulation of these pathways has the potential to develop novel therapeutics against RSV and other respiratory viral infections. Therefore, further studies are required to develop therapeutics that could significantly impact the morbidity of RSV infection. Future antioxidant-based therapeutic interventions need to address the concerns regarding the route of administration, bioavailability, and tissue distribution as antioxidant supplementation would only be effective if the drug was available at the site of infection/inflammation.

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Reactive Oxygen Species: Friends or Foes of Lung Cancer?

14

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Abstract

Reactive oxygen species (ROS) are important biological radicals essential for determining different stages and phenotypes of cells from quiescence to proliferation, differentiation, self-renewal and even apoptosis. Low ROS favours quiescence and self-renewal in contrast to high ROS that dictates proliferation, differentiation or apoptosis. Such wide variety of cell fates depends upon specific signalling pathways that regulate the cellular ROS, thus contributing to tissue homeostasis. Imbalance of ROS causes several pathological conditions including cancer which is associated with higher level of ROS that supports tumour development and progression. However, to restrain from the excessive oxidative damage of ROS, cancer cells efficiently control the antioxidative pathways, thus favouring its own survival and maintenance at the same time. Furthermore, importance of ROS has been an active field of research in 'cancer stem cells' (CSCs), a subpopulation of cancer cells with stem cell-like properties and features. CSCs possess low ROS level that make them resistant to the existing chemotherapy or radiotherapy that ultimately leads to cancer recurrence. Though several evidences have proved the role of ROS in self-renewal and stemness of CSCs, there is a lot to explore about ROS-regulated signalling mechanisms in CSCs. An understanding of ROS regulation in CSCs can provide an idea about the application of oxidative stress as a therapeutic strategy in treatment of cancer. In this book chapter, we have raised the debate as to whether ROS acts as 'friend or foe' for cancer cells. Moreover, exploring the significance of ROS and redox regulation in lung cancer stem cells has been our major focus. Finally, it is suggested that in order to get an effective treatment and recurrence-free survival, sensitization of the cancer stem cells to high ROS environment is a must.

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Reactive oxygen species · Cancer stem cells · Lung CSCs · Oxidative stress · Redox regulation

14.1 Introduction

Reactive oxygen species (ROS) are the by-products of general metabolic pathways of the biological system and are essential for cell signalling and maintenance of tissue homeostasis. These include several free radicals and a number of reactive molecules which are derived mostly from the molecular oxygen as produced in the mitochondrial electron transport chain. Generally, low ROS level favours growth and proliferation, whereas high ROS level is known to promote apoptosis and oxidative damages of the cells [1–3]. The excessive damage caused by ROS is neutralized by the cellular antioxidant pathways, thereby maintaining the redox balance of the cells. Several normal cellular activities are the manifestations of different ROS levels which comprise of proliferation, differentiation, cell cycle, apoptosis, etc. However, abnormal change in ROS level results in many deleterious effects such as oxidative modification of proteins, DNA damage and activation of signalling pathways [4, 5] which are linked to many pathological disorders and diseases.

In fact, oxidants and antioxidants imbalance in human system may lead to a plethora of lung-related ailments including lung cancer. Evidences have shown how ROS can channelize specific signalling pathways to induce apoptosis in non-small cell lung cancer [6, 7]. ROS level remains elevated in cancers due to higher rate of metabolism [8, 9] which allows tumour development and progression. But, aberrant level of ROS is responsible for cancer cell death [10]. To counteract the killing effect, cancer cells exhibit higher antioxidant capacity which remains under their intricate regulations. ROS, thus, acts as 'friend or foe' depending upon the amount of intracellular ROS which dictates whether it supports cell survival or demise. Cancer cells can bear the modest increase in ROS, which is supportive for cancer cell growth, for prolonged period of time [11] indicating the role of ROS as a 'friend' in this context. Also, the role of ROS can be regarded as oncogenic as it efficiently regulates multiple signalling networks contributing to cancer initiation, development, progression, invasion and metastasis. Induction of ROS and its promotion is appreciated as witty mechanism in various cancer therapies including chemotherapy and radiotherapy [12, 13]. In this regard, ROS acts as a foe for cancer cells by showing its tumour-suppressive actions [11]. ROS-mediated cancer cell elimination has been made possible by using effective chemotherapeutics or pharmacological agents that can elevate ROS level beyond its normal threshold. However, in spite of the several breakthroughs in cancer treatment, the 5-year survival rate for all patients with lung cancer is 17% – a statistic that has not changed significantly in decades.

Years of research have introduced the idea of a special subpopulation of cancer cells within a tumour, known as cancer stem cells (CSCs) or tumour-initiating stem

cells [14]. CSCs are endowed with the properties of self-renewal and differentiation [15] and have been found in a large range of cancers [16, 17]. CSCs bear the potential of tumour development and metastatic dissemination and exhibit resistance to chemotherapy as well as radiotherapy, thus maintaining a rich pool of tumorigenic cells that gives rise to recurrence [18–20]. Doherty et al. [21] showed that exposure to chemotherapeutic agents or radiation though reduces the tumour bulk and enriches CSC repertoire, eventually causing cancer recurrence. This leads to the understanding that targeting only the cancer cells would not yield satisfactory therapy response, but treatment has to be specific for elimination of CSCs. Reports suggested that in contrast to the rest of the tumour cells, increased ROS scavenging systems and lower level of ROS in CSCs protect them from the cytotoxic effects of chemotherapy and radiation [22–24]. Furthermore, presence of low intracellular ROS is possibly attributable to the increased drug-resistant nature of the CSCs in comparison to the rest of the tumour cell population [25]. Several mechanisms contributing to lung cancer resistance include increased expression of drug efflux pumps, increasing drug inactivation by enzymes, defective apoptotic machineries, DNA repair systems, etc. [26–30]. Henceforth, it is worthy to understand the redox signalling of the CSCs in order to employ ROS in cancer therapy in a more effective manner. This could help design therapies that would effectively target the CSCs by manipulating the oxidant-elevating or oxidant-depleting pathways.

In this chapter, we shall discuss about the significance of these redox networks functional within lung CSCs and the possibilities of manipulating ROS to induce apoptosis in these highly resistant cells.

14.2 Generation of ROS and Its Role in Regulation of Tissue Homeostasis

14.2.1 ROS in Normal vs Cancer Cells

ROS are generated as a result of normal cellular metabolism and are known to regulate the redox homeostasis under physiological conditions within the cell. This function protects the biological system from oxidative stress via several redox-based mechanisms. A fine balance between oxidants and antioxidants is, therefore, essential to avoid a plethora of diseases including that of various pulmonary ailments, e.g. chronic obstructive pulmonary disease (COPD), lung cancer, etc.

ROS accounts for the reactive molecules which include superoxide radical ($O2^{-}$), hydroxyl radical (OH•), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxynitrite (ONOO) and ozone (O_3) [31]. These reactive species are generated from various endogenous sources as well as exogenous sources (Fig. 14.1). Epithelial cells, alveolar macrophages, endothelial cells and inflammatory cells account for endogenous ROS [32]. Several enzymatic reactions in the body play significant roles in ROS generation such as reactions carried out during mitochondrial respiration involving cytochrome P-450, cyclooxygenases, lipoxygenase, peroxidases, etc. Besides, radiation, particulate matters and chemical carcinogens act as exogenous

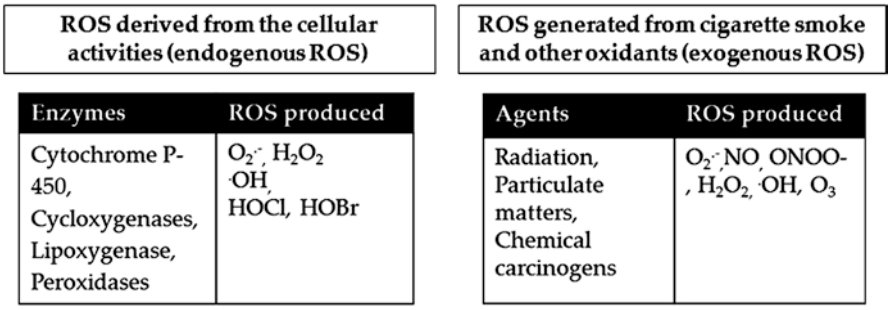

Fig. 14.1 ROS generated from different sources. Reactive oxygen species (ROS) generated from reactions catalyzed by various enzymes as endogenous sources of ROS (left) and ROS released from several exogenous sources including radiation and chemical carcinogens

sources of oxidative stress [33]. Electron transport chain of the mitochondrial respiratory system forms a basis of biochemical reactions for the oxidative metabolisms in living system. In the process of electron transfer and energy production, one-electron reduction product like O^{2-} (superoxide) is produced along with highly reactive ROS and RNS (reactive nitrogen species) [34]. Controlled functioning of such potentially destructive reactive species is carried over by different well-regulated antioxidant systems in normal cells. Normal cellular activities such as cell division, differentiation and proliferation are highly dependent on reactive species that act as redox signalling molecules. Normal stem cells remain quiescent at lower ROS level and self-renew, whereas increased ROS level is well-efficient to induce cellular differentiation, proliferation, senescence and even apoptosis in a dose-dependent fashion [34]. The redox signalling molecules are empowered to control a variety of signal transduction proteins and gene expression pathways. But, the redox by-products of oxidative metabolism are efficient to manipulate the antioxidant pathways resulting in changes in the genetic material of the living system. These alterations are reflected as mutations in the form of base damage, adducts or deletions to the DNA [34]. Though such mutations are repaired by high-fidelity proofreading machineries of the biological systems, some of the genetic damages easily escape and begin to accumulate with time, thus compromising the normal metabolic processes and enhancing the generation of reactive species. As a consequence of which, the accelerating mutated metabolic machineries lead to massive deterioration of biological structures and functions which manifest in the form of degenerative diseases and cancer too.

Carcinogenesis is highly associated with ROS-induced oxidative DNA damage that involves single- or double-stranded DNA breaks, modifications of purine and pyrimidine and DNA cross-linking, ultimately leading to cell cyle arrest, induction of protein synthesis, inflammation, etc. in many cancers [35]. Association of higher ROS production with genetic instability has been explained by Wallace [36] in nuclear and mitochondrial genes mutation within the components of ETC (electron transport chain). Also, Petros et al. [37] proved that increasing ROS is strongly

associated with cancer progression. Furthermore, reactive nitrogen species (RNS), such as peroxynitrites and nitrogen oxides, have also been implicated in DNA damage [37]. Higher amount of nitric oxide (NO) increases apoptosis in some cancer cells, whereas lower amount promotes vascularity and protects against apoptosis [38], as in case of lung cancer. Since ROS exhibits various functions depending on its level, it is believed to be regulated by intricate signalling networks within the cell. Cancer cells show elevated level of ROS due to their higher metabolic rates [8, 9]. In addition, they are able to evade and regulate the destructive level of ROS via their increased antioxidant capacities which would otherwise cause cancer cell apoptosis or necrosis [10]. Such a delicate balance of ROS level by the cancer cells allows their survival, which indicates that depending upon its concentration, ROS plays a dual role in cancer cell working either in its favour or against its survival, i.e. either as a 'friend' or a 'foe'. To gain an insight about the strategies how the cells maintain and regulate these reactive species in their favour, it is worthy to look at the various signalling networks and the associated components that actually modulate the main player 'ROS'.

14.2.2 Molecular Signalling Hubs Regulated by ROS in Normal and Cancer Cells

Low to high level of ROS is known to exert different pathological effects which are actually commenced via specific signalling pathways. In contrast to low ROS level which is particularly responsible for self-renewal proliferation and differentiation [39–44], increased level has detrimental consequences on normal stem cells. Slight enhancement in cellular ROS can impair self-renewal property but promote its proliferation and differentiation that lead to exhaustion of stem cells [45–47]. Further rise in ROS level even results in senescence through the redox-dependent activation of p38-p16 pathway [48]. Ultimately, excessive ROS production can induce stem cell apoptosis by activating p53 pathway following the DNA damage response [49, 50]. Though mounting evidences have suggested the involvement of wide variety of ROS-mediated mechanisms, they need detailed investigation. Activation of mitogen-activated protein kinase (MAPK) family member of proteins and downregulation of the pluripotent transcription factors Oct4, Nanog and Sox2 [51] have been shown to play role in ROS-mediated human embryonic stem cell (ESC) differentiation to mesendodermal lineage. Specific MAPK family members have been implicated in ROS-induced cardiovascular differentiation of ESCs [44, 52]. Among the various sources of ROS, NOX (nicotinamide adenine dinucleotide phosphate oxidase) is greatly required by cells for ROS production. Besides the classical NOX found in phagocytes, seven isozymes have been discovered in non-phagocytic mammalian cells [53]. Among the various NOX isoforms, lung tissue hosts NOX2, DUOX1 and NOX4 [53, 54]. It is reported that lung cancer tissue predominantly expresses NOX4 isoform [55, 56]. Additionally, presence of DUOX-1 and DUOX-2 has been suggested in airway epithelial cells [57], but their mRNA level remains downregulated in lung cancer tissues [58]. Most of the signalling mechanisms studied so far hire

ROS, e.g. H_2O_2 as a second messenger [59, 60] to support cancer cell activities. Such molecular signalling involves receptor tyrosine kinases, protein tyrosine phosphatases, transcription factors [61, 62], etc. that act as the targets for the reactive species. Some of the ROS-mediated biological pathways in cancer include MAPK/ERK axis, phosphoinositide-3-kinase (PI3K)/Akt-regulated signal transduction cascade, as well as the IκB kinase (IKK)/nuclear factor κ-B (NF-κB)-activating signalling axis (Fig. 14.2). For example, cancer cell proliferation is linked to H_2O_2-induced Erk1/2 activation [63, 64] where Ras may act as an upstream activator of Erk1/2 that undergoes oxidative modification [65]. Another study indicated increased ROS, resulting from the loss of mitogen-activated protein kinase phosphatase 3 (MKP3), elevates Erk activity [66]. Multiple cancers and studies in lung cells have also suggested connection between ROS and Erk1/2 [67, 68]. However, role of ROS in cancer cell survival is context-dependent [69, 70]. To be specific, in vitro studies proved that scavenging ROS promotes apoptosis [71, 72], whereas human glioma and pancreatic cancer cells are prone to death when treated with exogenous ROS which is due to their high basal level of ROS [73, 74]. PI3K/AKT signalling is another pathway controlled by the oxidants. Akt regulates cell survival via phosphorylation or inactivation of its substrates such as Bad, Bax, FOXO, etc. [75, 76]. Evidences suggest that ROS derived from oestrogen metabolism activates PI3K/Akt signalling pathway [77, 78], and H_2O_2 generated from EGF signalling in human ovarian cancer cells activates Akt and p70 S6K1 [79]. A highly studied redox sensor protein for oxidative stress is NF-κB [80] that is known to be activated by ROS [81].

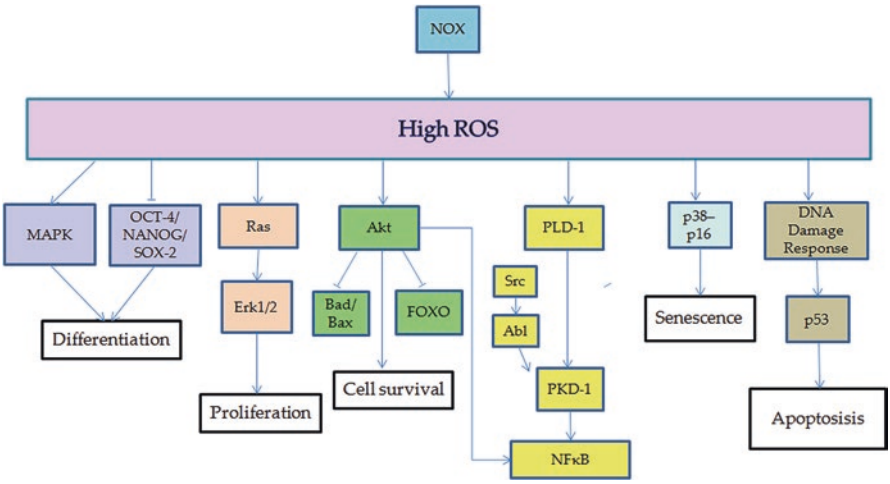

Fig. 14.2 ROS-mediated cellular signalling. NADPH oxidase (NOX)-activated ROS, regulated by growth factor signalling, induces important signalling cascades via MAPK (Erk1/2, p38, JNK), PI3K/Akt and NF-KB activation. These involve the activation of several kinases like src, abl and PKD-1 and transcription factor FOXO. ROS-mediated DNA damage induces p53-induced apoptotic pathway, whereas senescence is promoted via p38-p16 cascade. ROS-mediated regulation of Oct-4, Sox-2 and Nanog favours differentiation of human embryonic stem cells

ROS-dependent NF-κB activation has been observed in SOD-silenced carcinoma cells [82]. Some groups suggested IKK-dependent NF-κB-inducing signalling in elevated oxidative stress environment via various ways including that of inhibition of intracellular glutathione system [35, 83]. Here, PLD1 and many other kinases like Src, Abl, etc. regulate NF-κB via PKD1/IKK-β axis [35, 83–86]. Oxidant-regulated NF-κB-mediated IL-8 production has been reported in lung epithelial cells [87]. Moreover, mechanism of non-small cell lung cancer is linked to ROS-regulated HIF-1ūFC; expression [88]. The role of ROS-induced upregulation of HIF-1ūFC; activity in oxygenated condition in metastatic colonization of lung cancer has been suggested by Cho et al. and Zhao et al. [89, 90]. All these studies elucidate how intricately cellular signalling networks are dependent on ROS for their functioning (Fig. 14.3). In the next section, we have discussed different ROS-dependent functions in cancer cells.

14.3 ROS-Specific Functions in Cancer

A wide variety of cellular activities are modulated by oxidative stress-linked mechanisms. Such relevant ROS-regulated activities include cell survival, proliferation, cell cycle progression, apoptosis, cell motility, adhesion, etc. (Fig. 14.3).

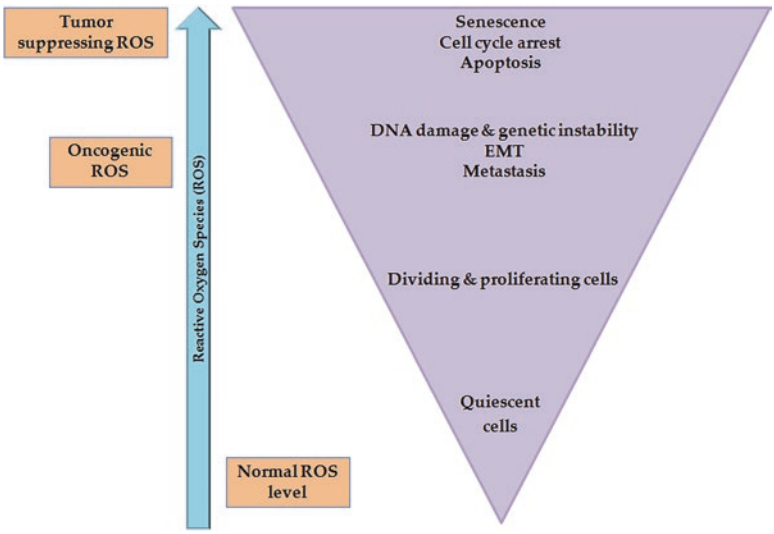

Fig. 14.3 Changes in cell behaviour with respect to varying ROS level. Normal cellular ROS at a low level maintains cells at a quiescent stage. Elevating ROS results in cell proliferation and survival. In addition, increased ROS level cause DNA damage and genetic instability; EMT and metastasis thus, acting as oncogenic. Excessive ROS generated in cancer cells can lead to cell cycle arrest, senescence or apoptosis

14.3.1 ROS: A Friend in Need

14.3.1.1 In Cell Proliferation

Mitochondria-generated ROS regulates proliferation as well as senescence via MnSOD (manganese superoxide dismutase) activity [91]. Lower MnSOD activity induces proliferation due to higher level of superoxide and lower level of hydrogen peroxide in contrast to higher enzymatic activity that favours quiescent stage in the presence of increased hydrogen peroxide [92]. ROS-mediated effect has been documented in cancer cell proliferation, where it involves the role of Erk1/2 MAPK activation and transcription factor CREB (cyclic AMP response element (CRE)-binding protein) [63, 92]. Such study explains how the change in cellular level of ROS is reflected in different physiological effects. Function of ROS in cell cycle regulation has also been well-observed. ROS controls a wide range of molecules including cyclin B2, cyclin D3, cyclin E1 and cyclin E2 in the event of G1 to S-phase transition [92]. Several cell types have witnessed the important attributes of ROS, where its effects have been either escalated or neutralized [93, 94]. Doubling time of cells is greatly dependent on various ROS-regulating enzymes and their activities which include endogenous MnSOD, Cu/ZnSOD, catalase and glutathione peroxidase [73, 95]. Moreover, ATM (ataxia telangiectasia mutated)-lacking individuals suffer critical oxidative damages which prove that ROS efficiently modulates ATM, an essential molecule for cell cycle control [96, 97].

14.3.1.2 In Metastasis

Reactive oxygen species exhibits interesting role in regulating motility and contributing to metastatic progression of cancer. Endogenous ROS at its higher level favours reduced motility of cancer cells. However, orthotropic tumours developed from these cells possess metastatic capacity [98]. Few evidences recommend that redox state can modulate the events of metastatic process such as cell adhesion to extracellular matrix (ECM), anchorage-independent survival, migration, invasion and so on. Adhesion of cells to ECM accompanies a spike in mitochondrial ROS, followed by increased cytosolic ROS that governs cytoskeleton remodelling [99, 100]. In this process of adhesion, substrates for mitochondrial ROS are SHP-2 and FAK (focal adhesion kinase), in contrast to cytosolic ROS which targets phosphatases LMW-PTP and SHP-2, receptor tyrosine kinases, Src-family kinases and many more [99]. Here, adhesion and migration are regulated by integrin receptors via cyclooxygenase-2 [101] and 5-lipoxygenases (5-LOX) [102]. An important role of ROS is evident in tumour cells where it protects them in adhesion-free environment. Non-transformed cells require ECM as a physical scaffold to execute its mitotic activities, whereas ROS facilitates such action [61, 99] through Rac-1-dependent pathway [103]. But, loss of contacts from the ECM results in cellular death in comparison to transformed cells, where increased ROS evades this phenomenon and allows their survival by controlling autocrine/adhesive signals, which are otherwise mediated by growth factor and integrin signalling in normal cells [103, 104].

14.3.2 ROS: A Foe Indeed

14.3.2.1 In Apoptosis

Involvement of ROS in promotion of apoptosis is due to several factors that include downregulation of antioxidant pathways, chemotherapy treatments, endogenous sources of reactive oxygen species, etc. Very well-known mechanism of apoptosis is through increased mitochondrial oxidative stress accompanied by cytochrome c release and caspase activation [105, 106]. Apoptosis induction can also be initiated via superoxide activated Rac-1/NADPH oxidase pathway [107]. H2O2 and NO are the activators of cJun N-terminal kinases (JNKs)-mediated cell death [105, 108], by downregulating Bcl-2 and Bcl-XL. Moreover, ROS-mediated JNK activation depends on Ask-1 (apoptosis signal-regulating kinase-1) signalling, regulated through a redox-regulated protein thioredoxin [109, 110]. Additionally, forkhead transcription factors, FOXO3a, p66Shc, p53, etc., have gained importance as inducers of apoptosis in the presence of ROS [75, 111]. Other than these, some of the receptor-mediated cell death signalling has also been observed that rely on ROS. TNF receptor I promotes ROS generation through mitochondria in the process of cell death [112]. In addition, TRAF4 (TNF receptor-associated factor4), a part of the TNF-α signalling axis, associates with NADPH oxidase complex to activate JNK [113]. Hence, such pathways indicate how receptor-based ROS induction plays a role in cellular apoptosis.

Precise signalling mechanisms that are context-dependent are essentially responsible for ROS inducing different cellular activities like proliferation, differentiation, cell cycle arrest, apoptosis and so on. Hence, from the above scenario and the evidences mentioned in the context of ROS-sensitive signalling mechanisms till now, it is clear that specific strategies are required to target the cancer cells.

14.4 Cancer Stem Cells and ROS: A Deadly Liaison

Although there have been mounting evidences for therapeutic strategies in favour of cancer reduction, a number of reports regarding the failure of treatment have also been suggested. One of the crucial reasons behind such failure is the presence of a subpopulation of cancer cells within the tumour known as cancer stem cells (CSCs) that are sparsely targeted by chemotherapy or radiotherapy. These CSCs are highly responsible for conferring tumour resistance and causing cancer recurrence too. Many researchers have reported that CSCs exhibit reduced ROS level in comparison to the non-stem cells in cancers [25]. Such an environment favours slow division of cells in CSC-enriched population in contrast to the highly proliferative cancer cells. Consequently, the effect of chemotherapy is suppressed and resistance develops. Additionally, large numbers of genes and proteins are present in CSCs that act in co-ordination with ROS defence, contributing to the therapy resistance. Henceforth, it becomes essential to understand the ROS-manipulation mechanisms of the CSC-enriched population in the tumour, in order to achieve an effective remedy for

cancer. In this regard, we have discussed the relevance of oxidative stress in cancer stem cell regulation in the following section.

14.5 Lung Cancer Stem Cells

The lung CSCs manifest robust endogenous resistance enabling them to survive the chemotherapeutic regimen. The enhanced survivability of this population has been attributed to numerous factors such as impeded apoptotic regulation, elevated DNA damage response and repair, increased function of drug efflux pumps, maintenance of redox homeostasis, etc. [114, 115]. Since the dawn of research in the field of CSC, the focus has remained on the identification of CSCs within the tumour population, which largely relies on specific biomarkers. Initial approaches for isolating lung CSCs involved identification by 'side population' (SP) phenotype [116] or by measuring enhanced ALDH activity [117]. Subsequently, identification of numerous membrane bound surface markers such as CD44 [118], CD 133, CD90 [119], etc., present in CSCs gained importance (Fig. 14.4). CD44 is an important redox mediator, and its crucial functions have been discussed in the latter sections. Lung

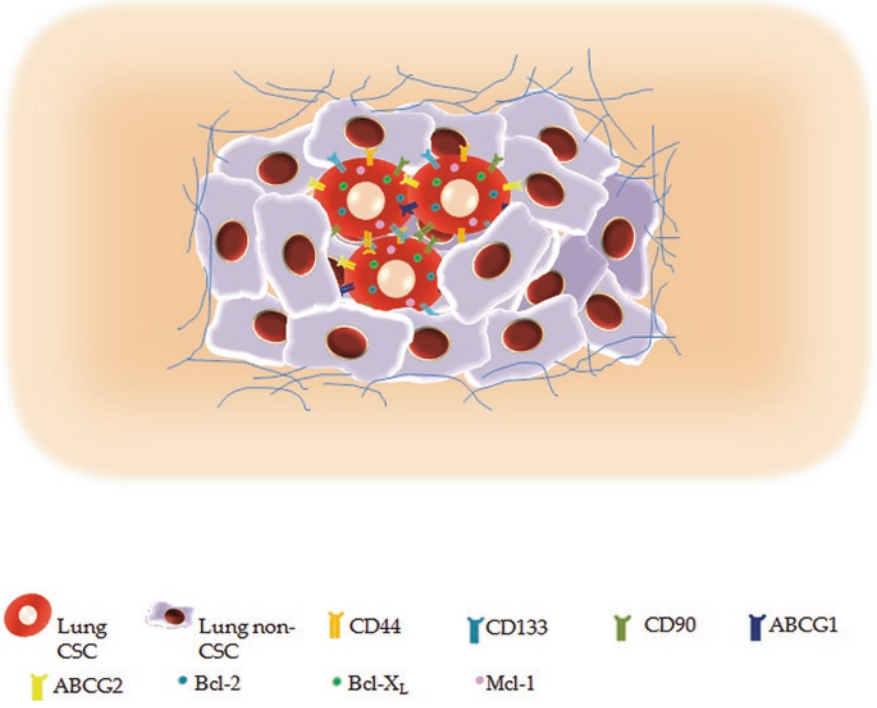

Fig. 14.4 Phenotypic characteristics of lung CSCs. Lung CSCs are characterised by specific surface biomarkers such as CD44, CD133 and CD90. They also harbour upregulated expression of drug efflux pumps such as ABCG1/2 alongside anti-apoptotic proteins such as Bcl-2, Bcl-X_L and Mcl-1

CSCs overexpress ATP-binding cassette (ABC) transporters which result in reduced intracellular drug levels, thereby protecting them from drug-induced cytotoxicity [116]. Both ABCG1 and ABCG2 are significantly upregulated in lung CSCs facilitating drug resistance [120, 121]. ABCG2 also manifests a protective role against oxidative stress [122]. Apart from this, lung CSCs mediate drug resistance by overcoming DNA damage inflicted by genotoxic drugs via efficient DNA repair pathways which is destined to reduce oxidative stress. Yu et al. [123] depicted enhanced DNA repair potential in lung CSCs which negated cisplatin-induced cytotoxicity and ROS generation. Also, the altered expressions of anti-apoptotic proteins impair cell death induction. Bcl-2, Bcl-X_L and Mcl-1, which promote cell survival, are upregulated in lung CSCs [124, 125]. Bcl-2 is also a redox modulator which is involved in maintaining reduced intracellular ROS levels [126]. Therefore, redox homeostasis is another crucial mechanism of survival depicted by the CSCs.

14.6 Role of ROS in Preserving Attributes of CSCs: Contribution of a Real Friend

ROS has been suggested to be intricately involved in various cellular networks and serves as a signalling molecule. Its critical role in tumour development has been discussed in the previous sections. Relevance of ROS in reference to CSCs is also noteworthy. Recent developments have recognized that stem cells inhabiting the niches are characterized by low ROS levels, which is particularly crucial for stemness maintenance. Subsequently, elevated ROS levels significantly enhance the differentiation potential (Fig. 14.5) [127]. CSCs also employ a similar mechanism of redox balance that helps in maintenance of self-renewal. They also maintain low level of inherent ROS as compared to the rest of the tumour mass which essentially contributes to resistance [25]. An increase in ROS levels results in cell differentiation with a subsequent decline in CSC population (Fig. 14.5). Also, ROS prevents β-catenin activity by perturbing its interaction with TCF4 which might ultimately block self-renewal of CSCs [128, 129]. Thus, high ROS has a negative impact on CSC survival. In CSCs, fructose-1, 6-biphosphatase (FBP1) is epigenetically silenced as a consequence of which there is increased glycolysis and ROS reduction, ultimately favouring β-catenin activation for CSC maintenance [130].Hence, low ROS levels are favoured by CSCs which critically aid in survival, and thus, it is also plausible that antioxidants may boost CSCs. In relation to this, the administration of antioxidants during cancer therapy still remains a disputable issue due to failure of clinical trials [131]. Therefore, it is conceivable that the cellular level of ROS acts as critical determinant for survival and functioning of CSCs, suggesting that manipulating the oxidant molecule may potentially help to eradicate this resistant population. Additionally, it becomes important to decipher the mechanisms of ROS regulation in CSCs and culling strategies to overcome the oxidative stress.

