Sajal Chakraborti · Tapati Chakraborti
Salil Kumar Das
Dhrubajyoti Chattopadhyay *Editors*

Oxidative Stress in Lung Diseases

Volume 1

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This book is dedicated to Prof. Padmanabhan Balaram, who was born on 19 February, 1949. Professor Padmanabhan Balaram is an outstanding Indian biochemist, an exceptional academic administrator, and a former director of the Indian Institute of Science (IISc), Bangalore, India.

He is currently the distinguished professor of Molecular Biophysics at the IISc, Bangalore, India. His main research interests are in bioorganic chemistry and molecular biophysics. He is the author of over 580 research papers with more than 16, 800 citations.

Prof. Balaram received his bachelor's degree in Chemistry from Fergusson College, Savitribai Phule Pune University, M.Sc. from the Indian Institute of Technology-Kanpur (1969), and PhD in Chemistry with Prof. Aksel A. Bothner-By from Carnegie Mellon University, Pittsburgh, USA (1972). During his PhD, he studied the use of negative nuclear Overhauser effect signals as probes of macromolecular conformations. He did his postdoctoral research at Harvard University with Nobel laureate Prof. Robert Burns Woodward. As a postdoc with Prof. Woodword, he worked on the synthesis of the antibiotic erythromycin.

Prof. Balaram is a fellow of the Indian Academy of Sciences, the Indian National Science Academy, and the Third World Academy of Sciences, Trieste, Italy. He has received many awards/honors in recognition of his work, of which mention must be made of the Shanti Swarup Bhatnagar Prize of the CSIR (1986), Alumni Award for Excellence in Research of the IISc (1991), G.D. Birla Award for Scientific Research (1994), Distinguished Alumnus Award of the IIT Kanpur (2000), and Padma Shri by the Government of India (2002). He also received the third highest Indian civilian honor of Padma Bhushan (2014). He has

served on many committees like CSIR, DST, DBT, DAE, DRDO, ICMR, and UGC of the Government of India and is currently a member of the Science Advisory Committee to the Union Cabinet and Scientific Advisory Council to the Prime Minister, among others. He has been the editor of Current Science *since 1995. He is currently serving in the editorial board of the following journals:* ChemBioChem, Biopolymers, *and* Protein Engineering, Design and Selection.

Prof. Balaram undoubtedly is a legendary figure in Indian Science. He has excellent ability to inspire and motivate young researchers. We feel honored to dedicate this book to him and wish him good health and success in his long fruitful activities.

Preface

"The aim of science is not to open
the door to infinite wisdom,
but to set a limit to infinite error."

Bertolt Brecht: Life of Galileo

The number of diseases in which detrimental oxidative stress has been proposed to play a causative or exacerbating role has grown steadily over the past two decades. Among them, the most prevalent one is the oxidant-mediated lung diseases.

As the lungs lie in the interface between environment and oxidative processes of tissues, it must possess several mechanisms to prevent excessive degree of stress. Enzymatic and nonenzymatic antioxidant systems can quench a wide range of ROS. Diseases like asthma, chronic obstructive pulmonary disease (COPD), and acute respiratory distress syndrome (ARDS) occur from a number of sources, including greater exposure to environmental prooxidants, airway infiltration of inflammatory cells, metabolic dysregulation, and reduced levels of antioxidants.

The oxidant burden in the lung is enhanced in smokers by the release of ROS from macrophages and neutrophils. Oxidants present in cigarette smoke can stimulate alveolar macrophages to produce ROS and to release a host of mediators, some of which attract neutrophils and other inflammatory cells in the lungs, thereby generating ROS via NADPH oxidase system. A marked increase in the numbers of neutrophils and macrophages was observed in the lungs of cigarette smokers compared with those of the non-smokers. Moreover, the lungs of smokers with airway obstruction have more neutrophils than smokers without airway obstruction. Circulating neutrophils from cigarette smokers and patients with exacerbations of COPD release more O_2^-. Cigarette smoke is associated with a marked increase in the production of myeloperoxidase (MPO) in neutrophils, which can be correlated with the degree of pulmonary dysfunction.

This book contains 23 state-of-the-art chapters contributed by established investigators working on the different oxidative stress-induced aspects of lung diseases. Each chapter of the book summarizes information from basic research that hints to possible pharmacological intervention. We hope that this book will be useful for the postgraduate students and biomedical researchers to better understand the

mechanisms associated with oxidant-induced lung diseases and to identify targets for drug development for different types of lung diseases.

Our sincere gratitude goes to all contributors for their considerable energy, time, and effort to accomplish a complete chapter with no *quid pro quo* benefit. We are thankful to Dr. Madhurima Kahali and Raagai Priya Chandrasekaran (Springer Nature) for their cooperation and support during the preparation of this book.

Kalyani, West Bengal, India Sajal Chakraborti

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Sajal Chakraborti is a Professor of Biochemistry at the University of Kalyani, West Bengal, India. His research covers the role of proteases, oxidant, and Ca^{2+} signaling in the pathogenesis of a variety of diseases. Prof. Chakraborti obtained his Ph.D. from Calcutta University and D.Sc. from Kalyani University. He did postdoctoral research at Johns Hopkins University, University of Utah, and New York Medical College. He received DBT-Senior Overseas Research Award for his research at the University of Florida, Gainesville. He has been engaged in teaching and research in biochemistry for the past 40 years. He has published over 120 original papers, 22 book chapters, and 15 review articles and also edited 12 books published by Springer.

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Part I

General Implications of Oxidative Stress on Lungs

Nutrition in Inflammatory Lung Diseases

1

Daisy Karina Aguilar-López, Carlos Olvera-Sandoval, Diego Estrada-Luna, Jeannett Alejandra Izquierdo-Vega, Ashutosh Sharma, and Gabriel Betanzos-Cabrera

Abstract

The lung is a specialized organ that facilitates the gas exchange between an organism and the environment. Its function is to supply the blood with oxygen that the body uses and eliminate the carbon dioxide produced by metabolism. Chronic inhalation of environmental contaminants can result in the overwhelming production of reactive oxygen species (ROS). Oxidative stress in tissues is a process of cellular deterioration dependent on the production of free radicals by an imbalance between ROS and antioxidant agents of endogenous. In the pathogenesis and evolution of numerous pulmonary diseases of high prevalence, inflammation and oxidative stress seem to coexist with an important degree of interaction between both. During the inflammatory process, increased production of ROS may induce damage to lipid structures, proteins, and DNA, the inhibition of apoptosis, and activation of proto-oncogenes when initiating signal transduction pathways. Diet and nutrition are becoming recognized as modifiable contributors to the development and progression of pulmonary diseases.

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Polyphenols are secondary metabolites of plants and are commonly present in the human diet. Because of their antioxidant and anti-inflammatory properties, polyphenols represent a potential to improve the treatments of pulmonary diseases. Several studies have shown the Mediterranean diet appears to benefit patients with airways disease, such as asthma and chronic obstructive pulmonary disease. However, more studies are needed to elucidate the molecular role of nutrition as well as more clinical trial interventions to assess the nutritional management of respiratory diseases and the prevention of these disorders.

Keywords
Nutrition · Inflammation · Oxidative stress

Abbreviations

OH	Hydroxyl radical
AG	Gallic acid
AGEs	Advanced glycation end products
APCs	Antigen-presenting cells
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
DCs	Dendritic cells
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FEV1	Forced expiratory volume in 1 s
GSH	Reduced glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
ICAM-1	Intracellular adhesion molecule
IgE	Immunoglobulin E
IL	Interleukin
iNOS	Nitric oxide synthase
IκK	Iκ kinase
LOX	Lipoxygenase
LPS	Lipopolysaccharides
LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MMPs	Metalloproteinases
MOD	Monoamine oxidase
MUFAs	Monounsaturated fatty acids
NF-κB	Nuclear factor κB
NK	Natural killer
NKT	Natural killer T cells

NLRP3	NOD-like receptor pyrin domain containing
NO	Nitric oxide
O_2	Molecular oxygen
$O_2^{\bullet-}$	Superoxide anion
PGE2	Prostaglandin E2
PPAR	Proliferator-activated receptor
PUFAs	Polyunsaturated fatty acids
RAGE	Receptor for advanced glycation end products
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SP	Surfactant proteins
TNFα	Tumor necrosis factor α
TREG	Regulatory T cells
VCAM-1	Vascular cell adhesion protein
WHO	World Health Organization
XO	Xanthine oxidase

1.1 Introduction

Chronic inflammation and oxidative stress are crucial in the pathogenesis of lung diseases, including chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome, and asthma (Caramori et al. 2013; Rahman and MacNee 2000), the high prevalence of which represent high health expenditures; besides that these diseases have remained the leading causes of death around the world (Roth 2014). Asthma is characterized by decreasing airway function, wheezing, shortness of breath, and tissue remodeling; usually, without parenchyma damage, allergic asthma is associated with airway hyper-responsiveness (Abramson et al. 2014; Murdoch and Lloyd 2010).

COPD is a chronic disease that causes obstructed airflow with an abnormal inflammatory response, an affectation of the lung parenchyma, poor reversibility, and a decrease in lung function. On the contrary, asthma is usually considered as a treatment-responsive and reversible inflammatory process (Barnes et al. 2003). COPD may increase the risk of developing lung cancer (Wang 2013), and diet seems to play a role as a trigger or inhibitor of inflammation in lung diseases (Romieu 2005).

1.2 Oxidative Stress

1.2.1 Free Radicals

In chemistry, a free radical is a molecule with an unpaired electron in the shells of the nucleus of the atom (Halliwell and Gutteridge 1985; Gutowski and Kowalczyk 2013). This results in a high reactivity of the molecule; hence, to obtain stability, the

same molecule pulls an electron from another molecule, which turns it into another radical. Thus, a chain reaction can occur. Most radicals are unstable and therefore highly reactive, causing cell damage (Gutowski and Kowalczyk 2013). Practically all molecules are targets of free radicals, but lipids, nucleic acids, carbohydrates, and proteins are the most affected because of their abundance in organisms (Kohen and Nyska 2002).

1.2.2 Oxygen Reactive Species

Molecular oxygen (O_2) is in the air, and it is essential for humans; its normal use might generate reactive oxygen species during cellular respiration. This formation of free radicals occurs continuously as a consequence of cell metabolism, which is conducted in the mitochondria by means of redox reactions of diverse enzymes, such as NADPH oxidase, lipoxygenase, cyclooxygenase, and peroxidases (Halliwell and Gutteridge 1985; Kohen and Nyska 2002; Liu et al. 2018a), although it should be noted that there are other endogenous sources of free radicals, e.g., phagosomes, microsomal oxidations, neutrophils, and auto-oxidation of substrates (Liu et al. 2018a; Simioni et al. 2018).

Nevertheless, endogenous free radicals play an important role in organisms as a defense mechanism against viral and bacterial infections (Kohen and Nyska 2002). They also participate in reticulocytes maturation and proteins destined for lysosomal degradation. Mitochondrial reactive oxygen species produced in muscle during exercise are required as main signaling pathways (Powers et al. 2011a, b; Jackson 2008; Yavari et al. 2015), and it has even been suggested that exercise-induced free radicals could promote insulin sensitivity (Yavari et al. 2015; Goldstein et al. 2005).

The most common oxygen free radicals involved in diseases are oxygen singlet, hydrogen peroxide, superoxide anion radical, hydroxyl radical, hypochlorite, nitric oxide radical, and peroxynitrite radical. These species are highly reactive and capable of reacting with biomolecules leading to the disruption of cellular homeostasis (Gutowski and Kowalczyk 2013).

Basically, the oxygen free radicals' formation occurs when O_2 is reduced by electrons that escape from the electron transport chain giving rise initially to superoxide ($O_2^{\bullet-}$), which can easily dismute to form hydrogen peroxide (H_2O_2) that in the presence of transition metals, e.g., Cu^+ or Fe^{2+}, produces a hydroxyl radical ($^{\bullet}OH$) by Fenton's reaction (Fig. 1.1) (Giustarini et al. 2009; Fenton 1894). Particularly, the hydroxyl radical has a short half-life (~10^{-10} s) and reacts quickly with neighboring molecules. Unlike the superoxide radical, where it can be eliminated by superoxide dismutase, the hydroxyl radical cannot be eliminated enzymatically (Kohen and Nyska 2002; Srivastava and Kumar 2015). This makes it a very dangerous radical causing immediate oxidative damage.

However, toxicity does not necessarily correlate with reactivity, meaning species with a longer half-life might have higher toxicity as long as the time is adequate to diffuse among the tissues or sensitive locations; thus, the damage is caused at long

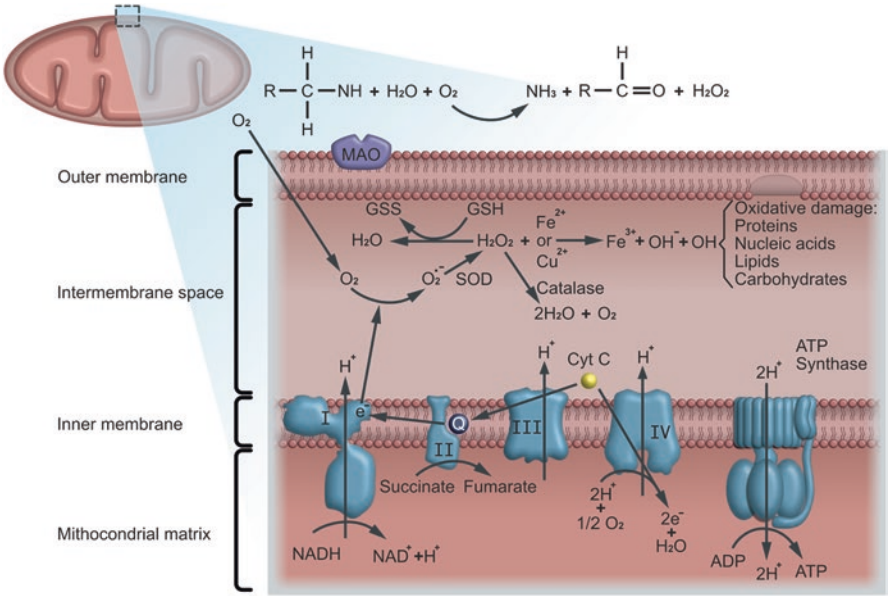


Fig. 1.1 Mitochondria as the main source of radical oxygen species (ROS). The respiratory chain comprises a series of complexes (these are embedded in the inner mitochondrial membrane) that donor-acceptor electron transfer across the inner membrane of mitochondria, where O_2 is used as a final electron acceptor in aerobic respiration. As a result, H_2O and ATP are formed by ATP synthase (oxidative phosphorylation). During the transference, some electrons can escape from the diverse complexes of the respiratory chain to reduce molecular O_2 forming $O_2^{\cdot-}$, which is metabolized by the superoxide dismutase (SOD) forming hydroxide peroxide (H_2O_2), which also can be formed by the action of monoamine oxidase (MOD). H_2O_2 can be eliminated biologically by catalase, reduced glutathione (GSH) or Fenton's reaction, these radicals can diffused into the cytosol or mitochondrial matrix causing oxidative damage

distances from its site of generation. For instance, the relatively long half-life of superoxide radicals allows them to move far away where they can interact with molecules; e.g., superoxide radicals produced in mitochondria diffuse toward the mitochondrial genome producing breaks in DNA (Srivastava and Kumar 2015; Lobo et al. 2010).

1.2.3 Oxidative Stress

Oxidative stress in the lung results when the capacity is overwhelmed through external exposures, such as altered oxygen tension or air pollution or internally. The lung has several mechanisms to prevent oxidative stress. Both enzymatic and non-enzymatic systems can buffer a wide range of ROS and other compounds with oxidative potential (McCord 2000; Holguin 2013). Under normal conditions, ROS concentrations are neutralized through the activity of antioxidant enzymes, such as glutathione peroxidase, superoxide dismutases, and catalase, and antioxidants, such

as vitamins A, C, and E, GSH, located both intracellularly and in the epithelial lining fluid of the lung (Domej et al. 2014). Oxidative stress in the lung results when the antioxidant capacity is depleted through exogenous sources, such as chronic diseases, high-fat diet, processed food, food conservatives, excess of alcohol, chemicals or air pollutants (ozone, cigarette smoking, herbicides, ultrafine $PM_{0.1}$), exposure to ionizing radiations (Simioni et al. 2018; Lobo et al. 2010; Liguori et al. 2018; Rogers and Cismowski 2018), altered oxygen tension, or internal activation of residents cells (Rogers and Cismowski 2018). This condition might lead to accumulation of oxidative damage in a wide range of biological molecules, including lipids, proteins, and nucleic acids (McCord 2000). Short-term oxidative stress can be generated in tissues under certain conditions, such as excessive exercise causing injury (Srivastava and Kumar 2015). The injured tissues produce radical generating enzymes (e.g., lipoxygenase, xanthine oxidase, and cyclooxygenase), activation of phagocytes, or a disruption of the electron transport chain producing an excess of ROS (Lobo et al. 2010; Rogers and Cismowski 2018; Harman 1992).

1.2.3.1 Implications of Oxidative Stress in the Organism

Based on reactivity, free radicals are expected to produce damage throughout life. Diverse radicals, including ROS, are suggested to be involved in diverse pathogenic processes, such as diabetes, cardiovascular disease, metabolic syndrome, rheumatoid arthritis, some forms of cancer, Parkinson's disease, and other diseases (Kohen and Nyska 2002; Otani 2011; Li et al. 2013). As mentioned, the antioxidant system can be overwhelmed under certain factors, such as unhealthy lifestyles, including overweight, obesity, smoking, and drinking, as well as those influenced by genetics (Simioni et al. 2018; Powers et al. 2011a; Yavari et al. 2015; Atitlán-Gil et al. 2017).

A highly significant correlation has been well established between the consumption of oils, fats, and death rates from malignant neoplasms, a condition well recognized by marked lipid peroxidation (Halliwell and Gutteridge 1985). Atherosclerosis may be due to free radical reactions where diet-derived lipids are deposited on the arterial wall and yield peroxides and other substances. This induces endothelial cell injury and produces changes on the arterial walls (Lobo et al. 2010; Harman 1992).

1.2.3.2 Oxidative Damage in Lung

Neutrophils are important in inflammatory processes because neutrophils release elastase and matrix metalloproteinases, which are capable of destroying extracellular matrix proteins. As it is known, pulmonary inflammation is a common sign in smokers (Boukhenouna et al. 2018; Salama et al. 2014; Hobbins et al. 2017). Neutrophils and macrophages release ROS as part of the action against the inflammatory process (Meijer et al. 2013). During the respiratory burst, neutrophil myeloperoxidase catalyzes the oxidation of chloride Cl^- by H_2O_2 to generate hypochlorous acid (HOCl) (Salama et al. 2014). HOCl has high reactivity and reacts with a variety of biomolecules; therefore, it cannot reach intracellular targets located far away (Boukhenouna et al. 2018; Pattison et al. 2009). Nevertheless, if HOCl reacts with amines, it can generate chloramines that are much more stable and can react at the long distances (Boukhenouna et al. 2018; Salama et al. 2014). In the case of

cigarettes, low molecular weight amines, such as nicotine, can form chloramines that can cross cellular membranes and mediate intracellular protein damage (Boukhenouna et al. 2018; Salama and Snapka 2012). Also, ROS induce lipid peroxidation producing malondialdehyde, which is a marker for oxidative stress and is one of the various reactive electrophile species in cells forming protein adducts causing protein inactivation (Siu and Draper 1982). Most importantly, this action may stimulate pulmonary inflammation (Rahman and Adcock 2006), promoting alveolar wall destruction. 4-Hydroxy-2,3-nonenal is other product of lipid peroxidation; its cytotoxic molecule seems to play a key role in cell signal transduction because it is able to induce pro-inflammatory cytokines and NF-κB expression, mitochondrial dysfunction, and apoptosis (Boukhenouna et al. 2018).

1.3 Inflammation in Lung Diseases

The lungs are key organs in maintaining internal stability making oxygen available for all tissues for metabolism and removing carbon dioxide, which is a final bioproduct from that metabolism. Owing to their wide surface area, the lungs are constantly being exposed to toxic compounds from the environment as well as viruses, bacteria, and parasites that reach the internal airway epithelium triggering lung-specific defense mechanisms. Regardless of the source of the lung damage, inflammation is a physiological event that aims to neutralize, confine, and remove harmful agents; in addition, inflammation promotes an immunological response to enhance recovery of damaged tissue. Understanding how inflammation works in lung diseases is necessary to describe the different cell types involved in this process and likewise how this organ responds according to the agent that produces it. Furthermore, the inflammatory response in the lung can be transitory or acute in some pathophysiological events or chronic if the resolution of acute phase is incomplete and combined with the presence of high amounts of necrotic debris and apoptotic cells (Cook and MacDonald 2016).

1.3.1 Inflammatory Cellular Response

In the inflammatory process, there is a family of cells that works together to mediate this physiological reaction to ensure the recovery of damaged tissue and the return to normal function. Some cellular types and their main participation in this process are described below. The dendritic cells (DCs), specialized antigen-presenting cells (APCs), are well distributed through blood circulation and practically reach all the pulmonary epithelium; this cell type represents the first barrier of defense against possible infection, acts as macrophages, and has the ability to mobilize the antigen into lymphoid tissue and present it to the T cells triggering a subsequent immune response (Cook and MacDonald 2016). DCs, in turn, can stimulate a second cell group that resides in the airways at all anatomical levels, the macrophages. Macrophages modulate both the acute and chronic phases of inflammation and,

together with dendritic cells, carry out phagocytosis of particles, bacteria, and apoptotic cells (Martin et al. 2014). Macrophages and epithelial cells are capable of modulating the immune response by promotion and secretion of cytokines, chemokines, and inflammatory mediators stimulating local neutrophil aggregation. When neutrophils reach the injured or infected area through capillary circulation, they accumulate and have phagocytic activity in airspaces; once phagocytosis is completed, they use lytic mechanisms, such as reactive oxygen species, antimicrobial proteins, and degrading enzymes, to destroy fungi, protozoa, bacteria, viruses, and tumor cells (Mantovani et al. 2011; Selders et al. 2017).

Another cell group found in the parenchyma and airways are lymphocytes, which are classified into two groups according to their immunomodulatory role: T-type lymphocytes, responsible for regulating cell-mediated immune function and B-type also with immunomodulatory activity but through the action of antibodies (immunoglobulins). CD4+ and CD8+ are the two main populations of T-type lymphocytes. CD4+ lymphocytes are in turn classified in two groups as T-helper cells with a specific function, Th1 and Th2 cells, both with different cytokine profiles. Through the release of cytokines, such as IFN-γ and TNF-α, Th1 cells mediate pro-inflammatory signals to counteract viruses, bacteria, parasites, and even cancer cells. Th2 cells increase the production and release of antibodies and cytokines, such as IL-4, IL-5, IL-9, and IL-13, which in turn control eosinophilic and IgE-mediated responses. To ensure an efficient immune response, there must be a balanced activity between Th1 and Th2 cells; any imbalance in this regard will result in chronic inflammation conditions. CD8+ T cells are well known for their cytotoxic actions against infected and tumor cells (Annunziato et al. 2015). Another subset of T cells implied in the inflammation process is the natural killer (NK) and NKT cells, the former with no antigen-specific receptors and the latter with functions similar to NK cells but specialized in combating bacteria, protozoa, and viruses (Marquardt et al. 2017).

Furthermore, mast cells are the cellular type that can be activated by multiple receptors. In the airways, mast cells are activated by their IgE receptors producing pro-inflammatory substances, such as cytokines, chemokines, histamine, and leukotrienes, that mediate the airway inflammation (Cruse and Bradding 2016). Eosinophils are characterized by the production of cytokines, basic proteins, bioactive lipids, and growth factors, which promotes mast cell production and function. These kinds of cells are related to a parasitic invasion, asthma, and chronic lung inflammation. However, inflammation is a complex process that requires not only the pro-inflammatory cells participation but also the local tissue cells, such as epithelial, endothelial, and mesenchymal cells (Tashkin and Wechsler 2018).

1.3.2 Biological Barriers in Lung

Several inhaled agents are constantly in contact with the airway epithelium, but the respiratory system through its epithelial cells has efficient mechanisms that indirectly avoid injury caused by toxic compounds and microbial attack. Cells that conform to respiratory epithelia synthesize and release substances (defensins, mucins,

lactoferrin, lysozyme, and nitric oxide) which protect the respiratory tract in a non-specific manner also provide pro-inflammatory profile cytokine (IL-1B, TNF-α) and, at the inflammation site, platelet-activating factor and inflammatory cells aggregation (Hiemstra et al. 2015).

Previously secreted cytokines stimulate the release of bioactive lipids from cell membranes, such as arachidonic acid and eicosanoids, which stimulate mucus secretion by goblet cells. Additionally, a family of surfactant proteins (SP A-D) synthesized at the alveoli level is essential to minimize lung surface tension and plays an important role modulating leukocyte activity, IgG production, and binding recognizing proteins to pathogens for further processing by phagocytes (Han and Mallampalli 2015). Plasma cells also counteract pathogenesis in the lung by secretion of IgA, which prevents bacterial adherence and inhibits the assembling processes of viruses. Moreover, the release of immunoglobulin E (IgE) stimulates the synthesis of histamine, prostaglandins, leukotrienes, and tryptase, which bind to IgE receptors expressed by B-type lymphocytes, mast cells, and other granulocytes. The release of these compounds produces immediate hypersensitivity in the respiratory tract and promotes inflammatory cell infiltration, an increase of vascular permeability, and bronchoconstriction. It produces immediate hypersensitivity in the respiratory tract and ensures vascular permeability, bronchoconstriction, and inflammatory cell infiltration. The presence of these biological barriers maintains the integrity of the lower respiratory tract and works as the first defense mechanism in the lung (Moldoveanu et al. 2009).

1.3.3 Acute and Chronic Lung Inflammation

Acute lung inflammation usually occurs under an infectious condition that can be caused by bacteria, viruses, and parasites. Neutrophils actions are associated frequently with acute lung inflammation, whereas chronic reactions involve macrophages and lymphocytes. Regardless of the source of lung damage, acute lung responses include the release of chemotactic factors driven by endothelial cells and posterior recruitment of neutrophils and leukocytes at the site of injury; this cellular aggregation produces chemokine release with the subsequent tissue granulation compromising the cellular integrity of endothelial cells, fibroblast, and leukocytes. Infections caused by bacteria regularly promote an acute inflammatory response when the microbial load exceeds the host's local defenses, while in chronic infection the appearance of damaged tissue is always a sign of failed acute response (Pragman et al. 2016). Under the cellular mechanisms previously cited, the first contact with the pathogen starts an inflammatory response beginning with the recognition of the microbial agent and its posterior phases (initiation, amplification, phagocytosis, and resolution), ensuring bacterial clearance. When the resolution phase in acute inflammation fails or is incomplete, chronic responses begin and are characterized by clearance of cellular debris, constant elimination of apoptotic cells, and heal/repair of injured tissue. In this stage, the main cellular cells involved in this process are macrophages and lymphocytes accompanied by cytokine production provided by immune and nonimmune cells. Chemokines are recognized by their

receptors located on lymphocytes, dendritic cells, and granulocytes, and in a chronic inflammation process they regulate cell displacement, angiogenesis, and neutrophil migration into functional tissue of lung (Lloyd 2002; Bagnato and Harari 2015). Apoptosis inducer or suppressor genes in the chronic inflammation process are slightly regulated to ensure the integrity and survival of immune cells and thus a continuous cell infiltration to the lungs and extended inflammation process.

This phenomenon has been described in chronic pulmonary affections as a chronic obstructive pulmonary disease (COPD) and asthma, and analysis of sample tissues from patients with those diseases reveal higher amounts of eosinophils infiltration and expression of genes that decrease apoptotic activity (bcl-2) in comparison with those that promote it (p53). Inhibition of apoptosis results in a high count of CD4+ cells in the asthmatic process and CD8+ T cells in COPD (Schmidt and Tuder 2010; Barnes 2016). Inflammation is a non-avoidable process in several pulmonary diseases presented either by infection or immunological conditions, such as asthma and COPD. The degree of the inflammation mediated by specific cell mechanisms is proportional to the severity of the lung disease. Currently, therapy aimed at minimizing the effects of inflammation is based on the identification of cell types and the cellular and humoral mechanisms they produce, and from a comprehensive understanding of this phenomenon, it is possible to develop innovative treatments that control the undesirable effects of inflammation.

1.4 Interaction of Nutrients with Inflammatory Elements

Inflammation is a normal process in several tissues that serves as protection from infection or injury. The early steps involve many cells and chemical mediators with adhesion properties (e.g., leukocytes) produced for signals, such as heat, pain, redness, and loss of cell function, triggering an increase of local blood flow and heightening levels of phagocytes. When deregulation of any factor exists it leads to inflammation and development of chronic diseases (Crascì et al. 2018). Another type of inflammation exists called metabolic inflammation that appears owing to consumption of certain nutrients and/or metabolic stress with the typical expression and activation of cytokines and several pro-inflammatory pathways on trigger tissues, such as adipose tissue (Kirwan et al. 2017). Inflammation is classified into two types: acute and chronic. Acute inflammation presents a recruitment of granulocytes to the tissue of injury and diverse biochemical reactions that allow the maturation of the inflammatory response and is usually resolved in hours or days. However, if the process continues, it becomes chronic inflammation, and it can lead to a loss of function and permanent tissue damage (Schmidt and Tuder 2010).

Normally, several chronic noncommunicable diseases show oxidative stress and inflammation as concomitant features because some pro-oxidant components activate the diverse inflammatory process. Inflammation presents four events: *(i)* increase of blood flow to the tissue; (*ii)* increased permeability of vascular wall; *(iii)* leukocytes migrate to the injury place increasing the number of chemo-attractants and other adhesion molecules, and *(iv)* mediators of different chemical composition,

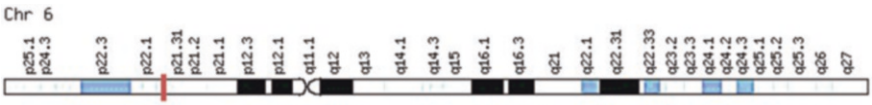
such as prostaglandins and leukotrienes (lipid-derived), chemokines and cytokines (peptides), $O_2^{\bullet-}$ (reactive oxygen species), histamine (amino acid), and matrix proteases (enzymes), are relocated into circulation triggering systemic effects. In this context, mainly tumor necrosis factor (TNF-α) and three types of interleukins (IL-6, IL-1β, IL-12) are released by astroglia, microglia, CD + 4 lymphocytes, natural killer cells, and neurons (Martinon et al. 2006; Walsh et al. 2014; Latz et al. 2013); another inflammatory component that stimulates the secretion of macrophages and releases cytokines is the NLRP3 inflammasome pathway and this, in turn, is activated by a second activation signal by reactive oxygen species (ROS) and potassium efflux (Jin and Flavell 2010; Martinon 2010).

Several pathways of inflammation recognize different antigens that activate signaling cascades, such as MAPK, NF- κB, and PPAR-γ pathways, with a reduction in the release of anti-inflammatory cytokines and regulatory T cells (TREG) (Bamias et al. 2017). PPAR-γ regulates NFκB in the nucleus that allows encoding pro-inflammatory molecules, such as interleukins, selectins, integrins, VCAM-1, ICAM-1, and cyclooxygenase-2, this factor is activated through extracellular inflammatory stimuli by endotoxin or phosphorylation of IκB in human monocytes (Dinarello 2010; Busso and So 2010; Calder 2013).

Recently, the inflammation studies have focused on traditional and natural treatments (Table 1.1), particularly about polyphenols that decrease inflammatory processes (Qiu et al. 2018; Zhao et al. 2018). Diverse meta-analyses emphasize diets rich in polyphenols revert the damage in chronic inflammatory diseases due to their antioxidant activity given by compounds, such as quercetin, naringin, procyanidins, catechins, proanthocyanidins, flavonoids, and their derivatives. This effect is mainly due to a direct mechanism.

The interactions of the biochemical structures or metabolites of polyphenols suppress the activity of receptors and cytokines pro-inflammatory and pro-oxidant enzymes, such as lipoxygenase (LOX), xanthine oxidase (XO), nitric oxide synthase (iNOS), and cyclooxygenase (COX), and increase activity of glutathione S-transferase (GST), superoxide dismutase (SOD), and catalase. In vitro some polyphenolic compounds have the ability to act as a scavenger of reactive nitrogen species (RNS) and mainly of reactive oxygen species (ROS) (Schell et al. 2017; Nogueira et al. 2017; Lockyer et al. 2017), as potent antioxidants play an important role in activating the NFκB mediated I-kappa B kinase complex (IκK) pathway. Another attribute of total polyphenolics, catechins, flavonoids, and total anthocyanins is the anti-glycation activity that results in a reduction of glycotoxins also known as advanced glycation end products (AGEs), which are linked to neurodegenerative diseases, diabetes, and cardiovascular disease, and under normal conditions the expression of their receptors (RAGE) is low but it is overexpressed in stress conditions. In processes of stress, the AGEs can up-regulate matrix metalloproteinases (MMPs) enzymes responsible for degrading the extracellular matrix, via the NFκB pathway. The MMPs can be classified into stromelysins, gelatinase, collagenases, and matrilysins, and some studies are related to their activity with ROS generation because ROS can act as second messengers. In vitro and in vivo studies have found that polyphenolic compounds can inhibit MMPs due to their anti-inflammatory and antioxidant properties (Crascì et al.

Table 1.1 Pro-inflammatory elements, gene location, and nutrients with anti-inflammatory capacity



Tumor Necrosis Factor (TNF)-α

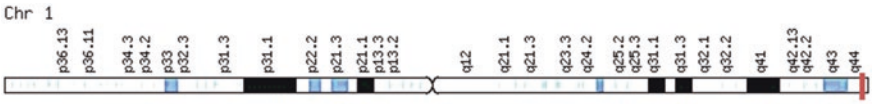
Gene (6p21.33) (GenBank accession bank: NM_000594.3) encodes a multifunctional pro-inflammatory cytokine associated with diseases such as asthma and malaria (Lockyer et al. 2017; Scoditti et al. 2012).
Honey aqueous extract contents apigenin and kaempferol that inhibits TNF-α –induced proteolytic activity in cutaneous inflammation and downregulation of MMP-9 expression (Lockyer et al. 2017), Luteolin flavonoid decrease TNF-α expression through NFκB signaling pathway (Scoditti et al. 2012), the contents of chlorogenic and chlorogenic acids in berries decrease cell proliferation and block TNF-α signaling (Babu et al. 2011).

Interleukin 6 (IL-6)

Gene (7p15.3) (GenBank accession number: NM_000600.4) encodes a cytokine that functions in inflammation and maturation of B cells, is produced in acute and chronic inflammation (Jia et al. 2015; Chang et al. 2018; Erta et al. 2012).
Genipin founded in gardenia fruit inhibit production of IL-6 in periodontal lesions (Kinra and Dutta 2013), flavonoids coming from *Saphora flavescens Ait*. (leguminosae) can reduce *in vitro* inhibit release of IL-6 (Majtan et al. 2013), tea polysaccharides inhibit the filtration of pro-inflammatory cells and cytokines expression (Jia et al. 2015).

Interleukin 1 beta (IL-1β)

Gen location 2q14.1 (GenBank accession number: NM_000576.2) the protein is produced by macrophages is an important mediator of inflammatory response, it´s related in process of proliferation and apoptosis (Ma et al. 2018; Liu et al. 2018).
Magnolia berry attenuates IL-1β induced expression and activity of MMP (Chang et al. 2018), procyanidins B2 inhibit IL-1β secretion in acute inflammation (Erta et al. 2012), phloretin, a flavonoid coming from apple shows anti-inflammatory activity reducing cytokine expression in cells Il-1β-stimulated (Neurath and Finotto 2011).

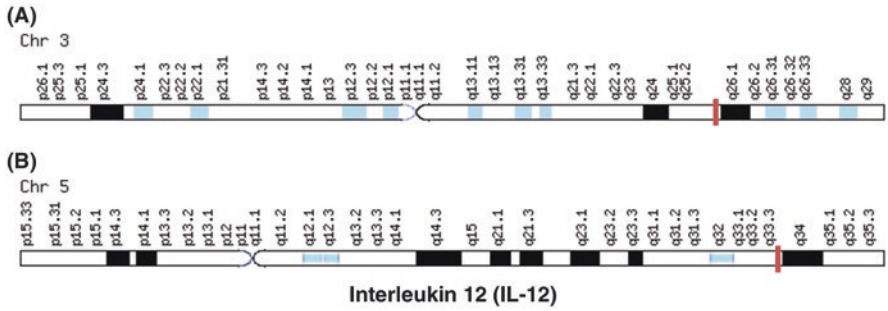


NOD-like receptors containing a PYD3 (NLRP3)

Gen location 1q44 (GenBank accession number: NM_001243133), the protein acts like upstream activator of NF-κB signaling, plays an important role in inflammation (Martinez-Micaelo et al. 2015).
Diverse bioactive compounds such as procyanidins (Tanaka et al. 2014) and resveratrol (Shindo et al. 2014) reduce multiple NLRP3 inflammasome activators; the spice curcumin modulates phosphorylation of some protein kinases (Ma et al. 2018).

(continued)

Table 1.1 (continued)



IL-12 participates in the early steps of inflammation, is a heterodimeric molecule composed by 2 monomers: subunit p35 or IL-12A (A) and subunit p40 or IL-12B (B) linked covalently by disulfide bonds (9597139, 12727921). The location of IL-12A is 3q25.33 (GenBank accession number: NM_001354582.1) meanwhile IlL-12 B location is 5q33.3 (GenBank accession number: NM_002187.2)

Sodium butyrate suppresses suppress the production of IL-12 and their receptors (Liu et al. 2018), fisetin flavonoid founded in diverse plants suppress the production of IL-12 and other costimulatory molecules related to inflammation (Shen et al. 2015).

2018; Scoditti et al. 2012). Gallic acid (GA) is another bioactive polyphenol that shows the ability to attenuate the RAGE expression and modulate some inflammation signaling pathways, possibly by its chemical structure like the presence of a double bond (Schmidt and Tuder 2010).

Furthermore, epidemiological studies have shown that populations whose daily diet included food rich in polyphenols present a lower incidence of inflammatory diseases. Procyanidins are found in grape seeds, cranberries, cherries, and other sources, and they present anti-inflammatory and antioxidant properties allowing the inhibition of NLRP3 inflammasome activation and pro-inflammatory cytokine expression in mice models (He et al. 2018; Ogura et al. 2016), presumably by the activation of scavenging ROS and inhibiting production of cytokines and inflammatory infiltration (Liu et al. 2017). Betaxanthins and betacyanins are part of the family of betalains, a water-soluble plant pigment that exhibits a potent antioxidant content that is related to their anti-inflammatory properties, can also reduce NO, inhibit the secretion of TNF-α and several interleukins and block NFκB activation, and could be in vitro degraded by peroxidase and polyphenol oxidase in studies (Huang et al. 2016).

Besides, different lipid compounds, including polyunsaturated fatty acids (PUFAs) and vitamin D, are important mediators in the inflammatory process. The eicosanoid receptors present a different biological potency, and the arachidonic acid receptor presents higher affinity and potency. Eicosanoid is released from inflammatory cells by the activation of phospholipase A_2 enzymes, and the free arachidonic acid acts as a substrate for pro-oxidant enzymes or cytochrome P450 enzymes increasing inflammatory stimuli. Studies in animals and humans showed a decrease in the levels of these enzymes after a treatment with omega 3 using several doses and different stages of inflammatory processes. A new family of lipids mediators, known as resolvins and produced from ω-3, involves COX and LOX pathways,

some members of this family include resolving E1, resolving D1, and protectin D1. These resolvins have the ability to inhibit migration and filtration of neutrophils into sites of inflammation, and together with other ω-3 decrease the production of pro-inflammatory cytokines in experimental models of inflammatory diseases such as asthma (Barbalho et al. 2017).

The simplest ω-3, α-linolenic acid is synthesized by desaturation from linoleic acid; however, animals and humans cannot synthesize this type of ω-3 because they do not possess the delta-15 desaturase enzyme, contrary to the plants that do have it, and this is why some plant oils, seeds, seafood, and nuts containing α-linolenic acid are the major source consumed in human diets (Johnson et al. 2018; Soveyd et al. 2017). Fish oil demonstrated a decrease in the recruitment of monocytes in inflammatory sites, and this mechanism may be related to a decrease in the expression of receptors for chemo-attractants. Something similar occurs with adhesion molecules expressed on the surface of endothelial cells or monocytes in subjects with metabolic syndrome and a reduction of concentration levels of soluble VCAM-1 and ICAM-1 in the bloodstream were found (Calder 2013).

Nowadays, diverse types of natural products and foods exist that can be used to counteract the inflammatory process through the inhibition of signaling pathways, receptors or pro-inflammatory molecules; indeed, the expression and activity of some mediators can be improved because of the interaction of their chemical structures and their antioxidant activity reducing oxidative stress slowing down the expression and production of pro-inflammatory oxidant compounds. However, despite this knowledge, more clinical trials are necessary to better understand the therapeutic mechanisms and molecules that interact in the inflammatory processes and to find new nutrients that could have a similar effect for improvement in diverse inflammatory and chronic diseases (Calder 2013; Qiu et al. 2018).

1.5 Role of the Diet in Lung Diseases

Lung diseases are closely related to oxidative stress and inflammation due to exposure to air pollutants, infections, and cigarette smoke (Valavanidis et al. 2013). Since drugs are extensively used for the treatment of lung diseases, they may cause side effects that complicate the favorable evolution of the patient. Consequently, it is important to look for alternative ways such as diet in the prophylaxis and treatment of lung diseases. However, further research is required to elucidate the specific function of each nutrient, and thus this option deserves to be explored (de Boer et al. 2017).

1.5.1 Mediterranean Diet

The Mediterranean diet comes from several ancient civilizations, such as Phoenicians, Romans, Greeks, and some others; it is considered by the UNESCO (United Nations Educational, Scientific and Cultural Organization) as Intangible Cultural Heritage of Humanity (Altomare et al. 2013). Keys et al. were pioneers in

the study of the Mediterranean diet, where seven countries (the United States, Yugoslavia, Holland, Japan, Italy, Greece, and Finland) showed the association between cardiovascular pathologies and dietary patterns, and the low incidence of cardiovascular diseases was attributed to the low intake of saturated fat (Altomare et al. 2013; Trichopoulou et al. 2014; Keys 1980). This fact began the study of the Mediterranean diet; although, it is necessary to point out that a way of life is more than just a diet.

This diet is based on the intake of whole grains, vegetables, fruits, fish, oilseeds, olive oil, moderate alcohol consumption, limited intake of red and processed meats, and the dietary pattern is rich in fiber (intake of complex carbohydrates), mono and polyunsaturated fatty acids, antioxidants, and low intake of saturated fat, cholesterol, refined sugars; in contrast to the western diet (Dussaillant et al. 2016; Castro-Quezada et al. 2014; D'Alessandro and De Pergola 2014) (Table 1.2).

The Cancer Prevention and Control Program of the University of South Carolina in Columbia designed the Dietary Inflammatory Index (DII) (because diet seems to plays an important role in the development of inflammation. Published studies showed the effect of dietary parameters on the levels of TNFα (TNF), IL-1β, IL-4, IL-6, IL-10, α and C-reactive protein (Shivappa et al. 2014; Maisonneuve et al. 2016), and this opened the possibility for DII to be used in diverse studies (de Boer et al. 2017). Smokers fed a high DII diet (pro-inflammatory diet) were associated with a higher risk of developing lung cancer, but not when smokers had Mediterranean diet high scores (indicate healthier diet) (Hodge et al. 2016). Subjects with chronic obstructive pulmonary disease (COPD) who followed a Mediterranean diet showed better pulmonary function (Yazdanpanah et al. 2016). In a cross-sectional study of 174 asthmatic adults, there was an association between adherence to a Mediterranean diet and asthma control understood as: forced expiratory volume in 1 s (FEV1) $\geq 80\%$ of predicted, exhaled nitric oxide (NO) ≤ 35 ppb, and Asthma Control Questionnaire score < 1; thus, patients were more likely to control the symptoms, pulmonary

Table 1.2 Traditional Mediterranean recommendations

Components	Frequency of consumption	Servings	Nutritional intake
Vegetables	Daily	6	Phytochemicals, fibers, ω-3-polyunsaturated fatty acids (PUFAs)
Fruits	Daily	3	Phytochemicals, fibers
Legumes	Weekly	3	Phytochemicals, fibers
Olive oil	Daily	3–4	Monounsaturated fatty acids (MUFAs), polyphenols
Fish	Weekly	5–6	ω-3-PUFAs
Red wine	Weekly	≥ 7 glasses	Polyphenols
Nuts	Daily	1–2	Polyphenols, fibers, ω-3-PUFAs
Unrefined cereals	Daily	1–2/main meal	Polyphenols, fibers

Adapted from Anania et al. (2018)

function, and exhaled nitric oxide (Barros et al. 2008). It has been suggested that the Mediterranean diet can attenuate inflammation in pathologies where chronic status is involved, e.g., lung diseases, arthritis, obesity, cancer, and diabetes (Gotsis et al. 2015; Oliviero et al. 2015), by means of the synthesis of anti-inflammatory cytokines, circulating antioxidants, and regulation in the expression of pro-inflammatory genes (de Boer et al. 2017; Gotsis et al. 2015; Marion-Letellier et al. 2015; Calder 2017). The positive effect of the Mediterranean diet in health is attributed to its high content of PUFAs (Ostan et al. 2015).