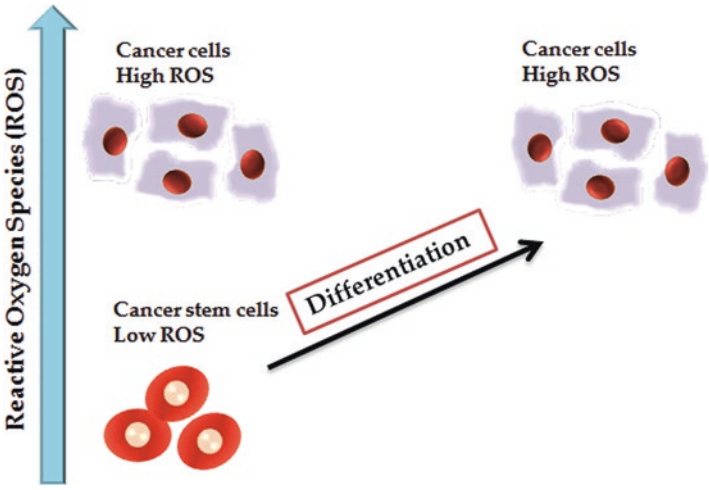

Fig. 14.5 ROS level is a critical determinant for CSCs. CSCs maintain a low level of cellular ROS when compared to cancer cells. An increase in ROS levels in CSCs stimulates its differentiation to cancer cells

14.7 How Do Lung CSCs Conspire with ROS to Overcome Oxidative Stress?

Redox equilibrium is essentially involved in the maintenance of self-renewal ability of stem cells along with its differentiation potential [132]. This low level of ROS in CSCs might be due to production of ROS at small level and/or elevated antioxidant system for scavenging ROS. The low ROS generation in CSCs may be attributed to the slow division process. The slowly proliferating CSCs act like 'quiescent' stem cells and maintain low inherent ROS levels [133]. Alternatively, upon investigation by Diehn and colleagues, it was determined that upregulation of glutathione (GSH) enabled detoxification of ROS and, thus, helped reduce oxidative stress inflicted upon CSCs (Table 14.1) [25]. Lan et al. critically observed that exogenous GSH enhanced cisplatin resistance in lung cancer [134]. Also, the variant form of the lung CSC marker CD44 is known to help enhance GSH biosynthesis by assisting cysteine uptake (Table 14.1) [135]. In line with this, peroxiredoxin II, an antioxidant enzyme, contributes significantly in redox regulation of lung CSCs [136–138]. The elevated level of this enzyme shows protective effect in response to oxidative stress. Chandimali et al. [139] have explored the role of peroxiredoxin II in attributing stemness characteristics to lung CSCs (Table 14.1). Also, it mediates redox regulation by facilitating JNK-dependent DNA repair, thereby protecting against DNA damage [140]. Therefore, peroxiredoxin II plays a critical role in maintaining stemness and provides protection against oxidative stress. In a similar manner, the lung CSCs overexpress the antioxidant proteins, thioredoxin and thioredoxin reductase,

Table 14.1 Factors facilitating redox regulation in CSCs

Factors	Functions
Glutathione (GSH)	Detoxification of ROS
CD44	Enhancement of GSH biosynthesis by assisting cysteine uptake
Periredoxin II	Protective effect against oxidative stress
Thioredoxin and thioredoxin reductase	Maintaining redox homeostasis
Nuclear factor erythroid 2-related factor (Nrf2)	Governs expression of various detoxification genes
	Upregulates glutathione peroxidases and GSH reductase
FOXM1	Downregulates ROS by triggering expression of ROS scavenging factors like catalase, manganese superoxide dismutase (MnSOD) and PRDX3
	Defense against oxidative stress

for maintaining redox homeostasis [141]. Following this, numerous ROS-responsive transcription factors help in maintaining the redox balance. The nuclear factor erythroid 2-related factor (NRF2) is one such critical regulator which governs the expression of various detoxification genes enabling protection against environmental stressors [142]. NRF2 is normally inhibited by Keap1, but alterations in Keap1 functions leads to activation of NRF2 which in turn has been observed to facilitate lung cancer growth [143]. In addition to this, recent evidences have clearly portrayed that NRF2 is involved in ROS modulation which ultimately facilitates CSC growth and resistance (Table 14.1). It functions as a redox modulator enhancing the expression of glutamate-cysteine ligase and cysteine transporter for elevating GSH levels. Also, NRF2 upregulates glutathione peroxidases and GSH reductase, thereby facilitating regeneration of reduced GSH. Other detoxification proteins such as thioredoxin 1, peroxiredoxin 1/6, thioredoxin reductase 1, etc. are also regulated by NRF2 [144]. NFκB is another crucial factor which is worthy to be discussed in redox regulation. It typically boosts cellular survival. ROS induction causes an NFκB response and the target genes function to attenuate the upregulated ROS enabling survival [145]. Another highly conserved transcription factor FOXM1 which is a member of forkhead box transcription factor family is a cardinal regulator of oxidative stress during carcinogenesis (Table 14.1) [146]. It is significantly overexpressed in lung cancer [147] where it downregulates ROS by triggering the expression of ROS scavenging factors like catalase, manganese superoxide dismutase (MnSOD) and PRDX3. Thus, FOXM1 works in a negative feedback loop, wherein the increased oxidative stress induces its expression which in turn counteracts the escalated ROS levels, thereby exerting protective function in the tumour cells [146]. Also, the critical role of FOXM1 has been discussed by Kwok et al. in human embryonic stem cells [148]. This transcription factor aids in defence against oxidative stress and its knockdown-impeded embryonic stem cell proliferation. Recent reports have indicated the role of FOXM1 in maintenance of lung CSCs, thereby enabling redox regulation within it [149]. Thus, numerous factors contribute to redox equilibrium in lung CSCs which essentially help them survive in response to oxidative stress.

14.8 Strategies to Break the Evil Friendship Between CSCs and ROS: A Friend Turned Foe

The redox homeostasis which is prevalent in lung CSCs contributes notably towards their survival. Thus, as a means of developing potential therapeutic strategies for targeting this resistant population, exploiting the redox status may serve as a novel therapeutic approach. Surpassing the inherent low ROS level of CSCs may improve oncologic therapies. Antineoplastic agents are known to selectively kill cancer cells by increasing ROS levels; however, it will be interesting to observe whether they can eliminate the CSCs. Induction of high ROS level in tumour niches may activate differentiation of CSCs, which may be a possible therapeutic strategy using chemotherapeutic agents [127]. Furthermore, CD44, a lung CSC marker, which, as discussed previously, plays a significant role in redox homeostasis, has been targeted recently by hyaluronan-based nanoparticles in lung cancer (Fig. 14.6) [150]. Also, a microRNA-based treatment strategy was used for targeting peroxiredoxin II. mir-122 successfully downregulated peroxiredoxin II and the associated stemness characteristics in lung CSCs, along with induced apoptosis (Fig. 14.6) [139]. In line with this, the thioredoxin and GSH systems also serve as potential targets for interrupting the redox homeostasis in CSCs [151]. Interestingly, Lagadinou et al. [152] observed that the CSCs having low ROS exhibit upregulated BCL-2 expression. Subsequently, BCL-2 inhibition also eliminates the CSCs via interruption of BCL-2-dependent oxidative phosphorylation (Fig. 14.6). In fact, the decreasing GSH level leads to increase in oxidative stress that ultimately kills the CSCs [152].

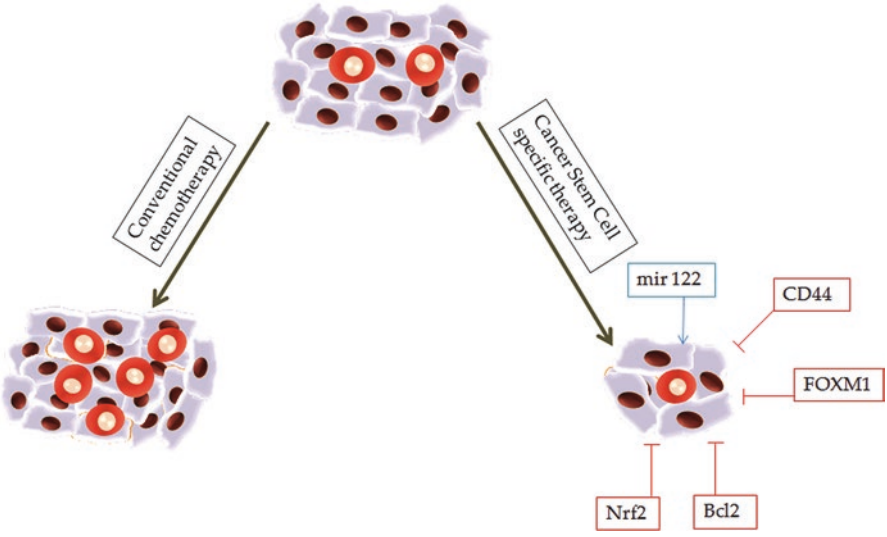

Fig. 14.6 Targeting CSCs via redox modulation. The conventional chemotherapy fails to conquer the CSCs. One of the strategies of overcoming therapy resistance in CSCs is by modulating its redox homeostasis. Downregulation of the redox factors such as CD44, Nrf-2, FOXM1, Bcl-2, etc. might help target the CSCs. Also, microRNA-based treatment strategy using mir-122 enabled disruption of redox balance

The role of NRF2 as a master regulator in protecting CSCs form oxidative stress has been discussed in the previous sections. Hence, targeting this regulator might provide means of eliminating the CSCs. Brusatol, which is an NRF2 inhibitor, promoted ROS generation and thereby sensitized lung CSCs to radiation therapy (Fig. 14.6) [153, 154]. Likewise, fenretinide was observed to exhibit cytotoxic effect towards CSCs. Fenretinide-induced cytotoxicity was linked to rapid ROS production along with repression of NFκB-associated genes [155]. Similarly, targeting FOXM1, another important redox regulator, may represent beneficial therapeutic strategy for lung CSCs [149]. Thus, numerous approaches are being developed for targeting the lung CSCs by redox modulation. However, there is still dearth of information on redox regulation in CSCs. Given the essential role exhibited by redox homeostasis in self-renewal and survival ability of CSCs, further studies in this direction may open greater avenues for treatment.

14.9 Conclusion

Reactive oxygen species essentially behave both as a 'friend' and a 'foe' of lung cancer. ROS regulates cellular signalling networks in lung cancer, thereby mediating numerous cellular functions such as cell proliferation, apoptosis, cell cycle progression, adhesion, metastasis and many more. The equilibrium between pro-oxidants and antioxidants is a critical determinant for cancer cell survival and progression. It is implicated that ROS-regulated networks manipulate the survival of cancer cells. A sudden spike in ROS enables cancer cell proliferation and boosts cell motility favouring metastasis. Furthermore, the dawn of the emerging field of CSCs has significantly revealed their important role in carcinogenesis and relapse. Hence, deciphering the redox networks in CSCs is vital. The CSCs harbour low inherent ROS facilitating their survival. Increase in cellular ROS level accelerates the differentiation of CSCs to cancer cells. Thus, surge in ROS may lead to hostile conditions for CSC survival and may prove beneficial from therapeutic viewpoint. Therefore, it is plausible to eliminate the resistant CSCs by manipulating its ROS content. Consequently, the differentiated CSCs can further be targeted by conventional chemotherapeutic agents which were previously ineffective against CSCs. Therefore, our discussion emphasizes on the omnipresent role of ROS in lung carcinogenesis. Modulating the redox homeostasis may enable us to target this disease by attacking multifaceted signalling networks which promote cancer cell survival. Hence, in the near future, we can plausibly discover newer therapeutic regimens in order to overcome the shortcomings of current treatment methods.

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Role of Noncoding RNA in Lung Cancer

15

Angshuman Bagchi

Abstract

Long noncoding RNAs are RNA molecules that typically are more than 200 nucleotides long. Though they are called noncoding RNAs, they do have the capacity to code for small peptides. Long noncoding RNAs play important roles in many of the cellular processes. They are found to be the causes of many of the diseases, and lung cancer is one such disease that is heavily influenced by lncRNAs such as EPEL. In this chapter, the relationship between lncRNAs and their influence on the onset of lung cancer is elucidated.

Keywords

Long noncoding RNAs (lncRNAs) · Lung cancer · EPFL · Mutations

15.1 Introduction

Long noncoding RNAs belong to the class of RNAs which are 200 or more nucleotides long molecules. Previously, they were considered to be junk molecules. These RNA molecules are very much abandoned in the genomes of the organisms. Majority of the lncRNAs are tissue specific [1–16].

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15.2 Some Important Characteristics of Long Noncoding RNAs

(a) They are generally 200 or more nucleotides long.
(b) They have conserved sequences.
(c) These RNA molecules are transcribed mostly from the intronic part of the genome. However, some lncRNAs are transcribed from the genomic portions which code for proteins as well.
(d) The nucleotide sequences of the lncRNAs remain somewhat conserved.
(e) The nucleotide sequences belonging to the lncRNAs' promoters are highly conserved. The nucleotide sequences of the promoter regions are more conserved than the sequences of the lncRNAs.
(f) The lncRNAs can be classified depending on their origin as (i) sense, (ii) antisense, (iii) intronic, (iv) intergenic, and (v) bidirectional.
(g) Though they are called noncoding RNAs, some of them do code for peptides.
(h) These RNA molecules have conserved secondary structures.
(i) The actual functions of the lncRNAs are still not very clear. However, these RNA molecules have their involvements in the following biological processes:
    (i) As signaling molecules for transcriptional regulators.
    (ii) As guides for protein localizations.
    (iii) As protein-binding scaffolds.
(j) These RNA molecules function in modification and remodeling of histone and chromatin, regulating the gene expressions and their silencing, methylation of DNA, heat shock response, and embryogenesis [17–48].

The lncRNAs are associated with lung cancer. Several lncRNAs are found to be either up- or downregulated in lung carcinoma.

15.3 A Few Words on Lung Cancer

The most commonly used term for lung cancer is lung carcinoma. It represents a tumor with uncontrolled growth in the lungs of a living being. The tumor in lung is characterized by uncontrolled growth of cells in lung tissues. The growth is so severe that it is able to spread to other nearby tissues by a process called metastasis. The lung carcinoma is categorized into two main classes: small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC) [49–58].

15.4 Signs and Symptoms

The most important symptoms of lung carcinoma are as follows:

(a) Symptoms pertaining to respiration: The patients have severe coughing and sometimes blood comes through cough. The patients feel shortness of breath.

(b) Symptoms pertaining to whole-body system: loss of weight, weakness. The patients have mild fever.
(c) Other symptoms: pain in the chest and bone, obstruction in superior vena cava obstruction. The patients might find it difficult to swallow food. Sometimes pneumonia might occur. The patient loses appetite [19–59].

15.5 Possible Causes

Though it is really very difficult to pinpoint the exact cause of the disease, still the following might be considered as useful guides to take preventive measures against the disease:

(a) Smoking: Consumption of tobacco is considered to be the root cause of the disease. Nearly 90% of deaths of the lung cancer patients occur due to smoking of tobacco.
(b) Radon gas: It is one of the important causative agents of lung carcinoma.
(c) Asbestos: It is another important causative agent of lung carcinoma. Tobacco smoking and asbestos both are equally important in the onset of lung carcinoma.
(d) Air pollution: Another source of the disease is air pollution. The chemicals released from burning of fossil fuels are the main causative agents. Sulfate aerosols and nitrogen dioxide are the substances that lead to the onset of the disease.
(e) Genetics: Though it is not fully established, about 8% of the total lung carcinoma is genetically inherited.
(f) There are certain other factors that also known to cause the lung carcinoma. Among them are compounds of metals like aluminum, cadmium, nickel, and beryllium; compounds of arsenic; incomplete combustion of coal; ionizing radiations; and toxic gases like methyl ether and sulfur mustard [59–73, 78].

15.6 Different Classes of Lung Carcinoma

Lung cancers are classified on the basis of histology. This classification is based on how the malignant cells look like and how big the cells are.

(a) **Non-small cell carcinoma:** This is the type of lung cancer that infects the epithelial cells. This type of carcinoma accounts for nearly 85% of the lung cancers. They are relatively insensitive to chemotherapy. There are several types of non-small cell carcinoma. Among them the most frequent ones are squamous cell carcinoma, large cell carcinoma, and adenocarcinoma. Lung adenocarcinoma is the commonest form of lung carcinoma in nonsmokers. It is mainly observed around the periphery of the lung tissues. On the other hand, tobacco

smokers generally have squamous cell carcinoma. It is more common in men than in women.

(b) **Small-cell carcinoma:** This is a type of carcinoma which is highly malignant. This type of carcinoma infects mainly the central airways leading mainly to narrowing of the bronchial passages [47–77]; Kumar et al. [60, 78, 79]; [80–85]; Usman et al. [86–88].

15.7 Disease Pathogenesis

As in the case of other types of cancers, lung cancer is also caused by oncogenic activations or the deactivations of tumor suppressor genes. Mutagenic carcinogens induce genetic changes in these genes leading to the onset of cancer.

The genes which are mainly involved in inducing lung cancer are K-ras, c-MET, NKX2-1, LKB1, PIK3CA, BRAF, and EML4-ALK. K-ras is a proto-oncogene and it is involved in mainly 10–30% of lung adenocarcinoma. On the other hand, EML4-ALK tyrosine kinase fusion gene is involved in the development of nearly 4% of non-small-cell lung carcinoma.

Apart from the direct genetic mutations, there are certain epigenetic changes which also induce lung carcinoma. Such, epigenetic changes are DNA methylation, modification of histone tail, regulations of noncoding RNA molecules, etc. It is also well known that cancer cells are resistant to oxidative damages. Under such conditions, the cancer cells remain unaffected by the cellular immune systems which would otherwise be able to destroy the cancer and tumor.

Another important protein is the epidermal growth factor receptor (EGFR). EGFR is known to regulate cell proliferation, apoptosis, angiogenesis, and tumor invasion. In non-small cell lung carcinoma patients, the EGFR is found to be heavily mutated.

Other possible routes associated with lung carcinoma involve abnormal activation of stem cells, neuro-dendritic cells, etc.

The most important aspect of lung carcinoma is the metastasis. It is the process that involves transitions of the epithelial cells to mesenchymal cells. In metastatic lung carcinoma, the following signaling pathways are activated: Akt/GSK3Beta, MEK-ERK, Fas, and Par6 ([74–77]; Kumar et al. [60, 78, 79]; [80–85]; Usman [86–88]).

15.8 Involvements of lncRNAs in Lung Carcinoma

Recently, lncRNAs are found to play important roles in several of diseases. One of such diseases is lung carcinoma. The following are the different types of lncRNAs associated with lung carcinoma:

(a) **E2F-mediated cell proliferation enhancing lncRNA (EPEL):** EPEL is also known as LOC90768 and MGC45800. This lncRNA was found to be associated

with the multiple occurrence and survival of patients suffering from lung cancer. This lncRNA is also associated with lung cancer cell proliferation via the activation of E2F target genes. In other words, this lncRNA is known to promote the cancer cell proliferation via the activation of E2F target genes. The knockdown of lncRNA EPEL is known to specifically downregulate the expression of cell cycle-related E2F target genes, including cyclin B1 (CCNB1), in lung cancer cells. However, it is not linked to apoptosis- or metabolism-related E2F target genes. The lncRNA EPEL is known to interact with E2F1. In this way, it regulates the expression of the E2F target genes. The lncRNA is known to make changes on the binding efficiency of E2F1 to the E2F target promoters. Therefore, it could be safely concluded that the expression levels of EPEL and CCNB1 both alone and together are prognostic biomarkers for lung cancer ([89] and references therein).

(b) **The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1):** The lncRNA MALAT1 was identified to be associated with patient survival. The lncRNA is known to affect the genes associated with cancer like cellular growth, movement, proliferation, signaling, and immunoregulation and so on. Furthermore, the amount of MALAT1 was found to be higher in brain metastasis. The lncRNA MALAT1 induces metastasis of lung cancer cells by enhancing epithelial-mesenchymal transition (EMT). The actual mechanistic detail of the involvement of MALAT1 in lung carcinoma is not yet fully understood. However, it was speculated that MALAT1 regulates DNA methylation. It also helps in the upregulation of Bcl2 and its interacting partners to induce non-small cell lung carcinoma ([89, 90] and references therein).

(c) **HOX antisense intergenic RNA (HOTAIR):** It is a 2.2 kilobase noncoding RNA. The lncRNA HOTAIR is known to facilitate the tumor development in non-small cell lung carcinoma. However, it is not linked to the carcinogenesis of non-small cell lung carcinoma. It was also revealed that HOTAIR is involved in the modification of the promoter of *p53* and thereby enhancing histone H3 lysine 27 trimethylation. This indicates a negative correlation between HOTAIR and *p53* in non-small cell lung carcinoma cells. Furthermore, HOTAIR is known to activate Wnt/β-catenin signaling pathway in esophageal squamous cell carcinoma ([90] and references therein).

(d) **HOXA distal transcript antisense RNA (HOTTIP):** HOTTIP is known to be an antisense noncoding RNA transcript. It is also known to be placed at the distal end of HOXA gene cluster. The expression of HOTTIP is found to be higher in non-small cell lung carcinoma than in the corresponding adjacent normal tissues. It thereby helps in contributing to cell proliferation and migration. The lncRNA HOTTIP also regulates HOXA13 and thereby functioning as oncogene ([90] and references therein).

15.9 Concluding Remarks

It has been reported in different recent studies that lncRNAs do play very important roles in the development of non-small cell lung carcinoma and thereby leading to lung cancer-related deaths. In this review I tried to analyze the link between lncRNA and lung cancer. Recent studies suggest that patients suffering from lung carcinoma have invariably differential expression patterns of different types of lncRNAs. However, the mechanistic details of the involvements of lncRNAs in the onset of lung carcinoma are not well described. Different lncRNAs like MALAT1, HOTAIR, etc. are constantly being targeted by scientists to analyze their effects in different disease conditions especially in lung carcinoma. The main aim of the review is to provide some insight into the effects of lncRNAs in disease onset.

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16 Reactive Oxygen Species (ROS): Modulator of Response to Cancer Therapy in Non-Small-Cell Lung Carcinoma (NSCLC)

Shamee Bhattacharjee

Abstract

Oxidative stress, caused by an imbalance between oxidants and antioxidants, is implicated in the etiology and progression of many types of cancer including lung cancer. The most common type of lung cancer, NSCLC, is the leading cause of cancer-related deaths worldwide. The lung tissue is particularly vulnerable to oxidative stress because of its direct interface with ambient air which exposes it to a variety of oxidants. In order to protect itself from oxidative stress, lung tissue is equipped with a robust endogenous antioxidant defense system mostly controlled by the redox-sensitive transcription factor Nrf2 which is negatively regulated by Keap1 protein. Lung cancer cells are reported to contain increased levels of ROS. However, administration of antioxidants has failed to show any obvious effectiveness in the prevention or cure of lung cancer. On the other hand, a pro-oxidant approach has been proposed to successfully kill cancer cells by generating ROS. Cancer cells, owing to their high basal ROS, are considered to be more vulnerable to the toxic effect of exogenous ROS-generating agents as opposed to normal cells. A major challenge in this mode of therapy is the acquisition of drug resistance in cancer cells. This is attributed to an elevation in the antioxidant system in cancer cells, leading to "redox adaptation," which facilitates survival under enhanced oxidative stress. Incidentally, lung cancer cells have been reported to exhibit constitutive overexpression of Nrf2. Therefore, impairment of the Nrf2/Keap1 antioxidant pathway might be a promising strategy to control NSCLC. In this chapter, the importance of ROS as a signaling molecule in regulating some of the hallmark feature of cancer, such as proliferation, apoptosis, angiogenesis, metastasis, etc., is discussed. Furthermore, the various ROS-modulating therapeutic approaches to treat NSCLC presently under investigation at experimental and clinical setting are also discussed.

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Lung cancer · NSCLC · ROS · Oxidative stress · Nrf2/Keap1antioxidant pathway

16.1 Introduction

Lung cancer accounts for majority of cancer deaths and is also the most common cancer diagnosed worldwide. Histologically, two main types of lung cancer are non-small-cell lung cancer (NSCLC) representing approximately 85% of all lung cancer cases and small cell lung cancer (SCLC) constituting the remaining cases [1]. To a large extent, lung cancer survival and treatment modality are governed by the stage of the disease [2]. The standard treatment recommended for stages I and II is surgical resection along with adjuvant therapy; radiation with chemotherapy is the mode of treatment for locally advanced inoperable stage IIIA tumors; for stage 3 IIIB and IV chemotherapy along with supportive care is the standard treatment. More recently, chemotherapy is being replaced by tyrosine kinase inhibitors (TKIs) as the first line of treatment [3]. Despite the evolution of targeted therapy, lung cancer prognosis is still very poor with a meager 17.8% 5-year survival rate [4] which is much lower than many other leading cancer sites. Approximately 50% of the lung cancer cases are detected at an advanced metastatic stage and the average median survival for such patients is 10–12 months [5] which have been found to marginally increase to 18–25 months with the use of TKIs [3]. Such dismal survival rates and poor prognosis are attributed to high recurrence, metastasis, and the development of drug resistance in majority of patients.

Oxidative stress is implicated in the pathophysiology of lung diseases including lung cancer [6]. An imbalance between production of oxidants and their neutralization by the antioxidant defense systems in our body is termed as oxidative stress. Elevated levels of intracellular ROS, which are by-products of aerobic metabolism, are frequently encountered during oxidative stress. The term ROS includes several free radicals, e.g., superoxide ($O_2^{\bullet -}$), peroxyl ($RO_2^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), and some non-radical oxidants such as hydrogen peroxide (H_2O_2). Considerable evidence suggests that redox imbalance and deregulation in redox signaling are associated with cancer progression and drug resistance [7]. Increased production of ROS has been associated with many human metastatic tumors [8] including lung cancer [9, 10] and is linked to tumor aggressiveness resulting in poor prognosis [11]. This persistent high level of ROS in cancer cells, resulting from genetic and metabolic alterations [12], promotes tumor growth and aggressiveness by regulating several key cellular processes such as proliferation, apoptosis, angiogenesis, invasion, and metastasis [13]. Therefore, it is logical to speculate that the molecular and biochemical alterations caused by elevated ROS in cancer cells can lead to the emergence of drug-resistant cells during disease progression [14]. Although drug resistance mechanisms are extremely complex and depend on the mode of treatment and genetic constitution of cancer cells, ROS-mediated

mechanisms can be assumed to have an important participation in the evolution of drug-resistant phenotype.

Paradoxically, as ROS are extremely reactive and can damage cellular macromolecules, the elevated intrinsic ROS in cancer cells provide a unique opportunity to selectively kill cancer cells based on their vulnerability to further oxidative stress. These contradictory roles of ROS have important implications in designing anticancer therapies based upon modulation of ROS levels. Therefore, modifying ROS levels by either enhancing or depleting their concentrations have been proposed for cancer treatment.

Herein the various signaling pathways through which ROS may modulate cancer phenotypes are described. The therapeutic implications of this escalated ROS levels in lung cancer is also discussed. Some clinical studies correlating ROS levels with tumor prognosis will also be detailed in this chapter.

16.2 Redox Signaling in Lung Tissue

The primary function of lung is to facilitate exchange of CO_2 for O_2. As compared to other organs, lungs are more vulnerable to oxidative stress because they are exposed to highest amount of oxygen [15]. Moreover, adult human lungs exchange between 10,000 and 20,000 l of air daily [16].This exposes the lungs to a wide variety of infectious agents and toxicants including mutagens and carcinogens. Therefore, in order to maintain the sterility of airways, lung cells have equipped themselves with NADPH oxidase (Nox) enzymes which are widely expressed in both upper and lower respiratory tracts [17] and participate in innate immunity and host defense of the lungs [16]. The Nox enzymes catalyze the reduction of molecular O_2 to superoxide ($O_2^{\cdot -}$) which is involved in maintaining lung integrity [17]. Expression of Nox and Nox-derived ROS are considered to participate significantly in the development of an oxidative environment which provides pro-oncogenic survival and proliferative signals [18]. Considerable studies now suggest a positive correlation between Nox expression/activity and lung cancer [19]. Apart from Nox, another endogenous source of ROS in lung tissues is polymorphonuclear neutrophilic leukocytes (PMNs) which are significantly higher in numbers in the pulmonary circulation than the systemic circulation [20]. Protection of lung tissue against such oxidative insult is mediated by a variety of endogenous antioxidants, for instance, glutathione S-transferase (GST), glutathione peroxidase, superoxide dismutase (SOD), catalase, thioredoxin reductase, and reduced glutathione (GSH) (L-γ-glutamyl-L-cysteinyl-glycine) [21]. The principal regulator of antioxidant enzymes is nuclear factor erythroid 2-related factor 2 (Nrf2) which plays a vital role in providing cytoprotection in response to oxidative stress.

Higher levels of ROS which play a positive role in carcinogenesis have been reported in lung cancer cells. To counteract the toxicity of such elevated ROS, lung cancer cells increase their antioxidant defense system (as shown in Fig. 16.1) [22, 23]. The augmented antioxidant level in response to oxidative stress is largely governed by the activity of the redox-sensitive transcription factor Nrf2. Under

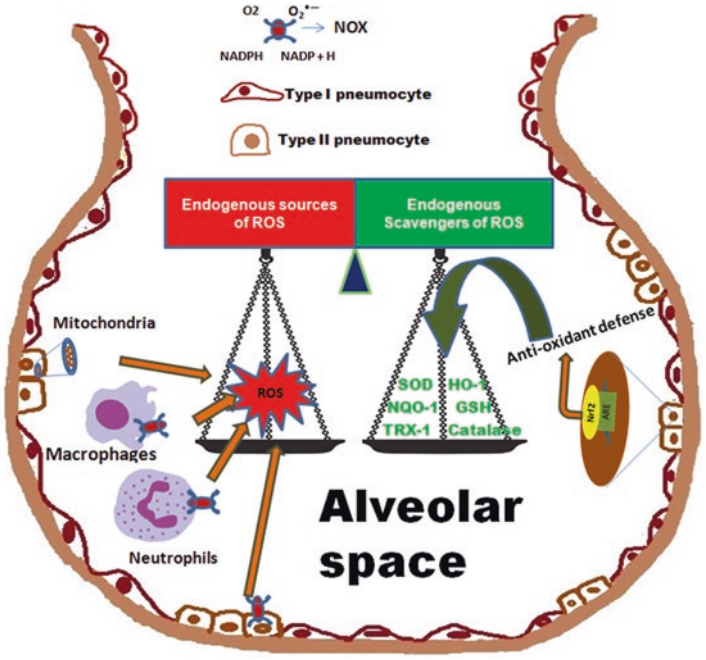

Fig. 16.1 Main sources and scavengers of ROS in the lung alveolar space. Nox family of oxidoreductases is widely expressed in different cell types of the lung alveoli such as type I and type II pneumocytes, alveolar macrophages, and neutrophils which create an oxidative environment in the lung tissue. Macrophages and neutrophils also generate ROS during oxidative burst. Lung tissue protects itself from this enhanced ROS by activating Nrf2-/Keap1-mediated antioxidative defense mechanism

unstressed condition, a cytosolic inhibitor of Nrf2, termed Kelch-like ECH-associated protein 1 (KEAP1), targets it for proteasomal degradation. Accumulating evidence indicate alterations of the Keap1-Nrf2 pathway in lung cancer, including somatic mutations, loss of heterozygosity, and epigenetic changes in the promoter region of Keap1 [24]. Thus, it has been postulated that cancer cells not only generate high levels of ROS but also can withstand these levels by activating antioxidant pathways, predominantly the Nrf2-Keap1 pathway, that drive their proliferation and survival [25].

16.3 ROS in the Regulation of Cell Behavior

16.3.1 ROS as a Mediator of Cell Proliferation

Binding of many polypeptide growth factors including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), etc., to receptor tyrosine kinases (RTKs) has been reported to induce ROS production

which facilitates growth-factor-induced receptor tyrosine phosphorylation [26]. Oxidation of cysteine (Cys) residues in proteins and other thiol-containing compounds in the cell by ROS is considered to play a vital role in a variety of signaling pathways [27]. Experiments have shown that H_2O_2 can oxidize and inactivate receptor-associated EGF-associated phosphatases such as protein-tyrosine phosphatase 1B (PTP1B) [28, 29]. Thus, inactivation of these phosphatases by H_2O_2 increases phosphorylation of tyrosine residues in the growth factor receptors thereby promoting activation of downstream signaling pathways such as mitogen-activated protein kinase (MAPK) pathway, signal transducer and activator of transcription (STAT), and phosphatidylinositol 3-kinase (PI3K)-Akt which are known to control cell proliferation, migration, and survival [30]. H_2O_2 can also promote the activation of Ras which in turn activates PI3/Akt/mTOR and MAPK/ERK pathways [7].

16.3.2 ROS as a Mediator of Cell Survival

As mentioned in the above sections, in response to oxidative stress, Nrf2, after being released from Keap1, escapes proteasomal degradation and transactivates the expression of genes related to cell survival. Among these are genes for antioxidant/detoxifying enzymes such as catalase, glutathione peroxidase, superoxide dismutase, thioredoxin, and glutathione S-transferase that can scavenge free radicals, thereby reducing ROS-induced cytotoxicity [31]. Nrf2 has also been shown to bind to the promoter region of the antiapoptotic protein Bcl-2 in response to oxidative stress. Nrf2-mediated upregulation of Bcl2 is reported to downregulate proapoptotic Bax and caspase 3/7 which protected cells from undergoing apoptosis and become drug resistant [32]. There are evidence of an association between increased accumulation of Nrf2 and augmented Bcl2 levels in lung cancer cells [32]. Another important transcription factor which promotes cell survival during oxidative stress is NF-κB [33]. NF-κB influences and is being influenced by ROS. Enhancing the expression of antioxidants, such as MnSOD [34], ferritin heavy chain [35], GST [36], and haemoxygenase 1 (HO-1), is one of the most important ways in which NF-κB moderates ROS levels [37].

16.3.3 ROS as a Mediator of Apoptosis

Apoptosis is mediated by extrinsic or intrinsic pathways. Extrinsic pathway involves the recruitment of death receptors on the cell surface, while the intrinsic pathway primarily pivots upon the mitochondrial outer membrane permeability. ROS is a critical inducer of apoptosis. H_2O_2-induced apoptosis is reported to be accompanied by an increase in p53, Noxa, Puma, and Bax expressions in several cell lines [38]. The activity of caspases is also known to be redox regulated as the cysteine residues located at the catalytic site of caspases are vulnerable to oxidation. In addition, susceptibility of procaspase-3, procaspase-9, and the active caspase-3 toward S-glutathiolation has also been observed [39, 40].

ROS have been intricately associated with mitochondrial pathway of apoptosis because it is the organelle where most of the intracellular ROS is produced. Mitochondria-derived ROS can damage mt-DNA which impairs transcription of electron transport genes, thereby compromising mitochondrial membrane potential [41]. These events ultimately result in intrinsic pathway of apoptosis. Additionally, ROS can also stimulate cytochrome c release from mitochondria and induce mitochondrial permeability transition pore (MPTP) opening by oxidizing protein components of MPTP [39].

Evidence indicate link between ROS and extrinsic pathway of apoptosis as well. Experiments have shown the involvement of ROS such as H_2O_2 in the activation of Fas-L, translocation of FADD to the plasma membrane, and activation of caspase 8 in many cancer cell lines [42, 43].

Newer evidence have recognized ROS-induced receptor clustering and lipid rafts as important redox signaling platforms for apoptotic induction. Death receptor-ligand binding has been reported to cause lipid raft formation which facilitated recruitment of Nox and ROS generation [44].

16.3.4 ROS as a Mediator of Angiogenesis

Exogenous ROS has been found to induce vascular endothelial growth factor (VEGF) in several types of cells and also promote the proliferation and migration of endothelial cells [45]. Upregulation of VEGF and its receptors are cellular responses under hypoxia which have been reported to trigger the generation of H_2O_2 by mitochondria [46]. ROS is also found to enhance the DNA-binding activity of the transcription factor hypoxia-inducible factor-1α (HIF-1α), which can activate the expression of genes such as VEGF in response to hypoxic stress. Intracellular ROS production mediated by NADPH oxidases, Nox2 and Nox4, has been reported to promote endothelial cell proliferation and survival via p38, ERK, and Akt signaling [47, 48].