Finally, the phytochemicals contained in the Mediterranean diet contribute to reducing oxidative stress, which is present in lung diseases (Hoffman and Gerber 2015) (Fig. 1.2).

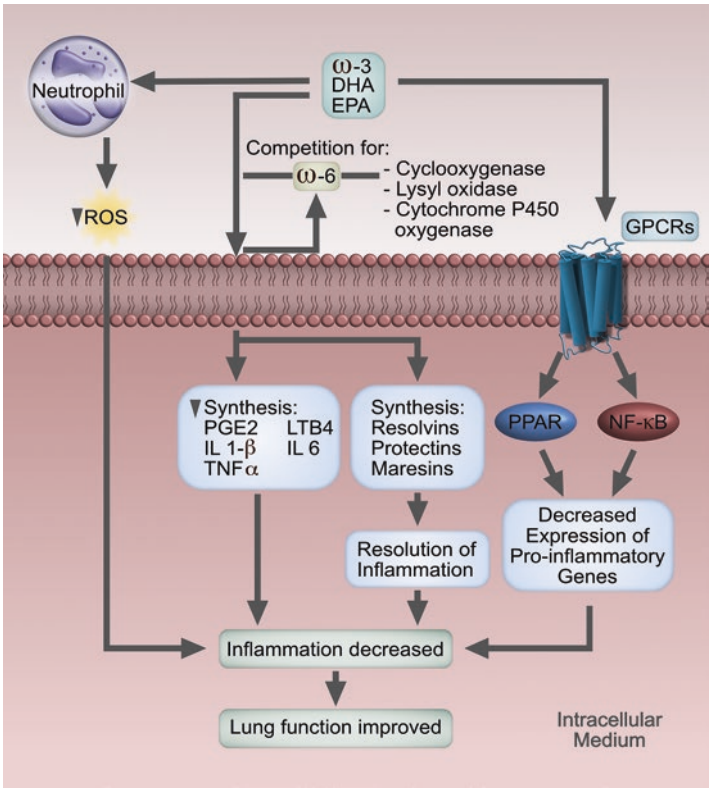


Fig. 1.2 Protective role of PUFAs in inflammatory lung diseases. Eicosapentaenoic acid (EPA) and ω-3 PUFAs docosahexaenoic acid (DHA) present in neutrophils reduce reactive oxygen species (ROS); they replace ω-6 PUFAs in the cell membrane, competing with them for the enzymes: cyclooxygenase, lysyl oxidase or cytochrome P450 oxygenase, decreasing synthesis of: PGE2, LTB4, IL 1-β, TNF-α; in plasma membrane or cytosol bind to G-protein-coupled receptors (GPCRs), activate PPAR and inhibit NF-κB, which has epigenetic implications, resulting in decreased expression of pro-inflammatory genes; also from the ω-3 PUFAs resolvins, protectins, and maresins are synthesized, which resolves inflammation, and thus decreases inflammation and improves lung function

1.5.2 Western Diet

Epidemiological studies suggest an association between diet and the development of diseases, especially in chronic non-communicable diseases (Zheng et al. 2016). The Western diet favors the consumption of refined grains, cured meat, fried foods, desserts, and is characterized by a high intake of simple sugars, low fiber, and high intake of saturated fat; therefore, evidently, it is a pro-inflammatory diet (Berthon and Wood 2015; Guilleminault et al. 2017). Literature reports that consumption of cured meat increased the risk of COPD, and high consumption (≥ 4 serving per week) is related to complicating asthma symptoms (Li et al. 2017). A high intake of fast food in children has been positively related to asthma (Berthon and Wood 2015), but there is a lack of studies in adults supporting a clear association between the Western diet and incidence and prevalence of asthma (Brigham et al. 2015; Lv et al. 2014).

1.5.3 Fruit and Vegetable Consumption

At least more than 400 g of fruits and vegetables is recommended by the World Health Organization as part of a healthy diet (Rodriguez-Casado 2016); bioactive compounds found in these foods have antioxidant activity against oxidative stress and anti-inflammatory effect (Berthon and Wood 2015; Szarc vel Szic et al. 2015; Zhang et al. 2015; Saqib et al. 2018) (Fig. 1.3).

Antioxidant vitamins have been shown to have a beneficial effect in obstructive pulmonary diseases; they may modulate the development of the disease and the decrease of lung function due to their ability to decrease pro-oxidant substances.

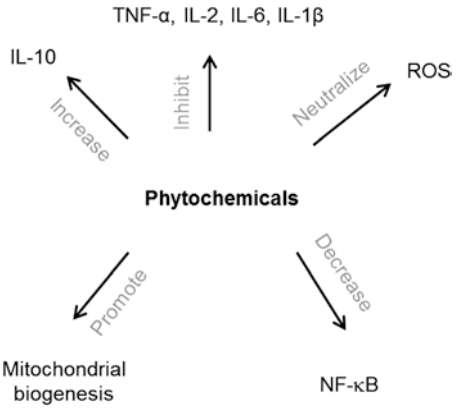


Fig. 1.3 Mechanisms of some phytochemicals. Flavonoids present in fruits, nuts, seeds, vegetables, tea, wine, and coffee neutralize ROS, inhibit TNF-α, and decrease the activation of NF-κB, which is involved in the regulation of inflammatory pathways. Lupeol found in strawberries, grapes, mangoes, tomatoes, and cucumbers inhibits TNF-α, IL-2, IL-6, IL-1β, but increases IL-10 (anti-inflammatory cytokine). Resveratrol present in grapes and peanuts promotes mitochondrial biogenesis

The consumption of fresh fruit has been linked to a decrease in the risk of carcinogenic airway obstruction (Romieu and Trenga 2001). High intake of fruits and vegetables (400 g/day) was inversely related with the risk of lung cancer, and no greater effect was observed when increasing this amount (Vieira et al. 2016); some studies have reported that this association is greater in women (Wang et al. 2015). Isothiocyanates from the hydrolysis of glucosinolates found in the cruciferous vegetables have shown a protective effect against lung cancer (Kumar et al. 2015; Abdull Razis and Noor 2013).

An imbalance of oxidants/antioxidants leads to COPD development, and a significant decrease in plasma levels of vitamins A,C,E, alpha- and beta-carotene and total carotenoids in Taiwanese subjects with COPD has been shown (Lin et al. 2010).

It has been suggested that there is an association between the decrease in the serum concentration of antioxidant vitamins A, C, and E and a higher risk of lung cancer (Dela Cruz et al. 2011).

On the other hand, the increased intakes or serum concentrations of these vitamins are associated positively with FEV1 (Hanson et al. 2013). Fruits and vegetables are high in fiber, and adequate fiber intake has been linked to a better lung function and lower risk of COPD (Fonseca Wald et al. 2014). Medical actions should be focused on reducing the risks of lung diseases, promoting the reduction of smoking habits and increasing the consumption of fruits and vegetables (Sorli-Aguilar et al. 2016).

1.6 Conclusion

Owing to oxidative stress, cells are susceptible to damage by oxidizing nucleic acids, proteins, carbohydrates, and lipids. ROSs are involved in the pathogenesis of many diseases and biological processes, such as inflammation in lung. Future research focused on evaluating the molecular mechanisms of new nutrients that will serve for the design of nutritional interventions that can prevent lung diseases associated with inflammation is required.

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2 Oxidative Stress and Smoke-Related Lung Diseases: A Tentative Approach Through the Blood, Lungs, and Gut

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Abstract

Respiration renders the lungs vulnerable to infectious agents, smoke, and hazardous material that are inhaled in the process. During respiration, oxidants deposit in the lung which can cause oxidative stress and impair the defense mechanism. Due to the subsequent tissue damage, the antioxidant system is not always able to tackle the reactive oxygen species (ROS). When oxidative stress results in tissue damage, progenitor cells try to replace the tissue damaged by the ROS, where the regenerative capacity of the lungs plays a crucial role in preventing further lung damage or disease. Studying molecular pathways of lung cell regeneration is essential in the study of regenerative biology, although

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regeneration might fail to entirely replace the resulting tissue damage and lead to pathophysiological conditions. The lungs are prone to tissue damage due to continuous exposure to both endogenous and exogenous oxidative mediators and other oxidants, making the process of lung repair extremely important. Some of the damaged tissue can't be repaired as the repair process cannot match up to the high levels of oxidant exposure resulting in disturbed oxidant and antioxidant balance, thus impacting the normal physiology. Concurrently, ROS and RNS cause oxidative damage and tissue dysfunction in pathological conditions of the lungs. They are responsible for the declining cellular function and compromised mitochondrial system. Prolonged exposure to tobacco and cigarettes is one of the main causative substances in the progression of oxidative stress, a detrimental process that can even lead to lung carcinogenesis. The knowledge in mechanisms of oxidative stress in the lung could lead to improved pharmacological manipulation of antioxidants in lung inflammation as well as injury. Tobacco carcinogens are involved in the upregulation of the redox-sensitive transcription factors and proinflammatory gene expression. Antioxidant defense mechanism consisting of antioxidant enzymes, proteins, and small molecules are impaired in most lung pathologies. Recent clinical investigations aim to develop precise treatment modalities targeting the functioning mechanism of antioxidants against oxidants. This will help in therapeutic management and improved treatment of respiratory diseases.

Keywords
COPD · Oxidative stress · Antioxidants · Cigarette smoking · Oxidation · GSH

Abbreviations

CCSP	Secretion of Clara cell secretory protein
VEGF	Vascular endothelial growth factor
EMT	Epithelial mesenchymal transition
ROS	Reactive oxygen species
RNS	Reactive nitrogen species

2.1 Introduction

The lungs are primary organs of the respiratory system. They have an elastic property which makes them ideal for gaseous exchange, the most important function of lung (Domej et al. 2014). In this process, the lungs are exposed to infectious agents, smoke, and hazardous material inhaled during inspiration. During respiration, the lungs acquire many oxidants which can, in excess, cause oxidative stress despite the

defense mechanism (Matalon and Egan 1981). Under normal conditions, the lung upholds the normal redox state through the antioxidant system which helps to neutralize the oxidants generated during respiration (Zhu et al. 1998). The primary function of the lung is to excrete the cellular waste during exhalation and ensure supply of oxygen to the tissues through the exchange in blood. The lung is guarded by specialized epithelial cells which protect the lung from tissue injury and foreign infectious agents (Ischiropoulos et al. 1992). The lung is a vital organ, and though it can get affected by oxidative stress mediators, it has the ability to tackle the oxidant by exogenous and endogenous oxidation.

2.2 Exogenous and Endogenous Oxidation

Oxidation occurs in the lung in two main ways: endogenous and exogenous oxidation. Exogenous oxidation takes place in response to inspiration of foreign materials (e.g., smoke, air pollutants, arsenic, and other chemical hazardous materials). Endogenous oxidation is triggered by ROS and RNS molecules (e.g., free radicals, inflammatory mediators) that deposit secondary to infection and systemic diseases causing inflammation. As part of an immunological defense mechanism, the immune cells respond by producing increased quantities of ROS primarily from myeloperoxidases, eosinophil peroxidases, membrane-bound NADPH oxidases, and mitochondrial sources, and the products include superoxide ($O_2^{\cdot-}$), nitric oxide (NO), H_2O_2, and HOCl. Ideally, these products destroy the invading organisms and upregulate the appropriate signaling pathways to restore cellular homeostasis.

Then, the catalase enzyme scavenges H_2O_2 by forming water and oxygen from it. Thus, an equilibrium is maintained between the oxidants and antioxidants. However, more reactive oxygen species result from oxidative damage progression, and this leads to the various lung diseases such as COPD and asthma (Leibel and Post 2016).

Ultimately, oxidative stress leads to tissue damage when the antioxidant system falls short in neutralizing the ROS. Adult progenitor stem cells then work to replace this damaged tissue. This regenerative capacity of the lungs plays a crucial role in preventing further lung damage.

2.3 Regeneration of Lung Cells

The regenerative capacity of the lungs is rapid in the aftermath of damage but is minimal under normal physiological conditions (Demoly et al. 1994). With improved understanding, treatment strategies have been bettered, aiming to retard the loss of regenerative capacity of lung tissue. It is during the conditioning phase that regenerative changes occur in the lung tissue, including removal of scars, senescent cells, and other epigenetic modifications. This phase is lacking in diseased lung tissue. For normal lung tissue regeneration, key steps have been noted to be induction of lung regeneration forte and expansion, cell population, and new architecture elaboration (Akram et al. 2016; Beers and Morrisey 2011).

Table 2.1 Lung cell type and their function

Cell type	Function
Alveolar type II (AT2)	Secrete surfactant
Alveolar type I(AT1)	Permit gas exchange between alveolar and capillary network
Clara cells	Secretion of Clara cell secretory protein (CCSP)
Bronchoalveolar cells	Proposed to be a precursor for the Clara cells and AT2 cells
Basal cells	Less differentiated
	Progenitor for columnar epithelial cells
	Cytokeratin 14 expression is used for the characterization of these cells

In lung tissue, 40 distinct cell populations were found to be present. From which, only a few cell types have shown to possess regenerative potential such as bronchoalveolar cells, type II epithelium, Clara cells, and basal cells (Lau et al. 2012) (Table 2.1)**.**

Adult lung tissue reparative deeds fall within the injury response gamut. One is a highly regenerative type – tissues such as the epidermis, intestine, and hematopoietic cells. The other is the rarely regenerative type – organs such as the brain and heart – a type that can easily develop a scar following any injury because of its relatively inefficient repair systems. A third type is an intermediate between highly and rarely regenerative, such as the lung, pancreas, and liver. These three organ tissues show slow and steady regenerative capacity that becomes rapid during tissue injury response (Hogan et al. 2014). In reparative cell biology of the lung, there are three correlated concepts:

1. Respiratory epithelium contains a varied population of epithelial cells that act as adult stem cells, which function depending on its composition and organization.
2. Lung progenitor stem cells have the ability to differentiate, for example, AEC2 in the bronchioles of mice.
3. Lung tissues responses to damage markers which are differentiate into one cell type, but it has the ability to change its phenotype to develop into different cell types (Blanpain and Fuchs 2014; Fuhrmann et al. 2014).

Addressing molecular pathways of lung cell regeneration is integral to the study of regenerative biology. Studying and comprehending the molecular signaling pathways was essential for therapeutic purposes. One such pathway is a cross talk between mesenchymal stem cells and epithelial cells that is important for proper development. Endothelial cells have thrombospondin 1 which control the Sca1+, a self-renewing gene in the lung epithelial cells. Thrombospondin 1 limits or controls the expression of Sca1+. Bmp4 is responsible for the activation of thrombospondin. Only if this activation takes place, the Sca1+ gene expression is regulated.

Another pathway targets some signaling pathways such as Wnt and Notch pathway, playing a key role in lung repair and regeneration. Canonical Wnt signaling

activation leads to regrowth and regeneration in different booths of the lung (Al Alam et al. 2011; Aumiller et al. 2013; Flozak et al. 2010; Hashimoto et al. 2012; Zhang et al. 2008). Self-renewal capacity and differentiation of the basal cells into secretory lineage in response to SO2-mediated airway epithelial injury require Notch signaling (Rock et al. 2011; Xing et al. 2012). Reactive oxygen species (ROS) activates the Nrf2 which in turn activates Notch signaling (Paul et al. 2014).

2.4 Oxidative Stress in the Lungs

Oxidative stress is the underlying cause for an imbalance between systemic manifestation of ROS and the biological system's potentiality to swiftly detoxify reactive intermediates and to rectify the consequent damage. In the course of normal metabolism, ROS are released in multiple biological processes and signal cascades that include normal tissue homeostasis and cell signaling. However, rapid alterations to the local and global levels of reactive species directly promote the development of several human diseases. The ROS are reactive oxygen species produced in vivo by endothelial, inflammatory, as well as immune cells via various cellular pathways as a by-product of metabolic processes and exhibit predominant roles in oxidative stress and tissue injury, along with its participation in redox signaling (McGuinness and Sapey 2017).

The respiratory system primarily provides abundant supply of oxygen to all the tissues, in order to corroborate normal oxygen homeostasis and organ functions. In the lung, constant exposure to gaseous oxygen and ROS is quelled by nonenzymatic and enzymatic antioxidant defenses such as glutathione (GSH), superoxide dismutase, and catalase (Holz et al. 1999). The antioxidant defense system becomes overwhelmed and fails to effectively counteract the stress mechanisms, leading to the development of various pulmonary diseases. Chronic inflammation-induced production of ROS in the lung predisposes an individual to lung damage and cancer development. The generation of reactive species is not restricted to pathogen defense. The role of reactive oxygen species and free radicals in multiple intracellular signaling pathways occurs at the macromolecular level, whereby proteins interacting with proteins and ligands with receptors, along with shape and surface charge, are the key to specificity. One such example is protein tyrosine phosphatases (PTPs) which can be inactivated in the presence of hydrogen peroxide and is reversible with glutathione along with other thiols (Holz et al. 1999). A complete understanding of the mechanisms of oxidant stress and the fundamental role of oxidants in lung disease pathogenesis paves the way to the development of improved therapeutic strategies.

The reactive species trigger the pathogenesis of various lung diseases, such as acute respiratory distress syndrome, asthma, interstitial pulmonary fibrosis, COPD, and lung cancer. Exposure to cigarette and tobacco smoke majorly contributes to about 80–90% of the COPD and lung cancer cases (Peden et al. 1995).

In the past few decades, protease/antiprotease imbalance and inflammatory processes have been proposed to act as downstream effectors of lung destruction

following persistent tobacco and cigarette smoking. It is believed that apoptosis is a major step occurring in the alveolar cells that result in prominent lung damage. A study has reported the manifestation of oxidative stress, apoptosis, and excessive proteolytic injury in cigarette smoke-induced human lung fibroblasts as well as epithelial cells in vitro (Guan et al. 2016).

2.5 Oxidative Stress Markers

In the lungs of several smoke-induced pulmonary disease patients, ROS and reactive nitrogen species (RNS) are rapidly released from the inflamed leukocytes and macrophages. ROS on reacting with a variety of biological molecules can lead to cell death as well as damage to the extracellular matrix of the lung (Park et al. 2009a).

Samples taken from cigarette smokers such as bronchoalveolar lavage fluid (BALF), exhaled breath condensate (EBC), plasma, urine, etc. show the presence of increased amounts of oxidative markers such as superoxides, oxidized glutathione (in BALF), hydrogen peroxide, 8-isoprostane (in EBC), and other various products of lipid peroxidation in plasma and urine (Taito et al. 2017). These reactive species have the potentiality to induce oxidative damage to DNA, lipids, carbohydrates, and proteins and thereby initiate an array of downstream processes that promote the development and progression of COPD (Lin et al. 2008). More specifically, these set of events together lead to a vicious cycle of intense inflammation, accompanied by chronic oxidative stress, which leads to prolonged interference in the protease-antiprotease balance, disruption in the tissue repair mechanisms, enhanced apoptosis, and accelerated autophagy in lung cells, which in turn collectively hasten the pathophysiological events that lead to COPD. It has been reported that cigarette and tobacco smoke possesses the ability to accelerate the urinary excretion rate of 8-OHdG, resulting in further destructive oxidative DNA modifications (Waris and Ahsan 2006). In yet another extensive study, the consequential role of four oxidative stress markers has been implicated in the pathogenesis of cigarette smoke-induced lung cancer. Oxidative stress biomarkers such as pH, 8-isoprostane, hydrogen peroxide concentration, and antioxidant capacity between lung cancer patients and healthy individuals are found to cause mutational changes in the genome of cells and have been detected in high levels in the exhaled breath of smokers. Tobacco smoke is one of the most important causative factors of lung cancer as it leads to chronic airway inflammation and activation of cells, which directly result in the production of high levels of nitric oxide. Its metabolites interact with ROS and trigger the generation of other reactive products with potential carcinogenicity (Voltan et al. 2016). A recent experimental study examined the prospective function of two specific oxidative stress markers in the pathological process of lung cancer. Supposedly, urinary metabolites such as 8hydroxyguanosine (80HG) and 8hydroxy 2deoxyguanosine (80HdG) were identified as biomarkers of oxidative DNA and RNA damage as well as markers of cancer growth and development. The nitric oxide molecules regulate modifications in tumor necrosis factor alpha (TNFα).

They sensitize the lung tumor cells to TNFα-mediated cytotoxicity via inhibition of NF-κB activation and increase the occurrence of oxidative damage. They are primarily responsible for cytogenetic changes in the lung and increased cell proliferation (Markele et al. 2014). Researchers have quantified the presence of various metabolites in urine, blood, and breath which significantly unveil reliable information on human exposure to carcinogens in tobacco and cigarettes. Marina Sarkele (Markele et al. 2014) conducted an extensive study to analyze the link between the level of oxidative stress biomarkers and the consequential effects in patients with acute respiratory distress syndrome. They investigated the variance between oxidants and antioxidants in the initiation of acute respiratory distress syndrome. Lipid peroxidation provokes intracellular death and particularly modifies proteins and DNA. The main goal was to measure the levels of oxidative stress-related molecules such as malondialdehyde and 4-hydroxynonenal as well as antioxidative molecules including superoxide dismutase, glutathione peroxidase, and tocopherol and analyze their clinical significance (Clarkson and Thompson 2000).

2.6 Antioxidant Defense System

Antioxidant defense system contains a complex system of substances that detain, inhibit, or eliminate oxidative damage to a target molecule. The human body contains an enzymatic and nonenzymatic antioxidant defense system which is a highly interconnected network that depends on dietary intake of antioxidants and endogenous production of antioxidative compounds such as glutathione (Halliwell 2006). These free radical scavengers can act at distinct levels and by multiple mechanisms in the oxidative sequence (Duarte and Lunec 2005) . Antioxidants can be categorized into a number of diverse groups such as:

1. Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GSR).
2. Antioxidative proteins include hemoglobin, ceruloplasmin, transferrin, albumin, lactoferrin. Small-molecular-weight compounds including ascorbic acid (vitamin C), glutathione (GSH), tocopherols (vitamin E), uric acid, bilirubin, glucose ubiquinone (coenzyme Q-10), selenium, flavonoids, and protein sulfhydryl (SH) groups (thiols) (Espinosa-Diez et al. 2015).