Nox1-induced ROS production has also been shown to activate ras-induced upregulation of VEGF and angiogenesis [49]. Endothelial cell migration has also been reported to be facilitated by ROS. Mechanistically, this involves tethering Nox-2 to actin cytoskeleton at the leading edge of migrating endothelial cells by the actin-binding scaffold protein, IQGAP1, leading to ROS production which facilitate cell migration [50].

16.3.5 ROS as a Mediator of Invasion and Metastasis

Evidence suggest that exposure of various cancer cells to ROS enhance their migratory and invasive property [51, 52]. Metastasizing cancer cells have been reported to possess higher levels of cytoplasmic and mitochondrial ROS as compared to primary tumors. It is now widely established that detachment of cancer cells from extracellular matrix (ECM) causes a robust increase in ROS [53]. ROS is known to

induce cell–cell dissociation by regulating the activity of Src kinase [54] which mediates internalization of N-cadherin and activation of Rho/Rho kinase pathways via phosphorylation of p-120 catenin [55]. The effect of this elevated ROS on the viability of disseminated cells is an active area of research now. Subsequent studies have shown overexpression of SOD and CAT in ECM-detached cells promoted the survivability of these disseminated cancer cells [56] and facilitated evasion from anoikis [57]. A study conducted by Piskounova et al. have shown inducible increase in endogenous antioxidants glutathione and NADPH in metastatic tumors indicating that metastasizing cancer cells acquire adaptive response to counteract the elevated oxidative stress. In the same study, it was revealed that treatment with N-acetylcysteine (NAC) augmented the existence of circulating tumor cells [58]. This is in agreement with a previous study which showed that activation of Src by ROS, in addition to facilitate cell-cell dissociation (as mentioned above), also activates NF-κB resulting in MnSOD expression to decrease the oxidative stress [55]. Thus possibly oxidative stress is essential for the initial steps of metastasis, but not for the later stages of metastatic cascade. In other words, high ROS content in metastatic cancer cells as well as their ability to effectively mitigate the oxidative stress during migration through the blood vessels and colonization at secondary sites determines efficient dissemination and seeding of metastatic cancer cells [59].

Tipping the balance from ROS-induced tumor, promoting events to ROS-induced apoptotic signaling is a major challenge for designing effective therapeutic strategies.

16.4 Modulation of ROS as Anticancer Strategy against NSCLC

As compared to normal cells, cancer cells are known to possess an altered redox status which can be explored for potential therapeutic benefits. Cancer initiation and progression is frequently associated with oxidative stress. Therefore, antioxidants have been proposed to help fight against cancer. Alternatively, another approach to target cancer cells is to exploit the vulnerability of cancer cells to further ROS insults. However, it has also been observed that cancer cells become adapted to persistent increased intrinsic oxidative stress and develop an enhanced antioxidant defense system leading to drug resistance. Therefore, modulating such redox adaptation is yet another strategy to eliminate cancer cells (Fig. 16.2).

16.4.1 Targeted Therapy

FDA-approved tyrosine kinase inhibitors (TKIs) for the treatment of lung cancer patients such as gefitinib, erlotinib, etc., are specifically targeted against epidermal growth factor receptors (EGFR) [60]. As mentioned in the above sections, ROS-induced oxidative stress is involved in tumor progression mediated by EGFR. Oxidative stress has been demonstrated to activate EGFR in a

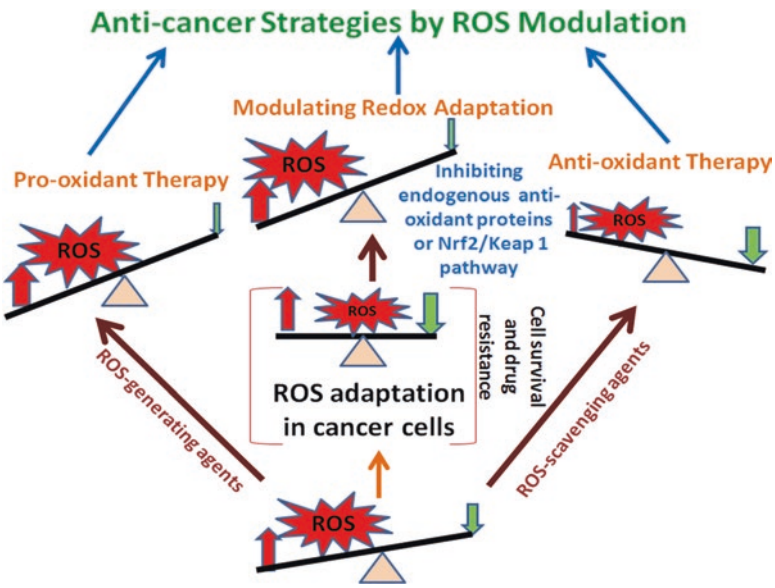

Fig. 16.2 Therapeutic modulation of altered redox status of cancer cells. There are mainly three ROS-modulating strategies to fight against cancer: (1) using antioxidants to scavenge the enhanced ROS in cancer cells, thereby inhibiting carcinogenesis, (2) treating cancer cells with prooxidants which would make them more vulnerable to ROS-mediated cell death, and (3) targeting and inhibiting the Nrf2-/Keap1-mediated endogenous antioxidant defense system in order to jeopardize redox adaptation of cancer cells

ligand-independent manner which does not involve receptor dimerization [61, 62]. Apart from functioning as mediators of the EGFR signaling pathway, ROS also act as regulators of the oxidation status and activation of the EGFR protein. Low and high ROS play contradictory roles in the EGFR signaling pathway. Oxidation of tyrosine phosphatases and/or specific cysteine residues in EGFR caused by mild level of ROS promotes the EGFR signaling pathway [63]. However, excessive ROS is reported to cause overoxidation of Met residue of $EGFR^{T790M}$, thereby inhibiting the survival signaling pathway [64]. One study has demonstrated that treatment with TKIs increased ROS in cells with intact Keap1 and loss of Keap1 inhibited this increase. Keap1 knockout leads to Nrf2 overexpression which caused increased cell survivability by reducing ROS levels in the presence of drug [65]. In line with this study, one group of researchers have shown that the histone deacetylase inhibitor, vorinostat, increases the efficacy of erlotinib or gefitinib against NSCLC when administered in combination by downregulating Nrf2 and upregulating Keap1 [66]. A natural naphthoquinone, shikonin, was shown to sensitize wild-type EGFR NSCLC cells toward the anticancer activity of TKIs which was associated with ER stress and ROS [67]. A combination treatment of resveratrol and erlotinib was also found to enhance ROS generation and cause apoptosis in NSCLC cells [68].

Acquired drug resistance during TKI therapy is a common problem which severely limits treatment outcomes and is responsible for poor prognosis of lung

cancer patients [69]. TKI-resistant NSCLC cell lines were reported to contain high levels of basal ROS [60, 64]. In addition, corroborating the high basal ROS levels, the same study showed high Nox-2 expression in clinical lung tumors which was associated with poor patient survival [60, 64]. ROS generated by chronic gefitinib treatment has been correlated with EMT which is a classical hallmark of drug-resistant tumors as well as alterations in mitochondrial structure and function [70]. In a separate study, NAC was found to inhibit EGFR-TKI resistance induced by ROS generated from cigarette smoke extract in NSCLC cell lines [62]. The findings from this study suggested that antioxidants might provide therapeutic benefit by scavenging TKI-induced ROS and EMT. However, contrary to these reports, other studies have shown induction of apoptosis in TKI-resistant NSCLC cells by upregulating ROS levels. Combined treatment with erlotinib and a flavonoid ampelopsin caused increased accumulation of ROS via upregulating NOX2 enzyme which resulted in apoptotic cell death in erlotinib resistance NSCLC cells [71]. Another natural compound dioscin is also reported to overcome TKI resistance in NSCLC by downregulating transcription of Src homology 2 (SH2) domain-containing PTP2 (SHP2) gene through p53 induction in response to ROS generation [72]. A small molecule, sanguinarine, which can elevate ROS level [73], is also reported to kill gefitinib-resistant NSCLC cells by upregulating NOX3 resulting in EGFR overoxidation, degradation, and apoptosis [64]. Studies have also reported that exposure to oxidative stress in EGFR-overexpressed NSCLC cell lines resulted in TKI resistance.

16.4.2 Chemotherapy

Apoptosis induced by classical cytotoxic chemotherapeutic agents including DNA alkylating agents (e.g., cyclophosphamide), anthracycline antibiotics (e.g., doxorubicin), platinum compounds (e.g., cisplatin), etc., is known to be partly mediated through generation of ROS [74]. However, due to the nonselective cytotoxicity of these agents which compromises the viability of normal cells as well, research is now focused in identifying agents which can selectively target cancer cells. The sustained oxidative stress in cancer cells owing to constitutively enhanced ROS generation or impaired antioxidant systems can selectively increase the sensitivity of cancer cells toward prooxidant cancer therapy which has been observed by several groups [14, 75]. In other words, cancer cells with high basal ROS levels are more susceptible to the toxic effects of additional exogenous ROS [76]. The approach to selectively kill cancer cells by ROS-mediating mechanisms is being explored to develop effective selective therapeutic agents. A lot of preclinical studies have established that various agents can induce apoptosis in NSCLC by exerting oxidative stress. Teroxirone, a triepoxide currently undergoing clinical trial, exerted oxidative stress on human NSCLC cells by disrupting the mitochondrial membrane permeabilization, generating ROS, and promoting ultimate apoptotic cell death [77]. Riluzole, an amino acid channel blocker, has been found to increase ROS beyond the tolerance limit of cisplatin-resistant NSCLC leading to cell death [78].

Rotenone increased the sensitivity of NSCLC cells toward apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) through ROS generation. Many phytochemicals such as sotetsuflavone, withaferin A, physalin A, and withasteroids have been reported to cause apoptosis or cell cycle arrest in the NSCLC cell line A549 by increasing the amount of intracellular ROS [79–82].

Chemotherapeutic agents such as motexafin gadolinium and anthracyclines (doxorubicin and daunorubicin) may react with flavoenzymes, viz., NAD(P)H:quinine oxidoreductase (NQO1) and cytochrome P450 reductase, to produce superoxide in the presence of reduced NADPH. These agents are, hence, known as redox cyclers [83, 84].

Conversely, antioxidants have also been proposed to induce reductive DNA damage leading to cell death. Such a reductive damage to DNA was shown to be mediated by the green tea flavonoid epigallocatechin gallate (EGCG) due to the donation of its weakly bound electron to DNA which resulted in A549 cell death [85].

16.4.3 Radiotherapy

Radiotherapy is an important mode of treatment for unresectable advanced human lung cancer. In radiotherapy, high- and low-linear energy transfer (LET) radiations are delivered to cancer cells which induce damage to cellular macromolecules including DNA, ultimately leading to cancer cell killing. X-rays, γ-rays, and β-particles constituting low LET radiations and electrons, protons, α-particles, and other heavy ions comprising high LET radiations can damage DNA directly or indirectly via free radicals such as OH°, O_2^{-}, and H_2O_2 generated from ionization of cellular water component [86, 87]. However, treatment outcome is severely limited due to two factors: undesirable toxicity to normal cells and development of radioresistance in cancer cells [88]. Therefore, there has been a two-pronged attempt to improve treatment outcome of radiotherapy: (1) identifying radioprotective agents which would offer protection to normal tissues without hindering the tumoricidal activity of radiotherapy and (2) identifying potent radiosensitizers which would overcome radioresistance.

Whole-grain flaxseed was found to provide protection against radiation-induced lung injury in murine model without inhibiting tumoricidal doses of radiation [89]. Subsequently, the same group has identified lignan complex of flaxseed as the bioactive component which upregulates antioxidant enzymes such as HO-1 and NQO1, thereby exerting radioprotective effect [90]. Contrary to the effect of radioprotectors which are mostly antioxidants, radiosensitizing strategy includes induction of DNA damage through ROS generation or inhibiting antioxidant defense system.

Oridonin, a natural diterpenoid compound in combination with radiation, was found to greatly enhance ROS generation and DNA damage, thereby increasing apoptosis in the H460 NSCLC cell in vitro [88]. Luteolin pretreatment of NCI-H460 and H1299 NSCLC cells before irradiation with γ-ionizing radiation-enhanced apoptotic cell death through p38 mitogen-activated protein kinase (MAPK) phosphorylation and ROS accumulation [91].

X-ray irradiation when administered in combination with coroglaucigenin, isolated from stems of *Calotropis gigantea*, increased radiosensitivity in human lung cancer cells (NCI-H446, NCI-H460, A549) accompanied by a reduction in Nrf2 levels [92]. Blocking Nrf2-dependent antioxidant activity by various agents such as brusatol [93], 4-(2-cyclohexylethoxy)aniline (IM3829) [94] have been found to enhance the effectiveness of radiotherapy in several human NSCLC cells through generation of ROS.

16.4.4 Conquering Drug Resistance by Tempering Redox Adaptation in Cancer Cells

Although preferential killing of cancer cells by using exogenous ROS-promoting agents has shown success in the experimental systems [11], it has not tasted widespread success in the clinical setting. Moreover there are reports of high ROS levels in chemotherapy- and radiotherapy-resistant tumor cells [95, 96]. A study has reported elevated ROS in cisplatin-resistant cells including those derived from patients [78]. Decreased expression of thioredoxin-1 (TRX1), which is a major endogenous antioxidant peptide, was found to be an important mediator of ROS in cisplatin-resistant lung cancer cells. One of the reasons might be that a few cancer cells have become adapted to this high intrinsic ROS by elevating antioxidant defense system. This upregulated antioxidant defense not only ensures survival of cancer cells under enhanced intracellular ROS but also endows cancer cells with a mechanism to resist the action of anticancer drugs. Endogenous antioxidant defense comprising SOD, GST, and GSH have all been implicated in the development of drug resistance. Overexpression or hyperactivity of these antioxidants can neutralize chemotherapy-induced oxidative stress, thereby leading to drug resistance [97]. A growing number of studies reveal that impairment of Nrf2-Keap1 pathway, the master regulator of the transcription of these endogenous antioxidants, exists in lung cancer [24]. Loss-of-function mutation in Keap-1 and gain-of-function mutation in Nrf2 leading to increased Nrf2 activity have been reported in lung cancer by various groups [98–100]. Such aberrant activation of Nrf2 is known to contribute to resistance against oxidative stress induced by chemotherapy or radiotherapy in cancer cells [100, 101]. HO-1, a transcriptional target of Nrf2, is overexpressed in NSCLC [102]. One study has reported decreased chemosensitivity of lung cancer cells toward cisplatin treatment through MAPK-dependent activation of Nrf2 leading to upregulation of HO-1 [103]. In line with this study, inhibition of Nrf2 has been shown to enhance the cytotoxicity of anticancer drugs. Administration of a PDGF inhibitor CP-673451 with cisplatin was found to cause synergistic anticancer effect against NSCLC in vitro through Nrf2 inhibition and consequent ROS production [104]. Inhibition of Nrf2 nuclear translocation was also found to augment the toxicity of adaphostin, a dihydroquinone derivative, in NSCLC cell line NCI-H522. Brusatol, which has been identified to be an inhibitor of Nrf2 pathway, is found to sensitize A549 lung cancer cells to cisplatin [105]. Therefore, targeting Nrf2 may be important for those drugs which kill or sensitize cancer cells to apoptosis through the generation of ROS.

16.5 Redox Modulators in Lung Cancer Therapy: Human Intervention Studies and Clinical Evidence

ROS are mutagenic thereby promoting tumor growth. Under this condition, antioxidants have become popular among general population and in the scientific community as agents that can protect and fight against cancer. However, clinical trials with antioxidants have failed to show beneficial effects against cancer.

Genomic analysis of 178 lung squamous cell carcinomas reveals that genes related to oxidative stress response were frequently altered pathways in lung cancer. Mutations or copy number alterations were observed in 34% of cases [106]. Another study has shown that biallelic inactivation of Keap1 is a frequent genetic alteration in NSCLC leading to constitutive activation of Nrf2-dependent antioxidant enzymes [98]**.** These findings suggest that modulation of redox state presents an important therapeutic opportunity in lung cancer.

16.5.1 Scavenging ROS

In order to find out the effect of dietary supplementation with α-tocopherol or β-carotene or both on the incidence of lung cancer, a randomized double-blind placebo-controlled study, termed the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study, was conducted in Finland. During the trial, 847 new cases of lung cancer were diagnosed. Among these newly diagnosed lung cancer patients, there wasn't any reduction in lung cancer incidence in men receiving alpha-tocopherol as compared to those who did not. Ironically, consumption of β-carotene led to a higher incidence of lung cancer in men as compared to nonconsumers [107]. In yet another randomized trial, supplementation with retinyl palmitate and/or N-acetyl cysteine for 2 years caused no difference in overall survival of head and neck or lung cancer patients [108].

16.5.2 Enhancing ROS

Anticancer drugs such as platinum coordination complexes (carboplatin, cisplatin) generate huge amount of ROS which can induce apoptosis in cancer cells. Combination of these drugs with inhibitors of the enzyme poly (ADP-ribose) polymerase (PARP), e.g., veliparib or olaparib, has yielded promising results in different types of cancer including lung cancer. Inhibiting the activity of PARP, which is involved in maintaining DNA integrity during genotoxic stress including oxidative stress, can compromise the capacity of tumor cells to respond to oxidative stress. Phase I/II clinical trial of olaparib in combination with carboplatin or cisplatin and other drugs (e.g., topotecan, vinorelbine, paclitaxel, gemcitabine, bevacizumab, or radiation) is underway for NSCLC patients [109].

Another drug which is approved for clinical trial in lung cancer is NOV-002 which mimics endogenous glutathione disulfide (GSSG) and hence alters the redox status by manipulating intracellular GSSG/GSH ratio [110, 111].

Increased levels of GSH protect cancer cells from apoptosis and are associated with drug resistance [112]. Inhibitors of another thiol-based antioxidant, thioredoxin, have been shown to have promising antitumor activity. Drugs such as motexafin gadolinium are thioredoxin reductase inhibitors that can sensitize cancer cells to oxidative stress. This drug has shown potent radiosensitizing activity in NSCLC patients with brain metastasis undergoing whole-brain radiation therapy in a phase III clinical trial [112].

16.6 Discussion

Conventional therapies in cancer, viz., radiotherapy and chemotherapy, cause substantial increase in cellular ROS levels so as to induce tumor cell apoptosis [113]. However, the main limitation to these therapies is their nonselective cytotoxicity toward normal cells of the patient. Several studies have reported increased oxidative stress and decreased antioxidant enzyme in lung cancer patients undergoing chemotherapy [114]. Therefore, there was a general notion that antioxidant supplementation during cancer therapy might help alleviate ROS-induced damage to normal cells, but clinical evidence supporting the efficacy of antioxidants and vitamins in cancer prevention or cure are inconsistent and therefore inconclusive. For instance, in a Phase I/II clinical trial with high-dose intravenous vitamin C and cytotoxic chemotherapy, a 73-year-old female stage IV lung cancer patient had stable disease after 2 treatment cycles, but after further 2 more cycles, a new nodule was observed in the lung which suggested disease progression. Usually, stage IV lung cancer patients experience progressive disease after the first-line chemotherapy itself. Therefore, the brief enhancement in the period of stable disease of this patient might be attributed to vitamin C. However, no such benefit was noted in case of another lung cancer patient whose condition deteriorated very fast [115]. Thus, no conclusive evidence regarding the therapeutic benefit or harm of using vitamin C in conjunction with cytotoxic chemotherapy in patients with advanced cancer could be obtained.

It might seem quite perplexing that despite the proven implication of oxidative stress in lung diseases including lung cancer, antioxidant therapeutic approach has failed to improve outcome in lung cancer patients. Rather, some studies have reported increased risk of lung cancer following administration of antioxidants [107, 116, 117]. Various reasons, e.g., inadequate dose, inadequate tissue delivery, or selection of a proper timing for an antioxidant, have been attributed to this failure of antioxidant therapies [118]. Another very important point that needs to be considered is that ROS are essential signaling molecules in normal cells. Therefore, antioxidants may indiscriminately scavenge ROS, thereby interfering with the normal physiologic roles of ROS leading to undesirable patient outcome. In addition, antioxidants also have been reported to mitigate the effect of chemotherapy on cancer cells which is dependent on ROS-induced cytotoxicity [119]. Due to the overall negative impact of antioxidant therapies, alternative approaches to target cancer cells by exploiting the fact that cancer cells have increased basal level ROS than

normal cells are being explored extensively. Interestingly, in order to withstand the impact of increased oxidative stress, cancer cells elevate their endogenous antioxidant defense system. According to Prof Jim Watson, incurability of cancer partly results from a heightened antioxidant levels in the cancer cells which can block the effect of prooxidant therapy [120]. The impairment of antioxidant defense system therefore provides a new strategy to selectively target cancer cells.

As discussed in the preceding sections, one of the main mechanisms involved in NSCLC progression and chemotherapy resistance is constitutive overexpression of Nrf2 leading to activation of cellular antioxidant and xenobiotic defense system [98, 121]. A study reported Nrf2 overexpression and decreased Keap1 expression to be common abnormalities in NSCLC which associated with poor clinical outcome [122, 123]. Further, siRNA-mediated inhibition of Nrf2 was found to increase sensitivity of tumor cells toward chemotherapy in a NSCLC mouse xenograft model [123]. Therefore, targeting the Nrf2-/Keap1-mediated antioxidant defense system in NSCLC might be a promising strategy to control tumor growth and overcome therapy resistance.

To develop effective ROS-modulating therapeutic agents, a more patient-specific approach may be needed which would require knowledge of individual genetic variation in the antioxidant defense system. Many antioxidant and xenobiotic detoxifying enzymes have been reported to be polymorphic which definitely would have an impact on disease progression and drug response [118]. Hence, such background patient information will help clinicians select patients who will be benefitted from treatment with redox-modulating agents.

In conclusion, the crucial role played by ROS in tumor progression and drug resistance is well proven. Therefore, manipulating ROS levels may have significant therapeutic implications. The dual role of ROS as cellular signaling and toxic molecules renders it to promote cancer development, on one hand while, on the other hand, causes damage to cellular macromolecules leading to apoptosis. This leaves scope for the development of both antioxidant and prooxidant-based therapeutic strategies against cancer. However, at present, there are a lot of discrepancies in designing anticancer therapies which involve either of the above therapeutic strategies. A promising new approach in this direction is to target the redox adaptation in cancer cells. This is especially important in the context of NSCLC where there is constitutive activation of Nrf2-/Keap1-mediated antioxidant pathway which correlated with poor patient prognosis. Moreover, in order to effectively and selectively kill cancer cells, it is important to comprehend and appreciate the multifarious redox adaptations in cancer cells, especially in cancer stem cells which are considered to play pivotal role in cancer relapse cancer and drug resistance.

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Lung Cancer: Old Story, New Modalities!

17

Urmi Chatterji

Abstract

Lung diseases, leading to lung cancer, are incommodious maladies often leading to dire patient prognosis and death all over the world. Lung cancer 5-year survival rate is only 15%. Death due to lung cancer alone is comparable to deaths due to breast, pancreas, colon, and oral cancers together. Lung cancer preferentially occurs in people who are 65 or older. A minority of patients diagnosed with the disease are younger than 45. Different treatment modalities have been implemented for treatment of lung disorders over the past three decades, and modernization of strategies has brought hope for the affected. Counteracting oxidative stress has been of major concern for treating lung cancer, though with limitations. Cancer stem cells, a subpopulation of cells within a tumor, are believed to confer resistance to standard chemotherapy and radiotherapy. Several studies have investigated the specific mechanisms of tumor recurrence driven by cancer stem cells; however, oxidative stress and cellular metabolism are often neglected attributes. Metabolism of cancer stem cells is still poorly understood and constitutes a promising area in cancer research. Distinct metabolic phenotypes in these cells depend on the type of cancer, the model system used, or the experimental design; however, controversies still need to be resolved. Specific alterations in metabolite levels and metabolic enzymes that regulate cancer stemness need to be verified, as does the long noncoding RNAs which modulate the expression of several factors which modulate oxidative stress. Identifying the role of metabolism in conferring resistance to therapy, mostly by the presence of cancer stem cells, is an opportunity for designing novel therapeutic targets, which will eliminate this resistant population, and additionally eradicating the whole tumor to a relapse-free condition and better patient prognosis.

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17.1 Introduction

Cancer continues to be a major cause of illness along with immense personal, social, and economic burden. The numbers of cases increase yearly due to modern screening and enhanced detection methods. However, deaths arising from cancer are typically due to malignant and metastatic disease. Malignant tumors are capable of invading and spreading to surrounding tissue and to distant body sites, in a process known as metastasis, through the circulatory or lymphatic system, giving rise to secondary tumors [1]. At initial diagnosis, more than 50% of patients will have clinically detectable metastatic disease [2], rendering challenging treatment and unfavorable prognosis. Metastatic tumors are largely responsible for cancer mortality; therefore, early tumor detection can improve patient prognosis and enhance survivability [3].

Disorders of the lung are extensive. It includes pneumonia, lung abscess, pulmonary fibrosis, tuberculosis, cystic fibrosis, pulmonary hemorrhage, and aspergilloma, to name a few. The quality of air we breathe determines the health of the lungs and definitely, with the increased incidence of lung disorders, the quality has been severely compromised. The most fatal outcome is lung cancer, which contributes to the highest number of deaths due to cancer all over the world, and the 5-year lung cancer survival rate is only 15% [4]. Sadly enough, even the nonsmokers are not spared, since air pollution is the most significant threat to health worldwide. Lung cancer is the leading cause of cancer-related deaths among men (26% of all deaths) and women (25% of all deaths), mostly occurring in people at an average age of 70 years [5].

A total of 10,000 l of air enters the lungs every day from which 420 l of oxygen is used for human survival and function. The quality of air we breathe therefore determines the health of the lungs, as well as other organs. Indeed clean air is a basic requirement of good human health and well-being. However, air pollution continues to pose a significant threat to health worldwide. The World Health organization (WHO) reported that in 2012 around 7 million people died due to exposure to air pollution, substantiating that air pollution is now the world's largest single environmental health risk [6]. More significant was the fact that household air pollution was the leading risk factor for poor health in South Asia. The quality of air inside homes, public buildings like offices, schools, day care centers, malls, and healthcare facilities where people spend a large part of their life is an essential determinant of healthy life and well-being. Hazardous substances emitted from indoor equipment due to human activities, such as combustion of fuels for cooking or heating, also lead to a broad range of respiratory disorders.

Air Pollution and Impact on Health in India As one of the world's most populous countries, India too has seen a significant rise in incidence of respiratory diseases such as asthma and COPD over the last decade. COPD in India has been estimated to be about 15 million (males and females contributing to 9.02 and 5.75 million, respectively), causing about 500,000 deaths per year. Use of spirometry to define COPD has shown a twofold higher prevalence of COPD [7]. India has 20–28 million asthmatics, with a prevalence of 10–15% among children, aged 5–11 years. Indian study on epidemiology of respiratory symptoms, asthma and chronic bronchitis in adults, a landmark epidemiological study conducted by the Indian Council of Medical Research, GoI, found the overall prevalence of asthma and chronic bronchitis to be 2.05% in adults of 15 years of age and 3.49% in adults aged more than 35 years, respectively. The national burden of asthma and chronic bronchitis in this study was estimated at 17.23 and 14.84 million, respectively. Epidemiological survey in hospitals over three decades (1979–2009) in children below 18 years showed a steady rise in asthma prevalence from 9% to 25.5%, attributed not only to genetic predisposition but also, more significantly, to urbanization, air pollution, and environmental tobacco smoke. Indeed, the rising prevalence of asthma, allergies, and COPD over the past few decades has been attributed to an increase in environmental pollution – a price being paid for rapid industrialization, socioeconomic development, urbanization, and changing lifestyles [8].

17.2 Lung Disorders of Concern

Chronic Obstructive Pulmonary Disease (COPD) COPD is characterized by progressive and irreversible air flow obstruction. Worldwide, COPD affects an estimated 380 million people [9] and is the third leading cause of death. Although often referred to as a "disease," COPD encompasses a spectrum of disorders with two predominant phenotypes: chronic bronchitis and emphysema. Chronic bronchitis predominantly affects the airways and is characterized by mucus hypersecretion, which functionally leads to airway obstruction and a productive cough [10]. In contrast, emphysema is an anatomical condition characterized by the permanent destruction of the alveolar walls, resulting in parenchymal destruction [11]. Despite the fact that chronic bronchitis and emphysema can present independently of one another, it is now widely accepted that most individuals with COPD often exhibit characteristics of both chronic bronchitis and emphysema to varying extents [10].

Although COPD is predominantly caused by smoking cigarettes, other environmental risk factors include inhalational exposure to ambient (e.g., air pollution) and occupational (e.g., coal mines, pulp, and paper manufacturing) toxicants [12–14]. Moreover, only 15–20% of smokers go on to develop COPD, indicating that factors beyond exposure to inhalational toxicants are important. These include genetic factors [15] and the only established genetic risk factor for COPD is a deficiency of alpha-1 antitrypsin [16], which occurs in 3–10% of individuals with COPD [17].

However, COPD is a heterogeneous disease with many interrelated pathogenic mechanisms including inflammation, oxidative stress, and cell death; there is also evidence demonstrating that the aryl hydrocarbon receptors (AhR) attenuate several of these mechanisms that ultimately contribute to the development of this disease.

A hallmark of the emphysema component of COPD is the loss of lung structural cells [18]. This includes loss of alveolar epithelial cells responsible for gas exchange and fibroblasts that synthesize the extracellular matrix necessary for lung structure and elasticity. Cigarette smoking induces apoptotic cell death in all major lung cells, including bronchial and alveolar epithelial cells, fibroblasts, endothelial cells, and airway smooth muscle cells [19, 20]. Furthermore, humans with emphysema exhibit heightened pulmonary apoptosis [21]. Experimentally, intratracheal injection of the apoptotic protein cleaved caspase-3 induces epithelial cell apoptosis and airspace enlargement in the murine lung [22], consistent with the notion that lung parenchymal destruction is linked to cell death.

Smoking additionally promotes pulmonary inflammation in that the number and proportion of immune cells in the lung shifts in response to cigarette smoke exposure. Human cigarette smokers have heightened levels of pulmonary neutrophils, macrophages, and $CD8^+$ T lymphocytes [23]. In COPD subjects, the quantity of these cell types is further increased compared to smokers without COPD [24]. Although macrophages and $CD8^+$ T lymphocytes are the predominant inflammatory cell types in the lungs of humans with COPD, neutrophilia is also common [25]. Elevated neutrophil numbers are also seen in the bronchoalveolar lavage of mice exhibiting a COPD-like phenotype [26].

Oxidative Stress in Lung Disorders Oxidative stress is another mechanism linked to COPD pathogenesis [27]. In the healthy lung, reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anions, and hydrogen peroxide, are counterbalanced by the production of endogenous antioxidants, including superoxide dismutase (SOD), catalase (CAT), and the glutathione (GSH)/glutathione peroxidase system. When ROS production exceeds the capabilities of these antioxidant defenses, oxidative stress ensues. Inhalational exposure to toxicants results in heightened ROS production in the lungs. Additionally, ROS production by recruited immune cells, like neutrophils and macrophages, represents another major oxidant source [28]. There is evidence to support that AhR attenuates cigarette smoke-induced oxidative stress. Additionally, AhR-deficient mouse lung fibroblasts exhibit an impaired induction of the antioxidants following in vitro exposure to cigarette smoke relative to AhR-expressing mouse lung fibroblasts. However, factors other than strictly cigarette smoking likely contribute to the oxidative stress observed in COPD, such as greater lipid peroxidation is observed in individuals with COPD that have never smoked relative to subjects without COPD. Moreover, COPD subjects also have significantly reduced antioxidant expression (e.g., SOD and GSH levels) relative to smokers without COPD [29].

17.3 Lung Cancer

Tobacco smoking is the most important risk factor for lung cancer and by itself is responsible for over 80% of all lung cancer cases. Interestingly, for lung cancer, women who smoke like men die like men. Since the 1960s, convergence of smoking patterns among men and women have led to the convergence of relative risks for men and women [30]. It was estimated that the rate of death in current smokers was about three times that among those who had never smoked; the rise in mortality among smokers was mostly because of neoplastic vascular respiratory diseases [31]. Lung cancer is a progressive disease and advances through various stages from a benign to a malignant state (Fig. 17.1).

Several other factors contribute to the development of lung cancer, such as environmental exposure to radon and asbestos; indoor emission of fuel burning; some metals such as chromium, cadmium, and arsenic; air pollution; and some organic chemicals [32]. Studies in different countries have reported that particulate matter air pollution contributes to the incidence of lung cancer [33]. Since these risk factors may be prevented by restricting smoking and adopting clear air initiatives, appropriate preventive strategies will therefore reduce lung cancer incidence and mortality.

17.3.1 Types of Lung Cancer

Lung cancer is divided into two major types based on histopathological observations. The two general types of lung cancer (Fig. 17.2) include:

Small cell lung cancer: Small cell lung cancer, the less common of the two, accounts for almost 15% of lung cancers and occurs almost exclusively in heavy smokers.

Non-small-cell lung cancer (NSCLC): Non-small-cell lung cancer accounts for 85% of lung cancers. NSCLC can be categorized into, generally, adenocarcinoma (AC 40%), squamous cell carcinoma (SQCC 25–30%), large cell undifferentiated carcinoma (10–15%), mixed subtypes (adenosquamous), and the far less common sarcomatoid carcinoma [14].