Among the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx or GSH-Px) play a crucial role. These enzymes are proteins which catalyze several important reactions to prevent damage from the reactive species (Kurutas 2015). There are numerous different isoforms of SOD which exist with multifold active metals in the center and different amino acid constituency. Studies have found that there are three different forms of SOD such as a cytosolic-CuZn-SOD, mitochondrial Mn-SOD, and extracellular SOD in humans. These scavengers primarily convert the free radical superoxide species into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) which are further

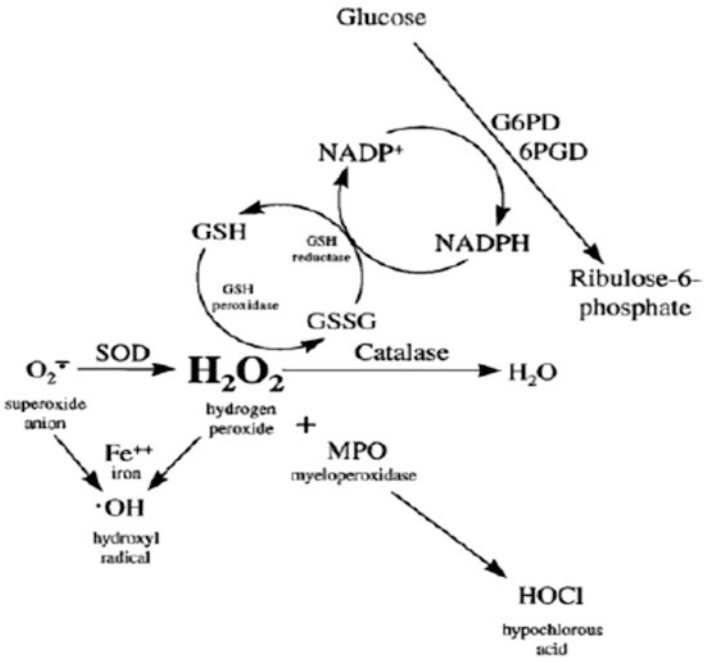

Fig. 2.1 Basic oxygen radical and antioxidant chemistry

catalyzed into by-products (Fig. 2.1)**.** One of the most well-recognized adaptive antioxidant defense system in the body is the thiol and glutathione complex. It functions as a conventional redox buffer against toxic free radicals for maintenance of cellular homeostasis and function. Most living organisms produce glutathione, which is a tripeptide containing a glutamate side chain with an amine group of cysteine at its carboxylate side which in turn is attached to a peptide glycine. GSH is a nonprotein sulfhydryl in the cells and efficiently contributes to the maintenance of the cellular redox status. Extensive research has determined the multifold properties of glutathione such as the protective defense mechanism against oxidants, cell proliferation, catalytic and metabolic functions, protein synthesis, and transport (Barrera 2012). The primary role of GSH is to induce the detoxification of lipid peroxidation products and protect against radiation-induced cell damage and other adverse events (Kinnula 2005). There are different forms of glutathione such as the reduced and disulfide (GSSG) form, mixed disulfides combined with various proteins, leukotrienes, and other metabolites. This defense system is one of the most efficient, particularly in association with GSH peroxidase (GPX), GSH reductase (GSR), and the hexose monophosphate shunt system (Wang et al. 2007). They are stimulated by various manipulation mechanisms of the cellular GSH/GSSG levels in reaction to various oxidative stresses, including hypoxia and inflammatory mediators, such as tumor necrosis factor (TNF)-α and lipopolysaccharide (LPS), in lung cells. Glutathione plays a critical role in the Nrf2-inducible glutathione-*S*-transferase system that catalyzes the specific fusion of GSH with endogenous and exogenous

electrophilic compounds (World Health Organization). Overall, the antioxidant-scavenging system can be enhanced by altering the nutrition intake, particularly vitamins, trace elements, and important amino acids that have either direct antioxidant effects or serve as precursors or cofactors for antioxidant enzymes.

2.7 Lung Pathology and Oxidative Stress

The vulnerability of lungs to tissue damage makes the repair process of the lung tissue essential. But some damaged tissue can't be repaired resulting in a disturbed oxidant and antioxidant balance. This leads to tissue damage, yielding pathological states, i.e., normal physiology is disturbed. This ultimately leads to lung diseases which count among some of the major diseases worldwide. (1) COPD, (2) bronchial asthma, (3) idiopathic pulmonary fibrosis, (4) lung cancer are four major causes of death worldwide due to lung ailments.

2.7.1 COPD

Chronic obstructive pulmonary disease (COPD) is a chronic pulmonary disease that results in chronic bronchitis and emphysema with associated difficulty in breathing. The leading cause of COPD worldwide is tobacco smoke, followed by exposure to high levels of air pollution. COPD increases the inflammatory mediators. Few inflammatory mediators are listed in Table 2.2 (MacNee 2006).

2.7.2 Bronchial Asthma

Bronchial asthma affects approximately 300 million individuals worldwide with significant global impact and is characterized by inflammation and

Table 2.2 Levels of inflammatory mediators in COPD

Inflammatory mediators	Their levels	Description about the inflammatory mediators
Leukotriene B4	Increased	It is produced by neutrophil, macrophages, and epithelial cells
CXC chemokine IL-8	Increased	It is the chemoattractant of T-cell and neutrophil
TNF α	Increased	Produced by epithelial cells and neutrophils
IL-1β	Increased	It attracts the cells from circulation and amplify the inflammatory response
IL-6	Increased	Proinflammatory cytokines
TNFβ	Increased	Proinflammatory cytokines
		Proinflammatory cytokines
		Responsible for the cause of fibrosis which can be directly or indirectly (activating the other cytokines)

hyperresponsiveness of the lungs; however, the mechanism and causes for the disease are still poorly understood (Barnes et al. 1998). The airway mucosal-mediated inflammatory response in asthma is cough, whereas other responses include increased vascular permeability with edema of airway walls, mucus hypersecretion with small airway plugging, and infiltration by inflammatory cells. In children who have asthma, an increased level of eosinophils and mast cells were observed when compared to normal children (Krishna et al. 1998). In adult asthma patients as well, raised eosinophils levels were seen (Krishna et al. 1998). It is characterized by the presence of increased levels of ROS and RNS in sputum and breath condensates, which further increase mucus secretion, enhance epithelial permeability (Hoshino et al. 2008; Jarjour and Calhoun 1994), and induce smooth muscle contraction. It can be exacerbated by exposure to ozone or allergens, causing an even further increase in oxidative stress (Holz et al. 1999; Peden et al. 1995).

2.7.3 Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is the formation of scar tissue within the lung without any incitement. Symptoms like exercise-induced breathlessness and severe dry cough are prominent features, and chronic diseases manifest over several years. Pathogenesis of IPF is initiated by the formation of lesions that vary with age and activity, and the resulting parenchymal fibrosis is directly caused by chronic inflammation in IPF. During the course of IPF, inflammatory response is a key factor for the disease progression, Th2-type immune response being the main type (Furuie et al. 1997; Hancock et al. 1998; Kunkel et al. 1996; Marrack et al. 2001). There are increased levels of Th2 cytokines such as IL-4 and IL-13 and eosinophils and mast cells (Briggs et al. 1991; Whyte et al. 2000). It was confirmed in murine models that Th2 response is more predominant than Th1 response in the lungs following an injury, and up to 3% demonstrates a genetic basis although the exact mechanism is still not clear (Kim et al. 2017; Schwartz et al. 1991a, b; Wells et al. 1997), 75% of the idiopathic pulmonary fibrosis patients being either smokers those who smokers formerly. This makes smoking an important contributory factor in the development of idiopathic pulmonary fibrosis. There is also an increased angiogenic activity and redolent tumorigenesis in pulmonary fibrosis, resulting from an imbalance between the angiogenic chemokines (IL-8, ENA-78) and angiostatic chemokines (IP10) (Strieter et al. 2007).

2.7.4 Lung Cancer

Lung cancer is both malignant and a cancer with high incidences. It is also the leading cause for cancer-related deaths worldwide, estimated at 1.5 million deaths per year (Jemal et al. 2009; Youlden et al. 2008). Among the reported cases of lung cancer, 90% have advanced to a metastatic stage and are rarely presented at stage I or stage II due to lung cancer being highly difficult to diagnose clinically until the later stages (Pignatelli et al. 2001). Metastasis in lung carcinoma is an end-stage

Table 2.3 Lung diseases and their classification based on the countries income

S. No.	Countries	Type of lung diseases	Rank	Percent of total deaths
1	World	COPD	4	7.8
		Lower respiratory tract infection	5	3.5
		Lung cancer	6	3.1
2	High-income countries	COPD	5	4.1
		Lung cancer	6	3.6
3	Middle-income countries	COPD	3	12
		Lung cancer	5	4.5
4	Low-income countries	COPD	4	5.5
		Lower respiratory infection	5	5.1

manifestation of the disease, i.e., stage IV. Higher oxygen tension is needed for cell migration in metastasis so increasing the pull toward higher oxygen tension areas of the cell, thereby changing its structure or resulting in epithelial mesenchymal transition (EMT). It is highly linked to environmental exposure and oxidant stress (Kim et al. 2017; Pignatelli et al. 2001). Since smoking remains prevalent and biomass fuels are still frequently used in China, epidemiological studies in that country have identified strong links between these exposures and cancer risks, indicating that 75% of lung cancer deaths are attributable to combined exposure (Guan et al. 2016; Lin et al. 2008). Interestingly, transformed cancer cells express increased levels of antioxidants, which contribute to their accelerated cell division and have elevated overall redox capacity. Occurrence of lung cancer is higher in cigarette smokers than non-cigarette smokers; it is being the most significant causal risk feature for developing lung cancer. For males and females, smoking causes over 90% and 70% of lung cancer deaths, respectively, in very high HDI countries; while in high−/median−/low-HDI countries, it is approximately 65% among males and 25% among females (US Department of Health and Human Services 2004; Pirie et al. 2013; Ezzati and Lopez 2003).

2.8 Lung Disease Statistics

Lung disease occurrence throughout the world is classified based on the income of the countries, that is, high-income countries, middle-income countries, and lower-income countries, and shows (Table 2.3) that it is higher in the lower- and middle-income countries than in the high-income countries (Mathers and Loncar 2006).

2.9 Molecular Mechanism of Lung Diseases

Cigarette smoking is primarily responsible for most of the lung diseases as it activates the inflammatory pathways which result in impaired lung physiology and also affects the ECM texture. Cigarette smoking directly leads to generation of reactive

oxygen species (ROS) resulting in oxidative stress (Domej et al. 2014; Ischiropoulos et al. 1992). When this antioxidant to oxidant ratio is disturbed, it results in oxidative stress (Fig. 2.2**).** Principal environmental factors concerning COPD is cigarette smoke causing decline in the pulmonary function, the extent of which varies from patient to patient. In COPD, oxidative stress plays a vital role in the development and pathogenesis of diseases and directly injures the DNA, protein, and lipid and indirectly activates the proteases system which leads to damage of the ECM's elastin and results in emphysema. In addition, it also activates the proinflammatory genes such as IL-1β, TGFβ, and TNFα which lead to the fibrosis of lung and other inflammatory diseases (Løkke et al. 2006). Respiratory epithelium gets activated by cigarette smoke which causes the production of proinflammatory mediators in the epithelium and subsequent activation of neutrophils and macrophages: IL-1β, granulocyte-macrophage colony-stimulating factor, IL-8, and leukotriene (Cosio et al. 2009). Cigarette smoking also results in direct DNA damage through 8OHdG (8-hydroxy-2′-deoxyguanosine) expression which creates the imbalance between the damage/repair reactive oxygen species. It is expelled during the repair of DNA damage by exonucleases, and it is a marker of oxidant-induced DNA damage (MacNee 2001). Cigarette smoke which contains 10^{14} free radicals not only affects lung epithelial tissue but also causes breakdown of connective tissues. Products of epithelial injuries act as ligand to TLR to activate the proinflammatory mediators. Cigarette smoke and oxidative stress trigger the activation of innate immune response in this manner (Anderson and Bozinovski 2003; Sundar et al. 2013) (Table 2.4**).**

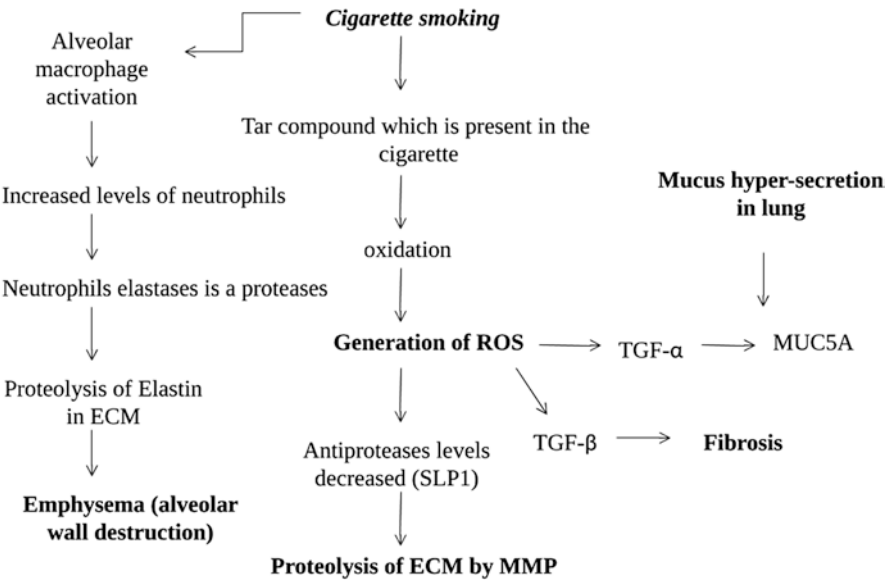

Fig. 2.2 Cigarette smoking and various lung-related diseases

Table 2.4 **COPD** (chronic obstructive pulmonary disease); DLCO (diffusion lung capacity for carbon monoxide); ↑ it denotes the levels increases

Characteristic	Tobacco smoke COPD	References
Reduced DLCO	↑↑↑	Schwartz et al. (1991a)
Oxygen saturation at rest and during exercise	↑↑↑	Schwartz et al. (1991b)
Bronchial hyperreactivity on methacholine challenge	↑	Wells et al. (1997)
Goblet-form cell hyperplasia	↑↑↑	Strieter et al. (2007)
Emphysema	↑↑↑	Jemal et al. (2009)
Airway wall thickening	↑	Youlden et al. (2008) and Pignatelli et al. (2001)
Anthracosis	↑	US Department of Health and Human Services (2004)
Pulmonary artery intimal hyperplasia	↑	Pirie et al. (2013)

2.10 Impact of Smoking on Lung Diseases

Tobacco or cigarette smoke contains over thousands of harmful toxins, carcinogens, and chemicals that enter the bloodstream through inhalation and thereby travel to every other organ in the body. The inhaled smoke reaches the bronchial tubes through the windpipe. The toxins found in the smoke possess the ability to cause inflammation and cell damage. In chronic smokers, the leukocyte count in the blood is constantly high in order to fight against the consequential damage (Hecht 1999). The immune system of a smoker is weaker and less efficient in counteracting the inflammatory response triggered by cigarette smoke. Prominent metabolic alterations and changes in morphological and morphometric parameters have been studied in the polymorphonuclear neutrophils of chronic smokers. Over a period of time, the carcinogens present in tobacco and cigarette can lead to mutagenic and genotoxic effects. Both the passive smoker and the habitual smoker are susceptible to DNA damage and are at risk of tumor development and cancer. There are different types of toxic agents found in tobacco and cigarettes, including organic and inorganic chemicals such as nicotine, hydrogen cyanide, carbon monoxide, formaldehyde, benzene, toxic metals, radioactive toxic metals, ammonia, and polycyclic aromatic hydrocarbon (http://www.who.int/news-room/fact-sheets/detail/chronic-obstructive-pulmonary-disease-(copd)). These chemicals collectively exert their properties as they enter into the body via the bloodstream and cause serious health problems such as difficulty in breathing, bronchial asthma, fatigue, weakness, chronic bronchitis, and severe irritation in the eyes, ears, and nose and can cause lung and throat cancer. According to reports published by the World Health Organization, smoking is the foremost cause of chronic obstructive pulmonary disease (COPD), and this includes smoke from cigarettes, cigars, and pipes as well as secondhand tobacco smoke exposure (Lee et al. 2012). The molecular mechanisms of these diseases include specific modifications in key biological structures such as

alveolar epithelial cells, which are vital to the maintenance of normal alveolar architecture and function. Frequent exposure to cigarette smoke subsequently results in particular adjustments in the alveolar epithelial cells that stimulate a rapid increase in epithelial permeability, a decrease in surfactant production, inappropriate production of inflammatory cytokines, and growth factors, provoking an increased risk of lung cancer. Supposedly, cell death was observed to be the most detrimental effect of cigarette smoke on alveolar epithelial cells, by either apoptosis or necrosis. The toxic smoke-induced cell death mechanisms largely enhance oxidative stress. This cigarette smoke contains and further generates free radical species that is destructive to the alveolar epithelial cells (Phaniendra et al. 2015). Recent experimental studies indicate that the apoptosis of alveolar epithelial cells and alveolar endothelial cells is involved in the pathogenesis of lung diseases such as pulmonary emphysema and asthma. The disruption of alveolar maintenance is a result of cigarette smoke-induced oxidative stress that will cause inflammation, apoptosis, autophagy, proteases/antiproteases imbalance, and oxidant/antioxidant imbalance. Cigarette smoke activates multiple signaling pathways which favor the progression of diseases. Cigarette smoking induces ROS, which downregulate Nirf2, a transcription factor responsible for the activation of SOD, catalases, and GSH (Boutten et al. 2010; Kensler et al. 2007; Malhotra et al. 2008). Cytokines, such as IL13 which are essentially responsible for causing emphysema, are dependent on MMP9 and MMP12 and result in a destructive effect on lung tissue by inhibiting the antiproteases system (Zheng et al. 2000). Cigarette smoking not only affects the oxidative imbalance but is also responsible for aging since klotho, an antiaging gene, expression has been seen to have reduced after exposure to cigarette smoke. There is a senescence marker protein (SMP)-30 which can protect the lung from enlargement and smoking-induced lung damage and also prevent alveolar septal damage that results from smoking. Collapse of these regulations led to emphysema and aging along with playing a significant role in smoke-induced lung damage (Takahashi et al. 2006; Sato et al. 2006) (Fig. 2.3)**.**

2.11 Lung Disease and Gut Ecosystem

Smoking also affects bowel mucosa and mucin expression (Allais et al. 2016) and gut microbiota, and several studies reported that smokers have such alteration as compared with non-smoker (Lee et al. 2018). This study suggested that smoking doesn't reduce the microbiota density nor affect alpha diversity but, rather, significantly modify beta diversity. This study reported also that there was no difference in the microbiota profile between non-smokers and former smokers, but when both groups were compared to current smoker, it appeared a significant variation in gut microbiota profile. Although the exact molecular mechanism linking smoking and gut microbiota is not fully known, it appears on an experimental level that cigarette smoking may alter in in vitro model epithelial transient receptor potential (TRP) channel expression, namely, the components on TRPV1 and TRPV4 (Allais et al. 2017) and the microbiota, via affecting the gut-brain axis signaling (Chi et al. 2017),

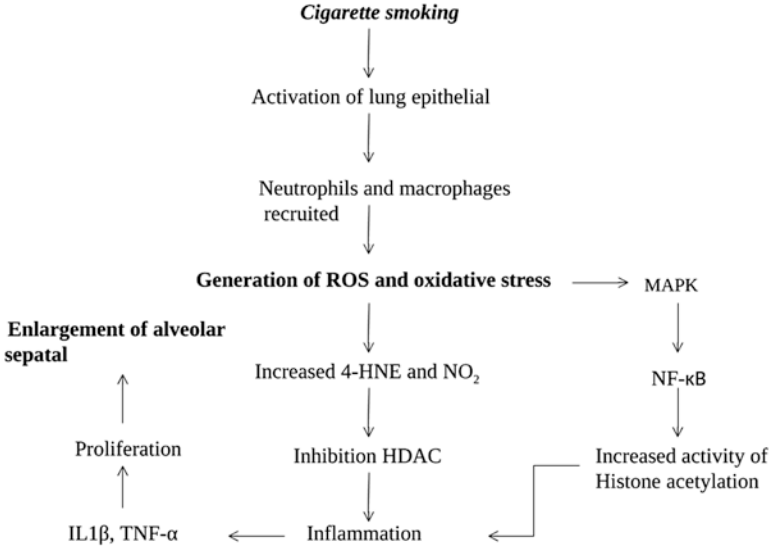

Fig. 2.3 Cigarette smoking and their effects on lung alveolar cells

while clinical data suggest that smokers display an increased ratio of *Bacteroidetes* over *Firmicutes* and increased *Proteobacteria* and *Clostridia* (Savin et al. 2018) as compared with non-smokers. This and the observed decreased *Actinobacteria* and the genera *Bifidobacteria* and *Lactococcus* mimic a proinflammatory profile observed in inflammatory bowel disease and weight gain (To et al. 2016). It is well-known that the toxicant of cigarette smoking affects the immune system, and the readers are suggested to refer to a recent excellent review on this complex topic (Qiu et al. 2017). Briefly, either innate (macrophages, DCs, and NK cells) or adaptive immune (regulatory T cells and B cells, cytotoxic CD8+ T cells, and D4+ Th cells), locally and systemically located, is significantly impaired by cigarette smoking where, depending on the triggering pathomechanisms, hyperresponsiveness or depressed function can take place via regulating NF-κB and MAPK signaling as well as histone modification. It is well-established the major role of gut in the immune system localization and that short-chain fatty acids associated with members of the phylum *Bacteroidetes* are relevant regulator of immune cell function. Studies have reported the beneficial role of probiotics and prebiotic intervention in immune system impaired by smoking (Reale et al. 2012; Seidel et al. 2007), thus posing the rational for their role in pulmonary disease management (Mortaz et al. 2013).

Provided that smoking cessation remains an unquestionable priority, these studies on smoking and gut ecology offer a platforms for new potential avenues aimed to implement protective/preventive and therapeutic strategies.