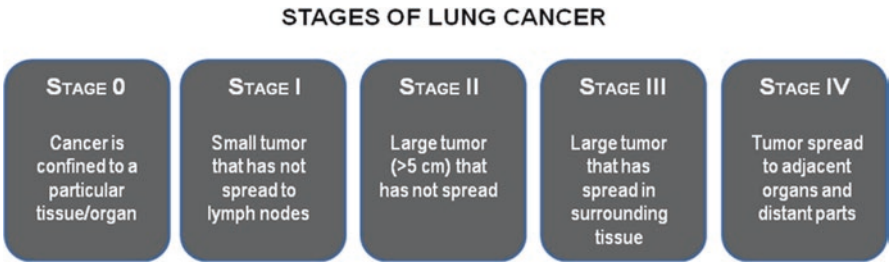

Fig. 17.1 Incidence of small cell and different non-small cell lung cancers

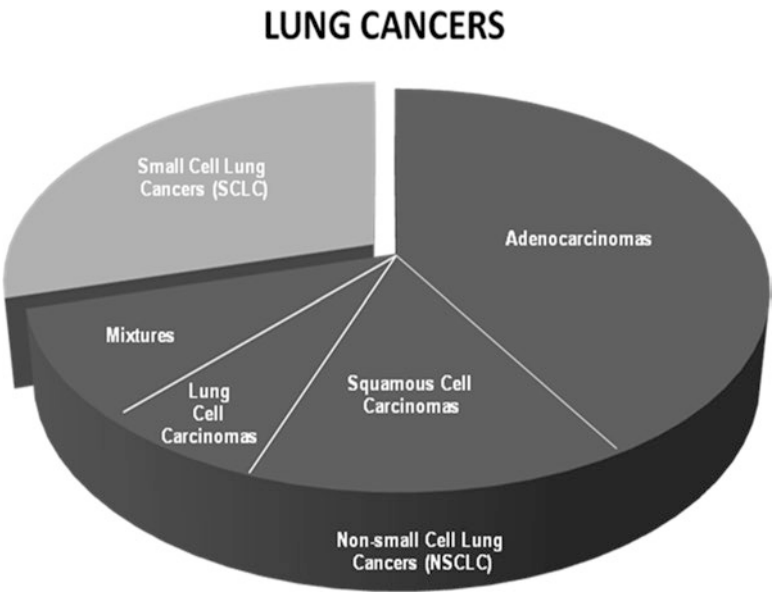

Fig. 17.2 Incidence of small cell and different non-small cell lung cancers

17.3.2 Lung Cancer Heterogeneity

Cancers are composed of mixed cell populations. They have diverse phenotypic, morphological, genotypic, and epigenetic characteristics. Tumor heterogeneity is observed in different patients with the same tumor subtype (interpatient heterogeneity), among tumor cells within one host organ (intratumor heterogeneity), between the primary and the metastatic tumors (intermetastatic heterogeneity), and among tumor cells within the metastatic site (intrametastatic heterogeneity) [34]. Heterogeneity is not only determined by intrinsic cell mechanisms but also by the dynamic tumor microenvironment (e.g., fibroblasts, angiogenesis, immune parameters) [35]. Heterogeneity of lung cancer is also due to altered metabolic activity at the macro-level as well as at the single-cell level [36, 37]. Genome sequencing in NSCLC has identified several mutations in subclonal fractions which increase with tumor grade [38] and, in primary tumors, predict early postsurgical relapse [39]. For example, smokers have tenfold more mutations than nonsmokers, with distinct driver mutations, such as EGFR versus KRAS [40]. In addition, chromosomal instability, which drives intratumor heterogeneity, is associated with anticancer drug resistance and poor outcome in NSCLC. Taken together, such diverse levels of heterogeneity in tumors are of clinical relevance in tumor progression, treatment response, and relapse. One of the main drivers of tumor heterogeneity and disease relapse is cancer stem cells, which create and maintain a tumor cell hierarchy [41].

17.4 Prevention: Novel Technologies, New Promises

Till date, there are limited ways to prevent lung cancer, but risk can be reduced if one:

(i) Doesn't smoke ever or stops smoking.
(ii) Avoids secondhand smoke.
(iii) Tests for radon levels at home.
(iv) Avoids carcinogens at work.
(v) Eats a diet full of fruits and vegetables.
(vi) Exercises regularly.

Remarkable progress has been made in evaluating the molecular abnormalities underlying lung cancers in the last two decades, which have led to the development of targeted therapies, including a new generation of immunotherapy, resulting in an improvement of clinical outcomes. However, major challenges still remain unresolved, including (a) the identification of new drugs and of combination therapies based on rational pharmacological associations; (b) the detection of new biomarkers, which would be capable of predicting the clinical responses to immunotherapy; (c) the recognition of new driver mutations in order to expand the population of patients who can benefit from targeted therapies, particularly for NSCLC; and (d) a better understanding of the cellular and molecular mechanisms underlying resistance to targeted therapies, so as to prevent and eventually to bypass such resistances with the identification of more active single or drug combinations. The development achieved and those that could be made will necessitate an integrated view of various aspects of lung cancer at cellular and molecular level, involving analysis of clonal evolution of tumor cells during spontaneous disease progression and under effect of various treatments, analysis of genetic and epigenetic abnormalities of tumor cells, identification of cells capable of initiating and maintaining the tumors, and development of suitable animal models simulating human tumors.

17.4.1 The Lung Cancer Genome: Identifying Targets in NSCLC

A complex pattern of driver mutations, including 200 non-synonymous mutations, have been identified from the whole genome sequencing of lung cancers, which can distinguish smokers from nonsmokers and predict patient outcome [40, 42, 43]. KRAS mutations are seen to occur in 25% of NSCLC. Despite preclinical efforts, till date there are no clinically approved drugs that effectively target KRAS. In lung adenocarcinoma, 10–15% mutations in the epidermal growth factor receptor (EGFR) occur which can be effectively targeted with tyrosine kinase inhibitors, such as erlotinib, gefitinib, and afatinib, and monoclonal antibodies, such as

cetuximab. In squamous cell carcinomas (SQCC NSCLC), most tumors carry mutations in TP53, RB1, and CDKN2A. Interestingly, SQCC differentiation genes such as SOX2 and TP63 (TP53 homolog) are commonly altered, in addition to amplification of EGFR, FGFR1, and PI3K pathways [44]. Antiangiogenic therapies aimed at the vascular endothelial growth factor (VEGF) or its receptor (VEGFR) may be developed as alternative approaches to target the tumor microenvironment.

17.4.2 Drug Resistance in Non-Small-Cell Lung Cancer: A Paradigm for Treatment Failure

One of the major causes for therapeutic failure in lung cancer, leading to tumor recurrence and disease progression, is drug resistance. Intrinsic mechanisms of resistance include activation of pro-survival and anti-apoptotic pathways, changes in the expression of drug transporters, as well as influences of the tumor microenvironment. It is evident that tumors are composed of a heterogeneous population of cells with different genetic, epigenetic, and phenotypic characteristics resulting in diverse responses to therapy and trigger the appearance of resistant clones. This heterogeneity is implicated to subpopulations of cells within a tumor, called cancer stem cells (CSCs), which are highly self-renewing, have tumor-initiating capabilities, and retain the ability for lineage-specific differentiation. CSCs have been identified in NSCLC and have been implicated to be the major players in conferring chemo- and radiotherapy resistance. Pathways controlling stem cell are frequently deregulated in cancer and are associated with recurrence after treatment. Different signaling pathways, such as NOTCH, HEDGEHOG, WNT, and TGFβ, contribute to stem cell maintenance in lung cancer, and targeting these pathways to overcome resistance to chemotherapeutic and targeted agents is of immense importance [45]. Mechanisms of resistance include (i) alteration of the drug target such as alternative splicing, resistance mutations, and gene amplification, as well as (ii) activation of alternative oncogenic pathways. Tumor cells harboring such resistance-creating mutations can be present at the onset of treatment (primary resistance) or emerge during treatment (secondary resistance). Other mechanisms of resistance, such as metabolic inactivation, drug interactions, or inefficient drug delivery, also play an important role in therapeutic outcome.

17.5 Lung CSCs

Cancer stem cells (CSCs) are tumor-initiating cells responsible for cellular hierarchy and tumor heterogeneity. They are maintained by means of self-renewal and are capable of multipotent differentiation [46]. Tumor heterogeneity arises due to the plasticity of CSCs which allows them to differentiate reversibly into different types of cells under specific environmental cues [47]. Furthermore, during disease progression, differentiated cancer cells may be reprogrammed to a more stem cell-like phenotype under specific conditions (e.g., hypoxia induces OCT4 and NANOG) [48] and eventually contribute to tumor recurrence. Furthermore, chromosomal

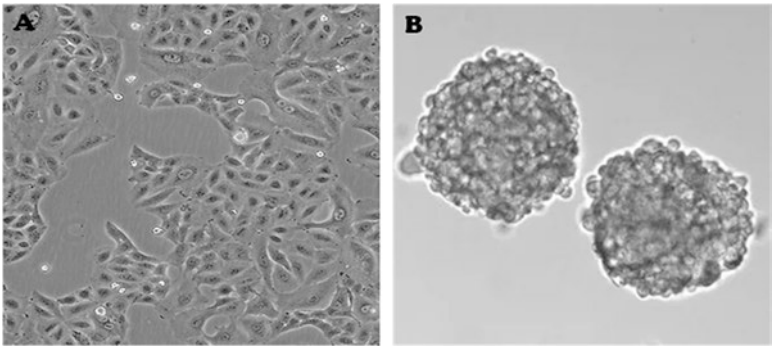

Fig. 17.3 Lung cancer cells grown as adherent cultures (**a**) and as tumorspheres (**b**) harboring the lung cancer stem cells

instability along with external environmental factors may lead to CSC heterogeneity and metastasis. In contrast to adherent cancer cells, CSCs characteristically grow as spheres when plated in nonadherent serum-free conditions (Fig. 17.3).

Cancer stem cells have the ability to form colonies in soft agar and are highly tumorigenic in vivo [49]. They can be identified and isolated by virtue of Hoechst dye efflux (the side population, SP), immunophenotyping, or ALDEFLUOR assay, using specific markers and flow cytometry. CSCs express multidrug ATP-binding cassette (ABC) transporters and are resistant to multiple chemotherapeutic agents [50]. One of the best characterized CSC markers for NSCLC is the CD133, a cell surface protein. CD133-expressing lung cancer cells are self-renewing tumor cells that are present in low numbers in human NSCLC but are highly tumorigenic. Moreover, when $CD133^+$ CSCs differentiate, their $CD133^-$ progeny is no longer tumorigenic [51]. It therefore seems plausible that combination therapy specifically targeting the cancer stem cells along with the non-stem cells would be required to successfully eradicate cancer [52]. It is therefore plausible to target normal stem cell pathways such as WNT, NOTCH, and HH, which are deregulated and mutated in cancer and CSCs [53].

17.6 Pathway-Targeting Inhibitors

Cell cycle arrest has been a major criterion for drugs developed from natural products. Taxanes, like docetaxel and paclitaxel, and vinca alkaloids, such as vinblastine, vincristine, and vinorelbine, are known to interfere with microtubule function either by preventing depolymerization (taxanes) or by disrupting microtubule formation (vinca alkaloids), ultimately blocking cell cycle progression through mitosis. However, many such drugs often prove to be ineffective due to overexpression of ABC drug transporters, multidrug resistance 1 (MDR-1) protein, or P-glycoprotein (P-gp), which confer resistance of the cells toward taxanes and vinca alkaloids [54]. A direct regulator of MDR-1 protein is microRNA miR-451.

Overexpression of miR-451 induces chemosensitivity, while loss of miR-451 results in taxane resistance in NSCLC. NOTCH1, through the activation of AP1, an early transcription factor necessary for progression through G_1 phase, downregulates miR-451. Therefore, inhibition of NOTCH using gamma-secretase inhibitors increases miR-451 and reduces MDR-1, thereby sensitizing tumors to taxane-based treatment [55]. MiR-451 is downregulated in docetaxel-resistant lung cancer cell lines, causing inactivation of glycogen synthase kinase 3 (GSK-3β), Snail activation, and epithelial-to-mesenchymal transition (EMT) [56]. Studies have shown that a γ-secretase inhibitor, BMS-906024, could sensitize the NSCLC cell lines to paclitaxel, and both drugs synergized preclinically by targeting the paclitaxel-induced increase in NOTCH1 in a TP53-dependent manner [57].

It is well established that factors from the stroma, the immune system, and cancer cells, which are secreted by drug-resistant lung adenocarcinoma cells, may develop acquired drug resistance by promoting cell proliferation and evading apoptosis. Glucose deprivation, however, reduces the secretion of some of these cell-growth-promoting factors. For instance, FOXO3a promotes cross-resistance to 5-fluorouracil and cisplatin via glycolysis-mediated upregulation of ABCB1. Repression of cellular energy supply by targeting glycolysis may, on the other hand, overcome acquired drug resistance [58]. Genes that encode proteins involved in the uptake of glucose and in glycolysis – conversion of lactate to pyruvate – and those that repress the tricarboxylic acid cycle are found to be direct transcriptional targets of NOTCH signaling. Upregulation of NOTCH in breast cancer cells leads to increased glycolysis via activation of the PI3K/AKT pathway, whereas endogenous NOTCH signaling reduces mitochondrial activity and induces glycolysis in a TP53-dependent manner [59].

The hedgehog signaling components are also implicated in the etiology of lung cancer. Sonic hedgehog (Shh) components are known to be active during the early stages of embryogenesis and organogenesis [48, 60]. It is the major regulatory pathway which designs limb formation and number of fingers and induces the dorsoventral axis on the neural crest [61]. In the lung, Shh pathway is involved in bronchial budding. The lung epithelial cells secrete Shh which then have a paracrine effect on mesenchymal cells, acting as a spatial regulator of bronchial bud formation. Shh pathway inhibition using mice models ($Shh^{-/-}$, $Ptch1^{-/-}$, $Gli2^{-/-}$, and $Gli3^{-/-}$ knockout) induces severe lung malformations, with hypoplasia and tracheal malformations and often nonviable phenotypes [62, 63]. Several lung diseases were thus found to be related to activation of Shh, such as lung fibrosis, which is characterized by epithelial lesions and shows an induction of epithelial-to-mesenchymal transition (EMT) [64]. In idiopathic lung fibrosis, hedgehog pathway components, such as Shh, Smoothened (Smo), Patched (Ptch), and Gli1, are found to be overexpressed in lung tissues [65, 66]. High levels of Gli1 and Shh protein expression are observed in SCLC cell lines and patient samples [67]. Inhibition of Smo has been shown to inhibit tumor growth in vivo [68]. Recent studies confirmed the importance of the Shh-dependent activation of the pathway in SCLC [69]. Szczepny et al. showed that Shh is necessary for the progression of SCLC in mice. In NSCLC, Shh pathway is activated, with a good correlation between each protein, suggesting a canonical

activation of Shh pathway [70]. Therefore, an intricate relationship exists between the Shh pathway and lung disorders and can eventually be a target for therapeutic intervention.

CSCs, which are contributors to tumor initiation, proliferation, and recurrence, maintain a low proportion in tumors [71]. They are responsible for resistance to anticancer therapeutics and are responsible for spreading of tumor cells and metastasis through EMT. Several signaling pathways are closely associated with CSCs; in particular the Hh, wingless-type (Wnt), and Notch pathways are mainly activated in CSCs and responsible for their maintenance. Stem cell characteristics and expression of signaling pathway components were concomitant in human lung fibroblasts isolated from parenchymal tissues of nonsmokers/non-chronic obstructive pulmonary disease (COPD), smokers with non-COPD, and smokers with COPD, who were undergoing surgery for lung tumor resection [72]. Additionally, lung fibroblasts expressed differentiation ability, mesenchymal stem cell markers, and immunosuppressive potential; these properties were however altered in lung fibroblasts from smokers and COPD patients. In NSCLC, Shh pathway actively contributes to tobacco-induced oncogenesis and inhibition of the Shh pathway can prevent the tobacco-induced tumor phenotype in cell lines [73]. Similarly, nicotine exposure can activate the Shh pathway [74], and vismodegib treatment of NSCLC cell lines completely inhibited tumor xenografts in nude mice [75].

17.7 Current Imaging Modalities and the Need for Personalized Imaging

Presently, medical imaging modalities rely on ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI), for visibility of tumors when compared to adjacent tissues. To assess tumor biology, positron emission tomography (PET) has typically incorporated radiolabeled fluorodeoxyglucose (FDG) due to the increased glycolytic rate, the Warburg effect, exhibited by the majority of malignant tumor cells [76]. PET imaging provides information on anatomical location of tumors by exploiting the increased metabolic rate of tumors and the use of radiolabeled FDG readily explains this fundamental principle. Despite advances in PET, there are challenges in molecular imaging. A major factor in molecular PET imaging is that it relies on the uptake of FDG to measure glucose metabolism by pathological cells. While pathological cells have an increased uptake of FDG, physiological uptake by normal tissues or inflammatory cells such as macrophages is highly variable, and in some instances, the accumulation cannot be predicted [77]. As FDG can accumulate in normal tissues or inflammatory cells, thus decreasing availability of tracer uptake by pathological tissue, this can also lead to false-positive detection and misdiagnosis. Furthermore, FDG uptake relies on malignant cells to be metabolically active. However, some tumor cells can enter a dormant state [78]. To improve the sensitivity of diagnostic imaging, agents are being developed to target malignant cells or their products, to enable molecular imaging of a tumor.

17.7.1 Peptides, Peptidomimetics, and Aptamers for Effective Detection

Peptides are short organic polymers. Amino acids are conjugated to each other and form a bond between multiple amino acids called peptides. Peptides may possess their own biological function or be a structural and functional part of protein molecule. Artificial peptides are also available with covalent bond between amino acid molecules [79]. Peptides are produced relatively easier than mAbs, are quicker to penetrate tumor sites, and are rapidly excreted from the body [80, 81]. Peptides can be used for the delivery of cytotoxic drugs and radioisotopes, as well as vaccines and hormones. Common difficulties of using peptides as a drug delivery carrier are limited stability due to proteolysis by peptidases, low oral bioavailability, meager transport properties through cell membranes, rapid excretion, and reduced target specificity due to flexible nature of peptides. In contrast, peptidomimetics (also called peptide mimics) can be constructed by the modification of an existing peptides or artificial introduction of alpha and beta amino acids in peptide structures. Peptidomimetics have better transport properties through biological membranes, fewer cross-target interactions, resistance to immune responses, and improved resistance to degradation by peptidases [82, 83]. [^{68}Ga]Ga-NODAGA-THERANOST™ is a αvβ3 integrin antagonist first used in humans for lung and breast cancer diagnosis.

Aptamers, also known as "chemical antibodies," are short (20–100 bases) single-stranded RNA (ssRNA) or DNA (ssDNA) oligonucleotides that bind to targets with high affinity and selectivity [84, 85]. Aptamers have the ability to fold into three-dimensional structures and bind to their target in a similar manner to their antibody protein counterpart via shape recognition [86]. In comparison, aptamers hold many advantages over antibodies. Unlike antibodies which cannot regain function after being denatured, aptamers are more stable and resistant to changes in pH and temperature, which also enables them to be easily chemically modified [87]. Antibodies require in vitro or in vivo production which can increase variation between batches, whereas this variation is reduced in aptamers as they are synthesized chemically. Due to their nucleic acid composition, aptamers are generally nonimmunogenic and nontoxic. Lastly, an important advantage of aptamers is their size (5–15 kDa) in comparison to large monoclonal antibodies (approximately 150 kDa) [88]. As aptamers are much smaller than antibodies, aptamers have superior tissue penetration (greater capabilities to be internalized by tumors) [89]. Furthermore, the smaller size of aptamers also enables them to bind hidden epitopes which cannot be accessed by the larger antibodies. Thus, given the numerous desirable properties exhibited by aptamers, the development of aptamers as molecular imaging probes is more promising than antibodies in diagnostic imaging.

Although cancer is a leading cause of mortality globally, early diagnosis and detection can improve treatment outcomes due to early surgical, curative intervention. The challenges lie in present medical imaging and diagnostic techniques in oncology. While current medical imaging modalities can identify tumor masses, they are unable to specifically detect micrometastases before their angiogenesis stage, due to the minimum number of cells required for detection. PET molecular

imaging has been able to improve detection of malignant cells; however, the typical use of FDG for pathological cells is nonspecific for a disease, as normal tissues can uptake FDG which increases the background signal relative to the tumor.

The prognosis of most lung cancer patients is quite poor with limited survival. Mostly, symptoms of stage-specific lung cancer are not evident. CT and some serum tumor markers, like carcinoembryonic antigen (CEA) and squamous cell carcinoma antigen (SCCA), have been used for early diagnosis of NSCLC. However, cumulative radiation damage and low sensitivity and specificity have limited traditional detection methods. Therefore, novel biomarkers with high sensitivity and specificity are required for precise molecular diagnosis and prognosis; this can be achieved through a more thorough understanding of the molecular mechanisms of NSCLC.

17.7.2 Immunotherapy: A Remarkable Boon

Superior to chemotherapy, remarkable patient survival has been observed using immune checkpoint inhibitors as first-line treatment in both squamous and non-squamous NSCLC [90]. Checkpoint inhibitors block the antitumor adaptive immune response by targeting the CTLA4 receptors which are expressed on immune and tumor cells and by suppressing the cytotoxic T-cell response, a theme which fetched the Nobel Prize in Medicine in 2018. Although several factors that would enable clinicians to resolve the response to checkpoint inhibitors remain to be discovered, a high mutation load creates immunogenic tumors and is strongly associated with response to checkpoint inhibitors [91]. Based on promising results with immunotherapy, treatment with either platinum-pemetrexed or anti-PD1 (nivolumab) + anti-CTLA4 (ipilimumab) is being offered to naïve MPM patients [92] (Fig. 17.4). Unfortunately, many NSCLC patients remain unresponsive to such immunotherapies, indicating resistance to checkpoint inhibitors [93]. Since lung cancer is highly heterogeneous disease at the genetic, epigenetic, and metabolic levels, it is imperative that personalized medical approaches can provide a more comprehensive treatment strategy for patients with lung cancer.

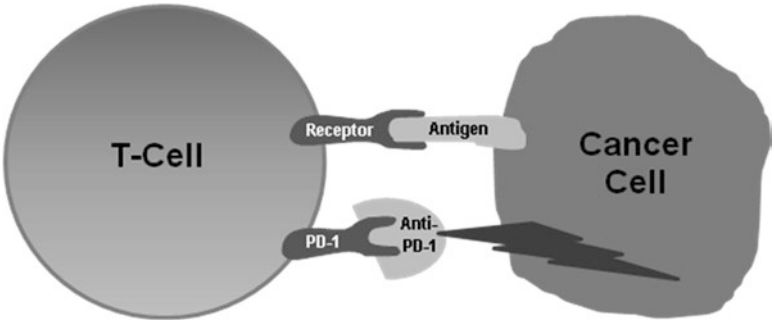

Fig. 17.4 Immunotherapy for lung cancer cells

17.7.3 LncRNAs Related to NSCLC Diagnosis and Therapy

Although current markers for the diagnosis of NSCLC are primarily proteins, relatively stable noncoding RNAs (ncRNAs) are prevalent in various body fluids [94], and their characterization is an emergent area of research for clinical diagnosis. The stability of long noncoding RNAs (lncRNAs) is similar to mRNA, while their tissue specificity is higher than mRNA. LncRNAs are detected in various body fluids such as blood, urine, and saliva and may serve as appropriate clinical indicators [95]. LncRNAs are known to act as tumor diagnosis biomarkers since many abnormal lncRNAs have been identified in NSCLC. For example, MALAT1 has been evaluated as a potential biomarker in body fluids and is associated with lung cancer. The expression of plasma MALAT1 could distinguish 45 NSCLC patients from healthy controls [96] and is considered a promising diagnostic biomarker due to its stability, high specificity, and minimal invasiveness. In addition, exosomal MALAT1 expression was positively associated with lymphatic node metastasis and TNM staging [97].

Similarly, the lncRNAs HIF1A-AS1 and XIST, which show enhanced expression in lung cancer, play significant regulatory roles in tumor pathology in contrast to the diagnostic values of their proteins in NSCLC, since they show significantly lower expression in serum. Furthermore, the combination of HIF1A-AS1 and XIST had higher diagnostic value [98]. Consequently, scientists selected 21 known lncRNAs as potential targets in order to improve diagnostic efficiency [99], such as NEAT1, ANRIL, and SPRY4-IT1, since their circulating levels were significantly higher in plasma samples of NSCLC patients compared to control set. A combination of novel lncRNA biomarkers could therefore have great diagnostic value for NSCLC detection [100]. Using gene microarray, Zhao et al. identified 72 lncRNAs to distinguish between lung squamous cell carcinoma (lung SCC) and lung adenocarcinoma [101], whereas White et al. discovered 27 lung cancer-associated lncRNAs as markers for the differential diagnosis of lung SCC and lung adenocarcinoma [102]. Since lncRNAs promote proliferation and cellular growth, have significant functions in epigenetic regulation, and result in uncontrolled and progressive tumor growth and metastasis, combination of multiple lncRNAs is effective in the diagnosis of NSCLC because of their specificity and sensitivity.

The present treatment regimen for NSCLC includes surgical excision, chest radiotherapy, and chemotherapy. However, these therapies are limited in their approach to cure cancer due to their poor therapeutic efficacy. Novel approaches are therefore required to be explored and applied for improving patient survivability and quality of life [103]. Significantly, deregulated lncRNAs are now being associated with many treatments, including molecular-targeted therapy and chemotherapy. For these reasons, lncRNAs are used as new therapeutic targets for NSCLC by sensitizing cancer cells to chemotherapeutic drugs [104].

17.7.4 RNAi-Mediated Gene Silencing Therapy

RNA interference technologies, such as siRNA, shRNA, and antisense oligonucleotides, are the most credible approach for selectively inhibiting target lncRNAs.

LncRNA-targeted RNAi has shown to be effective in cell lines; however, stable conditions are imperative to carry the siRNA to their targets in vivo. Accordingly, several lncRNAs have been recognized as prospective therapeutic targets. For example, silencing HOTAIR through RNAi reduced invasiveness and viability of lung, as well as breast and pancreatic cancer cells [105]. Additionally, it rendered lung adenocarcinoma cells resistant to cisplatin, by downregulating expression of p21 [106]. Similarly, shRNA-mediated knockdown of MALAT1 significantly reduced cell invasiveness and migration in NSCLC. However, the problems associated with delivery of siRNA and its off-target effects often limit its application, and therefore, in vivo inhibition of lncRNAs remains a challenge [107]. Recently, several strategies are being developed to overcome this shortcoming, such as conjugate-based delivery, polymer-based delivery, and lipid-based nanoparticle delivery [108, 109] for potential molecular treatment.

17.7.5 Antisense Oligonucleotide (ASO)-Based Treatment

ASOs are short single-stranded DNA which can be used to induce lncRNA degradation via RNaseH and are used for lncRNA regulation and silencing. In contrast to siRNA, ASOs show higher specificity and less off-target effects. In some instances, ASOs were shown to target MALAT1, inhibition of which undermined malignant phenotypes via cycle arrest in lung and cervical cancer cells [110]. Animals treated with MALAT1 ASO showed significantly reduced tumor volume and nodules in the lung compared to control ASO-treated animals. Thus, MALAT1 ASOs could prevent metastasis of NSCLC, rendering a novel therapeutic approach for treatment of patients with NSCLC [111].

17.7.6 Plasmid-Mediated Targeted Therapy

Plasmid-mediated targeted therapy is another fascinating approach for the treatment of cancer. H19 has high expression in lung and breast cancer, among others. The plasmid BC-819, which harbors the gene for the A subunit of diphtheria toxin, may be utilized for studying the tumor-specific expression of H19 lncRNA [112]. In addition to NSCLC, BC-819 also plays a role in the treatment of pancreatic, ovarian, and colon cancers [113]. LncRNAs are regarded as important regulators of diverse cellular processes, including cell growth, migration, stem cell maintenance, and apoptosis, and are involved in different signaling pathways [114]. Therefore, selectively targeting deregulated lncRNAs could provide a new therapeutic strategy for NSCLC treatment.

17.8 Altered Metabolism as a Hallmark of Cancer

A classical feature of cancer is the reprogramming of cellular energy metabolism, mainly used by cancer cells to sustain their highly proliferative status [115]. Under aerobic conditions, nonproliferating cells use glycolysis in the cytoplasm to form

pyruvate, which is eventually oxidized in mitochondrial oxidative phosphorylation (OXPHOS) to generate energy, in the form of adenosine triphosphate (ATP). Under anaerobic conditions, glycolysis-derived pyruvate is mainly directed to lactate production. On the contrary, cancer cells rely more on glycolysis for energy production even in the presence of oxygen. This phenomenon, first observed by Otto Warburg, was termed "aerobic glycolysis" or "the Warburg effect" [116, 117]. This metabolic adaptation, which generates ATP more rapidly, is, however, far less efficient than OXPHOS, finally resulting in abnormally high glucose uptake to sustain ATP production. The high requirement of glucose by cancer cells, mediated by the upregulation of glucose transporter 1 (GLUT1), was the main criteria behind development of fluorodeoxyglucose positron emission tomography (FDG-PET) techniques for cancer detection and disease monitoring even after treatment [118].

Cancer metabolism can be reprogrammed by oncogenes, tumor suppressors, and the tumor niche by directly regulating specific metabolic enzymes. Mutations in phosphatidylinositol 3-kinases (PI3K) are known to promote metabolic reprogramming by enhancing AKT (PKB) signaling, which drives glycolytic metabolism by increasing cellular glucose uptake and inducing activation of phosphofructokinase 1 [119–121]. Subsequently, AKT stimulates the mammalian target of rapamycin (mTOR) pathway, which eventually promotes glycolysis and the pentose phosphate pathway by regulation of hypoxia-inducible factors (HIFs) [122]. In the same way, deregulation of Myc in cancer induces glycolytic gene expression, enhanced glucose consumption, as well as biomolecule production, via nucleotide and lipid synthesis [123–126]. The tumor suppressor p53 has been shown to inhibit glucose transporters and activate the upregulation of TP53-induced glycolysis, eventually reducing fructose 2,6-bisphosphate levels and inhibiting PFK1 [127]. In addition, expression of the gene encoding the synthesis of SCO2, a cytochrome c oxidase protein, is stimulated by p53 [128]. Therefore, loss of p53 endorses a shift in ATP production from OXPHOS to glycolysis but renders cancer cells more sensitive to metabolic stress [128]. One of the mechanisms tumor cells use to trigger the switch from OXPHOS to glycolysis or from glycolysis in cancer cells to OXPHOS in CSCs is the upregulation of HIF-1α and HIF-2α under hypoxic conditions (Fig. 17.5). Specifically, HIF-1α is known to induce the expression of GLUT1 and glycolytic enzymes such as lactate dehydrogenase A with concomitant activation of pyruvate dehydrogenase kinase 1, a negative regulator of pyruvate dehydrogenase [64, 129, 130].

Moreover, stemness can be controlled by cellular metabolism. Interestingly, when reprogramming somatic cells into induced pluripotent stem cells, upregulation of glycolytic genes preceded expression of pluripotency markers, indicating that the metabolic switch from OXPHOS to glycolysis is an early event during stem cell reprogramming [131–132], and contrary to what was hypothesized, CSCs do not recapitulate the metabolic pattern of adult stem cells. In fact, depending on the type of tumor and stimuli from the tumor niche which trigger cell plasticity and metabolic reprogramming, CSCs may depend either on glycolysis or on OXPHOS for their maintenance [133]. CSCs generally show increased glucose uptake and lactate production, together with reduced mitochondrial respiration, compared to non-CSCs.

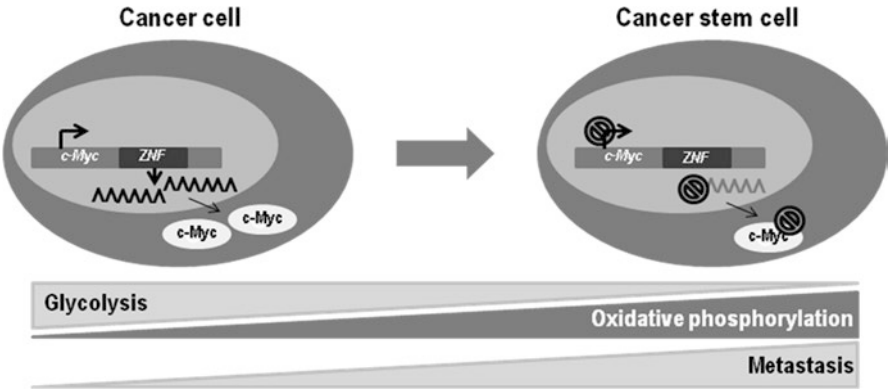

Fig. 17.5 Incidence of small cell and different non-small-cell lung cancers

Besides, CSCs demonstrate increased activity of key glycolysis enzymes, such as pyruvate kinase M2, LDH, and glucose-6-phosphate dehydrogenase. Subsequently, treatment with 2-deoxyglucose (2-DG), a glucose analogue that inhibits hexokinase 2, preferentially decreases the proliferation of CSCs compared with mature cancer cells, indicating that glycolysis is absolutely essential for CSCs [134].

17.9 Metabolism as a Therapeutic Target for CSCs

The main drivers behind drug resistance of CSCs are cancer cell plasticity and the acquisition of a quiescent state. It has been reported that residual dormant clones, which resist chemotherapy, can be activated under conducive conditions and cause tumor relapse [135–138]. Concomitant with characterization of the molecular mechanisms that govern stemness, novel therapeutic approaches are being developed and tested for the elimination of the CSC population. However, till date, no anticancer stem cell therapy has been successful in terms of effectiveness and specificity, in order to be approved for clinical use. Therefore, new therapies which would target the metabolic networks mediating cancer cell stemness would prove to be innovative and efficient strategies to target CSCs. Mouse models of cancer have also shown that targeting oxidative metabolism, the main source of energy for CSCs, sensitizes this population to known anticancer drugs, thus obliterating them for a more comprehensive cure.

The metabolism that contributes to the maintenance of CSCs is a promising concept for innovative therapeutic approaches which may provide novel, metabolism-related targets, albeit significant questions remain to be addressed. Specifically, the role which the tumor microenvironment plays to control metabolic plasticity of CSCs and elucidate whether CSCs themselves are metabolically heterogeneous and modulate tumorigenesis according to specific stimuli needs to be defined. It is therefore essential that future studies ameliorate the experimental conditions used so far and preserve the in vivo structure of tumors. Figure 17.6 summarizes the change in treatment strategies for better patient prognosis over several decades.

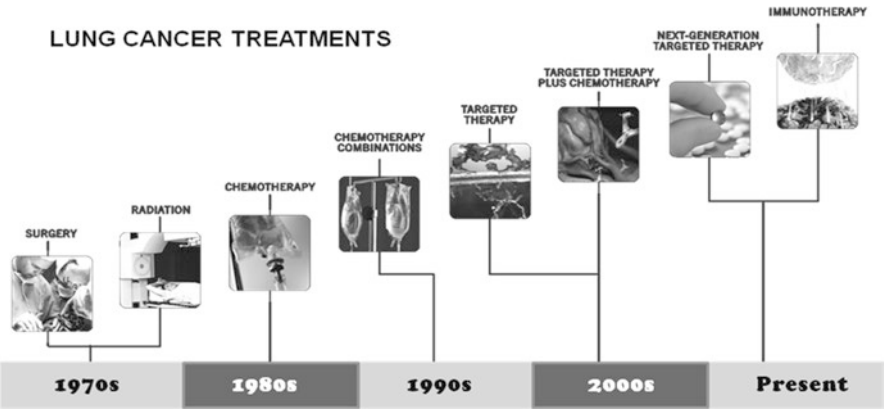

Fig. 17.6 Progress in different modalities for lung cancer treatment over the last five decades

17.10 Conclusion and Perspectives

Non-squamous NSCLC is the most common form of lung cancer and a deadly disease. Regardless of thorough information on tumor driver mutations and multimodal treatment regimens including surgery, chemotherapy, targeted agents, immune therapy, and radiotherapy, resistance to treatment by a tumor, eventually leading to disease recurrence, is common. NSCLC is a highly heterogeneous disease and continually attempts to survive by acquiring new favorable pro-survival conditions. There is growing evidence implicating CSCs in the process of relapse, along with the dynamically changing tumor microenvironment, which in turn results in tumor progression, metastasis, and therapeutic resistance. CSCs thus provide an interesting and invaluable therapeutic target to tackle tumor resistance.

Taken together, lung cancer may be classified as a complex heterogenous disease with interpatient, intratumor, and inter−/intrametastatic heterogeneity. Successful treatment options are therefore likely to arise from personalized precision treatment. Biomarkers will be necessary to advance from preclinical to clinical options in order to design reliable therapeutic strategies. Finally, although attention is being directed toward the development of specific drugs to target tumor progression and treatment resistance, efforts should also be made to identify synergistic interactions of inhibitors with clinically approved systemic treatments since such combinations will possibly lead to rapid clinical implementation and better patient prognosis in the future.

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Part IV

Prevention and Therapeutics

The Use of Ozone as Redox Modulator in the Treatment of the Chronic Obstructive Pulmonary Disease (COPD)

18

Emma Borrelli

Abstract

Chronic obstructive pulmonary disease (COPD) is a complex and progressive disease associated with an overproduction of reactive oxygen species (ROS), circulating pro-inflammatory cytokines, and acute-phase proteins. This generalized oxidative/inflammatory status is accompanied by a downregulation of the cellular antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2).Therapeutic agents that activate Nrf2 may have a pivotal role in the rebalance of the altered redox system. This chapter explores how the ozone can act as an endogenous redox modulator in the integrated treatment of COPD.

Keywords

Oxidative stress · Ozone therapy · Antioxidants · Reactive oxygen species · Chronic obstructive pulmonary disease

18.1 Introduction

There is no doubt that oxygen (O_2) has unique chemical and thermodynamic properties, and the evolution of aerobic respiration enabled cells to produce more ATP than anaerobic respiration [1]. However, in complex multicellular organisms, oxygen interaction and utilization via oxidative phosphorylation resulted in the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and superoxide anion radical ($O_2^{\bullet-}$). ROS are recently recognized, in submicromolar doses, as essential signaling molecules for the cells but, if abnormally present, are able to produce oxidative stress, defined, according to Halliwell,

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as “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage” [2–4].

The lung has in the human body the physiologic role to take the oxygen from the environment in proper amounts and to remove the carbon dioxide from the blood. This process needs a breathing volume of about 10,000 – 20,000 liters of air daily. Thus, the tissue in the lung is continuously exposed to oxygen, and, consequently, it is highly susceptible to injury mediated by oxygen-derived ROS [5].

Under normal conditon ROS concentrations in pulmonary system are keeping in a physiologic range through the activity of the antioxidants enzymes such as Superoxide Dismutase (SOD) and Catalase (CAT), in association with direct antioxidants such as Vitamin E, reduced Glutathione (GSH) and Ascorbate located both in the cell and in the epithelial lining of the lung [6–8].

In the lung, the altered redox balance may be caused by external factors such as environmental pollution or abnormal oxygen tension or by endogenous overproduction of oxidants, for example, during the activation of inflammatory cells after a tissue damage.

An increased number of evidences showed the implication of oxidant products in pulmonary pathologies like COPD, asthma, acute lung injury, pulmonary fibrosis, and lung tumor [9–11].

As regards COPD, it seems that both environmental exposures to noxious agents (mainly tobacco smoke and air pollutants) and the activation of the resident or inflammatory cells cause an increased amounts of reactive oxygen and nitrogen species (ROS and RNS) in the lung tissue. The abnormal levels of oxidants may have multiple consequences such as increased production of proteases, reduced defense mechanisms, and induction of inflammatory and growth factors and autophagic processes [12, 13].

More interestingly, COPD is now widely recognized as not simply an inflammatory/destructive lung disease but also a chronic oxidative systemic disease with extrapulmonary manifestations like cardiovascular disease, skeletal muscle dysfunction, osteoporosis, and neurological degenerations [14].

At this purpose, experimental works showed that during the inflammatory response in lungs affected by COPD, the pivotal mechanism for the systemic progression is the translocation of pro-inflammatory mediators like IL-6 and oxidants into the circulation [15].

Thus, it appears clear that, in COPD, a knock-on effect of persistent inflammation and prolonged oxidative stress in the lung cells begins locally but rapidly becomes a chronic challenge for the organism, if the process is not properly controlled or neutralized.

Several clinical and experimental studies also demonstrate the presence of an evident connection between the impaired antioxidant defense system controlled by Nrf2 and the development of oxidant-mediated lung diseases [16]. In the pulmonary tissue as well as in many other cells of various organs, the transcription factor called nuclear factor erythroid 2-related factor 2 (Nrf2) is expressed in large amounts. Nrf2 represents an important orchestrator in the induction of several enzymes implicated in the antioxidant defense. This factor under basal resting condition is tied in cytosol

to its repressor Kelch-like ECH-associated protein 1 (Keap1) and Cullin3-dependent E3 ubiquitin ligase. Keap 1 is a protein containing three main domains, and two of this domains have been shown to contain key reactive cysteine residues. The modification of Cysteine residues causes the disruption of the Nrf2-Keap1 complex and the activation of Nrf2. The free Nrf2 reaches the nucleus and binds, as heterodimer with small Maf proteins, to the antoixidant response element (ARE) in the upstream promoter region of antioxidant and phase II detoxifying enzymes genes and initiates transcription and expressions of these proteins [17, 18].

After chronic cigarette smoke, genetically Nrf2 null mice develop increased alveolar destruction, apoptosis, and inflammation with respect to mice with normal Nrf2 genetic expression. In addition, deprivation of Nrf2-positive regulator DJ-1 and posttranslational modifications of the Keap1-Bach 1 stability can produce the downregulation of Nrf2 and consequently a decrease in glutathione levels in macrophages and in the lung tissue of patients with COPD. DJ-1 is an Nrf2 stabilizer that plays a protective role against oxidative stress. The decrease of DJ-1 protein associated with a decrease in heme oxygenase 1, glutathione peroxidase, and NADPH quinone dehydrogenase 1 (NQO1) was found in lung tissues of patients with COPD and emphysema. Other findings reported a clear decline in Nrf2-associated detoxification enzymes in COPD further emphasizing the close relationship between lung damage and Nrf2 decrease [19–22].

Moreover, several studies reported a clear link between a dysfunction of Nrf2 signaling and the presence of COPD [23].

All together, these findings strongly suggest that molecules interacting with Nrf2 pathway through dissociation of the Nrf2-Keap1 complex, specifically via the oxidation of cysteine residues and the subsequent changes in the Keap1 conformation, could exert an important therapeutical role in COPD.

18.2 The Use of NRF2 Activators in COPD

On the basis of the previously exposed knowledges on the COPD pathogenesis, it has been suggested that all mechanisms able to decrease in the lung the ROS overproduction or to increase the amounts of antioxidants could be used as therapeutic agents in order to counteract the pulmonary damage and the consequent systemic polymorbidity [24, 25].

As support of its important role, it has been reported that Nrf2 activators may restore the altered phagocytosis of bacteria in alveolar macrophages and increase the defense system against viral attack in cells, potentially helping to reduce the viral exacerbations in patients affected by COPD [26].

Several exogenous antioxidants and Nrf2 activator agents have been tried in the prevention and treatment of COPD, including thiol compounds (N-acetylcysteine, carbocysteine), vitamins (vitamins C and E), and food-/diet-derived nutraceutical (curcumin, sulforaphane, lycopene) [27]. Unfortunately, none of the products could be shown convincingly to reduce the COPD progression and symptomatology. Probably the presence of several subphenotypes and chronic comorbidities in COPD

patients may reduce the probability of efficacy of a single therapeutic agent. As a result, further studies were performed on the combination of oral administration of several antioxidants able to increase both endogenous antioxidant enzymatic and nonenzymatic protection and/or widely cut down the formation of ROS in cells [28].

However, it has been reported that antioxidants, if exogenously oversupplied, can cause an "antioxidant stress" and damage the cells because ROS play a pivotal role during the redox homeostasis processes and the full suppression of this molecules in the cell would interrupt rather than prolong the normal function of the organism. It appears evident that ROS can become protective or harmful depending on the fine and complex equilibrium between ROS production and scavenging at the proper time and side [29, 30].

It seems that a more useful approach in the therapy of chronic oxidative diseases (like COPD) should be the endogenous upregulation of Nrf2 and a consequent increase of antioxidant and detoxifying enzymes without completely neutralizing the presence of oxidants as essential intracellular signal molecules.

To better achieve the therapeutical effect, the oral substances able to activate the Nrf2 pathway were used in experimental and clinical trials at high dose for months. Unfortunately Nrf2 activation, if prolonged and excessive, may have also detrimental effects. For example, overexpression of Nrf2 could promote cancer cells and also protect the cancer cells from potential toxic effects of chemotherapy in lung cancer. These negative effects of Nrf2 overexpression could be critical in patients with COPD and associated lung cancer [31–34].

Based on these considerations researchers are looking for a redox modulator substance, i.e. a molecule able to activate the Nrf2 and the intracellular antioxidants generations with an "on and off" mechanism that preserves the cells from the detrimental consequences of a prolonged Nrf2 stimulation.

Ozone has the ability to elicit and calculate a transient "oxidant shock" to restore a normal redox system of the body without side effects.

In the next paragraphs, we will explain how ozone can act as a redox modulator in an integrative approach to COPD therapy.

18.3 The Ozone as Redox Modulator and Its Use in Medicine

18.3.1 The Discovery of Ozone: The Initial Local Application and the Systemic Use with the Major Ozonated Autohemotherapy Procedure

Christian Friedrich Schonbein, a German chemist, in 1839 reported that the electrolysis of water produced a characteristic odor, and he proposed for this new gaseous substance the name ozone (from the Greek ozein = odorant). He described the ozone as a strong oxidant but also as a powerful disinfectant. The ozone was first used in clinical practice in the past century by Dr. Wolff and other German physicians for the treatment of posttraumatic gangrene and infected wounds in German soldiers during the First World War. Indeed, ozone has good disinfectant and

antibiotic properties when used directly to kill bacteria in skin or mucosa infections. Dr. Hans Wolff was also the first physician to expose blood directly to a gas association of ozone and oxygen.

The first reliable ozone generator for medical use was produced in 1958 by Dr. Hansler; since then, Prof. Bocci's studies better elucidated especially in the past 20 years the biochemical reactions of ozone after its contact with human fluids, particularly during the therapeutic way of administration called major ozonated autohemotherapy (MOA) that consists of ex vivo exposure of a precise volume of patient's blood to a calculated ozone dose for a few minutes followed by the reinfusion of ozonated blood in the patient [35–37]. This procedure is able, in chronic oxidative diseases like COPD, arteriopathy, and neurodegenerative disorders, to induce the production of antioxidant enzymes in the cells and restore the redox balance, as reported in clinical studies [38–41].

Unfortunately ozone is also present as an air pollutant, and, when inhaled, it can cause harmful effects on the respiratory tract: however, ozone, like other substances, can be therapeutic or toxic depending upon its concentration and location [42].

The next paragraphs will explain how the ozone acts and the correct use of ozone in medicine for the treatment of COPD.

18.3.2 Major Ozonated Autohemotherapy: A Brief Description

The major ozonated autohemotherapy (MOA) consists of exposing "ex vivo" 100–200 ml of blood of patient (it depends on body mass) to a precisely calibrated ozone dose for a few minutes followed by the reinfusion of activated blood in the donor.

More detailed, 100–200 ml of peripheral venous blood of the patient is collected in a sterile vacuum glass bottle containing sodium citrate for anticoagulation (ratio 9:1 blood/sodium citrate). In the bottle added is a corresponding volume (100–200 ml, 1:1 blood/gas ratio) of oxygen ozone gas mixture with an ozone amounting from 10 to 50 micrograms/ml of gas mixture depending on disease. After 3 min the blood is reinfused in the patient. In this way, ozone can trigger a large number of biochemical pathways in blood without producing acute or chronic toxicity, but it is important to observe that a correct ozone concentration is critical to obtain a therapeutical effect: too low concentration of ozone is useless and too high may be harmful. We need to produce a brief and calculated increase of ROS and LOP which, in turn, is able to elicit a transitory oxidative stress capable of triggering an adaptive response in the redox cell regulation [43].

18.3.3 Major Ozonated Autohemotherapy: How Ozone Acts During the Contact with Human Blood – The Ozone as a Redox Modulator

A large series of studies showed that in the blood, the antioxidant system can counteract the ozone in a range concentration from 10 micrograms ozone/milliliters of gas per milliliters of blood up to 50 micrograms ozone/milliliters of gas [44]. The

blood is composed of about 55% of plasma and 45% of cells, mainly erythrocytes. The plasma is mostly water (92 % of volume) and contains many substances: (a) dissolved ions such as HCO_3 and PO_4, essential for the control of the pH within the range of 7.3–7.4; (b) hydrophilic and lipophilic substances like glucose, ascorbic acid, cysteine, uric acid bilirubin, carotenoids, vitamin E, and lycopene; (c) an amount of about 5 g of lipids (triglycerides, cholesterol, phospholipids, and lipoproteins); (d) proteins, mainly albumin (4.5 g/dl), fibrinogen, and other globulins such as transferrin or ceruloplasmin that bind Fe^{++} and Cu^{++}; and (e) coagulation factors and hormones.

The antioxidant activity of plasma is maintained thanks to a large variety of substances such as uric acid, ascorbic acid, GSH, and albumin.

Erythrocytes are the cells with a high level of GSH, thioredoxin, and enzymes like catalase, glutathione reductase, glutathione peroxidase, glutathione/thioredoxin system, and SOD. All these antioxidants can rapidly neutralize a high concentration of oxidant molecules such as OH, H_2O_2, OCl^-, and ONOO [45, 46].

During major ozonated autohemotherapy, ozone, owing to the high solubility in plasma (0.02 M), immediately reacts with hydrophilic antioxidants (ascorbic acid, uric acid, albumin). At the same time, the small amount of molecules of ozone that remains in blood performs the peroxidation of albumin-bound unsaturated fatty acids, which is a preferential substrate. The peroxidation of PUFA causes the production of H_2O_2 and lipid oxidation products such as 4-hydroxy-2E-nonenal (4HNE):

$$\mathrm{R}\quad CH=CH\quad \mathrm{R}+\mathrm{H_2O}+\mathrm{O_3}\leftrightarrow 2\mathrm{RHCO}+\mathrm{H_2O_2}$$

These reactions are very fast, and ozone dissolves, from the gaseous phase, in the plasmatic water and immediately reacts. After 2–3 min, in the glass bottle containing ozone and blood, ozone is totally consumed with both a slightly reduction of hydrosoluble antioxidants and the contemporaneous plasmatic rise of ROS and LOPs (mainly 4HE and hydrogen peroxide).

Despite the fact that ROS have a life span of less than a second, they are able to harm essential cell constituents, and therefore it appears very important to regulate the ROS production in order to trigger a biological effect without cell damage. For this purpose the ozone therapists can manage the ozone dose (ozone concentration as microgram/milliliter of gas per milliliter of blood in 1:1 ratio) against the antioxidant capability of blood. To better evaluate this crucial issue, experimental studies showed the changes of total antioxidant status (TAS) after blood ozonation under low, medium, or high ozone dose (10 micrograms/ml, 30 microgram/ml, 50 microgram/ml). TAS levels decreased, respectively, from 10, 20, and 35 % in the first minute after ozonation, but then they raised and restored the original concentration within 20 min, confirming the large efficiency of blood to regenerate oxidized antioxidants like dehydroascorbate and GSH disulfide. Similarly, after blood ozonation, the intraerythrocytic GSH has been found oxidized to GSSG of only about 20% after 1 min, but it was completely reduced to normal after 20 min. All these results clearly demonstrate that the ozonation in therapy modifies for a short time and reversibly the redox homeostasis in the cells.

Hydrogen peroxide, as a principal ROS, enters in the blood cells and activates several reactions:

1. *In erythrocytes*, an increase of 2,3-diphosphoglycerate (2,3-DPG) was measured which produces a shift to the right of the oxyhemoglobin dissociation curve. This process facilitates an increased release of oxygen into tissues with ischemia. Also a transitory increase of ATP was observed. H_2O_2 is therefore rapidly neutralized by GSH.
2. H_2O_2 mildly activates, *in lymphocytes*, the nuclear factor K-beta (NFk-beta), which in turn allows a transitory increase of synthesis of cytokines, mainly tumor necrosis factor alpha, interferon gamma, and interleukins 2 and 8. This change may improve the immune status in patients.
3. *Platelets* showed an increase of platelet-derived growth factors A and B (PDGF A and B), transforming growth factor beta 1 (TGF beta1), and interleukin 8 (IL-8), with a possible positive effect in chronic limb ischemia.

As far as LOP productions following peroxidation of PUFA, in several studies an increase of heterogeneous compounds after blood ozonation has been observed: it was found in lipoperoxides, alkoxy radicals, lipohydroperoxides, isoprostanes and alkenals, and, among which, 4HNE and MDA. All these compounds are constitutionally toxic and must be produced in very low amounts. They are in vitro far more stable than ROS but fortunately, upon blood reinfusion, they have a brief half-life owing to these mechanisms: (a) a great dilution of these components in body fluids and a rapid decrease of their concentration, (b) an excretion via urine and bile, and (c) a detoxification due the interaction with detoxifying enzymes such as aldehyde and alcohol dehydrogenases, aldose reductase, and GSH transferases. Only a submicromolar concentration of 4HNE reaches the cells of the body of patients affected by chronic oxidative stress; these cells are unable to trigger their antioxidant system and thus are destinated to death. The cytoplasm of the stressed cells contains the Nrf2-Keap1 inactive complex. Luckily the 4-hydroxynonenal enters into the cell from the blood and binds two cysteines on Keap1 allowing the release of Nrf2 protein. The free Nrf2 is then able to move into the nucleus of the cell and, after a connection with a Maf protein, can stimulate the antioxidant response element (ARE) with the consequent induction of about 230 genes belonging to detoxification response and phase II antioxidant response [47–50].

Table 18.1 summarizes the biochemical steps during the ozone-blood contact, from the O_3 solution in plasma to the antioxidant enzyme production in the cells.

Several studies reported the possible antioxidant action of other Nrf2 activators like triterpenoids, dimethyl fumarate, and curcumin, but ozone alone has the unique property of generating a transitory and calculating stimulation of Nrf2. In fact, as reported by Pecorelli et al. [51], endothelial cells incubated in the presence of ozone and 4HNE showed a rapid and transient Nrf2 activation. This observation supports the hypothesis that ozone therapy is able to activate Nrf2 for the production of defense enzymes, and at the same time, it is unable to prolong this activity after about 40–60 min. Thus, the mechanism of transient activation is very useful for the

Table 18.1 The biochemical steps during ozone-blood interaction

The main steps of the ozone-blood interaction
(1) Oxygen-ozone gas mixture dissolves into plasmatic water
(2) Ozone reacts with several substances in plasma, mainly antioxidants and PUFA
(3) The result of this interaction is the generation of a little concentration of ROS and LOPs
(4) ROS are responsible for the rapid response (ex vivo), whereas LOPs are important for the late response (in the patient's body)
(5) LOPs (and in minimal amount ROS) are the principal molecular signals of the Nrf2 activation and gene induction of antioxidants when the ozonated blood returns into circulation upon reinfusion. *The Nrf2 stimulation is rapid and transient*
(6) Nuclear induction of 230 genes (detoxification response and phase II antioxidant response) in body cells

cells undergoing oxidative stress in COPD for restoring a normal redox system without the risk of cellular proliferation subsequent to a continuous prolonged Nrf2 stimulation [52].

18.4 Clinical Application of Ozone Therapy in COPD

The first application in Siena of ozone therapy on COPD patients was 10 years ago. Two patients affected both by age-related macular degeneration and COPD underwent major ozonated autohemotherapy and improved their quality of life and exercise's resistance. They continued the treatments for years with satisfaction.

It is important to remark that ozone therapy, in the COPD treatment, is used as integrated therapy, and it cannot substitute the standard orthodox therapy. However, the added ozone action as redox modulator plays a pivotal role in restoring a large amount of biochemical functions in all organs during chronic pulmonary diseases.

On the basis of the preliminary observations, 50 patients affected by moderate/severe COPD were enrolled in a case-control clinical trial (standard therapy + ozone therapy vs standard therapy alone) at the University of Siena. All patients were all ex-smokers, and they were admitted in the study during a stable phase of the disease, without signs of exacerbations in the 4 weeks before starting the protocol and under regular pharmacologic therapy (inhaled long-acting beta2-agonists/corticosteroids LABA/ICS and/or tiotropium in all patients).

The following measures were performed in all patients prior to the start and after the conclusion of the study: (a) pulmonary function test, (b) resting arterial blood gas analysis, (c) 6-min walking test (6MWT), (d) dyspnea index by the Borg scale, and (d) St. George's Respiratory Questionnaire (SGRQ).

The design of the study and the results were reported in [39].

Patients were randomized in two groups: group 1 (25 patients) received a cycle of MOA twice a week for the first 5 weeks and thereafter a single treatment every week for another 10 weeks. Group 2 (25 patients) served as the control and didn't undergo ozone therapy. All patients of both groups 1 and 2 received standard therapy with inhaled beta2 long-acting bronchodilators and/or corticosteroids.

After the cycle of ozone therapy, patients of group 1 significantly increase the walking distance, and the degree of dyspnea during physical effort was reduced according to the Borg dyspnea scale. The SGRQ showed an improvement of the daily activity and on overall quality of life.

The control group patients didn't show any difference in walking distance and SGRQ score at the end of the study (Figs. 18.1 and 18.2).

Six-minute walking test is an important measure of functional capacity during exercise in COPD patients. An association between distance walked and clinical outcomes has been reported such as hospitalization and mortality. The changes in 6MWT are used to evaluate the efficacy of various therapeutic interventions such as pharmacological therapy or rehabilitation [53]. After ozone therapy, the patients showed an increase in walking meters greater than 25 m that is considered the minimal clinically important difference for COPD patients [54].

St. George's Respiratory Questionnaire is a specific instrument to measure the quality of life for COPD. In this study there was a significant improvement in total scores, in activity domain scores, and in impact domain scores in patients that

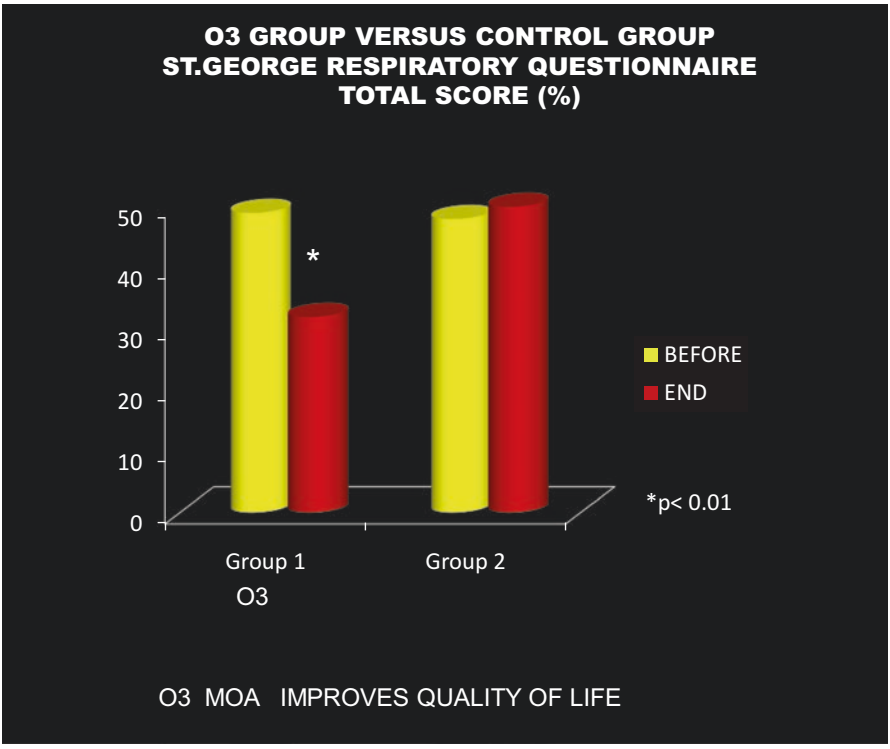

Fig. 18.1 St. George's Respiratory Questionnaire score before and at the end of the study in ozone-treated patients (group 1) and in control patients (group 2). $*p < 0.01$

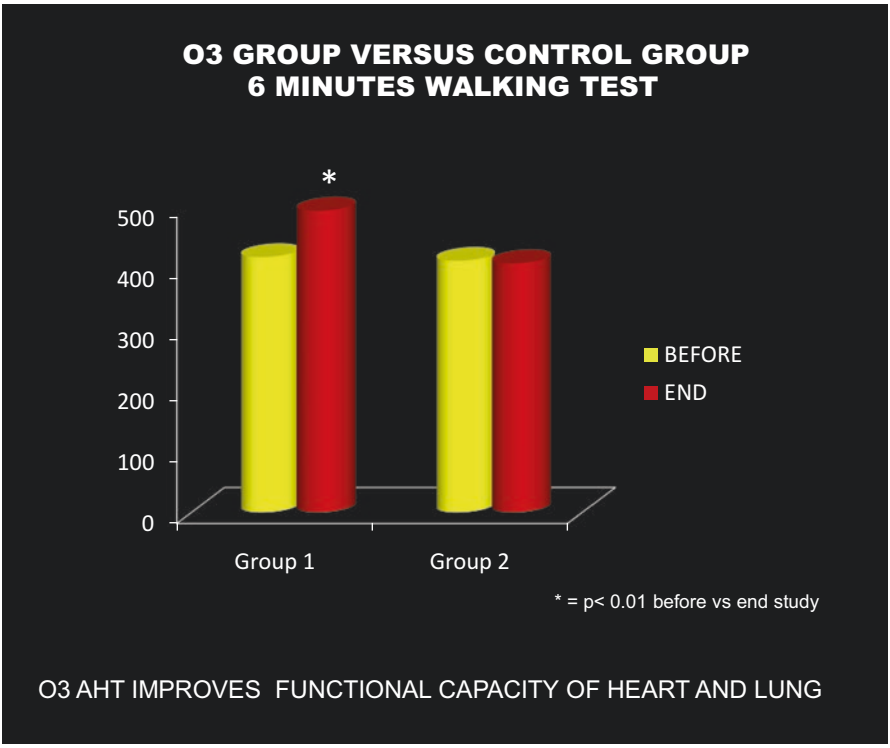

Fig. 18.2 Modifications of 6-min walking test at the end of study in ozone-treated patients (group 1) and control (group 2). $*p < 0.01$

underwent ozone therapy (Table 18.1). These results suggested an objectified positive effect of ozone therapy on different aspects of health status in COPD patients and in the overall quality of life [55].

The adverse effects observed in ozone treated patients are a temporary face redness during the MOA treatment (3%). On the contrary, upon ozone therapy some of the patients reported an enhancement of the general efficiency, concentration and cognitive functions as demonstrated by the improvement of the Saint George Respiratory Questionnaire score (Table 18.2).

To better evaluate the action of ozone therapy on oxidative stress, in the next investigation, the values of plasmatic reactive oxygen metabolites (dROM test, Diacron International, Grosseto, Italy) in two groups of COPD patients have been analyzed. Again, 20 patients received standard and MOA therapy, and 20 patients received the standard therapy alone.

Results showed a significant decrease of dROM plasma values in COPD patients after a cycle of ozone therapy (dROM baseline value, 375 ± 30 UCARR; after MOA, 281 ± 24 UCARR, $p < 0.05$). In control patients, dROM values remain

Table 18.2 Tabular results of 6MWT, SGRQ, and Modified Dyspnea Borg scale in groups 1 and 2

	Group 1	Group 2	*p*
6MWT initial (m)	417.5 ± 107.3	411.4 ± 109.2	ns
6MWT end (m)	493.8 ± 105.4	407.3 ± 104.4	$P < 0.01$ a, b
DYSPNEA BORG SCALE initial	4.1 ± 2.4	4.3 ± 2.1	ns
DYSPNEA BORG SCALE end	3.0 ± 1.2	4.3 ± 2.1	$P < 0.01$ a
SGRQ			
Activity initial (%)	65.4 ± 16.3	66.1 ± 17.4	ns
Activity end (%)	54.2 ± 15	62.4 ± 16.6	$P < 0.01$ a
Impact initial (%)	38.4 ± 18.2	37.6 ± 17.1	ns
Impact end (%)	30.1 ± 16.4	34.5 ± 17.9	$P < 0.01$ a
Total score initial (%)	48.9 ± 16	47.3 ± 16.4	ns
Total score end (%)	32.2 ± 12.1	49.9 ± 14.3	$P < 0.01$ a, b

a = intragroup comparison before vs end study, b = intergroup comparison Group 1 vs Group 2, end of study

unchanged at the end of the study (baseline values, 371 ± 28 UCARR; at the end of the study, 374 ± 32 UCARR, ns difference, unpublished data).

All patients in group 1 asked to continue the therapy after the end of the study; no adverse effects were noted after 6 years of therapy.

18.5 Conclusion

The updated report of the Global Initiative for COPD (GOLD) describes COPD as a "preventable and treatable disease" but also underlines the importance, in the overall clinical scenario, of the concepts of "exacerbation" and "comorbidity" [56]. In fact, the repeated episodes of exacerbations associated with comorbidities in COPD patients cause frequent hospitalization, an increase of drug prescriptions, and an impairment of the patient's quality of life. However, COPD progression, exacerbation, and comorbidity seem to be all together caused by a cross talk between oxidative and inflammatory stress mediators, leading to a pulmonary/systemic damage [57, 58].

For these reasons, along with the current pharmacological (inhaled beta2-agonists, antimuscarinic, corticosteroid) and nonpharmacological interventions (such as rehabilitation, noninvasive mechanical ventilation, and psychological counseling), growing evidence supports, in the management of COPD, the integrated use of Nrf2 activators to better counteract the above reported pathogenetic mechanisms by inducing cytoprotective genes, decreasing oxidative stress, and reversing corticosteroid resistance [59].

Ozone, when used as molecular redox signal, has many advantages: it activates Nrf2 with a transient and calculates stress; it is relatively inexpensive and atoxic when used properly; after ozone therapy, patients reacquire a well-feeling and an

objective improvement of the quality of life [60]. Obviously the above reported data needs to be repeated in a large trial, but an increasing number of studies confirm the efficacy of ozone therapy in chronic oxidative and degenerative diseases, and it is hoped that the world health authorities could promote the clinical research and application of ozone in medicine.

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Oxidative Stress and Therapeutic Development in Lung Cancer

19

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Abstract

Oxidative stress is associated with the pathogenesis of many lung diseases including lung cancer. The main goal of this chapter is to provide an overview of how reactive oxygen species (ROS) and antioxidants are related to normal physiological function and the pathophysiology of lung cancer and its therapeutic strategies. In this chapter, ROS are first characterized, followed by the role of oxidative stress in progression of different lung cancers, and a brief overview of therapeutic strategies developed gradually over the decades for better therapy of lung cancer is described. Limitations of current strategies and failures of clinical trials have also been discussed, and finally development of new therapy which may be helpful in the treatment of patients with lung cancer has also been addressed as future direction.