2.12 Rational Basis for a Systemic Antioxidant and Parenteral/Transmucosal Oxygen-Ozone Tentative Interventional Trial: The Cell-Energy Modulation Biotech, CMB® Protocol

While the primary trigger for cigarette smoking-induced pathophysiology is oxidative damage, this has also a crucial counteracting redox mechanism role to induce apoptosis so to limit the cancerogenesis process and progression (Ryoo and Bergmann 2012; Fernald and Kurokawa 2013). In this regard, it is worth reporting the experience of a large national survey on antioxidant protection from COPD (Park et al. 2016). By a multivariate analysis, it appeared that old age, male gender, heavy smoking, and a low intake of vitamin C were significant risk factors for COPD. On the contrary, there was a significant reduction of over 75% in COPD risk when examining the third quartile of vitamin C intake. These data are also corroborated by earlier epidemiological studies (Ochs-Balcom et al. 2006; Schünemann et al. 2001). On an interventional level (Pirabbasi et al. 2016), it has been shown that vitamin C supplementation improves antioxidant status in COPD (Biswas et al. 2013). Other authors have proven protective effect of vitamin C in COPD and also alleviating the flare-ups of the disease in a RCT (Isbaniah et al. 2011; Hu and Cassano 2000) and yielding better pulmonary function tests (Koike et al. 2014; Gupta et al. 2016). Recently, it has been shown on experimental ground that vitamin C could inhibit either the tobacco smoke-induced lung protein iNOS/NO-induced nitration as well as activation of pulmonary Rtp801, a key proinflammatory factor linked to cigarette-induced lung damage (Benedikter et al. 2017). Moreover, also the main lung protease involved in the proteolysis, i.e., matrix-metalloproteinase-9, involved in cigarette-induced emphysema, is partly downregulated. Vitamin C oral interventions have also been integrated with N-acetylcysteine, which has been shown to be depleted in these cases (Gupta et al. 2016) for its powerful anti-inflammatory effect, as shown in vitro (Valdivieso et al. 2018; Bocci and Paulesu 1990). The systemic oxygen-ozone therapy has been pioneered by Prof. Bocci at the Physiology Department of the University of Siena (Hu and Cassano 2000), a world-recognized authority who has the merit to have elevated a once a bit obscure and naively practiced procedure to a prime ranking scientific area. After that, some scientifically grounded and research-involved Italian associations of oxygen-ozone have been set up such as FIO, while some others have to be disregarded being purely commercially based and biased by producing their own bulky outdated (washing machine size) devices and old-style bags to collect blood requiring unsafe multiple manipulations. As Prof. Bocci recently suggested, systemic oxygen-ozone is now emerging as one of the potential weapons to treat COPD in its initiating mechanisms as well in its progression (Borrelli and Bocci 2014). In a clinical study in COPD patients undergoing autologous infusion of ozonated blood therapy, they recorded a significant improvement of the 6-minute walking test and with remarkable improvement of quality of life together with a better response to given medications.

Briefly, the principle of this treatment in this context is to act as a quick hermetic stressor (Bocci et al. 2009) able to generate a rebound upregulation of the defense

system (enhanced GSH/GSSG ratio, synthesis of antioxidant enzymes catalase, SOD, GSH-peroxidases, GSH reductase, NADPH-quinone oxidoreductase, cytochrome P450, and HSP70) which is as a whole pathologically under siege in the event of COPD. One minute following the infusion, the highly soluble ozone reacts with plasma via H_2O_2 and 4-hydroxynonenal by forming alkenal adducts which will react with the cytosolic Kelch-like ECH-associated protein 1 (Keap 1)-NF-E2-related factor 2 (Nrf2) system or with glutathione, and this immediately triggers the antioxidant defenses throughout all body cells. Interestingly, the alkenal interaction with either Cys 272 or 288 of Keap-1 favors the release of Nrf2 while enabling it to skip proteasomal degradation and binds to the nuclear antioxidant response element (ARE). Moreover, oxygen-ozone treatment may contribute to the inhibition of cytokine-mediated inflammation via leukotriene B4 reductase induction. Under a protocol approved by ReGenera R&D International for Aging Intervention, so far 46 long-lasting (over 10 years) cigarette smokers (m/f: 20/26, age range: 41–66 years) have been recruited. After thorough dietary and life questionnaires, a CMB® score (coming from a proprietary algorithmic analysis of redox parameter, BMI, blood tests, heavy metal test, and others) was applied at entry and after a personalized blend, as for quali-quantitative composition and session frequency, of oxygen-ozone procedures (using only a modern device) with sequential IV therapy with antioxidant, methyl-donor agents, and inhaled antioxidants. Follow-up at 1 and 3 months showed a significant improvement of CMB® score, of Pittsburgh Sleep Quality Index and pulmonary function tests. Larger study is ongoing. Transmucosal rectal route of oxygen-ozone is also underway with possible synergizing noninvasive pulsed electromagnetic field technology to increase the splanchnic blood flow and maximize the microvascular bed distribution. This is also in view of potentially extending another beneficial action of oxygen-ozone, i.e., the upregulation of heme-oxygenase 1 which, by allowing the interaction of trace quantities of CO in combination with NO, enables a vasodilatory response. As a matter of fact, we are also conducting studies on capillaroscopy to check on this integrative approach. Thus, a proper personalized antioxidant acute intravenous supply conjugated with a stepwise gradual, oxygen-ozone-induced hormetic oxidative stressors may conjure up to build a foreseeable strategy to yield an adaptive beneficial response for dealing with COPD pathophysiology.

2.13 Using Natural Antioxidants and Nutrigenomic Cytoprotective Agents to Prevent Oxidative Stress Damage in Lung Caused by Cigarette Smoking

Natural antioxidants have beneficial effects on oxidative stress-induced damage, especially phytomarine compounds isolated from *Rhodiola* plant extract which exhibited antioxidant activity along with singlet oxygen scavenging, H_2O_2 scavenging, and protein thiol protection activities (Chen et al. 2008). In addition, *Rhodiola rosea* extract was noted to be inhibiting the production of intracellular ROS and increasing the activity of antioxidant enzymes such as catalase, SOD, glutathione

peroxidase, and glutathione reductase. We have recently shown that a specific bioactive fraction (SBF) of *Rhodiola* associated with a patented marine lipoprotein extract from *Trachurus* sp. (R-L compound) could significantly and beneficially modulate cell proliferation rate in normal cells and also in cells under oxidative stress induction. It has also shown effects at the gene level. From a complete characterization of the R-L compound, it can be confirmed that there is a robust antioxidant nutrigenomic effect that is responsible for prevention of cell damages caused by both extrinsic and intrinsic factors. Antioxidant and antiaging property of R-L compound extract helped to maintain cell integrity throughout its lifespan, thus potentially proving to be valuable tools in efficient cellular geroptection. From our ongoing studies, it appears that R-L phytomarine compound is effective on the normal lung cells (L132) but not on lung cancer cells (H522).

Recent studies indicate that hydrogen coadministration in smoke-induced COPD-like lung disease in a rat model slows down the development of the disease. Results showed that not only did inhalation of hydrogen bring down the number of inflammatory cells in the bronchoalveolar lavage fluid, and the mRNA and protein expression levels of tumor necrosis factor alpha, IL-6, IL-17, IL-23, matrix-metalloproteinase-12, caspase-3, and caspase-8, but increased the tissue inhibitor of metalloproteinase-1 expression. Inhalation of higher percentages of hydrogen showed better outcomes (Liu et al. 2017). Both images have been sourced from the International Journal of Chronic Obstructive Pulmonary Disease (Liu et al. 2017). Mesenchymal stem cells have shown a lot of promise in combatting COPD in animal models and though successful to a lesser degree in human phase I and II clinical trials, hopefully leading to a better understanding and specific treatment of COPD (Janczewski et al. 2017) (Figure 5). In yet another study, extensive research is also going on in attempts to isolate phytochemicals that act as antioxidants or aid in retarding oxidative stress on lung tissue. These efforts are in a bid to alter the poor quality of life that almost necessarily comes with lung-related ailments and provide more effective and specific treatment (Al-Awaida et al. 2014).

2.14 Conclusion and Future Direction

There is now considerable evidence supporting an increased risk of lung diseases, including lung cancer in chronic cigarette or tobacco smokers. According to the WHO reports, smoking causes a staggering amount of seven million deaths annually. Chronic smoking habits are majorly responsible for most of the lung diseases as they are found to cause morphological changes to the respiratory tract (Park et al. 2009b). The numerous toxic chemicals present in the smoke directly damage the first line of defense in the upper respiratory tract and furthermore lead to reduced cell viability, enhanced inflammatory processes, and induction of apoptosis in the respiratory hair cells. There exists a definite complex relationship between duration of smoking, oxidative stress, and the onset of pulmonary diseases. The complex fusion of highly concentrated soluble and gaseous electrophiles aggravate the risk of protein and lipid oxidation, abnormal ceramide metabolism, ER stress, and cell

death in the lungs. Increased levels of oxidative stress are shown to occur in patients with chronic obstructive pulmonary disorder, acute lung injury, and lung cancer. Normal cellular metabolism releases oxygen-based reactive species which are known to act as second messengers in various signaling cascades involved in cell proliferation and differentiation. These reactive oxygen species principally regulate cell growth and maintain the redox status under homeostatic physiological conditions in order to prevent oxidative damage to the cells. The reactive nitrogen species (RNS) are highly reactive molecules precisely derived from nitric oxide and superoxide anion under the influence of enzymes such as nitric oxide synthase 2 (NOS2) and NADPH oxidase, respectively. Concurrently, ROS and RNS cause oxidative damage and tissue dysfunction in the cases of pathological conditions. They are responsible for the declining cellular function and compromised mitochondrial system. Prolonged exposure to tobacco and cigarettes is the main causative agent in the progression of oxidative stress-induced damages up to lung carcinogenesis. Understanding of the mechanisms of oxidative stress in the lung could possibly lead to improved pharmacological manipulation of antioxidants in lung inflammation and injury. Tobacco carcinogens are involved in the upregulation of the redox-sensitive transcription factors and proinflammatory gene expression. The antioxidant defense mechanism consisting of antioxidant enzymes, proteins, and small molecules is impaired in most lung pathologies (Figure 6). Recent clinical investigations aimed to develop tentative treatment modalities (Figure 7) targeting the functioning mechanism of antioxidants against oxidants. Discovery of various novel molecular compounds that is defensive and specific to oxidant signaling and possesses the ability to suppress the potent free radicals derived from the tobacco smoke is under clinical trials (Rahman and Kinnula 2012).

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Oxidative Stress in Neonatal Lung Diseases

3

Ru-Jeng Teng

Abstract

Neonates experience abrupt surge in oxygen (O_2) tension immediately after birth when their antioxidant defense system is not yet fully established. The more than fivefold increase in O_2 tension causes immediate oxidative stress, and the change can be more exaggerated when neonates are born with respiratory distress that requires supplemental O_2 to maintain tissue metabolism. This perinatal transition-induced oxidative stress is apparently very different from those experienced by other age groups. There are more than 1 in every 100 neonates who suffer from respiratory distress at birth. When mechanical ventilation is used for respiratory distress, which sometimes can be complicated with infection secondary to the invasive treatments, more oxidative stress will be generated. Mechanical ventilation, oxygen use, and infection are the three major contributors to neonatal chronic lung disease (CLD) – bronchopulmonary dysplasia – and all of them are associated with the generation of reactive oxidants. Persistent pulmonary hypertension of the newborn (PPHN) which occurs 1 in every 500 live births is another common neonatal lung disease mainly due to the persistence of high pulmonary vascular resistance. Increased endogenous oxidative stress has been shown to play a mechanistic role in the decreased vasodilation in PPHN. Inhaled nitric oxide and high concentration oxygen are used to reduce the pulmonary vascular resistance in PPHN. Since the lung is the first organ to confront this dramatic perinatal change, the cells within the lung need to cope with the oxidative stress. Cells respond to the oxidative stress with unfolded protein response, autophagy, and other adaptive mechanisms to survive this challenge by sacrificing their normal functions. When the oxidative stress persists too long, or is too overwhelming, then cell growth will be impaired with the development of chronic lung

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disease as a complication. The first 2–6 years of life is the most important period for lung growth, so any injury during this critical period can have major long-term impact to adult lung function. Neonates who survive chronic lung disease usually need months or even years of O_2 support; this obviously will further prolong the oxidative stress of their lungs. Pulmonary hypertension can complicate the CLD which significantly increases the mortality rate. The CLD survivors are also prone to develop chronic obstructive pulmonary disease which is the major leading cause of death worldwide. Although oxidative stress plays a vital role in causing lung injury, the antioxidant treatment, however, has never shown clinical efficacy indicating that more complicated mechanisms are involved. Roughly 20,000 neonates suffer from chronic lung diseases each year in the USA. New therapeutic strategies are apparently in need to help these neonates with a better lung growth.

3.1 Introduction

Perinatal period is most critical for postnatal organ development. The body sustains an unfathomable biological change within the first few minutes of life. This is especially challenging in prematurely born neonates with their body not fully prepared for facing this impact. With the advance in neonatal care, we are now taking care of infants barely viable at the gestational age of 22–24 weeks (55–60% of full term). With improved survival rate for these extremely premature infants, we can expect to encounter more patients suffering from the complications of their premature birth. Not surprisingly, almost all the prematurity-associated morbidities are related to oxidative stress. Bronchopulmonary dysplasia (BPD) is such a morbidity that affects more than 10,000 infants each year (Van Marter 2006), and the medical cost for their care is estimated to be more than $2.4 billion each year (Bhandari et al. 2016). Unfortunately, even after so much effort has been put into understanding its pathophysiology, we have not seen decrease in the incidence of BPD for the past 10 years (Patel et al. 2015). BPD is not just a neonatal lung problem because survivors with this problem tend to have long-term lung function deficit and probably develop chronic obstructive lung disease (COPD) at an earlier age. Understanding how oxidative stress injures neonatal lungs may help us to design new strategies in protecting lung function in at risk adults.

Neonates face a dramatic surge of oxidative stress immediately after birth. The highest oxygen (O_2) content in fetus is about 30 torr (SpO_2 ~80%) in the umbilical vein and 25 torr (SpO_2 ~65%) at aortic arch (Fig. 3.1). The O_2 content in alveoli reaches 150 torr, a fivefold increase when compared to the fetal blood, and can reach 700 torr (~24-fold) when pure O_2 is used during respiratory support. The antioxidant capacity of fetus is low since the fetus stays in a low O_2 environment (Hayashibe et al. 1990). This low O_2 tension may help blood vessel formation in utero by stabilizing the hypoxia-inducible factor (HIF) and increasing the expression of vascular endothelial growth factor (VEGF) (Fraisl et al. 2009). Antioxidant

capacity slowly builds up after around 36 weeks of gestation in human fetus and keeps going up afterward (Davis and Auten 2010; Frank 1998). The nonenzymatic antioxidants (Mirończuk-Chodakowska et al. 2018), mainly the thiol-containing compounds (Turell et al. 2013), established in neonates can help neonates to cope with the rapid increase in oxidative stress (Buhimschi et al. 2003) and are usually deficient in premature infants. So, if neonates are born prematurely, their antioxidant capacity will not be ready to cope with the drastic surge in oxidant formation, and they will be susceptible to oxidative injury.

Different from systemic vasculature, O_2 is a vasodilator for pulmonary arteries and is the primary support for tissue oxygenation (Lakshminrusimha 2012). Resuscitation with 100% O_2 used to be the standard practice under the assumption that failure to reverse the primary apnea in depressed neonate fast enough will lead to secondary apnea and irreversibly cause organ damage. Dr. Saugstad first raised the concern that using 100% O_2 for neonatal resuscitation may cause excessive oxidative stress and lead to more organ damage (Ramji et al. 1993). Several recent studies have demonstrated the safety of using lower O_2 concentration in resuscitating depressed neonates with equivalent or even better outcomes (Vento et al. 2001, 2003). Although the definite efficacy using lower O_2 concentration for neonatal resuscitation remains to be determined, the relationship between O_2 use and high oxidative stress to neonates has been clearly shown by those researches (Saugstad 2004). The new Neonatal Resuscitation Program guideline recommends using room air and 30–40% as the initial resuscitating gas for term and premature infant, respectively.

High concentration of O_2 treatment inhibits alveolar formation (O'Reilly and Thébaud 2014), uncouples endothelial nitric oxide synthase (eNOS) (Jing et al. 2017), causes endoplasmic reticulum (ER) stress (Teng et al. 2017) and macrophage (Jankov et al. 2003) and neutrophil infiltration (Auten et al. 2001), impairs blood vessel formation (angiogenesis), and probably causes insults to other distant organs through circulating exosomes (Lal et al. 2018). Decreased brain cortex and retinal thickness (Poon et al. 2016) and nephrogenesis (Popescu et al. 2013) have been shown in neonatal animals exposed to hyperoxia. Kidney fibrosis has also been reported in hyperoxia-exposed neonatal rats (Jiang et al. 2015). These reports suggest that oxidative stress is not limited to the lung but involves other organs and systems as well.

In some neonatal lung diseases, the endogenously generated oxidative stress plays a major mechanistic role such as the persistent pulmonary hypertension of the newborn (PPHN). In PPHN there are phenotypic changes in pulmonary vascular cells with increased formation of reactive oxygen species (ROS) (Teng et al. 2009), eNOS uncoupling (Konduri et al. 2007), impaired mitochondrial superoxide dismutase (MnSOD or SOD2) shuttling into mitochondria (Afolayan et al. 2012), and altered prostacyclin synthesis (Mahajan et al. 2015). These findings all point out the important role of oxidative stress in neonatal lung disorders. The association between PPHN and neurodevelopmental deficit has been explained by the aggressive treatments, including the use of extracorporeal membrane oxygenator, but one recent study has shown some evidence of decreased blood vessel formation in the

brains right after birth indicating a systemic effect of this neonatal lung disease (Cohen et al. 2014).

Oxidative stress generated by O_2 use (Freeman and Tanswell 1985), mechanical ventilation (Chapman et al. 2005), and inflammation damage neonatal lungs through multiple mechanisms. Exposing lung to high O_2 environment increases free radical formation and leukocyte infiltration (Auten et al. 2001), decreases nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Walters et al. 2008), uncouples eNOS (Jing et al. 2017, et al.), and not only affects lung function acutely but also has long-lasting impact. Bronchopulmonary dysplasia (BPD) is a common complication in infants who sustained a severe oxidative stress-induced lung injury (Baraldi and Filippone 2007). The dependence on O_2 use further prolonged the oxidative stress in BPD. Several studies have shown that BPD infants are inclined to develop hyperactive airway disease (Eber and Zach 2001), impaired lung function (Filippone et al. 2009), and chronic obstructive pulmonary disease (COPD) (Wong et al. 2008). Evidence also suggests neonatal exposure to hyperoxia is associated with impaired lung growth and decreases the tolerance to oxidative stress later in life (Yee et al. 2011). It is obvious that effect of oxidative stress on neonatal lungs can redirect the lung growth trajectory.

3.2 Perinatal Transition

Fetus grows in a low O_2 environment with the O_2 supply coming solely from the placenta. The umbilical vein has the highest O_2 content, and only small amount (10–15%) of fetal circulation enters the uninflated lungs. We can expect the O_2 tension in fetal lungs to be extremely low (Fig. 3.1). With uterine contractions before delivery, the cortisol and catecholamine levels in fetus increase (Hillman et al. 2012) that upregulate the amiloride-sensitive epithelial Na + channels (ENaC) in the developing lung to pump the alveolar fluid into the interstitial space (O'Brodovich et al. 1993). Fail to do so, especially in the situation when cesarean section is performed without contraction, the alveolar sacs will be filled with fluid and cause a problem called transient tachypnea of newborn (TTNB) or retained fetal lung fluid (Jain and Eaton 2006). After neonate takes the first few breaths with most of the alveolar fluid removed, ambient air enters the alveoli; the O_2 tension in the blood increases precipitously within a few minutes to reach the adult level (Rabi et al. 2006). With this abrupt increase of O_2 tension in circulation, there is an increase in ATP generation (Konduri et al. 1993) which causes pulmonary arteries to relax via eNOS-generated NO (Konduri and Mital 2000). The pulmonary vasodilation decreases the blood flow through the ductus arteriosus. The increased pulmonary blood flow increases blood return to left atrium and helps to close the foramen ovale. Usually within 2–7 days, the pulmonary vascular resistance will drop to adult level, and the ductus arteriosus closes to complete the establishment of extrauterine circulation.

Premature lungs with insufficient production of surfactant, decreased expression of eNOS in pulmonary arteries, and deficit in alveolar surface area will limit O_2

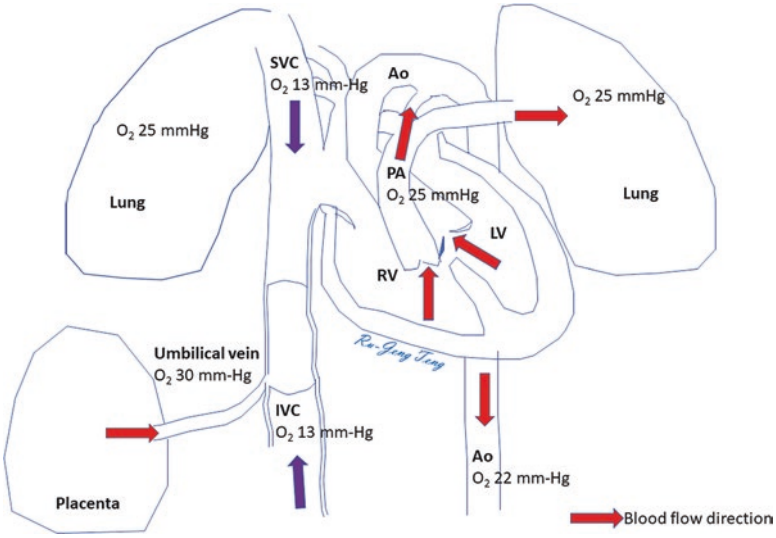

Fig. 3.1 Oxygen (O_2) content in fetal circulation. The highest O_2 content is 30 mm Hg with saturation at 80% in the umbilical vein from the placenta. The oxygenated blood mixes with deoxygenated blood from the inferior vena cava (IVC); then the O_2 content decreases down to 25 mm Hg with saturation of 65%. Most of the blood in right ventricle (RV) goes through the ductus arteriosus into descending aorta (Ao) with only about 10–15% of the RV output goes into the lungs which explains the low O_2 content in the fetal lungs. The O_2 content in the descending Ao is about 22 mm Hg with saturation at about 60%

extraction secondary to the ventilation-perfusion mismatch (Smith and Jones 2001). This condition is called respiratory distress syndrome (RDS). The limited O_2 availability to the pulmonary vasculature leads to the persistence of high pulmonary vascular resistance (PVR) which aggravates the already impaired oxygenation. The surfactant deficiency decreases lung compliance, and the alveoli are prone to collapse during expiration. It becomes extremely difficult to open the lung once it collapses, so there is a typical "retraction" during inspiration. Premature infants combat against the problem by partially closing their vocal cord hence causing the "grunt." To prevent the lung from collapsing, positive inflation pressure is commonly implemented to increase the functional reserve capacity of the lung.

Term infant with TTNB fails to clear the fluid in the alveoli, so O_2 cannot be extracted by the pulmonary blood which similarly causes the persistence of high PVR. Meconium aspiration syndrome or neonatal pneumonia, with either meconium or inflammatory exudate in the lungs that occludes the small airway and prohibits air from entering the alveoli, or impairs the function of surfactant, will lead to poor lung compliance with persistence of the PVR. All these neonatal pulmonary problems require supplemental O_2, possibly also the mechanical ventilator, and hence sustain the oxidative stress.