Keywords

Oxidative stress · Reactive oxygen species · Lung cancer · Non-small cell lung cancer · Monoclonal antibody therapy · Immune therapy

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19.1 Introduction

Oxidative stress is characterized by an overproduction of reactive compounds that are not compensated by the exploitation of antioxidants and also as a consequence of perturbation of cell redox balance [1]. Usually, low levels of these reactive compounds are essential to activate many cell-signalling molecules as well as signal transduction pathways before their elimination in normal cells. Conversely, high level of these compounds is associated with different disease states. Based on the main atom involved, these reactive compound/species can be divided into four groups: reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive sulphur species (RSS) and reactive chloride species (RCS). Among these reactive compounds, ROS are the most abundant and they could have short to long half-life depending on their molecular stability. ROS includes singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), ozone (O_3), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH^-$) [2]. On the other side, the most abundant RNS is nitric oxide (NO^-), which has the ability to react with certain ROS producing peroxynitrite anion ($ONOO^-$), which is also a reactive compound and lethal for cells and tissues. The biomedical study with oxidative stress is popular in current research because of its association with a wide variety of human diseases, such as cardiovascular disease, for example, muscular dystrophy; neurodegenerative disease such as Parkinson's disease and Alzheimer's disease; lung diseases such as pulmonary fibrosis and pulmonary hypertension; inflammatory diseases, for example, rheumatoid arthritis; and along with allergies, diabetes, immune system dysfunctions, aging and cancer. In this chapter, we will bring to the reader an apparent view of how oxidative stress is connected with lung cancer in both molecular and clinical level.

19.2 Oxidative Stress and Lung Cancer

Nowadays, one of the most deadly diseases is lung cancer, and it is the primary cause of morbidity and mortality worldwide. Histologically, lung cancer is divided into four subtypes; however, depending on the responses to chemotherapy and radiation therapy, it is mainly divided into two major subgroups – small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). SCLC accounts for about 15% of all new cases of lung cancer. On the other hand, NSCLC is predominant and accounts for about 85% of all lung cancer. NSCLC is subdivided into three groups, and they are adenocarcinoma, squamous cell carcinoma and large-cell carcinoma. The main strategy for the betterment and increase of the survival time of SCLC patients mainly depends on how they individually response to chemotherapy or radiation therapy or in combination of both therapies. In contrast, the main strategy for the betterment and increase of the survival time of NSCLC patients mainly depends on the resectability of the tumour at the time of presentation. Up to date, several prognostic factors have been identified based on different tumour grades and stages in both SCLC and NSCLC patients. These factors mainly include the overexpressed oncogenes that show more malignant behaviour and metastatic property or

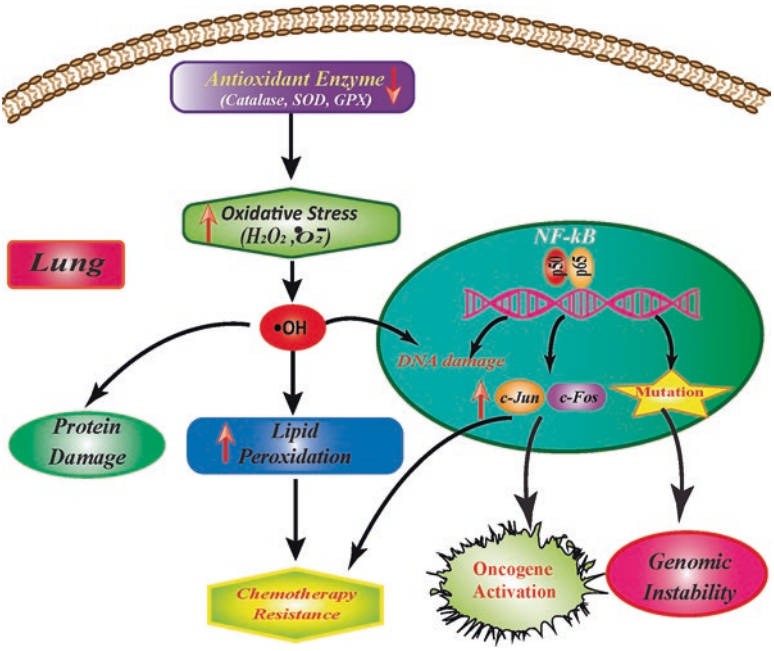

Fig. 19.1 Schematic presentation of oxygen- and free-radical-mediated chemoresistance and oncogene activation in lung cell

lowly expressed tumour suppressor genes which show anti-proliferative properties. Beside this, various genes are also overexpressed and many signalling molecules are activated as a consequence of resistant behaviour of cells during drug therapy. Both ROS and RNS have a strong connection with the onset of lung cancer because they were found to be associated with the regulation of the activation of the signalling molecules and expression of those oncogenes. Figure 19.1 depicts how deregulation of antioxidant-oxidant balance induces oncogene expression or chemotherapy resistance in lung cancer cell model.

19.3 Oxidative Stress and SCLC

Application of surgical removal of tumours is sporadic in SCLC patients, since SCLC has a tendency to metastasize early, and thus combination chemotherapy (CT) is the pragmatic choice for the treatment of SCLC [3, 4]. SCLC is chemosensitive and combinations of different chemotherapeutic drugs, such as Adriamycin with vincristine and cyclophosphamide (CAV) or etoposide with cisplatin, have been considered as standard treatment for SCLC since long back [5, 6]. However, various new therapeutic treatment approaches have been continuously investigated

to improve treatment outcome. One of the important molecules or pathways through which these chemotherapeutic regimens act and exert their effects is reactive species or oxidative stress. These drugs are mainly anthracyclines, such as epiadriamycin, Adriamycin and daunorubicin [7]. One of the well-known chemotherapeutic drugs, Adriamycin, mainly shows its anticancer activity with the interaction with topoisomerase II, leading to DNA fragmentation and cell death [8]. It also exerts its effect by enhancing protein oxidation [7]. Erhola et al. previously suggested that anthracycline-based chemotherapy shows their effects by producing oxidative stress [9]. They proved it on the basis of the evidence that after combination chemotherapy in SCLC patient, a decrease in total antioxidant capacity (TRAP) occurred in the plasma [10]. Other than SCLC, there are also many reports documented that shows reduced levels of different antioxidants have been observed during combination therapy using chemotherapeutic drugs in patients with different carcinoma [11, 12].

One of the known lipid peroxidation products that results from oxidative stress is malondialdehyde (MDA) mainly found in body fluid, and it has been found to induce growth arrest in a human large-cell lung carcinoma cell-line model [13]. There are also other lipid peroxidation products which are produced during oxidative stress from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and have the ability to inhibit growth of different lung cancer cells lines [14]. Additionally, superoxide dismutase and glutathione peroxidase that are known to suppress lipid peroxidation have been shown to be associated with lower survival of lung adenocarcinoma patients [15]. Therefore, it is obvious that chemotherapeutic drugs could show their anticancer activity by inducing the peroxidative damage of different biomolecules including lipids [16–18]. There are many reports showing an increased level of lipid peroxidation end products have been observed after using of various chemotherapeutic drugs in different cancer patients [19]. Nowak et al. in their study investigated the level of the lipid peroxidation products in serum after a combination chemotherapy using vincristine, carboplatin and etoposide in SCLC patients. They found increase of lipid peroxidation products in serum after chemotherapy which was strongly associated with the survival of SCLC patients [20], and as a conclusion it can be suggested that oxidative stress is strongly connected with SCLC involving lipid peroxidation.

19.4 Oxidative Stress and NSCLC

ROS are one of the major causes of NSCLC development and cigarette smoking (CS) is prevalent to this because it is the main source of ROS [21–23]. The tissue oxidant and antioxidant balance is altered and chronic inflammation is found to be persistent due to production of these oxidant compounds by inflammatory cells such as polymorphonuclear neutrophilic leucocytes (PMNs) and non-phagocytic cells [24–26]. Nicotinamide adenine dinucleotide phosphate oxidase (NOX) is essential in this scenario because CS induces the activation of NOX which then initiates ROS production. NOX family enzymes are inflammatory components of host defence and several NOX isoforms are found to be expressed in lung tissue

including NOX2, DUOX1 and NOX4 [27, 28]; however, the classical NOX are found to be present in structural non-phagocytic mammalian cells [29, 30]. In NSCLC patients, NOX activity was found remarkably higher in malignant patients compared to non-malignant patients [31, 32]. Moreover, elevation and depreciation of NOX and myeloperoxidase (MPO) activities are interrelated and tightly regulated in NSCLC [33, 34]. Interestingly, one of the major as well as strongest carcinogen, benzo[a]pyrene (B[a]P), has been found in CS that produces oxidative stress. B[a]P induces ROS-mediated activation of epidermal growth factor receptor (EGFR) signalling pathway which has shown to increase the proliferative potential of lung adenocarcinoma cells by upregulating phosphorylated EGFR and inducing EGFR ligand expression such as amphiregulin and epiregulin [35]. Khan et al. also showed that the exposure of CS to human lung epithelial cells activates EGFR which may prolong the signalling to contribute the uncontrolled lung cell proliferation and growth [36].

Both predictive and prognostic significance for aberrant histone post-translational modifications is found in case of NSCLC [37, 38]. Chronic obstructive pulmonary disease (COPD) and NSCLC are both tightly connected with oxidative stress through various genes and their downstream cellular response pathways [39] like glutathione peroxidases (GPXs), superoxide dismutases (SODs), glucocorticoid receptors (GRs), hypoxia-inducible factor-1 (HIF-1) and heme oxygenases (HOs). Importantly, epigenetics play a vital role in the regulation of such genes and their downstream signalling pathways in COPD and NSCLC. Since dysregulation of such genes manifests the diseases, therefore, epigenetics are selected as the potential targets for therapeutic strategies for the treatment of both diseases. It is well known that histone deacetylases (HDACs) form a protein complex, remove acetyl groups (O=C–CH3) from a ε-N-acetyl lysine amino acid on histone, and inhibit transcription, thereby regulating several gene expressions [40]. An increase in level of many HDAC isoenzymes has been found in different types of malignancies [41]. Particularly, HDAC1 mRNA levels have been found to be increased in NSCLC at stage III or IV [42]. Altered form of this HDAC multi-protein complex has also been observed in NSCLC such as mSin3A, a corepressor of this complex, and has been downregulated in NSCLC [43]. Therefore, HDAC inhibitors are part of a new therapeutic strategy in NSCLC which acts by blocking the suppression of tumour suppressor genes as well as antioxidant genes induced by HDACs [44].

Chronic inflammation is very actively associated with diverse type of cancer [45]. Pro-inflammatory signalling pathways have robust impact in NSCLC via oxidative stress responses [46] and NFκB has been found as a critical mediator of pro-inflammatory cascade in this scenario [47]. It has been found that oxidative stress and TNF-α both stimulate histone acetyltransferase (HAT) activity resulting in an increase in histone acetylation in a lung cancer cell line model. The increased histone acetylation further induces DNA binding of NF-κB leading to elevation of pro-inflammatory IL-8 levels [48]. Therefore, the activation of lysine acetyltransferases triggers NF-κB DNA binding where lysine acetyltransferases, the coactivators, act as key regulators in NF-κB-driven gene expression [49–51], and thus, oxidative stress induces an increase in the expression of pro-inflammatory genes such as IL-8 and IL-6 via associations of RelA/p65 and KAT3A, KAT3B subunit or p50 and

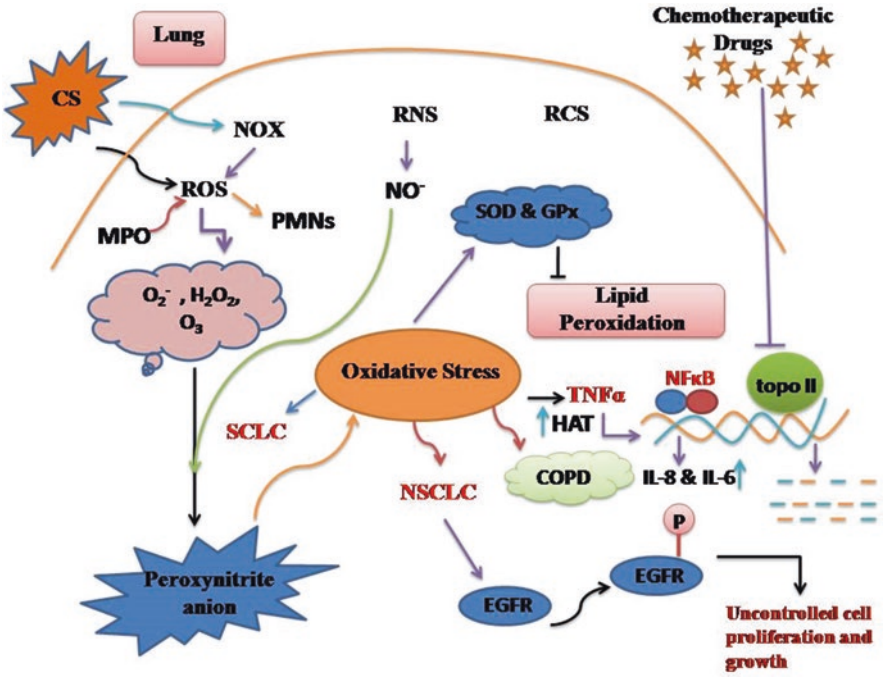

Fig. 19.2 Diagrammatic presentation of lung carcinogenesis mediated by reactive species that are induced by cigarette smoking and of chemotherapy mediated by biologically active small molecule

KAT13A leading to inflammation which may induce development of lung cancer [52–55]. As a consequence, two main epigenetic therapeutic strategies are available for the treatment of NSCLC – one is via targeting DNA methylation through DNA methyltransferase inhibitors (DNMTi) and the other by histone acetylation via HDAC inhibitors. In line with this, the expressions of critical genes in NSCLC have been found downregulated after the use of DNMTi [56–60]. Figure 19.2 depicts a schematic representation of how CS initiates production of ROS that induces an increase in the level of pro-inflammatory molecules stimulating lung cancer cell growth and finally leading to progression of lung cancer.

19.5 Current Therapeutic Option for Lung Cancer

Recently, very specific targeted therapies are developed for the betterment of lung cancer. With the breakthrough in molecular biology and immunotherapy, these current developments have got attention because of their more efficient and specific treatment in lung cancer patients. But, the leading challenge to be considered as a goal is to investigate the therapeutic resistance mechanisms which develop within a year after the onset of therapy and need urgent attention and further elucidation.

Surgery, chemotherapy, radiotherapy, immunotherapy and targeted therapy are the major therapeutic strategy for the treatment of lung cancer currently. In this following section, we will provide a brief discussion about the therapies in lung cancer and how oxidative stress is related to these treatment therapies are described.

19.6 Chemotherapies

Chemotherapy is one of the cancer treatments that use drugs to abolish cancer cells and it is well known as 'chemo' also. These chemotherapeutic drugs target cells that grow and divide faster, like cancer cells. Unlike radiation or surgery, which is specific to target areas, chemo can work throughout the body; however the most disadvantageous part of chemotherapy is its nonspecific nature because it can also affect some fast-growing healthy cells, like skin, hair and bone marrow, and mostly shows side effects due to the treatment. The main functions of chemotherapeutic drugs are (1) to destroy cancer cells, (2) to stop spreading of cancer cells and (3) to slow the growth of cancer cells. Chemotherapy can be given alone, or to improve the outcome of the treatment, it is given in a combination with other treatments.

Chemotherapies function in patients during their treatment cycle by producing oxidative stress. Oxidative stress production was found noticeably during chemotherapy application and it was evident by the increase of the level of lipid peroxidation; the significant reduction of tissue glutathione (GSH) levels; decrease of antioxidants level such as β-carotene, vitamin C and vitamin E in plasma; and the decrease of the level of total radical-trapping capacity of blood plasma that occurs during chemotherapy [61–63]. Finally, chemotherapy treatment generates nuclear DNA adducts, which leads to cell death because of the blockage of DNA replication and transcription [64]. Agents that are well known to produce high levels of ROS include platin-based drugs (e.g. carboplatin, cisplatin), anthracycline drugs (e.g. epirubicin, doxorubicin and daunorubicin), alkylating agents, camptothecin drugs (e.g. irinotecan and topotecan) and epipodophyllotoxins (e.g. etoposide and teniposide), which are mentioned in Table 19.1. Till date the anthracyclines and taxanes,

Table 19.1 Chemotherapeutic drugs used in NSCLC patients

Generic name	Brand
Carboplatin	–
Cisplatin	Platinol
Docetaxel	Taxotere
Etoposide; etoposide phosphate	Etopophos
Gemcitabine hydrochloride	Gemzar
Paclitaxel	Taxol
Paclitaxel, albumin bound	Abraxane
Pemetrexed	Almita
Vinablastine sulphate	–
Vinorelbine tartrate	Navelbine

for example, docetaxel, or vinca alkaloids, for example, vincristine, are known to generate the highest and lowest levels of oxidative stress, respectively. However, the mode of action of all of these drugs acts in the same manner by producing ROS leading to apoptosis of the cancer cells. The potential mechanism for these chemotherapeutic drug-induced apoptosis involves the release of cytochrome-c from mitochondria and simultaneously electrons are moved from the electron transport chain (ETS) to oxygen by NADH dehydrogenase and reduced coenzyme Q10 and subsequently formation of superoxide radicals [65].

Since most of the chemotherapeutic drugs used in cancer therapy cause oxidative stress, it is good to reduce the level of oxidative stress by applying antioxidants which may improve the efficacy of the treatment because the anticancer drugs would kill normal cells along with cancer cells. Thus, many antioxidants are used with the chemotherapeutic drugs to prevent chemotherapy-induced side effects (e.g. glutathione is used for cisplatin-induced nephrotoxicity and coenzyme Q10 is used for anthracycline-induced cardiotoxicity).

Therefore, even though these chemotherapeutic drugs are effective, however new approaches are obligatory to minimize the side effects and enhance their efficacy. Also, new combination approaches should be explored with targeted therapies to attain maximum clinical benefit.

19.7 Targeted Therapy

Targeted therapy, as the name suggests, works on specific targets that play a major role in tumour cell proliferation. Potential targets of such therapies are mostly oncogenes which have undergone genetic alteration mostly with potential driver mutation leading to tumour growth. In NSCLC the most common oncogenic driver gene alterations include EGFR, ALK, ROS1 and BRAF and RET rearrangement. Targeted therapy is often different from chemotherapy because in chemotherapy, the effect is more global on the cells due to which the normal cells are also affected, whereas in targeted therapy the off-target effect is less and the tumour cell killing is more with fewer side effects on healthy cells. Monoclonal antibodies, tyrosine kinase inhibitors (TKIs) and immunotherapies, which are more common, are extensively used as a targeted cancer therapy in the past recent years. However, these targeted therapeutic compounds are shown often less specific and show deadly antagonistic effects. Thus, combination of these therapies is more common in a clinical trial experiment nowadays [66]. During the initial period when the idea of targeted therapies was first developed, these were accepted as promising magic bullets with single targets [67, 68]; later on their off-target effects and development of drug resistance mutation on the gene led us to focus on the different mechanisms of action of these targeted therapies. ROS has been found to play a substantial role in producing such off-target effects during targeted therapies. Currently, there are nearly 15 approved TKIs for the treatment of NSCLC and the list is expanding rapidly (Table 19.2).

Table 19.2 Small molecule inhibitors and mAb used in NSCLC patients

Target name	Generic name	Brand name
EGFR	Erlotinib hydrochloride	Tarceva
EGFR	Gefitinib	Iressa
EGFR	Osimertinib	Tagrisso
EGFR and HER2	Afatinib	Gilotrif
ALK	Brigatinib	Alunbrig
ALK	Ceritinib	Zykadia
ALK	Alectinib	Alecensa
BRAF V600E	Dabrafenib	Tafinlar
BRAF V600E	Vemurafenib	Zelboraf
MEK1 and MEK2	Trametinib	Mekinist
RET	Vandetanib	Caprelsa
EGFR	Cetuximab	Erbitux
HER2	Ado-trastuzumab	Kadcyla
VEGF	Bevacizumab	Avastin
VEGF	Ramucirumab	Cyramza

19.8 Tyrosine Kinase Inhibitors (TKIs) as Targeted Therapy

Gefitinib was approved as the first TKI selectively for EGFR to treat NSCLC. EGFR mutations, the major mutation in lung adenocarcinoma, have been in Asia (approx. 40–50%) and in Western countries (approx. 10–15%). The most common mutations are exon 19 deletions (account for approx. 45% mutation) and L858R point mutation in exon 21 (account for approx. 40–50%) in EGFR-mutated lung cancer, while G719X and S768I are found in exon 18 and exon 20, respectively, and only <10% of cases of mutation is shown in exon 20 insertion. All of these mutations in EGFR make EGFR overexpress and this constitutively active EGFR leads to overactivation of the anti-apoptotic Ras signalling cascade, finally leading to uncontrolled cell growth. The mechanism of action of TKIs is to bind to the mutated active ATP-binding site of EGFR leading to the inhibition of RAS signalling cascade and thus activates many pro-apoptotic pathways and consequently causes tumour cell death [69]. However, regardless the vivid response to these EGFR-targeted TKIs, most of the patients have been shown to develop acquired resistance to these drugs. In a recent publication, gefitinib has been found to produce oxidative stress in a dose-dependent manner. It has also been found that this increased oxidative stress induces epithelial-to-mesenchymal transition (EMT) and cardiotoxicity [70, 71]. However, another study suggested that Prx II mRNA as well as protein level has been increased via demethylation of the Prx II gene in a gefitinib-resistant A549 cells in comparison to gefitinib-sensitive A549 cell line, and as a result, ROS level went down leading to inhibition of apoptosis and induction of cell progression and subsequently increase colony formation [72]. Thus, Prx II appeared as a potential target for overcoming gefitinib resistance and theoretically this could also be applied to other EGFR-targeted TKIs. Erlotinib was also added to the list of TKIs as EGFR-targeted

therapy later after approval of gefitinib. In line with the cytotoxic effects of other EGFR-targeted TKIs, erlotinib also shows cytotoxicity through the generation of oxidative stress while using it to treat many types of cancer, and after a certain time period, the patients become resistant to that drug [73]. Afatinib is another TKI targeted to EGFR and used in less, but chronic oxidative stress has also been found to be associated with the development of afatinib resistance as well [74]. The mechanisms of resistance to TKIs can be broadly divided into two categories: first, intrinsic resistance, and second, secondary EGFR mutations (e.g. T790M mutation). Osimertinib (OSI), also known as AZD9291, is a third-generation TKI targeted to EGFR that has been approved for the treatment for the patients harbouring T790M mutation. Lu et al. [75] have shown that treatment of OSI induces accumulations of cytoplasmic vacuoles and increase the expression of phosphatidylethanolamine-modified microtubule-associated protein light-chain 3 (LC3-II) and the formation of GFP-LC3 puncta in various cancer cells. It has been clearly shown in their study that OSI increased ROS generation accompanied by autophagy which causes decrease in cell viability and induced apoptosis in NSCLC cells. Currently, there are nearly 15 approved TKIs for the treatment of NSCLC and the list is expanding rapidly (Table 19.2).

19.9 Monoclonal Antibodies as Targeted Therapy

Monoclonal antibody-based therapy of cancer has been established as one of the most effective targeted therapeutic strategies in the last two decades. Initially, combination of serological techniques for the discovery of receptor proteins on cancer cell surface and hybridoma technology for the generation of antibodies led to a series of landmark clinical trials and subsequent clinical successes. Optimization of antitumour immune responses through Fc modifications also put a major contribution to clinical efficacy. The modification of the complex interplay between immune system and tumour cell environment through targeting of T-cell receptors has been developed as a powerful new therapeutic approach for cancer therapy.

Bevacizumab is a recombinant, humanized monoclonal antibody (mAb) that targets vascular endothelial growth factor (VEGF), leading to inhibition of ligand binding to VEGF receptor (VEGFR), and finally blocks neoangiogenesis and vascular leakiness.

This mAb is used mainly for the treatment of colorectal cancer (CRC) and NSCLC. The mode of action of this antibody is to produce hypoxia condition in tumour cell. Many antioxidants such as L-cysteine, l-cystathionine and GSH levels were decreased during the treatment of this mAb indicating an increase of oxidative stress levels. In a report with retinal cells, it was implicated that exposure of H_2O_2 and bevacizumab to the cells decreased bcl-2 mRNA and protein level and subsequently caused apoptosis with dose-dependent increase of bevacizumab, suggesting that oxidative stress enhances the effect of bevacizumab on apoptosis [76].

Cetuximab is a recombinant monoclonal antibody designed to target specifically EGFR. US FDA approved cetuximab for the treatment of HNSCC, CRC and NSCLC. In NSCLC, EGFR mutation is second most abundant with ~25% among

the total NSCLC patient pool. In addition to MAPK signalling pathway blocking, EGFR inhibition opened up additional mechanisms that affect cell survival and reveal a new way of NSCLC treatment. Lu et al. recently published a paper describing how cetuximab has an effect on the downregulation of glutamine transport protein-mediated complex in the cell cytoplasmic membrane [77]. It has also been reported that oridonin and cetuximab combination therapy suppressed phosphorylation of EGFR leading to an increase of ROS and apoptosis in laryngeal carcinoma cells [78].

19.10 Other Small Molecules as a Targeted Therapy

Vemurafenib was discovered as the first BRAF inhibitor that is used for the treatment metastatic melanoma. The mode of action of vemurafenib is to target the most common genetic alteration in melanomas, BRAF V600E. By binding to BRAF, vemurafenib inhibits the signalling cascade RAS/MEK/ERK and subsequently reduces cell proliferation leading to inhibition of tumour growth. Additionally, vemurafenib induces $NO^{\bullet}$ and $O_2^{\bullet -}$ production and depolarization of mitochondrial membranes in BRAF V600E-mutated melanoma cells leading to apoptosis and growth inhibition [79]. Although most of the studies are published in melanoma, vemurafenib has also shown a good survival in some NSCLC patient pool.

19.11 Immunotherapies

Immunotherapy is also referred to as biologic therapy or biotherapy. It is an area of cancer treatment that stimulates the inherent ability of our immune system to attack tumour cells. Scientists in the field of cancer research believe that immunotherapy can enhance the efficacy of drug treatments and reduce or eliminate the devastating adverse drug side effects that often come with traditional chemotherapy. A variety of strategies are continuing to evolve in the laboratory and in the clinic and include cytokines, cancer vaccines and, most notably, certain monoclonal antibodies identified as checkpoint inhibitors. Immunotherapy is the most successful and challenging cancer therapy used nowadays to combat with cancer, and US researcher James Allison and Japanese researcher Tasuku Honjo have won the 2018 Nobel Prize for Physiology or Medicine for their breakthrough work on modulating the immune system for the battle against cancer. In connection with oxidative stress to immunotherapy, one of the recent studies observed using uncouplers as ROS generators with immunotherapy have synergistic effects with inhibition of PD-1 and subsequently blocking of tumour growth. The mechanism was assumed to be the generation of hypoxia by the synergistic effect of the uncoupler with chemotherapy [80, 81] because using of uncoupler alone did not show any significant effect on the growth or gene expression of tumour cells. The drugs used as immunotherapy in NSCLC are described in Table 19.3.

Table 19.3 Drugs used as immunotherapy in NSCLC

Type	Generic name	Brand
PD-L1 inhibitor	Atezolizumab	Tecentriq
PD-L1 inhibitor	Durvalumab	Imfinzi
PD-1 inhibitor	Nivolumab	Opdivo
PD-1 inhibitor	Pembrolizumab	Keytruda

19.12 Conclusion and Future Direction

Inflammation is accepted increasingly as an important factor involved in the development of various types of cancer including lung and also a vital mediator in response to therapy. A critical interaction between immune cells and tumour cells as well as other stromal cell types and tissue components is shown in the NSCLC microenvironment. ROS or RNS generation is crucial for the regulation of normal cell metabolism and is essential for many important events for the development of the organism. In contrary, overproduction of ROS and RNS is toxic for cells and presumably stimulates chronic inflammatory conditions and thus contributes to the progression of lung cancer. Therefore, a well and apparent discussion how ROS or RNS play role in the development of lung cancer may reveal new mechanisms associated with lung cancer and open possibilities to design new therapeutic strategies for the prevention of lung cancer.

Leading a healthy and proper lifestyle is considered to be an indispensable factor to overcome the difficulties of treatment for many diseases and may extend the lifespan of patients diagnosed with several life-threatening diseases such as cardiovascular disorder and cancer. Nowadays a regular physical exercise routine is advised as a crucial factor for good and healthy act of living. A well-planned and moderate physical exercise has often been prescribed in addition to medicine for adult as well as elderly people. Regular exercise compensates the adverse effects resulted by free radicals and brings many health benefits, including reduced risk of all-cause mortality and death in elderly people. Although, there is a debate that physical exercise also induces oxidative stress and inflammation. Moreover, significant increase of ROS level has been found during physical exercise that cause damage to the cell membranes and show harmful effects on skeletal muscle performance. A progressive and persistent physical activity enhances detoxification of a large amount of ROS of body cells, and this has been recommended for both in adult and elderly people to keep them safe from oxidative damage and prevent from age-related disorders. Several studies have been performed to identify natural compounds and micronutrients that are capable of preventing or attenuating the exercise-induced oxidative stress and inflammation. Many types of natural compounds individually or in a mixture, which act as exogenous antioxidants, are usually provided within the diet to boost our endogenous antioxidant systems, and they

are used as important ergogenic factors to increase the strength in physical exercise, both in young and elder population.

There is enormous number of evidences suggesting exercise can help in a different way for improvement of lung cancer patients. Exercise can be effective for patients at any stage of lung cancer as it increases strength and endurance and decreases emotional issues. A recovery fitness program is prescribed for lung cancer patients, which provides guidance on breathing, stretching, aerobic exercise and strength training. Therefore, a regular progressive physical exercise with healthy lifestyle can be a good option to keep ourselves safer from lung cancer.

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Regulation of Oxidative Stress by Nitric Oxide Defines Lung Development and Diseases

20

Suvendu Giri, Sumukh Thakar, Syamantak Majumder, and Suvro Chatterjee

Abstract

Development and maturation of the lung airways primarily take place in two different phases: first during embryonic days and second during postnatal days. During postnatal development, rapid angiogenesis and alveolarization are necessary to attain the capacity of the lung to support the need of the baby. During lung development, alteration in ROS level may significantly compromise maturation of the alveolar structure. We have employed a unique approach to achieve alteration in ROS level in the chick embryos to ascertain ROS function in early lung development. We have used a known ROS quenching nitric oxide (NO) donor and a ROS inducer called thalidomide, a known teratogen. Using next-generation high-throughput sequencing (NGS) analysis, we have performed the transcriptomic analysis of the NO- and thalidomide-treated chick embryos. Using STRING database, we have identified a set of lung-associated developmental genes that were significantly altered upon NO and/or thalidomide treatment and thus providing evidence that interplaying with cellular ROS level could possible alter the set of genes involved in early lung development. In conclusion, the current study shed light that alteration of ROS level could modulate the expression of early genes which are required for normal lung development and maturation.

Keywords

Alveolarization · Angiogenesis · Lung · Nitric oxide · Reactive oxygen species · Thalidomide

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20.1 Introduction

Lung structure and functions are incredibly interesting given the marathon task it performs through the years of one's life. Alveolar airways of the lung known as alveoli sacs are designed to have enlarged surface area to promote gas exchange between blood and inhaled air [1]. Each lung contains around 300 million alveoli, the main gas-filled air sacs, where the major gas exchange takes place. Developments of these alveoli take place in two major steps: foetal stage and postnatal stage after birth. Foetal lung development consists of three main stages: pseudoglandular, canalicular, and saccular. Bronchial tree formation along with epithelial differentiation follows the next. In contrast, postnatal lung development follows classical and continued alveolarization. The hallmark of this phase is the microvascular maturation and vascular integration with airway machinery for forming an efficient gas exchange module. Interfering with this process of lung development could severely compromise lung function that have a long-term consequence on one's life.

Several lung-associated complications like pulmonary hypertension, bronchopulmonary dysplasia (BPD), chronic obstructive pulmonary disease (COPD), and asthma are driven by reactive oxygen species (ROS) level. For instance, in response to vasodilators, ROS impairs the normal relaxation of pulmonary artery [2]. ROS level is also shown to play role in the remodelling of pulmonary arteries that increases the severity of pulmonary hypertension [3]. High oxidative stress resulting from environmental exposure, primarily cigarette smoking, facilitates the onset and progression of COPD [4]. The exogenous ROS generated from cigarette smoking causes oxidant/antioxidant imbalance, whereas the ROS released in the course of mitochondrial stress and inflammation may contribute to the disease progression. ROS may affect discreet biomolecules, viz., DNA and proteins of alveolar epithelial cells (AEC), thereby causing epithelial cell apoptosis [5]. In pathologies associated with early lung development as in case of BPD, generation of free radical is largely recognized as one of the major factors of damage to the airways. The link between inflammation and ROS involves the direct activation of inflammatory cells, especially granulocytes, which potentiates the inflammatory reaction during progression of BPD. Supplementation with antioxidants in preterm newborns particularly exposed to ROS who are at risk for BPD showed improved airway function and better lung health in long term [6].

In the present study, we have specially investigated into the early lung development that primarily takes place during embryonic stages. We have used thalidomide, the notorious teratogen, which causes a wide range of morphological and developmental errors in foetus including lung development. Thalidomide interferes with different signalling pathways related to embryonic development. However, it globally exerts oxidative stress in the embryo [7]. We have used thalidomide to exert oxidative stress in the chicken embryo and used nitric oxide (NO), which is known for its superoxide quenching property, to reduce the propensity of oxidative stress in the developing lung. We investigated the modulation of genes, which are closely linked to lung development in the early phase of life. We demonstrated that thalidomide-mediated ROS modulation leads to drastic changes in the expression of

lung-associated developmental genes that could likely abrogate normal lung maturation.

20.2 Background

20.2.1 Stages in Lung Development

In broader context, the lung development can be divided into two major stages: organogenesis that takes place during the stages of foetal lung development followed by differentiation that takes place during rest of the stages of the foetus as well as during postnatal lung development. During organogenesis formation of major airways and bronchial tree along with the birth of acinus occurs, while in next stage differentiation and formation of mature lung epithelial cells (both type I and type II) along with formation of air-blood barrier and expansion of the airspaces take place. Cellular differentiation to organize more alveolar and microvascular structures continue through the postnatal stages of lung development. The developments complete by three months after birth however possible continue up to two years to adulthood. The phases of lung development are primarily based on the morphological criteria as depicted (Fig. 20.1 [1, 8]).

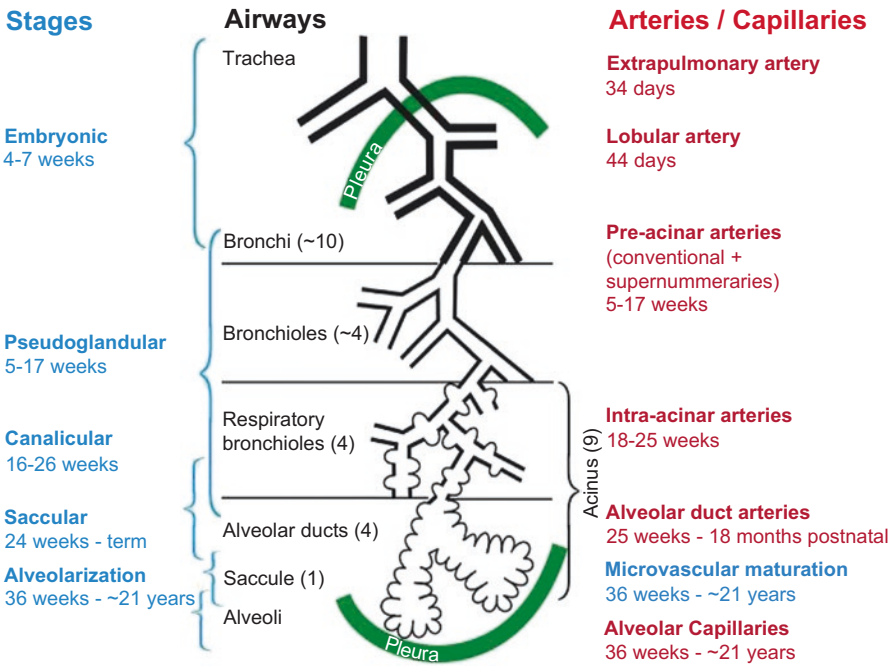

Fig. 20.1 Stages of lung airways and arteries development

20.2.2 Late Development of Lung

Increased luminal pressure and attraction of septa towards chemoattractant signals are critical to regulate late lung development. At birth, it can be attributed to the large increase in pulmonary recoil pressure resulting from the creation of an air-liquid interface and associated intrapulmonary surface forces [9]. The ability of the lung to increase its intra-alveolar area after partial pneumonectomy showed that this requires the presence of a mechanical force as it was increased after higher inspiratory efforts seen by an increase in the intraplueral pressure of the healthy lung [10]. Earlier studies in foetal sheep and postnatal lambs show that relative to the lungs of postnatal lambs, those of the late-gestation foetus are highly distended [11]. At birth, when an air-liquid interface is created within the lung, the lung partially recoils because of increased intrapulmonary surface forces, lowering lung capacity and creating a more subambient intrapleural pressure [12]. During the latter one half of gestation in foetal sheep, the lung luminal volume increased progressively which can be attributed to a large increase in pulmonary recoil pressure due to an increase in the expiratory pressure after birth so as to increase the surface area and help in the formation of an air-liquid interface. Rhythmic stretch of 10%, 0.8 Hz given to foetal sheep lungs for a period of 24–48 h increased the proliferation of type II AEC and also stimulated the incorporation of choline into intracellular phospholipids and increased adenosine 3′,5′-cyclic monophosphate levels [13].

20.2.3 Contribution of Reactive Oxygen Species in Lung Development

ROS is generated in the developing lung from sources which act as the first line of defence to counter infection, viz. alveolar macrophages and phagocyting cells. Alike neutrophils, superoxide generated by the membrane NADPH oxidase forms the prime source of ROS [14]. Alveolar macrophages are the major source of ROS under physiologic conditions. Inflammatory cytokines enticed peripheral monocytes-macrophages that cause a substantial damage to alveolar sacs leading to pulmonary diseases. Recently, it has been shown that after invigorating and differentiating these cells into macrophages, they become capable of producing superoxide by xanthine oxide reductase (XOR) that plays a significant role in acute lung injury (as opposed to invading neutrophils, where XOR is silent) [15]. The presence of mast cells in the lungs is rather conjectural. However, there are irrefutable findings which prove their ability to generate ROS. Type II pneumocytes with their dynamic metabolism yield surfactants and form a part of alveolar epithelium. These cuboidal cells are shown to function as precursor cells for type I pneumocytes in the case of increased destruction of alveolar epithelial cells [16]. Recent studies show that even type II epithelial cells possess enzymatic properties for production of ROS and modulation of these properties may lead to pathologies such as associated with asthma or COPD. Superoxide reacts rapidly with NO and forms peroxynitrite (ONOO), thus attenuating NO-mediated vasodilation and inactivating other

enzymatic pathways by the generation of nitrotyrosine [4]. Exposure to hyperoxia was also found to result in decreased angiogenesis, affecting VEGF transduction through ROS, suggesting that ROS generation affected preterm individuals. ROS was further found to induce vasoconstriction in pulmonary hypertensive foetal lamb lungs via signalling of NF-kB and activation of Nox 4 suggesting a role in early lung pathogenesis [17]. eNOS protein levels were also found to be decreased at such circumstances. Long-term oxygen therapy is given to premature infants with BPD, but exposure of the immature lung to elevated levels of oxygen may have adverse effects on lung development due to abnormal VEGF signalling and low eNOS levels and PH [18, 19]. Regarding ROS generation itself in the foetal lung during alveorization will help one to understand the signalling mechanisms behind it.

20.2.4 Sources Aiding in ROS Generation

NADPH oxidases, a class of membrane proteins, transfer electrons from NADPH to molecular oxygen, thereby producing superoxide intracellularly or extracellularly. Seven of them have been identified, with Nox1, Nox2, and Nox4 being important for mediating gene expression in vascular cells. Nox1 is expressed in vascular smooth muscle, endothelium, and adventitia and is circumscribed to plasma membrane, caveolae, and endosomes. Overexpression of Nox1 in vascular smooth muscle was found to increase ROS levels, causing eNOS uncoupling due to formation of BH2 and decreasing NO bioavailability and injuring the lung [20]. Mouse lung cell lines showed an increase in Nox1 expression on a 72-h exposure to hyperoxia. Similar effects being seen in mice affected with pulmonary hypertension suggest that Nox1 may pathologically engage in degeneration of alveolar foetal lung [21, 22]. In contrast, Nox2 is expressed in cells constituting the vasculature and also in the phagocytic cells. However, the isoform is activated by pathways very similar to Nox1. This requires the congregation of various protein subunits, including p22phox, p47phox, p67phox, and Rac. Nox2 when present on plasma membrane inhibits NO signalling by secreting superoxide into the extracellular space. In lamb lungs, increased ROS produced by Nox2 expression was found to alter vascular responses leading to vasoconstriction during neonatal pulmonary hypertension. Nox4 is expressed more copiously in vascular cells than Nox1 and Nox2 and localizes to the mitochondria, endoplasmic reticulum, and nucleus. Nox4-derived H_2O_2 may contribute to impaired vasodilation in persistent pulmonary hypertension in the neonate (PPHN) lambs via decreased eNOS and sGC expression and increased PDE5 activity [3]. Cyclin D1 expression was found to be higher in PPHN lungs and in pulmonary arteriole smooth muscle cells (PASMC) isolated from rats treated with monocrotaline. However, Nox4 small interfering RNA decreased the expression of cyclin D1 in PPHN and PASMC, and intra-tracheal catalase also decreased the expression of cyclin D1 in the lungs of ventilated PPHN lambs indicating a link between increase in ROS generation and activation of cell-cycle promoters leading to remodelling of pulmonary vasculature during pulmonary hypertension [18]. NF-kB activation causes increase in Nox4 expression leading to ROS generation

upon pulmonary hyperoxia. It was found downstream of ROS activated via the phosphorylation of IkB and enabled NF-kB translocation into the nucleus, thus regulating the transcription of target genes. This may propose a feed-forward mechanism in PPHN by which Nox isoforms assist ROS generation, and thereby, raises the activities of key transcription factors such as NF-Kβ resulting in sustained expression of Nox itself [23].

20.2.5 Nitric Oxide Implications in Alveorization and Angiogenesis

NO as first discovered as endothelial-derived relaxing factor was later extensively studied in many cellular processes including cell migration, proliferation, survival, and cell death. Majority of the research studies support that eNOS-derived NO plays many roles than just as a vasodilator. NO was found to enhance proliferation of endothelial cells (EC) by inducing expression of VEGF and fibroblast growth factor. In rat aortic EC, L-NAME or DAHP inhibited NO production from NOS, and VEGF fabrication was concomitantly reduced. Finally, when NO production was even marginally increased by L-arginine or BH4 supplementation, VEGF synthesis was also restored [24]. NO was also found to stimulate angiogenesis by downregulating angiostatin which helped to promote proliferation by decreasing activity of MMP-9 and MMP-4. In addition, NO through its canonical cGMP-PKG pathway promotes cell migration which is pivotal for endothelial cell migration to support sprouting angiogenesis [25]. Alveolarization, formation of new airway sacks that is essential for gas exchange between air-blood barrier, starts at week 36 of the human foetus and continues till adulthood. It consists of two major stages: (1) classical alveorization as evident by more branching by the AEC to form alveoli, the principal gas exchange units of the lung and (2) microvascular maturation that is associated with formation of more network of vascular structures having close connection with the alveoli structures to cause efficient gas exchange. Endothelium-derived NO plays a crucial role in postnatal microvascular maturation in the lung, mediating downstream signalling in response to classic angiogenic factors. Recent findings in eNOS-deficient mice point to a novel and previously unrecognized role of eNOS–NO pathway in foetal lung vascular development and lung morphogenesis [26].

20.2.6 Angiogenic Growth-Factor-Driven Angiogenesis during Alveolar Development, Injury and Repair

1. *Vascular endothelial growth factor*: VEGF binding to VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) on vascular endothelium activates receptor tyrosine kinases, leading to endothelial survival signalling. Localization of VEGF to distal airways was observed when rat lungs were injected with VEGF beads during late lung development and also showed an increase in vasculature development. During the development of a regular mouse lung, the mRNA expression of VEGFR-2 also

increases. It is closely confined to the pulmonary EC as opposed to the flourishing epithelium. The expression of VEGF 120, VEGF 164 and VEGF 188 rises during the canalicular stage (when most parts of the lung are vascularized) in alveolar type II cells. These levels then decrease gradually until postnatal day 10 (P10), and the expressions further increase to the levels that are maintained through adulthood [27]. Treatment with VEGFR-1 and VEGFR-2 blocker SU5416 to adult rats spontaneously causes enlargement of the airspaces, indicative of emphysema [28], suggesting that VEGF is essential to the maintenance of the pulmonary vessels and alveolar structures throughout adulthood. Conversely, lung overexpression of VEGF during normal lung development disrupts the lung architecture [19].

2. *Nitric oxide*: VEGF-induced lung angiogenesis is partially moderated by NO. Delivering SU5416, a VEGF inhibitor, downregulates neonatal eNOS protein and NO production, while subsequent treatment with NO (inhaled) revamps vascular and alveolar growth at neonatal stage in the BPD model [29]. *(iii) PECAM-1*: Administration of an anti-CD31 antibody that inhibits EC migration without affecting proliferation or survival in vitro also impairs septation in neonatal rats. Loss of CD31 function compromises postnatal lung development and provides evidence that inhibition of EC function, in contrast to loss of viable EC, inhibits alveolarization [30]. *(iv) Angiopoietin*: Ang-1 and VEGF expression in the lung was found to increase during gestation. In baboons conditioned for appropriate oxygenation and ventilation so as to maintain the standard arterial blood gases, VEGF was reported to be concentrated over the distal airspace epithelium through 140 days of gestation, thereby supporting the hypothesis that these angiogenic factors contribute to pulmonary microvascular development. Comparably, 125-day baboons showed a higher capillary density and decreased pulmonary microvasculature, showing premature lung development injury leads to disruption of angiogenesis [31, 32].

20.2.7 Disruption of Alveorization and Angiogenesis

A study performed on eight patients suffering from BPD showed a disruption of arterial vasculature and a decrease in percent medial thickness of pulmonary arteries indicating that pathological remodelling would follow abnormal vascular development, giving rise to lung disorders. The marked expansion of the pulmonary microvasculature in ventilated lungs was, at least partially, responsible for brisk EC proliferation. There was improved angiogenesis in ventilated long-term infants compared with age-matched post-term infants and also found to be nearly proportionate to the growth of the air-exchanging lung parenchyma, indicating that microvascular development when disrupted could lead to BPD [33]. PECAM1 protein levels decreased in BPD than control individuals indicating an inability to form arterial system, thereby decreasing the ability to form a gas-filled distal lung parenchyma that could lead to BPD in affected preterm infants [34]. In general, infants in brief ventilator phase were more affected than the infants in long-term ventilator phase. This could be associated with pronounced EC proliferation to support

angiogenesis. Many animal models were also found to exhibit effects similar to BPD and chronic pulmonary dysplasia when alveorization was disrupted by exposure to different oxygen levels. This observation indicates that angiogenesis and alveorization are dependent on each other.

By now, the link between NO to postnatal lung development in context to their role in lung microvasculature formation and alveolarization is strongly established. It is evident from many findings that NO is a modulator of cellular ROS, and such function of NO could possibly contribute to many physiological functions including those functions essential for organ or tissue development. In addition to its ROS modulation property, NO can also act through canonical sGC-cGMP-PKG signalling pathway which is well established as a driver of postnatal lung angiogenesis prerequisite for alveolarization during early lung development. Although such report of describing its role as a ROS modulator is well established, the molecular mechanism of NO-ROS axis during lung development is underexplored.

20.3 Methodology

20.3.1 Mice Tissue and IHC

The mice tissue sections were a part of the study from the University Rochester Medical Centre, Rochester, NY, USA. The mice lung tissues were processed for immunohistochemistry (IHC) using von Willebrand factor (vWF) antibody (1:1000; Dako) to stain the microvasculature in the tissue. IHC was executed on formalin-fixed and paraffin-embedded 4-μm-thick sections of the lung. After processing for IHC, tissues were counterstained with H&E and mounted with IHC mounting media (Vector Labs).

20.3.2 Chick Embryo Model, Treatment Conditions, and Next-Generation Sequencing and Analysis

Transcriptome data were acquired from the online gene expression database under the accession number GSE69159. Methods were adopted from Kumar et al. (2016) [35]. In brief, fertilized brown leghorn (*Gallus gallus*) eggs were commercially obtained from nearby poultry research centre, Potheri, Chennai. Cleaned eggs were incubated at 37 °C in 60% humidity chamber. At HH8 stage, a small window was made in the eggshell, and eggs were treated with 10 μM NO donor (SpermineNONOate) or 60 μg of thalidomide dissolved in 5% DMSO (1 mg/ml) or vehicle. At HH29 stage, total RNA was isolated using TRIzol® method. Illumina HiSeq 2500 platform was used for entire transcriptome sequencing and aligned against genome of chicken (Galgal4). Cuffdiff (v2.2.0) and TopHat (v2.0.8) programs were used to spot the genes that expressed differentially. STRING v10.5 was used to generate protein–protein interaction (PPI) network and to identify lung–associated gene sets.

20.4 Results and Discussion

20.4.1 Deformed Vascular and Alveolar Structures in Mice Lung

Crosssection of the lung tissues obtained from mice having premature lung development during early embryo days. Tissues were stained for vWF to visualize lung microvasculature and further counterstained with H&E to visualize the alveolar structure. These mice are known have defective lung vasculature and an increased ROS level. The representative images showed to have an increased alveolar space and less number of alveolar sacs per unit area in the diseased mice lung which is co-related with the reduced microvasculature structure (Fig. 20.2) which appears as brown (shown by red arrows).

20.4.2 Modulation in ROS Level through either NO or Thalidomide Treatment Leads to Alteration in the Expression of Lung-Associated Genes

Upon analysing the NGS data obtained from early chick embryos treated with either a quencher of ROS such as NO or a ROS inducer thalidomide lead to alteration in the transcript level expression of many lung-associated genes. We have selected these genes based on STRING network of genes that are suggested to be lung-associated gene sets. Using the analysis, we have identified the following genes (Fig. 20.3; Table 20.1) which are further elaborated on their role in lung development:

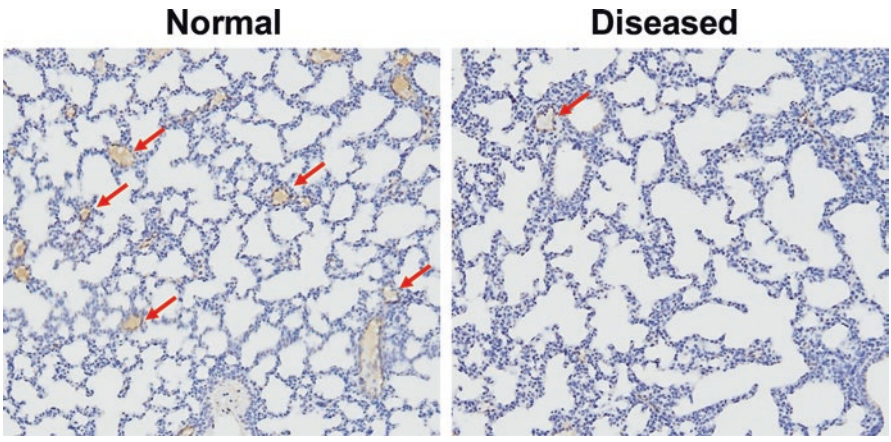

Fig. 20.2 Deformed alveolar structure in postnatal mice lung which is associated with less vascularization

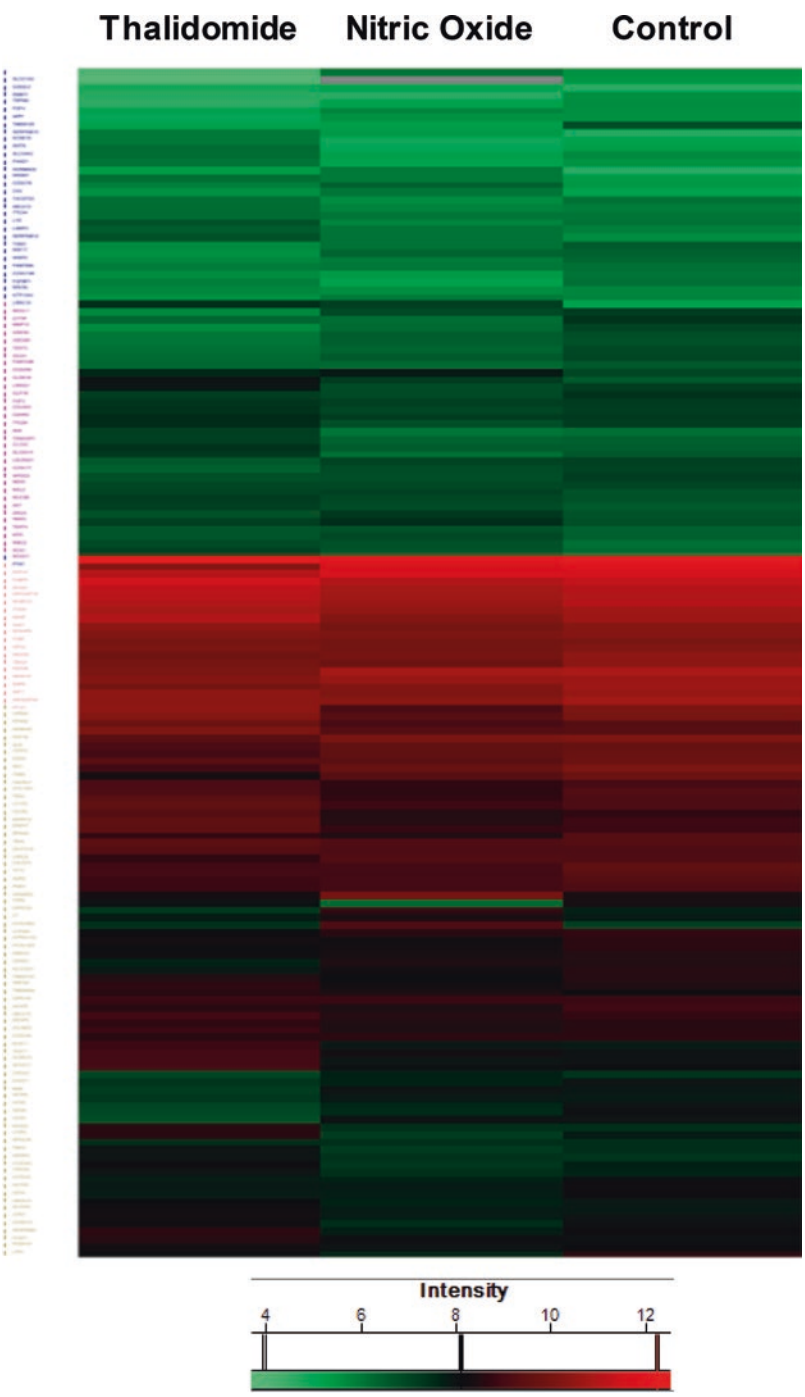

Fig. 20.3 The heatmap constructed from important genes selected from 1146 lung specific genes (Xiong et al. 2016)

Table 20.1 Selected from 83 lung specific genes, which are significantly altered upon either NO or thalidomide treatments

No	Gene name	Control Vs. NO (Fold change)	Control Vs. Thalidomide (Fold change)
1	LDLRAD1	0.787110391	2.056001295
2	TBX4	0.554617488	0.604089836
3	LRRN4	0.438591389	1.43430168
4	AKAP5	1.246258119	1.036239869
5	SLC34A2	0.872380683	2.690502357
6	CLDN18	3.031438668	1.957745998
7	WIF1	0.801372647	0.499163531
8	NKX2-5	0.829406266	0.781458907
9	MS4A15	1.128130407	0.482386605
10	SNTN	0.900075308	2.357064031
11	LAMP3	0.894054738	1.934642163
12	WNT3A	0.786988923	1.015645477
13	MUC5B	1.899059991	1.441340548
14	CDHR3	1.000645205	1.306729107
15	SLC6A14	1.264585444	2.632674818
16	RTKN2	0.60777849	1.361021748

1. *LDLRAD1*: NO treatment downregulated the expression of this gene by 21%, while thalidomide alone treatment induced the expression of these genes by 100%. LDLRAD1 is crucial in lung development as it takes part in lung organogenesis [36]. It has been also reported that the gene is associated with disease like childhood asthma [37] and chronic obstructive pulmonary disease [38].
2. *TBX4*: Thalidomide treatment inhibited the expression of TBX4 gene by 40%, while NO treatment as well reduced the expression of this gene by 45%. T-box transcription factor Tbx4 is shown to be associated with pulmonary arterial hypertension [39].
3. *LRRN4*: NO downregulated LRRN4 by 56%, while 43% elevation was observed under thalidomide treatment. The leucine-rich repeat neuronal 4 (LRRN4) is expressed in the lung and kidney [40]. It is reported to be associated with chronic obstructive pulmonary disease [41].
4. *AKAP5*: NO induced the expression of AKAP5 by 24%; contrarily thalidomide did not alter its expression in whole chick embryo. AKAP5 expression is significantly reduced in COPD [42].
5. *SLC34A2*: Transcriptomic data obtained through NGS revealed that thalidomide treatment induced the expression of this gene by 169%, while NO did not alter the expression. The sodium-dependent phosphate transport protein 2B responsible for the phosphate transport via Na + cotransport in pH-dependent manner expressed in the small intestine and lung [43]. Mutations in the gene can cause pulmonary alveolar microlithiasis (PAM) [44].
6. *CLDN18*: Both NO and thalidomide treatment elevated the expression of CLDN18 transcript level in chick embryos. Claudin 18 (CLDN18) protein contributes to tight junction. It gets mostly expressed in the stomach and lung alve-

olar epithelium [40]. Reduction in the level of CLDN18 can cause impaired alveolarization [45].

7. *WIF1*: Thalidomide treatment abrogated the expression of WIF1 by 50%. In contrary, NO treatment did not alter the expression of these genes. The gene regulates the Wnt signaling by WNT inhibitory factor 1, associated with reduced lung function in asthma [46]. It is also related to lung development in embryo [47].
8. *NKX2-5*: Thalidomide treatment downregulates the expression of NKX2–5 by 22%, without having any effect by NO treatment. This gene is responsible for the production of a homeobox-containing transcription factor that mostly related to heart development in foetus [48]. It has been also reported that it plays a major role in pulmonary myocardium formation [49]. Haploinsufficiency in the gene leads to several abnormalities like Choreoathetosis and pulmonary alterations [50].
9. *MS4A15*: According to our result, the expression of this gene increased by 12.8% in NO-treated embryos, while it decreases by 52% under thalidomide treatment compared to control. The product of the gene is Membrane-spanning 4-domains subfamily A member 15 (MS4A15) which is associated with lung adenocarcinoma [51].
10. *SNTN*: NO treatment did not alter the expression of this gene, while thalidomide treatment cause at least 2.3-fold increase in the expression of this gene. The protein sentan, cilia apical structure protein encoded by SNTN gene, is a major component of vertebrate motile cilia [52]. Directional induction of multiciliated airway cells from pluripotent stem cells is associated with increase in this protein level in human [53].
11. *LAMP3*: NO has no significant effect in this gene expression, but thalidomide induced its expression by almost 93%. It has been reported previously that Lysosome-associated membrane glycoprotein 3 (LAMP3) is associated with adaptive immunity [54]. The gene has been predicted to play crucial role in asthma [55].
12. *WNT3A*: The expression of WNT3A significantly reduced upon NO treatment, while thalidomide did not alter the expression of this gene in developing chick embryo. This is one of the major components of WNT signalling associated with differentiation and proliferation of mesenchymal cells in embryonic lung [56]. It has an important role in metastasis of lung adenocarcinoma [57].
13. *MUC5B*: NO treatment caused almost 90% upregulation in its expression, but 44% increase in the same has been observed under thalidomide treatment. The gene encodes one of mucin family of protein that functions for mucus secretion and airway defence [58]. The upregulation of the gene is linked with COPD [59] and idiopathic pulmonary fibrosis [60].
14. *CDHR3*: No change was observed in NO-treated tissue samples, while thalidomide treatment significantly increased the expression by 30%. Cadherin-related family member 3, expressed mostly in airway epithelium, is related to the calcium ion binding activity and associated to asthma [61].

15. *SLC6A14*: NO treatment caused almost 23% increase, but thalidomide treatment results in massive increase (about 163%) in the gene expression, observed in our study. The gene encodes the protein solute carrier family 6 member 14 (SLC6A14) which is responsible for the transport of neurotransmitter depending on sodium and chloride ion [62]. Previous study supports the fact that the gene is related to embryonic lung development [56]. It also been found that the gene gets highly expressed in chronic obstructive pulmonary disease patients compared to healthy subjects [63].
16. *RTKN2*: Under NO treatment, the gene expression decreased by about 40%, whereas thalidomide treatment elevated its expression by 36%.The protein rhotekin-2, described as lymphocytic Rho-GTPase effector, has been related to the lung function of patients suffering with idiopathic interstitial pneumonias [64].

20.4.3 Transcriptomic Analysis of the Lung-Development-Associated Genes in Chick Embryos Treated with ROS Modulators NO and/or Thalidomide

We further analysed our NGS data to identify the differential expression of the genes that are extracted from STRING database which is shown to be associated with lung development. STRING database identify 1146 genes under this category which either directly or through secondary messenger can control lung development. Through this analysis we have identified a set of genes that are significantly altered upon NO and/or thalidomide treatment suggesting that alteration in ROS pathways as achieved in our case either through NO or thalidomide treatment leads to changes in transcriptomic level of these lung-development-associated genes (Fig. 20.4; Table 20.2):

1. *CCDC40*: NO treatment did not alter the expression of CCDC40, while thalidomide elevated the expression by 32%. The CCDC40 gene is required for encoding coiled-coil domain-containing protein 40 that is very important for the migratory movement of cilia and left-right axis formation [65]. Mutations in this genes lead to the disease ciliary dyskinesia type 15 [66].
2. *HOPX*: Thalidomide alone inhibited the expression by 67%, while NO did not alter the expression. Homeobox only protein x (HOPX) is necessary for pulmonary maturation as well as regeneration of alveolar cells [67]. Its expression level is elevated during alveolar injury and during the repair process. It is believed that decrease in expression of HOPX may lead to end-stage idiopathic pulmonary fibrosis due to failed regenerative processes [68].
3. *ADA*: Again thalidomide treatment significantly abrogated the expression of ADA by 55% without having any effect by NO alone treatment. Adenosine deaminase (ADA) that catalyses the deamination reaction of 2-deoxyadenosine and adenosine is essential for cellular signalling in postnatal development [69]. Recent studies claim that regulation of the gene could be the important key for prevention of lung injury and progression of COPD [70].

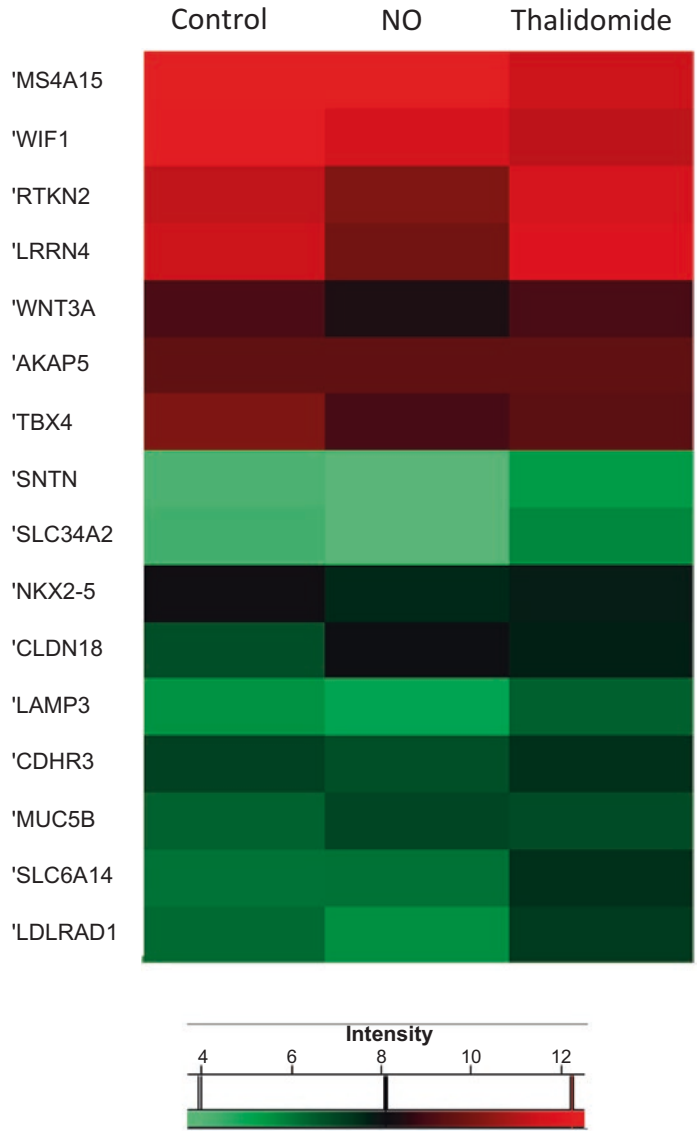

Fig. 20.4 The heatmap constructed from important genes selected from 83 lung specific genes (Xiong et al. 2016)

4. *FOXF1*: FOXF1 expression is not altered with NO treatment, while thalidomide elevated the expression by 70%. FOXF1 gene is responsible for the formation of transcription factor, forkhead box F1 (FOXF1) protein, that regulates many other genes activity related to lung lobe morphogenesis, alveolus development, vasculogenesis and trachea development in early development stage of embryo [71]. Commonly, it takes part in regeneration of lung after injury [72].

Table 20.2 Genes selected from 1146 lung specific genes, which are significantly altered upon either NO or thalidomide treatments

No	Gene name	Control Vs. NO (Fold change)	Control Vs. Thalidomide (Fold change)
1	CCDC40	1.0673	1.323051
2	HOPX	0.7478	0.330521
3	ADA	1.116421164	0.458733
4	FOXF1	0.8231	1.698857
5	EPAS1	0.9212	1.290014
6	TBX4	0.554617488	0.604089836
7	GPC3	1.1481	1.023282
8	PTK7	1.2961	1.728926
9	AGR2	0.7943	0.68004

5. *EPAS1*: NO did not alter the expression of EPAS1, whereas thalidomide elevated the expression by 29%. This gene encodes the endothelial PAS domain-containing protein 1 or hypoxia-inducible factor-2alpha (HIF-2alpha) which is essential in adaptation to high altitude at low oxygen pressure by regulation of erythropoietin level and angiogenesis [73].
6. *GPC3*: Under thalidomide treatment, there was no significant changes in the expression of this gene, while NO treatment augmented the expression by 14.8%. Glypican-3 is a heparan sulfate proteoglycan which is associated with various physiological signalling like Wnt, fibroblast growth factors and hedgehogs [74]. It is also associated with lung squamous cell carcinoma, and prediction has been made that it could be well used as a marker for the disease [75].
7. *PTK7*: Both NO and thalidomide treatments significantly increased the expression of PTK7 in early chick embryo. PTK7 is a coding gene for protein tyrosine kinase which is associated with mesenchyme development in the lung [76]. It involves many signalling pathways like ERK, Akt and Wnt signalling pathways [77].
8. *AGR2*: Thalidomide inhibited the expression of AGR2 by 32%, whereas NO treatment downregulated its expression by almost 21%. This gene encodes the anterior gradient protein 2 (AGR2) which is associated with mucus secretion and goblet cell differentiation in the lung along with other cellular transition and migration [78].