Sometimes fetus suffering from intrauterine distress, or exposed to nonsteroid anti-inflammatory drug (aspirin, indomethacin, ibuprofen, etc.) in utero (Alano

et al. 2001), can develop idiopathic persistent pulmonary hypertension of the newborn (PPHN). It is estimated that 1 in every 500 infants is born with PPHN (Konduri and Kim 2009). PPHN infants have difficulty progressing through perinatal transition due to either decreased blood vessel number and/or hyperplasia/hypertrophy of the smooth muscle layer of the pulmonary arteries which cause the persistence of high PVR (Teng and Wu 2013). In sheep PPHN model, our group has demonstrated an increased formation of reactive oxygen species (ROS) (Konduri et al. 2007; Teng et al. 2009) which can be attenuated by glucocorticoid (Chandrasekar et al. 2008; Konduri et al. 2013). Interestingly, the increased ROS formation is not due to O_2 treatment but is through multiple endogenous mechanisms as detailed below (Afolayan et al. 2012; Afolayan et al. 2016; Tadokoro et al. 2016; Teng et al. 2012).

3.3 Vasoactive Molecules Are Involved in the Perinatal Transition

Vascular resistance is determined by both vasodilator and vasoconstrictor. Nitric oxide (NO) and prostacyclin (prostaglandin I2, PGI2) are the major vasodilators, whereas endothelin-1 (ET-1) and thromboxane A2 (TXA2) are major vasoconstrictors. NO is the most extensively studied in neonates among the four vasoactive molecules and is believed to be the one that determines the basal vasomotor tone of the pulmonary artery. Oxidative stress can disturb the function of the enzymes that synthesize these vasoactive molecules.

3.3.1 Nitric Oxide (NO)

NO is synthesized by nitric oxide synthases. There are three isoforms of nitric oxide synthase (neuronal NOS, inducible NOS, and endothelial NOS) with the endothelial isoform (eNOS or NOS3) as the major one that modulates vasomotor tone. NO produced in the endothelial cells will diffuse into vascular smooth muscle cells to activate the soluble guanylate cyclase (sGC) in to generate cGMP (Stasch et al. 2011) which relaxes the smooth muscle cells. Phosphodiesterase-5 (PDE5) is the enzyme in the pulmonary artery smooth muscle cells that hydrolyzes cGMP. The expression and activity of PDE5 increase in the vascular smooth muscle cells under exposure to hyperoxia (Farrow et al. 2008; Heilman et al. 2015). It is believed that oxidative stress mediates the upregulation of PDE5 in the circulatory system and contributes to the development of pulmonary hypertension (Farrow et al. 2010; Lu et al. 2010).

3.3.2 Prostacyclin (PGI2)

Prostacyclin (PGI2) is one of the prostaglandins – a fatty acid derivative with 20 carbon atoms including a five-carbon ring – and is a potent vasodilator for

pulmonary circulation (Gryglewski et al. 1978). All prostaglandins are derivatives from arachidonic acid through two cyclooxygenases (COX-1 and COX-2) (Smith et al. 1996) to form an intermediate – prostaglandin H2 (PGH2) – which is a vasoconstrictor (Lin et al. 1994). COX-1 maintains the baseline levels of prostaglandins, whereas COX-2 is an inducible enzyme that produces prostaglandins under stimulation. The inducible COX-2 level increases in the hyperoxia-exposed neonatal lungs which may contribute to the inflammatory cell infiltration in those lungs (Britt et al. 2013). Prostaglandin H2 is the precursor for prostacyclin, prostaglandin E2, and thromboxane A2. Oxidative stress can modify the enzymes that synthesize each eicosanoid (prostaglandin or thromboxane) and affect their activities (Zou and Ullrich 1996). Interestingly, NO generated in endothelial cells enhances prostaglandin synthesis by upregulating the PGH2 synthase (Davidge et al. 1995). Synthesizing prostaglandins through PGH2 synthase will consume NADH or NADPH and generate superoxide (Kukreja et al. 1986). In sheep PPHN model, it was reported that nitration of prostacyclin synthase inhibits the enzyme activity, while nitration of thromboxane synthase does not affect the activity (Mahajan et al. 2015) which at least partially explain the impaired angiogenesis and vasodilation in PPHN.

3.3.3 Thromboxane A2 (TXA2)

As member of the eicosanoid family, thromboxane A2 is also a derivative of PGH2. TXA2 is unstable, so the level of its more stable metabolite – TXB2 – is commonly used to reflect the level of TXA2 in biologic fluids. The formation of TXA2 may be important for the closure of the ductus arteriosus (Yokota et al. 2014), but its level increases significantly in neonates born with the PPHN, suggesting its role in causing the increased PVR (Kääpä 1987). In perfused rabbit lungs, exposure to reactive oxygen species leads to an increased level of TXB2 in the perfusate indicating that oxidative stress encourages the synthesis of thromboxane. The TXA2 receptor can bind other ligands including the 8-iso-prostane, a stable lipid peroxidation product. Activation of TXA2 receptor promotes the vascular smooth muscle cell proliferation which may contribute to the development of pulmonary hypertension in the hyperoxia-exposed lungs. In premature infants born to mother with chorioamnionitis, the TXB2 level in tracheal aspirate is significantly higher in those who later developed BPD (Watterberg et al. 1996). These data suggest that TXA2 may play a role in the development of BPD and possibly also in the pulmonary hypertension developed in infants with moderate to severe BPD.

3.3.4 Endothelin

Endothelins are 21 amino acids peptides. There are three isoforms of endothelin (ET-1, ET-2, and ET-3) and two G-protein coupled receptors (ET-A and ET-B) (Davenport et al. 2016). ET-1 is the most abundant endothelin in endothelial cells. Endothelial cells continuously synthesize and release ET-1 into the circulation.

ET-1 binds to both ET-A and ET-B with equal affinity. After binding to ET-A, ET-1 becomes one of the most potent vasoconstrictors in human. The binding of ET-1 to ET-B can cause vasoconstriction or vasodilation. The vasodilatory effect of ET-1 is believed to be mediated by the release of NO from the endothelial cells. Plasma ET-1 level increases in mouse exposed to 100% O_2 (Habre et al. 2006). The increased ET-1 level can promote the proliferation of vascular smooth muscle cells through promoting the formation of reactive oxygen species (Fei et al. 2000) and probably through the activation of ET-B (Di Luozzo et al. 2000). The growth-promoting effect on vascular smooth muscle cells may contribute to the development of pulmonary hypertension. This leads to the recent studies of ET receptor as the therapeutic target to prevent or treat pulmonary hypertension in BPD.

3.4 Free Radical Generating Systems

Three free radical generating systems have been extensively studied in neonatal lung disorders.

3.4.1 Mitochondrial Electron Transport Chain

Oxidative phosphorylation is considered the most important source of ROS in the body. It is estimated that ~1–2% of O_2 consumed during physiological respiration is converted into superoxide when electrons prematurely leak out from the electron transport chain and are aberrantly transferred to the molecular O_2 (Chen et al. 2003). Complex proteins I, II, and III are the main superoxide-generating proteins among the five complex proteins (Guzy and Schumacker 2006). This mitochondrial superoxide formation probably provides the physiologic level of oxidative stress that serves a signaling purpose. Most superoxide formed inside of the mitochondria will be dismutated into H_2O_2 by the manganese superoxide dismutase (MnSOD or SOD2). Any disturbance to the electron flow in the respiratory chain can cause an increased superoxide generation (Waypa et al. 2010; Waypa et al. 2013) just like both hyperoxia and hypoxia cause increased reactive oxidant formation.

3.4.2 NADPH Oxidase (NOX)

NADPH oxidase (NOX) is first identified in neutrophil, with its deficiency impairing intracellular bacterial killing which is called chronic granulomatous disease (Suh et al. 1999). There are at least nine members of the NOX family reported in the literature (Sirokmány et al. 2016). Although there are some debates about which reactive oxygen species are formed from each isoform, in general superoxide is believed to be the main product. Evidence suggests that NOX4 generates mainly H_2O_2, while DUOX1/DUOX2 can generate both superoxide and H_2O_2. Some isoforms localize only in certain places to carry out their special mission (Meitzler et al. 2014). NOX isoforms 1, 2, 4, and 5 are all distributed in the vascular system

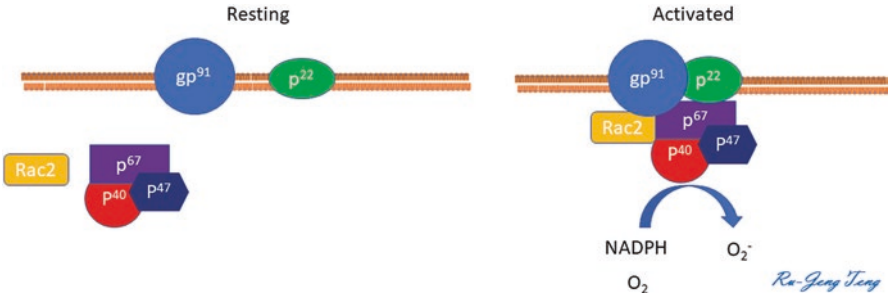

Fig. 3.2 Activation of NADPH oxidase 2 (NOX2) causes multimerization of the NOX2 subunits then generates superoxide from O_2 and NADPH. Only gp91 and P22 are membrane bound. Once activated, several cytosolic subunits (P40, p47, and p67) bind to gp91 and P22 and in the presence of Rac1/2 to complete the formation of the multimeric complex to generate superoxide

(Konior et al. 2014), while NOX2 is widely distributed in several cell types including endothelial cells. NOX2 is the modal isoform for the NOX family (Nauseef 2008) which is a multimer complex formed by gp^{91}, P22, P40, P47, P67, and Rac1/2 under activation then uses NADPH and O_2 to generate superoxide anion which is also called "respiratory burst" in neutrophils (Fig. 3.2). Reactive oxygen species formed during O_2 exposure (Kim et al. 2014), mechanical ventilation (Chapman et al. 2005), and inflammation are all resulted from the NOX activity.

3.4.3 eNOS Uncoupling

eNOS is a heme-containing enzyme which requires several cofactors (Ca^{2+}, FAD, FMN, BH4, NADPH) to convert arginine into NO. Since NOS uses arginine as substrate, so the depletion of arginine, or accumulation of arginine analogue, asymmetric dimethylarginine (ADMA) (Sibal et al. 2010), will decrease NOS function. Arginases (I and II) are the enzymes that hydrolyze arginine into urea and ornithine and on the other hand can competitively inhibit the NOS activity (Durante et al. 2007). With the existence of the cofactors, eNOS forms homodimer and associates with heat-shock protein 90 (HSP90) (Pritchard et al. 2001) to achieve adequate coupling (Fig. 3.3). Lacking any cofactor or inhibiting the association between eNOS and HSP90 will lead to eNOS uncoupling. eNOS is also modified by several posttranslational processes (Table 3.1) such as acylation, nitrosylation (Ravi et al. 2004), phosphorylation, acetylation, glycosylation, and glutathionylation that can modulate the activity (Reviewed by Heiss and Dirsch 2014).

Uncoupled eNOS releases superoxide anions instead of NO after activation. In sheep PPHN model, the dysfunctional GTP cyclohydrolase 1 (GCH1) activity in pulmonary artery endothelial cells causes eNOS uncoupling even without oxygen or mechanical ventilation support and persists after passages, indicating a true phenotypical change (Teng et al. 2011a, b). Interestingly, rat pups exposed to high O_2 environment also show decreased GCH1 level and activity in the lungs. GCH1 is the

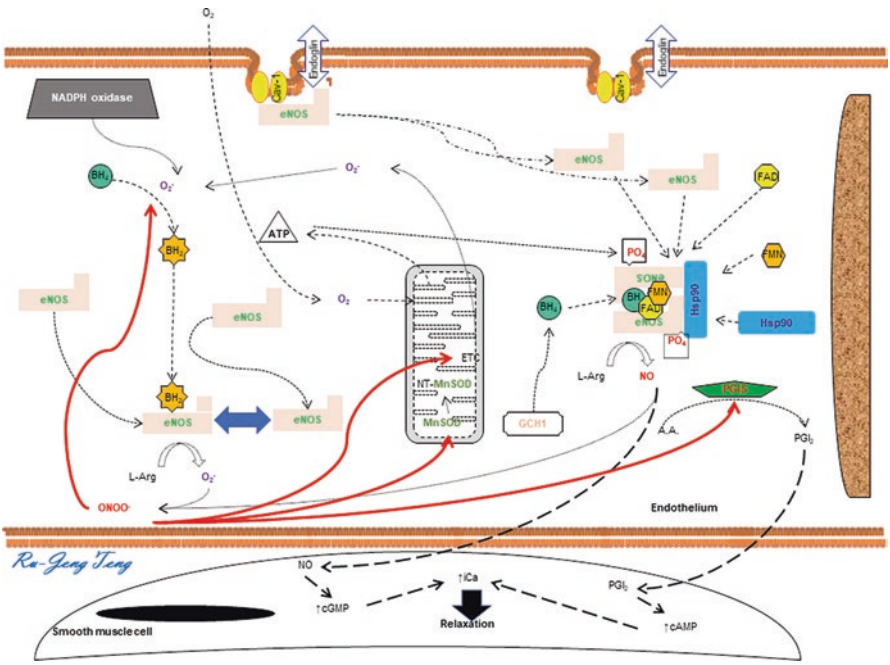

Fig. 3.3 Adequate eNOS activity (coupling) requires several cofactors, posttranslational modifications, and interaction with other proteins. The association between eNOS and caveolin-1 may inhibit eNOS coupling, but this association is required for the initiation of coupling process. eNOS protein contains both reductase and oxidase domains and forms a homodimer to complete electron transfer to generate NO. The homodimeric formation requires the presence of multiple cofactors including Ca^{2+}, FAD, FMN, BH4, etc. When BH4 is oxidized to BH2, BH2 will compete with BH4 for the biding site on eNOS and cause eNOS uncoupling. Uncoupled eNOS releases superoxide instead of NO which can further deplete NO by forming an extremely reactive free radical peroxynitrite. Peroxynitrite oxidizes BH4 and S-nitrosylated eNOS, to cause a vicious cycle of eNOS uncoupling. Peroxynitrite also nitrates prostacyclin synthase, SOD2, or electron transport chain complex to impair their activities
Red arrow: Peroxynitrite nitrates the protein and impairs the activity

rate limiting enzyme for the sythesis of tetrahydrobiopterin (BH4) which is the vital cofactor for eNOS coupling (List et al. 1997). The increased ubiquitin-proteasome activity explains the decreased GCH1 level which contributes to the impaired alveolarization and angiogenesis in the neonatal lung under hyperoxia (Jing et al. 2017).

Under oxidative stress the BH4 will be oxidized into BH2 which binds to eNOS as effectively as the BH4 but is incapable of maintaining eNOS catalytic function (Crabtree et al. 2008; Vásquez-Vivar et al. 2002). The exogenous oxidative stress-induced eNOS uncoupling may also contribute to the impaired blood vessel and alveolar formation seen in the BPD. NO also controls mitochondrial biogenesis (Nisoli et al. 2003). NO modulates mitochondrial function of the endothelial cells by mildly suppressing the electron transport chain complex which prevents the formation of too much superoxide anion from oxidative phosphorylation (Konduri et al. 2015).

Table 3.1 Various posttranslational modifications of the eNOS that affect function (Heiss 2012)

Type of modification	Site of modifications	Enzyme or compound involved	Activity change
Phosphorylation	*Serine*		
	114	AMPK, CDK5, PKC	↑↓
	615	AKT, PKA	↑↓
	633	AMPK, CDK5, PKA	↑
	1177	AKT, AMPK, CaMKK2, CHK1, PKA, PKG	↑
	Threonine		
	495	AMPK, rho kinase, PKC	↓
	Tyrosine		
	81	Src	↑
	657	PYK	↓
S-Nitrosylation			
	Cysteine		
	94		↓
	99		↓
	661, 802, 853, 976, 991, 1048, 1050, 1114, etc. totally 29		↓(?)
Acylation			
Myristoylation	*Glycine* 2	N-Myristoyltransferases	↑
Palmitoylation	*Cysteine*		
	15	Palmitoyl acyltransferases	↑↓
Depalmitoylation	26	Acyl-protein thioesterase 1	–
		Palmitoyl-protein thioesterase	
Acetylation			
	Lysine		
	497	Lysine acetyltransferase	↓
	507	NAD-dependent deacetylase sirtuin-1 (inhibitory)	↑
	610	Aspirin	↑
		Histone deacetylase 3 (inhibitory)	↓
O-glycosylation	*Similar to phosphorylation sites*	O-linked N-acetylglucosamine (O-GlcNAc) transferase	↓(?)
Glutathionylation	*Cysteine*		
	689		↓
	908		↓

AKT protein kinase B/Akt kinase, *AMPK* AMP-activated kinase, *CDK5* cyclin-dependent kinase 5, *CHK1* checkpoint kinase 1, *ERK* extracellular stimuli-regulated kinase, *PKA* protein kinase A, *PKC* protein kinase C, *PKG* protein kinase G, *PYK* proline-rich tyrosine kinase, *Src* Src kinase

3.5 Free Radicals in Neonates

Other than the hydrogen peroxide (H_2O_2), most of the free radicals contain an uncoupled electron singlet that interacts actively with the surrounding biomolecules. Free radicals can be broadly divided into two groups – reactive oxygen species (ROS) (Table 3.2) and reactive nitrogen species (RNS) (Table 3.3). Superoxide (●O_2^-), hydroxyl radical (●OH), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) are typical ROS (Ray et al. 2012), while peroxynitrite (●$ONOO^-$), nitrogen dioxide (●NO_2), and nitrosonium (NO_2^+) are the RNS (Patel et al. 1999). An adequate amount of ROS/RNS is needed for organ growth and homeostasis but can damage tissues when the level reaches certain threshold levels (Frank 1998) – hormesis (Calabrese et al. 2007).

H_2O_2 is a membrane-diffusible molecule which is reduced to H_2O by catalase in the cytosol. The most common source of H_2O_2 is from superoxide dismutation. Blood level of H_2O_2 can reach 20–40 μM during sepsis. Exogenous H_2O_2 can activate ROS formation through disturbing mitochondrial function (Park 2013). Interestingly, the increased formation of ROS in mitochondrion happens both under hyperoxic and hypoxic conditions when the electron flow is disturbed. Physiological level of NO can enhance the efficiency of oxidative phosphorylation and decreases oxygen consumption with equal amount of ATP production (Brown 2001; Clerc et al. 2007), but large amount of NO will irreversibly inhibit oxidative phosphorylation due to nitrosylation of the electron transport chain complexes.

Although hyperoxia exposure in neonatal mice increases mRNA and protein levels of eNOS due to hyperacetylation of H2aZac and H3K9ac (Chao et al. 2018), the function of eNOS is not increased correspondingly. This discrepancy can be explained by the depletion of BH4 either through oxidation by peroxynitrite (Kuzkaya et al. 2003) or degradation of GCH1 under hyperoxia (Jing et al. 2017).

Table 3.2 List of the ROS(Apak)

Symbol	Name
$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Superoxide anion radical
$^{\cdot}OH$	Hydroxyl radical
HOCl	Hypochlorous acid
$RO^{\cdot}$	Alkoxyl radical
$ROO^{\cdot}$	Peroxyl radical
H_2O_2	Hydrogen peroxide
LOOH	Lipid hydroperoxide

Table 3.3 List of the RNS

Symbol	Name
NO_2	Nitrogen dioxide
$ONOO^-$	Peroxynitrite
No^+_2	Nitrosonium
$ONOOCOO^-$	Nitrosoperoxycarbonate

O_2 itself directly causes superoxide formation in the lungs (Brueckl et al. 2006). Two other sources of ROS during neonatal period are the mechanical ventilation (Gitto et al. 2009) and inflammation (Mittal et al. 2014). NOX has been shown to play central role in both sources. Other potential contributors are xanthine oxidase/dehydrogenase, prostaglandin synthase, and the uncoupled eNOS.

Under certain pathologic conditions, the generated superoxide cannot be promptly reduced, so it can interact with the nitric oxide (NO) to form peroxynitrite. (Reiter et al. 2000). The inducible NOS in the inflammatory cells produces thousand-fold NO as compared to the basal NO formed by the eNOS. Inflammation and uncoupled eNOS are the two major sources of RNS. As an extremely potent free radical, peroxynitrite attacks almost every known biomolecules in its vicinity. Peroxynitrite preferentially oxidizes BH4 in the endothelial cells as compared to ascorbate and thiols (Kuzkaya et al. 2003). Under hyperoxia the BH4-forming enzyme GCH1 will be degraded by the proteasome-ubiquitin system (Zhao et al. 2012), and under pulmonary hypertension the binding between GCH1 and HSP90 decreases and also encourages the degradation through proteasome-ubiquitin system (Sun et al. 2011). The most well-known product of peroxynitrite is the 3-nitrotyrosine from any tyrosine-containing proteins or free tyrosine. The presence of Cu,Zn-SOD (Ischiropoulos et al. 1992) and bicarbonate/CO_2 (Tien et al. 1999) actually facilitates the tyrosine nitration by peroxynitrite. The fact that CO_2 facilitates peroxynitrite-induced damage seems to contradict the common belief that conservative ventilator strategy, allowing permissive hypercarbia in ventilating premature infants, can prevent ventilator-induced lung injury (Miller and Carlo 2007).

Inflammatory cells generate reactive oxidants through NOX2 or the release of myeloperoxidase (MPO) (Gaut et al. 2002). Infiltration of MPO-containing leukocytes in the lungs can be seen in rodent pups within a few days of hyperoxia exposure (Deng et al. 2000; Eldredge et al. 2016). Oxidative stress causes the so-called damage-associated molecular pattern (DAMP) (Kuipers et al. 2011) and releases inflammatory mediators including the high mobility group box-1 (HMGB1) (Tang et al. 2011; Entezari et al. 2014). HMGB1 amplifies the inflammatory response after binding to receptors (TLR2, TLR4, RAGE, CD24) and recruits more MPO-containing leukocytes into the damaged area (Lotze and Tracey 2005; Andersson and Tracey 2011). HMGB1 levels in tracheal aspirates are much higher in intubated premature infants who later develop BPD, and the level is not affected by the glucocorticoid (Aghai et al. 2010).

In the presence of H_2O_2 and NO_2^- generated during the inflammatory process, and chloride anion from the surrounding environment, MPO generates hypochlorous acid (HOCl) and nitrosonium cation to chlorinate and nitrate tyrosine-containing proteins (Zheng et al. 2005), respectively. This process leaves chlorotyrosine (Cl-Tyr) and nitrotyrosine (3-NT) as the footprints. Cl-Tyr has been widely used as the evidence for the existence of MPO (Kettle 1996; Winterbourn and Kettle 2000) including the relationship between its level in tracheal aspirate and the development of BPD (Buss et al. 2003). Taurine protects cells from MPO mediasted injury by scavenging the HOCl.

Free 3-NT, the end-product of RNS injury, is not a free radical itself, but its accumulation can disturb cell functions. Eiserich et al. first described that free 3-NT disturbs cell function by incorporating into the microtubules irreversibly (Eiserich

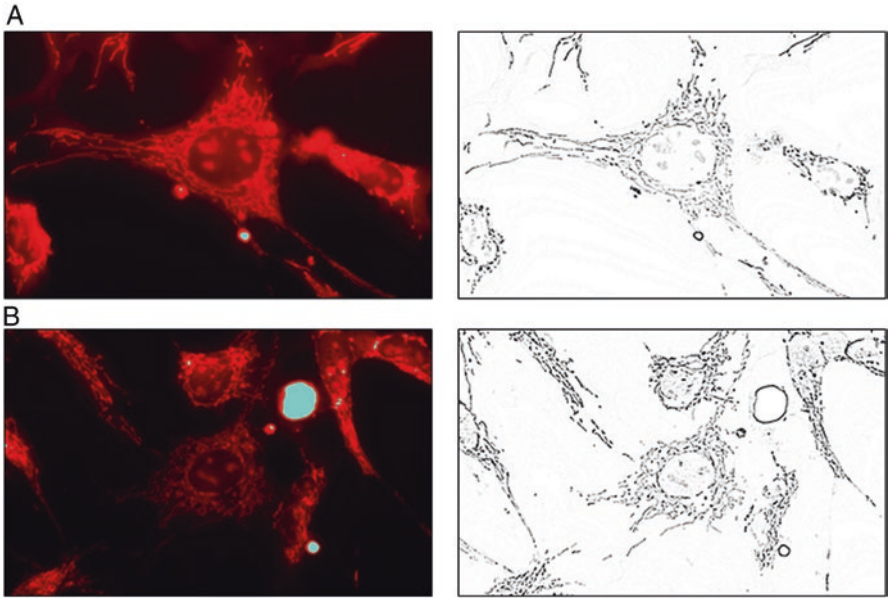

Fig. 3.4 Free nitrotyroine (3-NT) is more than an innocent tyrosine derivative. The accumulation of 3-NT causes mitochondrial fission (fragmented) in the pulmonary artery endothelial cells (PAEC). MitoTracker Orange was used to visualize the mitochondrial morphology. (A) Control PAECs; (B) PAEC cultured with 10 μM 3-NT

et al. 1999). Since microtubules intimately interact with mitochondria during mitochondria biogenesis, and protein shuttling within the cells, the incorporation of 3-NT into microtubules may affect mitochondrial and protein functions. Our study in endothelial cells showed that the 3-NT incorporation into microtubules is a reversible process, but its presence can cause mitochondrial fission (Teng et al. 2016) (Fig. 3.4) and eNOS uncoupling (Teng and Wu 2013). This adds another level of RNS-related impact to cells. Whether the accumulation of Cl-Tyr causes similar effect to cells remains to be determined.

3.6 Antioxidant System

3.6.1 General Aspects

There are two major categories of antioxidants in biologic system (Table 3.4). The enzymatic antioxidants usually have a defined location of distribution and reaction, while nonenzymatic antioxidants tend to be more widely distributed and less target specific. Most enzymatic antioxidants are not well established until 36 weeks of gestation (Berkelhamer and Farrow 2014), whereas most nonenzymatic antioxidants accumulate at the last 15% of gestation. It is apparent that premature born neonates will not be equipped with adequate amounts of antioxidants to protect themselves (Fig. 3.5). Manganese SOD (mitochondrial SOD, MnSOD, SOD2) is synthesized in

Table 3.4 Characteristics of the NADPH oxidase family

Enzyme	Interaction partner(s)	Cellular localization	Major tissue distribution
NOX1	NOXA1/P67	Cell membrane	Colon epithelium
	NOXO1/ P47phox		
	P22		
	Rac		
NOX2	P67	Cell membrane	Phagocytes
	P47		Endothelium
	P22		Smooth muscle cell
	Rac		Fibroblast
NOX3	NOXA1/P67	Cell membrane	Inner ear
	NOXO1/P47		
	P22		
	Rac		
NOX4	P22	Cell membrane, mitochondrial and nuclear membranes, ER	Kidney, ovary, brain
NOX5		Cell membrane, nuclear membrane, ER	Spleen, testis, lymph node
DUOX1	DUOXA1	Cell membrane	Thyroid, lung, prostate, testis
DUOX2	DUOXA2	Cell membrane, ER, vesicles	Thyroid, salivary gland, colon, pancreas

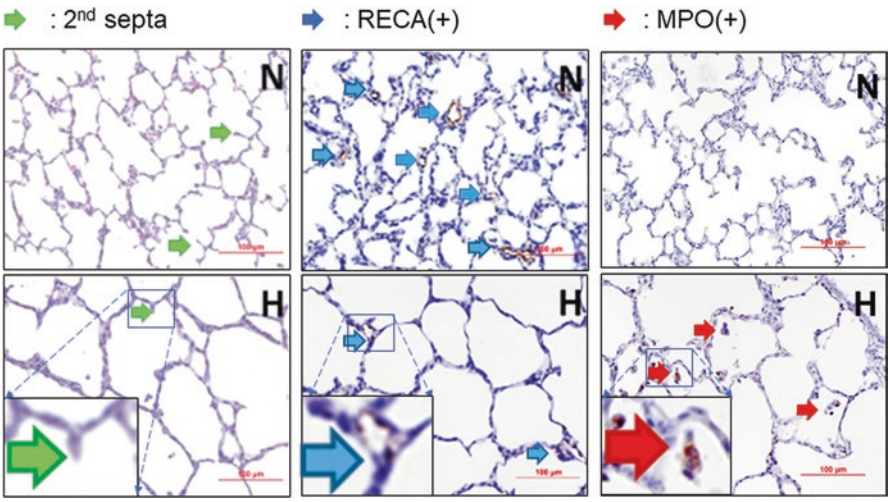

Fig. 3.5 Hyperoxia causes a characteristic BPD changes in rat pups. With the number of alveolar sac decreased and increased size of alveoli, the gas exchange surface area is decreased. The decreased number of secondary septum (green arrow) also contributes to the decreased gas exchange area. The number of capillary (blue arrow) decreased by hyperoxia indicates an impaired angiogenesis. Myeloperoxidase (MPO)-positive leukocyte (red arrow) suggests that hyperoxia can induce a sterile inflammation in the neonatal lungs
RECA, rat endothelial cell antigen

cytosol then chaperoned by heat-shock protein 70 and transported into mitochondria (Afolayan et al. 2014). Extracellular SOD (EC-SOD or SOD3) is predominantly synthesized by the vascular smooth muscle cell and most highly expressed in the lung (Oury et al. 1994; Oury et al. 1996). SOD3 comprises a large proportion of the antioxidant activity of the pulmonary vasculature and may also modulate the availability of NO to the vascular smooth muscle cells (Qin et al. 2008).

There are some non-antioxidant molecules which can affect antioxidant capacity such as selenium and zinc. Selenium functions as an antioxidant in the form of selenoproteins (Tapiero et al. 2003). There are at least more than 400 enzymes in the body that contain zinc. Zinc depletion causes a disease state known as acrodermatitis enteropathica with mucosal disruption, edema, and impaired immune system (Fan et al. 1996). Zinc induces the expression of metallothioneins that act as electrophilic scavengers, increases glutathione and catalase expressions, stabilizes protein sulfhydryls, and antagonizes transition metal-catalyzed reactions (Jarosz et al. 2017) (Tables 3.5).

There are a few abundant biologic molecules that carry antioxidant capacity but less commonly discussed by us. These molecules include albumin, bilirubin, and urate. Albumin is the most abundant sulfhydryl-containing protein (3–5 g/dl in plasma) in blood and is a multifunctional protein. Albumin acts as an antioxidant through free radical trapping and ligand binding (Roche et al. 2008). Albumin has one reduced cysteine and six methionines that provide antioxidant capacity to trap free radicals. Albumin is the major binding molecule to metal cations such as Fe^{2+} and Cu^{2+} to decrease the hydroxyl radical formation through the "Fenton reaction" (Winterbourn 1995). The albumin level in neonates is usually lower than adults, especially in premature infants. The albumin level sometimes can be as low as 2 g/dl in extremely premature infants which can significantly decrease the antioxidant capacity from this molecule.

Bilirubin is a metabolite from degraded heme molecules which is notoriously known for its insult to the central nervous system in infants. Most infants have a steady increase in their bilirubin levels after birth when they are experiencing the biggest challenge of oxidative stress in their life. The importance of its antioxidant property has been demonstrated for more than three decades (Stocker et al. 1987) but, unfortunately, this property has been overshadowed by its potential central nervous system toxicity. Bilirubin level at physiologic range for neonate provides potent antioxidant activity, especially at 2% O_2 environment which is the normal physiological O_2 tension at cellular level (Ziberna et al. 2016). As the end-product of xanthine oxidase, urate is a powerful antioxidant that scavenges singlet oxygen and radicals (Ames et al. 1981). Urate effectively scavenges the peroxynitrite to protect cell function (Teng et al. 2002).

3.6.2 Caffeine

For the past four decades, caffeine and related compounds have been used as the treatment for the apnea of prematurity (Dobson and Hunt 2013). Although it is not

Table 3.5 Antioxidants, their cellular distributions, and the reactions they carry out

Enzymatic antioxidant	Cellular location	Substrate	Reaction
Mn/cu/ Zn-SOD	Cytosolic (cu/ Zn-SOD), SOD1 Manganese SOD (MnSOD) or mitochondrial matrix, SOD2 Extracellular SOD (EC-SOD), SOD3	$O_2^{\cdot -}$	$O_2^{\cdot -} \rightarrow H_2O_2$
Catalase	Peroxisomes, cytosol	H_2O_2	$2H_2O_2 \rightarrow O_2 + H_2O$
Glutathione peroxidase (GSHPx)	Cytosol	H_2O_2	$H_2O_2 + GSH \rightarrow GSSG + H_2O$
Peroxiredoxin 1–6 (Prdx)	Nucleus, cytosol, endosomes, lysosomes, peroxisomes, mitochondrial, extracellular	H_2O_2 Organic hydroperoxides	$H_2O_2 + TrxS_2 \rightarrow Trx(SH)_2 + H_2O$

Nonenzymatic antioxidant	Function
Vitamin E	Chain breaking
β-Carotene	Scavenge ROS, singlet O_2 quencher
Coenzyme Q	Regenerates vitamin E
Transferrin	Sequesters iron and copper ions
Lactoferrin	Sequesters iron at lower pH
Albumin	Sequesters heme and copper, most abundant thiol in circulation
Ceruloplasmin	Scavenges superoxide radical, binds copper ions
Bilirubin	Scavenges peroxyl radical
Uric acid	Scavenges hydroxyl radical and reactive nitrogen species
Vitamin C	Scavenges hydroxyl radical, recycles vitamin E
Reduced glutathione	Binds free radicals, thiol group oxidized to disulfide group (GSSG)
α-Lipoic acid	Recycles vitamin C, glutathione substitute
Melatonin	Interacts with hydroxyl radical, hydrogen peroxide, singlet oxygen, peroxynitrite anion, and hypochlorous acid
	Upregulates activity of superoxide dismutase, glutathione peroxidase, and glutathione reductase
Taurine	Scavenge hypochlorous acid

an endogenous compound, caffeine is a commonly used medication in neonates especially in premature infants. It is hypothesized that by decreasing the apneic episodes in premature infant, caffeine can decrease the free radical formation by reducing the ischemia-reperfusion injury. Caffeine scavenges hydroxyl radical, •OCH3, and probably of alkoxyl radicals as an antioxidant (León-Carmona and Galano 2011). Other mechanisms for caffeine to reduce free radical formation are probably through the modulation of inflammation (Weichelt et al. 2013) or

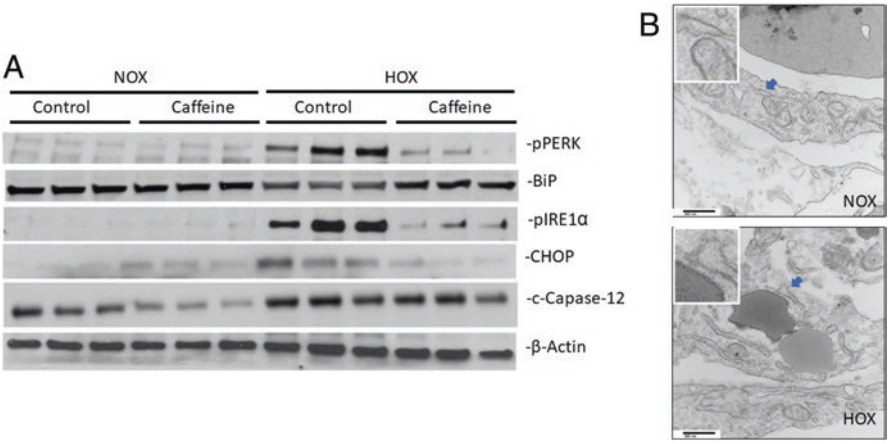

Fig. 3.6 The global endoplasmic reticulum (ER) stress is increased in the hyperoxic lungs. (a) All major ER stress markers (pPERK, pIRE1α, CHOP) are increased, and the cleaved pro-apoptotic ER stress related marker (caspase-12) is also increased in the hyperoxic lungs. Clinically used caffeine effectively attenuates the ER stress which may support the clinical report that caffeine decreases the incidence of BPD in premature infants. (b) Electron microscope of the endothelial cell shows dilation of ER in the hyperoxic lung indicating an increased ER stress by hyperoxia

attenuating the ER stress (Teng et al. 2017). Early caffeine treatment has been shown to decrease the incidence of BPD in premature infants (Schmidt et al. 2006), but the mechanism behind the protection remains to be determined (Dayanim et al. 2014) (Fig. 3.6). Pentoxifylline, as a derivative of caffeine with similar pharmacologic properties (Almario et al. 2012), on the other hand, has not been shown to provide significant clinical efficacy in preventing BPD (Schulzke et al. 2014).

3.6.3 Melatonin

The antioxidant activity of melatonin was detected not long ago. Melatonin, as an antioxidant, interacts with hydroxyl radical, hydrogen peroxide, singlet oxygen, peroxynitrite anion, and hypochlorous acid. It also upregulates activity of SOD, glutathione peroxidase, and glutathione reductase (Gitto et al. 2005; Reiter et al. 2003). Since melatonin is an endogenous compound produced in the pineal body, it might have a potential to be implemented clinically, but no report is available now.

3.6.4 Taurine

Taurine, a 2-aminoethanesulfonic acid, is a derivative of cysteine. Premature infants are taurine deficient, due to the lack of enzymes to convert cystathionine to cysteine,

so taurine is a conditional amino acid to them. In fact, taurine is usually fortified into infant formula and routinely added to the parenteral nutrition solution (Chesney et al. 1998) for premature infants to improve fat utilization (Ghisolfi 1987) even though there is no solid evidence to prove the efficacy (Verner et al. 2007). Taurine can function like an antioxidant by scavenging heavy metal-induced free radical formation in the body, which regulates mitochondrial electron transport chain complexes formation to assist the electron flow and indirectly decreases superoxide generation (Jong et al. 2012). Most importantly, taurine is the most potent scavenger for hypochlorous acid (Cunningham et al. 1998), protects neutrophils from the hypochlorous acid generated by themselves (Weiss et al. 1982), and reduces inflammatory reaction (Kearns and Dawson 2002).

3.7 Neonatal Lung Diseases

3.7.1 General Aspects

About one in every 200 term neonates require O_2 treatment for TTNB, PPHN, or neonatal pneumonia. Occasionally, neonates with congenital heart disease, congenital airway or lung anomaly, neurological problem, or surgical abdomen will need O_2 or ventilator support after birth. One in every 100 infants is born prematurely, and about 20% of them need some forms of respiratory support for their RDS. The respiratory support can add more oxidative stress to the already challenged lungs. RDS and PPHN are the two most extensively studied neonatal lung diseases.

3.7.2 Respiratory Distress Syndrome (RDS) and Bronchopulmonary Dysplasia (BPD)

There are five stages for fetal lung development – embryonic (0–8 weeks), pseudoglandular (8–16 weeks), canalicular (16–24 weeks), saccular (24–36 weeks), and alveolar (>36 weeks). Surfactant production usually matures at about postconceptional age of 36 weeks, while alveolar formation mainly happens after birth and persists at least until 2–6 years of age. Two processes, blood vessel formation (angiogenesis) (Abman 2001; Thébaud 2007) and alveolar formation (alveologenesis) (Frank et al. 2016), are the major players in lung development. A coordinated interaction between these two processes is necessary for adequate lung growth (Bourbon et al. 2005), and failure to do so will lead to BPD.

Classical BPD occurs in less premature infants (>30 weeks) before the era of exogenous surfactant (Northway et al. 1967) who have been treated by mechanical ventilator for their RDS and required O_2 for more than 28 days of life. Inflammation,

barotrauma, and fibrosis are typical histologic changes in the classic BPD. Most of the survivors suffered from reactive airway disease and lung hyperinflation when reached adolescence (Northway et al. 1990). With the introduction of exogenous surfactant, antenatal steroid use, and gentle ventilation strategy, more immature infants (<28 weeks) nowadays can survive the RDS. However, these immature infants born at late canalicular or early saccular stage will develop a different type of BPD. Some of these immature infants developed BPD even without O_2 use or mechanical ventilator in the first 4 weeks (D'Angio et al. 2016), suggesting a different mechanism from the "classical BPD." This "new BPD" is characterized by diffusely reduced alveolar development, with very mild airway injury, inflammation, and fibrosis (Jobe). Such a "new" form of BPD is interpreted as a disorder of developmental arrest (Mosca et al. 2011). Most of the BPD infants need prolonged supplemental O_2 for months, or even years, and about 25–40% will develop pulmonary hypertension (Berkelhamer et al. 2013). The presence of pulmonary hypertension doubles the mortality rate in BPD infants.

BPD is considered a multifactorial problem. Oxidative stress (Saugstad 2010) and inflammation (An et al. 2004; Groneck and Speer 1995; Hillman et al. 2011) are the central players for the BPD development. Efficacy of using lower O_2 concentration (30%) in resuscitating premature infants has been reported in decreasing oxidative stress, inflammatory response, and BPD (Vento et al. 2009). The initial RDS treatment elicits inflammation with increased HMGB1 (Aghai et al. 2010) and leukocyte infiltration (Auten et al. 2001; Deng et al. 2000). Superoxide released from neutrophils' respiratory burst can be reduced to H_2O_2 by SOD or interacts with NO to form peroxynitrite. MPO from the infiltrated leukocytes (Teng et al. 2017) mediates the formation of 3-NT and Cl-Tyr (Buss et al. 2003), indicating the existence of the potent oxidant – hypochlorous acid. Oxygen use also increases superoxide formation by disturbing respiratory chain electron transport. The impaired mitochondrial function is a known contributor to the BPD (Ratner et al. 2009). Exposure to hyperoxia in early neonatal life causes mitochondrial ROS generation which further induces NOX1 expression and aggravates the oxidative stress (Datta et al. 2015). Evidence of the ROS formation during the O_2 treatment is supported by the increased 8-OH-deoxyguanosine in BPD lung (Teng et al. 2017) as the result of DNA damage under oxidative stress.

To make thing even more complicated is the fact that clinical attempts using antioxidant to prevent BPD have never really worked (Welty 2001), but upregulating antioxidants by melatonin provides some benefit in rat BPD model (Pan et al. 2009). It is possible that some downstream reactions elicited by oxidative stress are the true culprit to the impaired lung growth. One of the possible mechanisms is the ER stress caused by oxidative stress (Malhotra and Kaufman 2007; Santos et al. 2009) (Fig. 3.6). ER is the subcellular organelle where most proteins are folded and modified before sending out to designated places for proper biologic function. ER also intimately interacts with the neighboring mitochondria to modulate their function. Increased ER stress has been demonstrated in neonatal rodent lung exposed to hyperoxia (Choo-Wing et al. 2013; Lu et al. 2015). Normal protein synthesis will be shut down during early stage of ER stress to survive the harsh condition but enhance

the synthesis of some proteins, such as VEGF, that are vital for survival (Ghosh et al. 2010; Pereira et al. 2010). But, prolonged ER stress can impair function and activity of several growth factor signaling pathways by de−/under-glycosylation of the growth factor or the corresponding receptor. VEGF and VEGF receptor-II (VEGFR2) are glycosylated in the ER, and de−/under-glycosylation of VEGFR2 can either destabilize VEGRF2 (Hosford and Olson 2003) or attenuate the VEGF signaling (Park et al. 2016). What causes this switch and when it happens in vivo remains to be determined.

Mitochondrial function can be disturbed under ER stress since the lipid moiety and calcium for mitochondrial metabolism come from the neighboring ER (Marchi et al. 2014). We have seen evidence of disturbed mitochondrial biogenesis and increased ER stress in hyperoxic rat lung (Teng et al. 2017) which might be a contributing factor for the poor lung development (Ratner et al. 2009). ER stress itself can also elicit inflammatory reaction in the lungs to magnify the oxidative stress injury (Choo-Wing et al. 2013; Zhang et al. 2006).

Oxidative stress inhibits cell cycle progression by causing DNA damage. Tumor suppressor protein, P53, is phosphorylated in the presence of DNA damage to prevent aberrant DNA replication or cancer transformation (Williams and Schumacher 2016). Cyclin-dependent kinase (CDK) inhibitor p21 (CDKN1A or $p21^{WAF1/Cip1}$) level increases after P53 phosphorylation and leads to either cell quiescence or replicative senescence (Abbas and Dutta 2009). P53 phosphorylation can also elicit apoptosis when phosphorylated P53 is refrained from entering the nucleus. Either cell quiescence, replicative senescence, or apoptosis prohibits cell replication. Increased P21 levels have been shown in the lungs of hyperoxic mouse pups (Dayanim et al. 2014). The increased P21 level and impaired mitochondrial biogenesis can explain the increased apoptosis in the hyperoxic lungs (Teng et al. 2017). Whether quiescence or replicative senescence involves in the BPD or not is, however, at this moment unknown.

The VEGF-eNOS-NO pathway is the major player for angiogenesis, and impaired angiogenesis is considered the root cause of BPD (Abman 2001; Thébaud 2007). The VEGF and eNOS phosphorylation decreased in the lungs after prolonged hyperoxia exposure. The level of VEGF recovers after recovery in room air, but eNOS remains uncoupled for a while (Jing et al. 2017). As eNOS coupling requires the existence of BH4, the decreased phospho- and total GCH1 explain the decreased BH4 levels and explain the eNOS uncoupling under hyperoxia. The changes to GCH1 and eNOS persist after recovery in room air indicating a long-lasting effect of hyperoxia to the VEGF-eNOS-NO pathway. Since NO mediates the VEGF effect on angiogenesis, it is possible that the impact of hyperoxia to the neonatal lungs will last longer than we expected.