20.5 Conclusion

The current study demonstrated that alteration of ROS level in developing lung predisposes alveolar epithelial cells and new developing microvasculature to injuries leading to deformities in lung development. By combining the study with next-generation sequencing study, we are able to identify a set of lung-associated developmental genes which are significantly altered upon modulation of ROS either through reducing its level using a quencher (NO donor) or by elevating its level by treating the embryos with ROS inducer thalidomide.

20.6 Future Direction

Further research in evaluating the role of these genes identified through the sequencing study will help in better understanding of these ROS-associated lung diseases besides identifying new treatment strategies for ROS-driven lung diseases especially those that are associated with compromised lung development such as BPD.

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Epidermal Growth Factor Receptor: Promising Targets for Non-Small-Cell Lung Cancer

21

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Abstract

Cancer-related mortality is a worldwide health issue; among those, lung cancer is devastatingly caused by regular smoking. It is quite surprising that a considerable category of patients with non-small-cell lung cancer (NSCLC) has never had a habit of smoking. In these patients, an elevated level of small drifts or mutations in the epidermal growth factor receptor (EGFR) is observed. The genetic makeup and development of EGFR tyrosine kinase inhibitors (TKIs) are considered as promising drug candidates for the treatment of wild-type NSCLC. The aim of this chapter is to summarize EGFR and its physiological and pathological roles, including the acquired resistance of various generations of TKIs with future perspectives of EGFR-targeted therapeutic approaches.

Keywords

Epidermal growth factor receptor · Tyrosine kinase inhibitors · Non-small cell lung cancer · Cancer chemotherapy

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21.1 Introduction

Oxidative stress is the major mechanism and secondary messenger behind the pathogenesis of various diseases including the neurodegenerative disorders, ageing and cancers and by regulating apoptosis, survival and their proliferation [1]. Among lung diseases, lung cancer (LC) is identified as the major cause of cancer mortality worldwide due to its poor prognosis result in high death rates [2]. Aberrance on cell apoptosis – a programmed cell death mechanism – leads to pathogenesis including cancer. Cancer research mainly focuses on a new discovery which improves uncontrolled cell apoptosis of cancer [3, 4]. According to the World Health Organization survey, lung cancer will be attributed as the seventh cause of death and will contribute to 3% of the total death by 2030 [5].

Clinical evidence document that among the small cell lung cancer (SCLC) and non-small-cell lung cancer [NSCLC], the latter is referred to be the primeval, i.e. lung malignancy (85%) which can form both central and peripheral tumours and which has a poor survival rate, with only less than 15% of patients surviving more than 5 years [6]. Different types of NSCLC develop in different locations of the lungs: squamous cell carcinoma (25–30%), adenocarcinoma (45–50%) and cell carcinoma (5–10%). Adenocarcinoma develops in the peripheral part of the lungs, e.g. alveolar type II cells, squamous cell carcinoma in the central lung areas and large cell carcinoma in the central and peripheral regions [7].

Recently, researchers extensively have identified a large number of structural-based changes such as mutations and amplifications that could affect tumour survival [8]. Cancer treatment protocol mainly focuses on molecular changes and on how to effectively increase the survival rate of patients in non-surgical stages [9, 10]. During cancer therapy, it is important to identify the genomic profile of the patient and appropriate drug dosage [11]. Among the various types of signalling pathways, epidermal growth factor receptor (EGFR) target-based mechanism plays a significant role in the treatment of NSCLC [12]. The EGFR tyrosine kinase inhibitors gained wide recognition as an adjuvant therapy because of its high efficiency that could potentially cure LC patients [13, 14]. This chapter represents the EGFR and its physiological and pathological roles, as well as future perspectives of EGFR-targeted therapeutic strategies.

21.2 Role of EGFR in the Treatment of Lung Cancer

EGFR is a member of the erbB family, intimately related to receptor tyrosine kinases, which embrace erbB1 (synonym of EGFR) and other three known homologous proteins like erbB2 (HER2), erbB3 and erbB4 (Fig. 21.1; reproduced by Elena et al. 2013, with permission) [15]. All the EGFR analogous have almost similar structures but have a difference in their tyrosine kinase activity. Its extracellular region comprises a ligand-binding domain, whereas intracellular region and transmembrane portion consist of tyrosine kinase and regulatory domains. The naturally functioning EGFR undergoes conformational changes upon binding with specific

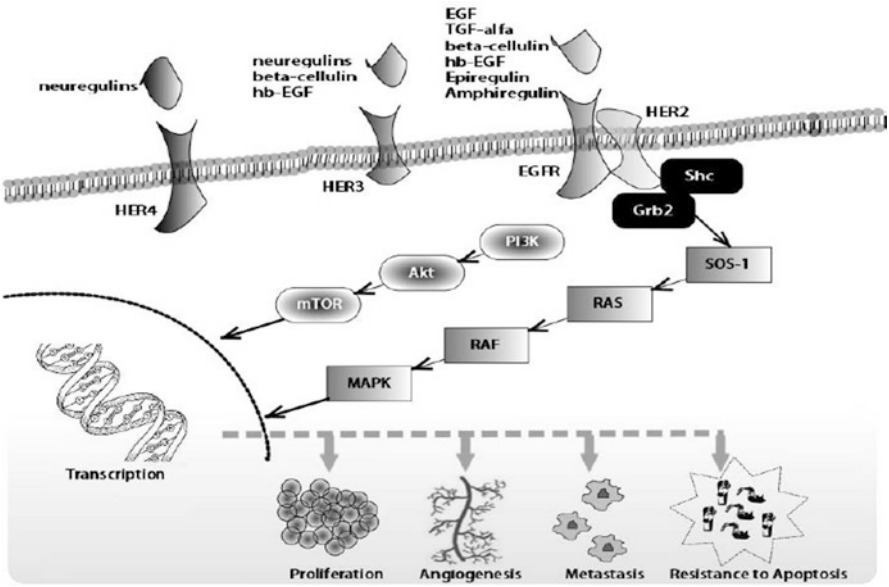

Fig. 21.1 EGFR signalling pathways

ligand (epidermal growth factor), followed by a series of events occurring in signal transduction pathways such as phosphatidyl inositol 3-kinase (PI3K/Akt) and mitogen-activated protein kinase (MAPK) with the help of factors like activator of transcription proteins (STATs) and Raf1-extracellular signal-regulated kinase. The outcome of cell proliferation and cell maintenance mainly depends on the above-mentioned signalling pathways [16]. In NSCLC, DNA mutations (often referred to as 'oncogene addiction') in EGFR expressed by polymerase chain reaction can take place both in extracellular and intracellular domains of the protein [17–20].

21.3 Architecture of EGFR

The architecture and physiology of EGFR show that among the 1186 amino acids, 621 residues belong to ectodomain and 4 to subdomains [21]. An earlier binding study revealed that inhibitor binding cavity (IBC) of EGFR is mainly positioned between amino acids of 294 and 543 which are stationed at domain III region. It further revealed that domain I also played a minor role in the efficiency of EGFR [22]. Three-dimensional X-ray crystallographic studies also assert that the three ligand sites are configured in domain I and III and small molecules situated in II and IV are linked together by one or two disulphide linkages.

NMR studies published that residues 626–647 are α-helical in structure and they continue into the juxtamembrane domain which has a number of regulatory functions including ligand-dependent internalization and receptor dimerization. It also clarifies that amino acid, tyrosine present in carboxy-terminal domain whose,

phosphorylation process and post-translational modifications is important to mediate the signal transduction.

21.4 EGFR Receptor Tyrosine Kinase (TK)-Targeted FDA-Approved Drugs for Treating NSCLC

The establishment of structure, biology, physiological functions, regulation and it's family members generated the development of new inhibitors of EGFR based cancer treatment. Rapid research in treatments targeting EGFR intensively progressed and resulted in the discovery of gefitinib, a first-generation TKI, in 2009, and erlotinib in the year 2013, which resulted in massive and rapid tumour volume shrinkage. On the other hand, patients developed secondary T790M gatekeeper resistance which introduced highly resembled second-generation irreversible inhibitors (TKIs) like afatinib, neratinib and dacomitinib. But their clinical efficacy is restrained due to narrow therapeutic window, which focused the research into third-generation EGFR TKIs. Their design explored the way for the introduction of a covalent bond with amino acid residue, Cys 797, for the effective management of T790M-associated resistance. Rociletinib, the first reported among third-generation compounds (WZ40028, also known as CO-1686), showed 30–100 times more potency against EGFR T790M than wild-type EGFR. But its further development halted due to FDA recall request for more randomized data in trials and accelerated approval. Clinical trial data also showed that rociletinib causes hyperglycaemia by inhibiting insulin growth factor receptor (IGFR) and insulin receptor kinases. In 2015, osimertinib, also known as AZD9291, became the first accorded TKI for T790 M EGFR-positive NSCLC patients. The Korea Ministry of Food and Drug Safety (MFDS) in 2016 approved olmutinib (HM61713) for the treatment of NSCLC with a mutation in T790 M. Currently, drugs in early-phase trials are nazartinib (EGF816, NCT02108964), avitinib (AC0010, NCT022743337) and naquotinib (ASP8273, NCT02500927) (Fig. 21.2) [22, 23].

21.5 Future Direction and Perspectives

Though targeted therapy has markedly improved the after effects of patients with non-small-cell lung cancer (NSCLC) who are prone to driver mutations, neoteric tactics are needed in the future to address invariable resistance. The current standard of care for patients with NSCLC which conceals genetic driver mutations is treatment with corresponding tyrosine kinase inhibitors (TKIs). The emergence of TKIs considerably improved outcomes for patients with NSCLC. EGFR inhibitors, for example, improved the median survival from about 1 year to 3–4 years. Conversely, acquired resistance occurs in more or less every patient and emphasizes the necessity for new approaches in the future to improve patient outcomes.

Current clinically actionable deviance includes EGFR, ALK, ROS1 and BRAF. But then again, newer targets need to be recognized to comprehend tumours

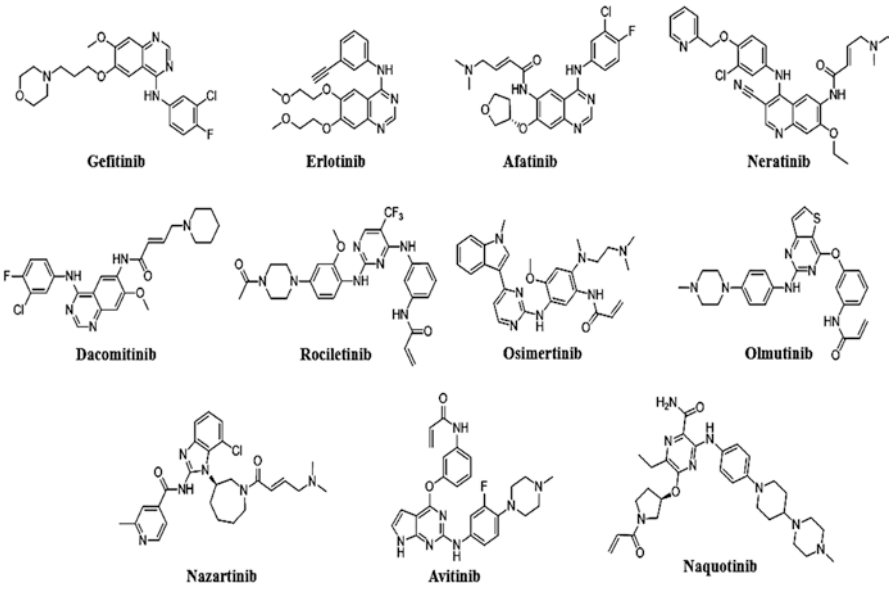

Fig. 21.2 Structures of the EGFR-targeted FDA-approved drugs

driven by other mechanisms. Inhibitors targeting the proteins of genetic deviance in RET, MET, HER2, NTRK1 and KEAP1/Nrf1 are in development and may become added options in the near future. KRAS is also frequently mutated in NSCLC; however, attempts to target this pathway have not yet been successful to date. In addition, more mutation-specific and wild-type-sparring TKIs are needed, as more specific TKIs have proven to be more successful than their less-specific equivalents. Osimertinib, an EGFR inhibitor that targets T790 M-resistant mutants, is more effective than former-generation EGFR inhibitors and is usually better tolerated. The ALK inhibitor alectinib is also more effective and well tolerated than others in this category. The heterogeneity of NSCLC signifies that combination regimens that comprise targeted therapy are a promising strategy to overcome resistance. Combination approach can target multiple pathways that may be involved in the disease.

21.6 Conclusion

The development of molecular-targeted anticancer drug moieties is considered remarkable due to their high exactitude in treating cancer malady. The pathways that target cellular signalling play a crucial role in cancer proliferation, survival and their development, which are genetically altered in cancer cells. Targeted-based therapy is a possible therapeutic strategy for patients with different malignancies. Chemotherapeutic drug resistance is a complex issue encountered in various kinds of cancer treatment, which is difficult to overcome. For example, even though

various research have been extended to study the feasibility of resistance mechanisms to EGFR blockade, it appears that numerous biomarkers and pathways were responsible for developing resistance to anti-EGFR therapy. New moieties that focused on single target were also found to have pronounced limitations like drug resistance in the treatment of cancer. As the prediction of resistance mechanisms is difficult, more complex prognostic methods are essential to improve the response rates to targeted therapy. It is concluded that robust knowledge and perceptions of cellular mechanisms underlying death and survival will lead to sensible approaches to overcome drug resistance. It is no doubt that, in the near future, there may be discovery of various aspects in governing the expression of the targeted drug resistance genes.

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Oxygenated Lipid Products in COPD and Asthma: A Clinical Picture

22

Debamita Chatterjee

Abstract

Lipids and lipid metabolism are essential for carrying out a wide range of physiological processes ranging from cellular integrity to storage and energy to signal transduction. It is increasingly being appreciated that lipid metabolism comprises of an interrelated set of pathways that orchestrate multiple cellular signaling processes, both in normal physiology and in aberrant pathological conditions such as inflammation. Oxygenated lipid products (oxylipins) are particularly suited to maintain the fine balance between a pro-inflammatory and an anti-inflammatory status inside the cell. Because lung diseases including COPD and asthma exhibit chronic grade inflammation, studying oxylipins in the context of such diseases is critical for the etiology and management of these diseases. Additionally, oxylipin profiles can be utilized as biomarkers to distinguish and diagnose different types of inflammatory respiratory diseases such as COPD and asthma.

Keywords

Lung · COPD and asthma · Inflammation · Lipid inflammatory mediators · Lipid oxidation · Oxylipin

22.1 Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are two of the most prevalent respiratory disorders. Both are responsible for increased morbidity and mortality and results in major economic burden globally [1–4]. Asthma is typically characterized by chronic airway inflammation [5–8]. COPD, on the other hand, is a

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progressively worsening condition characterized by fixed airway obstruction, and it terminally results in chronic bronchitis and emphysema [8–10]. Chronic bronchitis is mainly airway response to irritants characterized by mucus hypersecretion and bronchial wall thickening. Emphysema is characterized by damage to alveolar walls, resulting in loss of support and collapse of airways. The pathogenesis of both diseases has been documented extensively; the intensity of pathological changes increases as the disease progresses [11, 12].

In terms of statistics, COPD is the fourth-ranked cause of mortality globally and predicted to become the fifth-ranked cause of disability [13]. It affects about 10% of persons who are mid-aged or older [14]. Asthma is a chronic disease considered to affect approx. 339 million people worldwide and is 16th among the leading causes of years lived with disability [15]. A common mechanism of many respiratory disorders is chronic inflammation [16–19]. Inflammation is aided by a suite of inflammatory cells, cytokines, and other inflammatory mediators. These constituents are extensively studied as biomarkers both to predict and monitor disease outcome and to guide therapy [20].

22.2 Oxygenated Lipid Products

The inflammatory mediators in asthma and COPD include free radicals, cytokines and chemokines, and lipid mediators [19, 20]. Lipids have always been thought of as structural blocks conferring cellular integrity and as metabolic stores imparting energy in times of starvation. Apart from the usual role, lipids are now considered participants in cell signaling and cellular homeostasis [21]. Specifically, lipids are extensively required in inflammatory processes and innate immunity to survive in response to external insults and internal aberrations. However, chronic dysregulated inflammation leads to further cellular damage that leads to multiple diseases like asthma, COPD, cancer [22], Alzheimer's disease [23], and obesity [24].

One such class of lipids that are participants in inflammation are termed oxylipin. Oxylipins are oxygenated compounds synthesized from fatty acids: it includes eicosanoids synthesized from arachidonic acid as well as related compounds synthesized from other polyunsaturated fatty acids (PUFA – both long and short chain length) [25]. Such reactions involve at least one step of mono- or dioxygenase-catalyzed oxygenation. Cyclooxygenase (COX-1 and COX-2), lipoxygenase (5-LOX, 12-LOX, 15-LOX), and cytochrome P450 (CYP enzymes an CYPP450 microsome ω-hydroxylases) are three major enzymatic pathways for oxylipin biosynthesis [26, 27]. In contrast to pro-inflammatory oxylipins, a few oxylipins exert anti-inflammatory effects too [28–34].

22.2.1 Pro-inflammatory Oxylipins

22.2.1.1 Arachidonates

Arachidonic acid (20,4 n-6, 20, ω-6) is 20-carbon chain PUFA with four double bonds with the final double bond located 6 carbons from the terminal end of the aliphatic chain opposite the carboxyl group (ω-6-or n-6) [28, 30].

Prostaglandins (PGs)

PGs are eicosanoids with a 5-carbon ring. PGs are local autocrine and paracrine signaling molecules [31, 32] and are produced in most of the tissues. They attach to G-protein-coupled receptors like FP, DP1-2, IP1-2, TP, EP1-4, and CRTH2 [33, 34], and thus PGs act on a wide range of cells to produce a variety of effects. PGs are formed by sequential oxidation of arachidonic acid by cyclooxygenases and prostaglandin synthases [35, 36].

The most abundant PG is PGE2 which has a complex role in lung homeostasis. It has multiple functions that are regulated by a concerted action of different receptors and signaling pathways [35, 36]. PGE2 interacts with EP1 and EP3 to affect pain in inflammatory hyperalgesia while it interacts with EP4 to confer anti-inflammatory effects in allergic airways [37]. PGE2 has been shown to confer a bronchodilatory effect in allergic airways: it prevents allergen-induced bronchoconstriction [37, 38]. PGD2, on the other hand, interacts with DP and CRTH2 receptors and has been shown to be pro-inflammatory producing airway bronchoconstriction [38–40]. Studies in mouse indicated that COX-1 activity is bronchoprotective, while COX-2 promotes infiltration of inflammatory cells in the lung [41–43]. Interestingly, patients with asthma have lower levels of PGE2 [44], while COPD patients have increased PGE2 in respiratory secretions [29, 45]. Additionally, increased signaling in PGE2-EP2/4 axis has also been observed in fibroblasts isolated from the lung parenchyma of COPD patients [29].

Thromboxanes

Thromboxane A2 (TXA2), generated from PGH2, interacts with TP receptor [46] and is a potent bronchoconstrictor [47]. TXA2 is mainly formed from platelets wherein it promotes aggregation and vasoconstriction [48]. Additionally, it acts on bronchial and vascular smooth muscle cells. Because TXA2 affects bronchial hyperresponsiveness [49], inhibitors of TXA2 are considered as a therapy for asthma.

TXA2 is nonenzymatically degraded to TXB2 in the lungs for exhalation. Interestingly, TXB2 level was below detection levels in exhaled breath condensates (EBC) from COPD patients. However, the levels were found to be elevated in EBC from asthma patients [50, 51]. In addition to baseline levels, TXB2 levels were detected in airways of asthma patients when they are subjected to allergen stressors [52]. TXB2 was also found in other samples such as bronchoalveolar lavage (BAL), urine, and plasma from asthma patients [53–55]. A particular metabolite of TXB2, 11-dehydro-TXB2, has been found in urine samples from COPD patients [56].

Leukotrienes

Leukotrienes (LT) were first discovered in leukocytes [57]. LTs contain 20 carbons with 3 conjugated double bonds but lack the 5-carbon ring structure. LTs act through G-protein-coupled receptors [58] and peroxisome proliferator-activated receptors (PPARs) [59]. They can be both autocrine and paracrine signaling molecules.

There are two distinct types of LTs. The LTs conjugated to glutathione are termed cysteinyl-LTs (cys-LTs) and include LTC4, LTD4, and LTE4 [60–66]. Cys-LTs are both bronchoconstrictors affecting peripheral airways [67] and vasoconstrictors [68] affecting the contraction of smooth muscle cells [69–72]. LTC4 is converted to LTD4 by cleavage of a glutamic acid residue from the glutathione moiety [73, 74]. LTD4 is short-lived and converted by a dipeptidase to LTE4, which is then excreted in the urine [75]. Thus, urinary LTE4 levels can be utilized as a biomarker of asthma [76] as it reflects total cyst-LT levels in vivo [77, 78]. Interestingly, LTE4 levels are not elevated in COPD patients [50].

The other types of LTs are formed by a different pathway and is regulated by LTA4 hydrolase, which catalyzes the hydrolysis of LTA4 to LTB4 [79]. COPD patients have higher LTB4 levels in EBC compared to control subjects [79, 80]. Patients with mild asthma present elevated levels for both LTB4 and LTE4 in EBC [51].

In summary, oxylipin profiling from EBC and sputum can distinguish between subsets of COPD and asthma patients. COPD patients display higher levels of PG (specifically PGE2 and PGF2a) and LT (LTB4 but not cysteinyl leukotrienes) in EBC [55]. Asthma patients, on the other hand, display higher levels of thromboxane and cysteinyl leukotriene levels in EBC. However, non-cysteinyl leukotrienes such as LTB4 are found in sputum in asthma patients. Incidentally, LTB4 levels are elevated in both sputum and EBC during acute stress [81].

Hydroxyeicosatetraenoic Acids (HETEs) and Oxoeicosatetraenoic Acids (KETEs)

HETEs are monohydroxy fatty acids formed by two pathways: one via enzymatic LOX metabolism and second via nonenzymatic autoxidation. Reports indicate that asthma is accompanied by high activity of pro-inflammatory enzymes 12/15-LOX and corresponding 15- HETE levels [88–90]. 15-HETE also modulates the activity of anti-inflammatory peroxisome proliferator-activated receptor PPAR-ɣ [91–94]. Macrophages found in BAL showed a decrease in 15-HETE levels in asthma patients [95]. However, eosinophils isolated from BAL display significantly higher levels of 15-(*S*)-HETE in cases of severe asthma [96]. Likewise, chronic bronchitis patients also display 15-(*S*)-HETE levels in induced sputum [97].

HETEs are converted to 5-oxoeicosatetraenoic acid (5-KETE) via enzymatic oxidation [82]. 5-KETE has also been shown to be formed by airway epithelial cells during oxidative stress [83]. 5-KETE is a bronchodilator [84]. It has been shown in vitro that 5-KETE is effective in attracting eosinophils and neutrophils [85, 86]. It has also been reported that this response is greater in atopic asthma patients [87].

Cis-Epoxyeicosatrienoic Acids (EETs) and Dihydroxyeicosatrienoic Acids (DHETs)

The third enzymatic pathway for oxylipin biosynthesis is CYP monooxygenase pathway which results in cis-epoxyeicosatrienoic acids. EETs are formed from CYP metabolism of arachidonic acid. EETs are a series of regiospecific and stereospecific fatty acid epoxides [98–100]. EETs, in turn, can be transformed to their corresponding diols, dihydroxyeicosatrienoic acids (DHETs) [101, 102]. Nothing much is known about the contribution of CYP-derived eicosanoids in lung homeostasis. However, CYP2J2 is found to be expressed in airway epithelial cells, bronchial and vascular smooth muscle cells, and alveolar macrophages [103, 104]. Additionally, transcripts of CYP enzymes have been found in human lung RNA [105]. Thus, EETs might have a significant contribution to lung physiology and pathology. It has been indicated that 11,12-EET and 5,6-EET regulate bronchomotor tone [106–108] and pulmonary vasoconstriction [109]. They are also involved in the composition of airway lining fluid and diminish pulmonary inflammation [98].

Eoxins (EX)

Eoxins were discovered in eosinophils. Eoxins are produced by metabolizing arachidonic acid via 15-lipoxygenase to 15(S)-hydroperoxyeicosatetraenoic acid (15-HPETE). 15-HPETE can be dehydrated to 14,15- epoxy-eicosatetraenoic acid (14,15-LTA4) [110–112]. Similar to the cys-LTs, 14,15-LTA4 can be transformed further to 14,15-LTC4, 14,15-LTD4, and 14,15-LTE4. Thus, eoxins are 14,15-analogs of the 5,6-leukotrienes. It has been reported that eoxin 4 (14,15-LTC4) is found in BAL fluid isolated from the lungs of patients suffering from asthma as well as other respiratory syndromes such as Churg-Strauss syndrome, eosinophilic pneumonia, and sarcoidosis [113]. Interestingly, eoxins are significantly elevated in EBC of pediatric patients who are suffering from asthma and bronchial hyperresponsiveness [114]. Further studies are warranted to elucidate the contribution of eoxins to respiratory pathology.

22.2.1.2 Linoleates

Linoleic acid (18:2 n6, 18:2 ω-6) is an essential fatty acid that has to be ingested through diet as it cannot be biosynthesized [115]. Linoleic acid itself can be metabolized by ∧6 and ∧5 desaturases and elongases [115–117] thus acting as precursors to other ω-6 fatty acids. It can also be metabolized by the same enzymatic pathways as those used in case of arachidonic acid [117, 118]. The enzymatic pathways in the linoleic acid metabolism are (1) lipoxygenation by LOX enzymatic pathway specifically 12/15-LOX, forming 13-(*S*) and 9-(*S*)-hydroperoxyoctadecadienoic acid (HPODE), and (2) CYP enzymatic pathway forming leukotoxins, i.e., 9(10)- and 12(13)-epoxyoctadecenoic acids (EpOME). However, both the enzymatic pathways can be bypassed, and the same products can be formed via autooxidation.

Linoleic acid metabolites via LOX pathway such as 9-HODE, 13-HODE, 9-KODE, and 13- KODE have been reported to have binding affinity to PPAR-ɣ [119–121]. Thus, it could be that these molecules and their levels might affect respiratory physiology through inflammatory process-sensitive subgroups (e.g.,

smokers). Elevated levels of such leukotoxins are found in BAL fluid isolated from patients suffering from acute respiratory distress syndrome (ARDS) [122]. NO_2 and other oxidants can also metabolize leukotoxins by autooxidation in the lungs [123]. Thus, this class of oxylipins needs to be investigated in the context of respiratory pathology.

22.2.2 Anti-inflammatory Oxylipins

22.2.2.1 Arachidonate-Derived Oxylipins

Lipoxins

Lipoxins are short-lived eicosanoids that aid the resolution of inflammation [124–128]. Lipoxins are enzymatically formed from arachidonic acid, an ω-6 fatty acid, and contain three hydroxyl residues and four double bonds. Lipoxin LXA4 interacts with alpha lipoxin receptor [126], a G-protein-coupled receptor. There are three reported mechanisms by which LXA4 potentially aids in resolution of inflammation: (1) LXA4 inhibits synthesis of the cys-LTs [129], (2) LXA4 inhibits neutrophil aggregation, chemotaxis, and activation, and (3) LXA4 inhibits NF-κb signaling pathway thus dampening production and activity of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 [130]. Both LXA4 and 15-epi-LXA4 have been found to be beneficial to asthma patients [131, 132]. Airway hyperresponsiveness and inflammation is blocked upon administering a stable analog of LXA4 in the mouse model of asthma [131]. Additionally, LXA4 levels in whole blood are found to be significantly lower in severe cases of asthma [133]. Airflow blockage is negatively regulated by LXA4 levels. Also, LXA4 is found to be deficient in induced sputum isolated from patients suffering from severe asthma [134].

22.2.2.2 ω-3 Fatty Acid–Derived Oxylipins

Resolvins, Protectins, and Maresins

ω-3 fatty acids are fatty acids that contain a double bond three carbons from the terminal methyl group of the fatty acid chain. Oxylipins derived from ω-3 fatty acids, as opposed to ω-6 fatty acids, are known to be anti-inflammatory and thus aid resolution of inflammatory processes. As such they are increasingly being considered nutritionally beneficial [135–139]. These fatty acids have been documented as beneficial not just for inflammatory disorders [140] but also for cardiovascular disease [141].

Resolvins (Rvs) [136] are metabolic byproducts of eicosapentaenoicacid (EPA) and docosahexaenoic acid (DHA), as well as docosapentaenoic acid (DPA) and clupanodonic acid. Rvs are categorized into several classes based on their origin parent PUFA chain and/or their structural distinctiveness. Research is being done to elucidate the role of Rvs in restoring normal cellular function after tissue injury and inflammation.

Protectins [136, 142, 143] and maresins [144] are formed from omega-3 fatty acids, EPA and DHA. Protectins are similar to lipoxins in many ways. Both are protective by inhibiting production of the pro-inflammatory IL-13 and cys-LTs, although protectins are found to display protective properties at lower amounts than LXA4 [145, 146]. Maresins are so named because they are derived from macrophage mediator in resolving inflammation [144]. Maresin-1 is a potent pro-resolving mediator activating macrophage efferocytosis. COPD patients having defective macrophage efferocytosis might affect the resolution of inflammation and, therefore, it is conjectured that a stable therapeutic analog of maresin-1 might be beneficial to COPD patients [144].

22.3 Lipid Peroxidation Product

Oxidative stress is characterized by an excess of reactive oxygen species (ROS) than the antioxidant defenses can handle. This leads to extensive damage to lipids, proteins, and DNA. Oxidative stress is a critical feature in patients with COPD [147] activating inflammatory and immunity cells, including neutrophils, macrophages, and epithelial cells in the airways of patients with COPD. Oxidative stress leads to the production of a new class of prostanoid mediators formed via nonenzymatic oxidation of arachidonic acid. This new class is termed isoprostanes that can affect respiratory pathological functions including bronchoconstriction and plasma exudation [148].

22.3.1 Isoprostanes

Isoprostanes are lipid peroxidation products specifically of arachidonic acid. Due to their ROS-mediated origin, isoprostanes are assayed as oxidative stress markers in respiratory diseases [149–153]. Isoprostanes are both cytotoxic and pro-inflammatory. The levels of 8-iso-prostaglandin F2α – otherwise known as 8-isoprostane – are found to be increased in EBC isolated from smokers and from patients with COPD. This indirectly assays the degree of oxidative stress in such subjects [154, 155]. Measurements of 8-isoprostanes in the plasma and BALF are also increased in such subjects. Additionally, it was found that plasma F2-isoprostanes increased to a much higher degree in patients with acute COPD [155]. 8-iso-PGF2 displays potent smooth muscle constriction properties in vitro via several prostanoid receptors. 8-iso-PGF2α has been appreciated as the “gold standard” to study the oxidative stress in respiratory pathology [156]. Since smoking leads to oxidative stress thereby increasing risk factor for COPD, 8-iso-PGF2α levels have been assayed in the EBC of smoker and nonsmoker COPD patients [157] (Fig. 22.1).

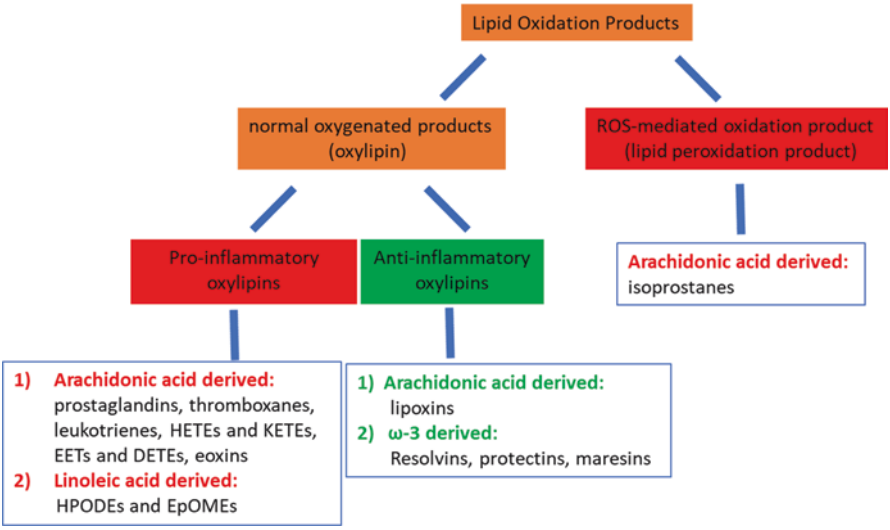

Fig. 22.1 The scheme above illustrates two branches of lipid oxidation products. The left branch shows lipid oxidation products that are produced under normal enzyme-mediated and autooxidation conditions. Normally oxygenated products are both pro-inflammatory (shown in red) and anti-inflammatory (shown in green). The right branch shows aberrant ROS-mediated oxidation products – lipid peroxidation products (also shown in red). The parent compound of most of these lipid products are derived from arachidonic acid

22.4 Conclusion

Lipid mediators are being increasingly appreciated as playing a significant role in physiological and pathophysiological inflammation. This is particularly prevalent in respiratory pathology. Identifying and quantifying the lipid pathways in lung disease such as asthma and COPD will greatly aid in the diagnosis, management, and therapy of the disease. In this context, high-throughput lipidomics along with sophistication in technology such as spectroscopic methods (ESI-MS and MALDI-MS) and refined data analysis is of paramount importance. Another important insight gleaned from lipid biology is related to nutrition. Recently it is considered beneficial to ingest a diet rich in particular type and quantity of lipid as well as balance the intake of ω −3 and ω-6 fatty acids. More research is needed to investigate the effects of such nutritional changes in mediating shifts in lipid balance, homeostasis, and metabolism.

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