Pulmonary hypertension is one of the complications recently identified to occur in 25–45% of premature infants with BPD (Check et al. 2013; Khemani et al. 2007). The presence of pulmonary hypertension complicates the BPD management with higher medical cost (Johnson et al. 2013) and longer hospital stay (Klinger et al. 2006). More importantly, the mortality rate can be as high as 50% in BPD infant with pulmonary hypertension (Khemani et al. 2007). Both rodent and

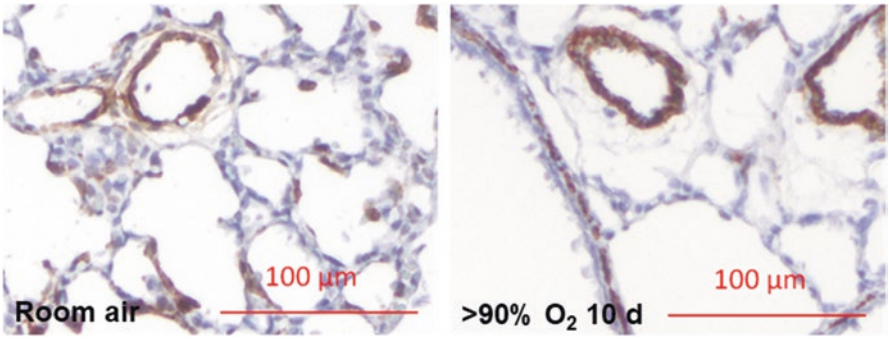

Fig. 3.7 Prolonged hyperoxia causes thickening of the pulmonary artery smooth muscle layer. The smooth muscle layer is more complete in hyperoxic pulmonary arteries which may contribute to the pulmonary hypertension in BPD

murine BPD models show thickening of the muscle layer of the pulmonary arteries (Fig. 3.7) which is believed to be the cause of the pulmonary hypertension. Hyperoxia disrupts cGMP signaling in both pulmonary artery (Lee et al. 2014) and right ventricle (Heilman et al. 2015) mainly with the upregulation of PDE5. This aberrant cGMP signaling persists even after the animals with pulmonary hypertension have recovered in room air (Heilman et al. 2015). PDE5 inhibitors have been used clinically to manage BPD infants with pulmonary hypertension. However, long-term efficacy of PDE5 inhibitor treatment in BPD pulmonary hypertension remains unclear.

3.7.3 Persistent Pulmonary Hypertension of the Newborn (PPHN)

PPHN is a common neonatal lung problem which occurs in one per 500 live births (Walsh-Sukys et al. 2000; Travadi and Patole 2003). This problem can be classified into three categories: (1) pulmonary artery constriction due to parenchymal lung disease; (2) poor formation of pulmonary vasculature; and (3) abnormal remodeling of pulmonary vasculature (Table 3.6). The mortality rate in severe PPHN used to be 30–40% decades ago until the invention of extracorporeal membrane oxygenator (ECMO) and inhalational NO. Neonates with moderate to severe PPHN routinely need mechanical ventilation with high concentration of O_2 support. Decades ago ECMO treatment was the last resort for neonates with severe PPHN. Unfortunately, 10–15% neonates will suffer intracranial hemorrhage under ECMO treatment (Teng and Wu 2013). With the introduction of inhalational NO treatment, the mortality rate has decreased to less than 10% for moderate to severe PPHN (Konduri et al. 2004), while less and less of them require ECMO nowadays. Inhalational NO ($\leqq$20 ppm) is now the standard treatment for PPHN in developed countries.

Intrauterine ductus arteriosus constriction of fetal lamb is the most commonly used animal model to study PPHN (Morin 1989). This model is very similar to the

Table 3.6 Classification of PPHN

Abnormally constricted pulmonary vasculature due to parenchymal disease
Meconium aspiration syndrome
Respiratory distress syndrome
Pneumonia
Hypoplastic pulmonary vasculature
Congenital diaphragmatic hernia
Lung hypoplasia
Normal parenchyma with remodeled pulmonary vasculature
Idiopathic PPHN
Congenital heart disease
Hypoxic-ischemic encephalopathy Chronic intrauterine distress
Others

idiopathic PPHN. Global increase in endogenous ROS formation has been shown in this sheep PPHN model (Wedgwood and Steinhorn 2014). The source of the ROS includes NOX2 (Brennan et al. 2003; Teng et al. 2009), eNOS uncoupling (Konduri et al. 2007; Konduri et al. 2015), mitochondrial electron transport chain (Farrow et al. 2010), and increased ER stress (Tadokoro et al. 2016). There is a cross talk between mitochondrial ROS and NOX (Dikalov 2011) which can amplify the oxidative stress. The O_2 treatment for PPHN can further increase the mitochondrial ROS generation (Farrow et al. 2010). The decreased antioxidant capacity also contributes to the oxidative stress in PPHN. MnSOD activity decreases in PPHN pulmonary arteries, not the whole lung, due to impaired mitochondrial transport and nitration (Afolayan et al. 2012). This impaired MnSOD activity contributes to the decreased eNOS expression and activity in PPHN endothelial cells. The MnSOD nitration is probably secondary to the eNOS uncoupling (Afolayan et al. 2016). eNOS activity is decreased in PPHN lungs and pulmonary artery smooth muscle cells (Wedgwood et al. 2011), potentially through the Nox4-derived H_2O_2 that oxidizes the copper at the active site of MnSOD (Wedgwood et al. 2013).

Recently a new mechanism that increases endogenous ROS formation has been identified – disturbed ER function (Cao and Kaufman 2014). Nogo-B and its receptor (NgBR) are resident membrane proteins of ER that help in maintaining the ER integrity and function (Miao et al. 2006). Both endothelial cell (Teng et al. 2014) and smooth muscle cells (Tadokoro et al. 2016) from PPHN pulmonary arteries show decreased expression of NgBR. NgBR depletion leads to ER stress with increased ROS formation in the pulmonary artery smooth muscle cells which explains the proliferative phenotype of the cells (Tadokoro et al. 2016). NgBR knockdown in pulmonary artery endothelial cell uncouples eNOS to generate superoxide instead of NO that impairs the angiogenesis (Teng et al. 2014). Overexpression of NgBR in PPHN pulmonary artery endothelial or smooth muscle cells is able to reverse those changes.

Uncoupled eNOS may be the most extensively studied source of ROS in PPHN. Several mechanisms are involved in the eNOS uncoupling in the pulmonary

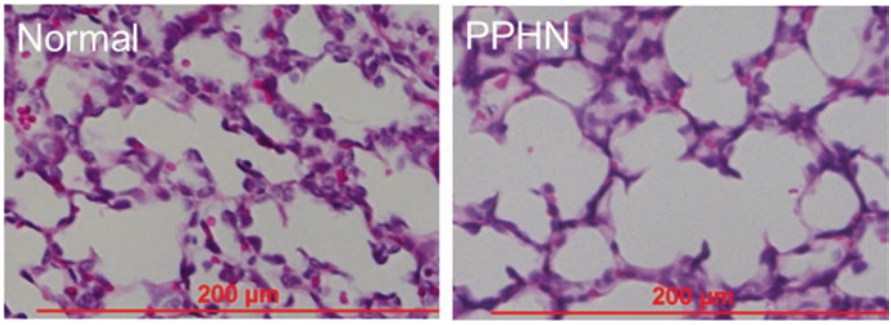

Fig. 3.8 The alveoli are less complex in PPHN fetal lung as compare to the sham-operated co-twin

artery endothelial cells from PPHN such as impaired association with HSP90 (Konduri et al. 2007) and decreased availability of BH4 (Teng et al. 2011a, b). Aberrant methylation of eNOS promoter area has recently been identified as a mechanism to decrease eNOS expression in PPHN (Ke et al. 2018). The decreased NO formation can impair mitochondrial function (Afolayan et al. 2016; Konduri et al. 2015), cause autophagy and apoptosis of the endothelial cells (Teng et al. 2012), decrease PGI2 formation (Mahajan et al. 2015), and impair angiogenesis (Teng et al. 2009). Since angiogenesis is vital for the postnatal lung growth, it is not surprising to have alveolar simplification in the PPHN lungs (Fig. 3.8). The impaired angiogenesis also contributes to the higher pulmonary vascular resistance in PPHN. The probability of enhanced PDE5 activity has (Farrow et al. 2010) led to the introduction of PDE5 inhibitors in PPHN treatment to embolden the efficiency of NO-cGMP pathway.

ET-1 is synthesized in vascular endothelial cells which is needed to maintain the proliferation and growth of the vascular smooth muscle cells (Kim et al. 2015). ET-1 promotes the proliferation of vascular smooth muscle cell through the generation of ROS (Wedgwood et al. 2001). The level of circulating ET-1 level is increased in the cord blood of PPHN neonates (Christou et al. 1997; Rosenberg et al. 1993), but the level comes down to normal by 5 days of life (Endo et al. 2001) regardless of the treatment (Christou et al. 1997). This leads to question of whether ET-1 plays a vital role in the high PVR or just a biomarker for PPHN. Although the role of ET-1 in PPHN remains to be determined, the clinical use of ET-1 receptor antagonist in PPHN neonates has been reported with reasonable response (Maneenil et al. 2018).

3.8 Conclusion

Free radicals are generated continuously by the body, and the antioxidant system keeps them in check. Basal ROS and RNS formations are normal and are used for either signaling or promoting organ growth. Dramatic change in O_2 tension during perinatal transition can cause oxidative stress to the lungs. When neonates are born

prematurely with low antioxidant capacity, or with lung problem that requires supplemental O_2, then the oxidative stress will be exaggerated. Some of the neonatal lung problems have increased endogenous oxidative stress that can further augment the exogenous oxidative stress from the treatment. The oxidative stress can elicit inflammatory process, inhibit cell growth, and impair angiogenesis then prevent the lung from adequate growth. Unfortunately, antioxidant treatment has so far not shown clinical efficacy. It is possible that the downstream effects of oxidative stress are playing more important role in causing the lung injury. Understanding how oxidative stress leads to lung growth inhibition will be important to provide the neonate a better lung growth trajectory.

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DNA Repair Protein OGG1 in Pulmonary Infection and Other Inflammatory Lung Diseases

4

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Abstract

In the last decades, extensive research has uncovered functional roles and underlying mechanisms of DNA repair enzyme 8-oxoguanine DNA glycosylase (OGG1) in the pathogenesis of inflammatory response in infection and other diseases in the lung. OGG1 excises 8-oxo-7,8-dihydroguanine (8-oxo-dG) lesion on DNA that is often induced by generation of reactive oxygen species (ROS) and has been linked to mutations, cancer development, and tissue damage. Most, if not all, environmental toxic agents and mammalian cellular metabolites elicit the generation of ROS, either directly, indirectly, or both, which is among the first cellular responses. ROS in combination with other oxidative molecules/moieties are recognized as a major factor for killing invading pathogens but meanwhile can cause tissue damage. ROS potentially modify proteins, lipids, and

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DNA due to the strong molecular reactivity. While oxidative stress causes increased levels of all types of oxidatively modified DNA bases, accumulation of 8-oxo-dG in the DNA has been singled out to be a main culprit linking to various inflammatory disease processes. Oxidatively damaged DNA bases such as 8-oxo-dG are primarily repaired by the base excision repair (BER) mechanism, in which OGG1, as the lesion recognition enzyme, plays a fundamental role in fixing this DNA damage. In this chapter, we summarize the roles and potential mechanistic analyses of OGG1 in lung infection and other inflammatory diseases.

4.1 Introduction

The lung is the vital organ in the body to supply oxygen and remove carbon dioxide and is vulnerable to exogenous sources of oxidative stress because of the continuous, direct exposure to the external environment (Vlahopoulos et al. 2018). Pulmonary infection often has a consequence of inflammatory response in the lung parenchyma including the terminal airway, alveolar cavity, and interstitial lung. A variety of pathogens including bacteria, viruses, and fungi are the most common causal agents in lung diseases (Stehle et al. 2018). Bacterial infection can lead to serious lung injury by initiating oxidative damage and inflammatory responses to activate a series of signaling pathways (Liu et al. 2018). Reactive oxygen species (ROS) contain a spectrum of oxidative products that aerobic cells produce during metabolism, including $O2^{-}$, H_2O_2, HO_2^{-}, -OH, and so on (Ewald 2018). A great deal of research has uncovered that low concentrations of ROS can beneficially regulate a number of signal transduction pathways in cells, while medium and high concentrations of ROS induce apoptosis and necrosis through cellular oxidative stress. The two-way regulation of ROS is essential for maintaining the metabolic balance of cells (Testa et al. 2018). Imbalance of ROS production will lead to tumorigenesis and disease progression (Yuan et al. 2017). The occurrence of lung disease is closely related to levels of ROS in vivo when exposed to environmental stimuli, such as bacteria, chemicals, oxygens, etc. ROS accumulation in cells induces DNA oxidative damage associated with lung diseases including pneumonia (Zhang et al. 2018). Among the several ROS-induced DNA base lesions in the genome, 8-oxo-7,8-dihydroguanine (8-oxo-dG) is one of the most abundant because guanine is extremely susceptible to oxidative attack. DNA repair enzyme 8-oxoguanine DNA glycosylase (OGG1) specifically repairs damaged DNA which arises when exposed to ROS to prevent 8-oxo-dG accumulation and maintain cell genetic stability. 8-Oxo-dG has been linked to the pathogenesis of bacterial infection and other inflammatory lung diseases (Ba et al. 2014). OGG1 repairs damaged DNA mainly by initiating DNA base excision repair (BER) pathway. First, OGG1, a DNA glycosylase/AP lyase, hydrolyzes N-glycosyl sites to release 8-oxo as a free base, which then cleaves the sugar phosphate backbone to form a depurination/depyrimidine (AP) site. OGG1 would then recruit depurination/apyrimidine endonuclease 1

(APE1) to remove the 3′-phosphate-α,β-unsaturated group and form a 3'-OH end. DNA polymerase β carries the correct guanine insertion gap and recruits ligase II/XRCC1 linkage. The cleaved 8-oxo-dG base is released into the cytoplasm and is bound by OGG1 to form OGG1·8-oxoG complex to activate GDP → GTP. These actions may require a series of regulators involving multiple DNA repair enzymes and signaling proteins within the cell (Wang et al. 2018). The expression of OGG1 is an important marker of lung infection and inflammatory processes resulted from accumulation of ROS levels or other potential stress factors (Zhang et al. 2017).

The dual role of DNA repair enzyme OGG1 in innate immunity and lung diseases allows rapid fixation of base damage caused by different types of bacteria and external stimuli. Our recent research showed that *Ogg1*$^{-/-}$ mice exhibit significantly challenged inflammatory response including cell infiltration after challenge of ovalbumin (OVA), a reagent that causes asthmatic pathology (Li et al. 2012). However, *Ogg1*$^{-/-}$ mice exhibited increased lung injury after infection with *P. aeruginosa* [12]. OGG1 is of importance in repairing oxidative DNA damage and activating a range of signaling pathways. In this section, we describe the roles and related mechanisms of what we call the DNA Repair Protein OGG1 Response (DRPOR) to lung infection and inflammatory diseases.

4.2 DNA Repair Protein OGG1 Response to Lung Infection

4.2.1 OGG1 Plays a Role in Bacterial Infection

4.2.1.1 *Pseudomonas aeruginosa*

A certain concentration of ROS induces apoptosis or necrosis through different signal transduction pathways, such as oxidative stress response. Oxidative stress responses lead to DNA fragmentation, DNA site mutation, and double-strand distortion, while DNA repair mechanism plays critical roles in host defense against oxidative stress resulting from various ruthless attacks. The opportunistic pathogen *P. aeruginosa* causes host ROS release during infection by releasing quorum sensing (QS) molecules and inducing pronounced DNA damage. As mitochondrial DNA (mtDNA) is highly sensitive to ROS/RNS (reactive nitrogen species), oxidative mtDNA was found to damage the lung and cause *P. aeruginosa*-induced acute lung injury(Lee et al. 2017). The *P. aeruginosa* caused oxidative mtDNA damage and then resulted in a feedback cycle of mtDNA damage and DNA damage-associated molecular pattern (DAMP) formation that leads to consequences of acute lung injury(Kuck et al. 2015). As OGG1 is the indispensable DNA repair enzyme and its expression and enzymatic activity was found to be strikingly changed in lungs, Wu et al. demonstrated that the OGG1 acetylation site mutation exacerbated Cockayne syndrome group B (CSB) expression and found that deletion of OGG1 results in lung injury during *P. aeruginosa* infection. Coincidentally, Cheng and colleagues confirmed that deacetylase sirtuin 3 (Sirt3) could slow down the degradation of OGG1 to protect mtDNA damage under oxidative stress(Wu et al. 2011;

Cheng et al. 2013). Thus, OGG1 likely plays a key role in response to *P. aeruginosa* infection via acetylation-associated signaling.

4.2.1.2 *Klebsiella pneumoniae*

K. pneumoniae is one of the human iatrogenic bacteria and one of the fundamental causes of pulmonary infections and sepsis. Huang et al. found that OGG1 overexerted lung cells to alleviate cytotoxicity during *K. pneumoniae* infection, suggesting that OGG1 affects DNA damage and repair through membrane lipid-mediated signal axis against *K. pneumoniae*. Mechanistically, infection-induced oxidation causes DNA damage following ROS induction in lung cells through activation of lipid rafts, which is from a totally new angle to explain how OGG1 fixes DNA damage through lipid raft-mediated pathways(Huang et al. 2013). Although more studies are required to further understand the role of OGG1 in coordinating a concerted regulation of immune response in *K. pneumoniae* lung infection, understanding of lipid raft-associated pathways may provide better views of OGG1's involvement in host response to *K. pneumoniae* infection.

4.2.1.3 Other Bacteria

Staphylococcus aureus pneumonia is an acute pulmonary purulent inflammation caused by *S. aureus. S. aureus* is highly toxic by producing hemolytic toxins, plasma coagulase, and deoxyribonucleic acid catabolism enzyme and has a serious threat to children's lives. Bartz et al. (Bartz et al. 2011) are a major team in studying OGG1 and mtDNA repair during *S. aureus* infection. Although their studies are directed to sepsis and tested tissue injury in the kidney and liver rather than the lung, the mechanism they found may reflect other organs including the lungs. In their studies, mtDNA repair and mitochondrial biogenesis were evidenced by increased levels of OGG1, nuclear respiratory factor (NRF), and mitochondrial transcription factor A expression (Bartz et al. 2011, 2014). NRF is well known to play key roles in regulating mitochondrial biogenesis, which is vital for ROS production during lung infection. Hence, the function of OGG1 may be important for host defense to control *S. aureus*-induced oxidative damage.

Helicobacter pylori is also a gram-negative bacterium like *P. aeruginosa*, mainly distributed in gastric mucosa tissues, and 67–80% of gastric ulcers and 95% duodenal ulcers are caused by *H. pylori* (Kate et al. 2013*)*. Earlier, *H. pylori* was thought to just exist in the stomach, but now studies have suggested that *H. pylori* also exists in the mucosa of the respiratory tract(Deng et al. 2013), which may be involved in some lung diseases including pulmonary tuberculosis and chronic bronchitis(Adriani et al. 2014). Although there is no study reported in OGG1 to *H. pylori* in the lung, OGG1 is constantly linked to repairing DNA under *H. pylori* infection in other organs (Touati et al. 2006; Mathieu et al. 2006) (Izzotti et al. 2007). It is possible that OGG1 plays a critical role in lung disease associated with *H. pylori* too. Finally, it will be interesting to understand the involvement and mechanisms of OGG1's function during infection by *Escherichia coli*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Legionnaires' disease*, which are associated with lung infections (Fig. 4.1).

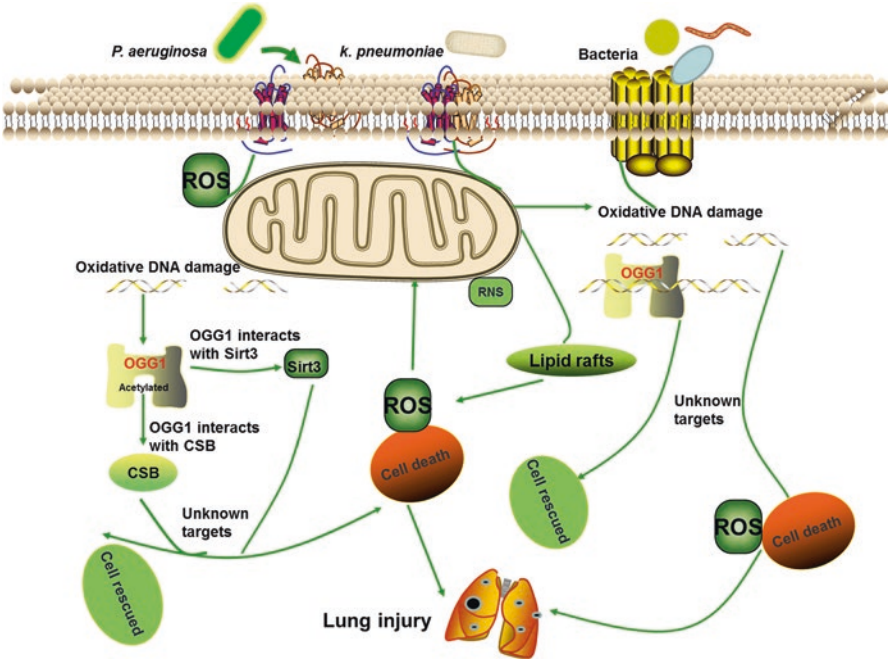

Fig. 4.1 OGG1 response *P. aeruginosa* and *K. pneumoniae* infection: *P. aeruginosa* infection induces ROS and subsequent oxidative DNA damage, which activates OGG1 acetylation. The acetylated OGG1 reacts with uncanonical CSB or Sirt3 pathways and repairs DNA damage to rescue host cells. In contrast, inhibition of OGG1 acetylation will result in cell death and lung injury. *K. pneumoniae*-induced oxidation elicits DNA damage following ROS production by activation of lipid rafts. This process can cause serious oxidative DNA damage that can be repaired by OGG1. The same mechanism is also implicated in other bacteria

4.2.2 OGG1 Affects Viral Infection

DNA damage-induced signaling has been recognized as a key factor in viral infection. Bovine herpesvirus-1 (BoHV-1), a bovine-derived virus that causes severe respiratory diseases and inflammation, can induce oxidative DNA damage and exhaust the expression level of OGG1 by activating the production of ROS. OGG1 can ameliorate DNA damage and inhibit viral replication to absorb the excessive ROS (Zhu et al. 2018). The titer of the hepatitis C virus (HCV) is inversely related to the expression of OGG1, as high viral burdens aggravate cellular DNA damage levels but suppressed damage-related DNA repair gene expression. qPCR and Western blotting showed that OGG1 levels were significantly upregulated in low viral level (LVL) cells but downregulated in high viral level (HVL) cells (Piciocchi et al. 2016). In patients with viral hepatitis, OGG1 polymorphism is associated with oxidative DNA damage and disease progression and patient survival rate, suggesting that OGG1 plays an important role in the pathophysiology of HCV and hepatitis B virus (HBV) infection (Jung et al. 2012). The proportion of HIV genes integrated

into host cells is significantly reduced in murine cells with deletions of the gene *Ogg1*, indicating that the activity of the repair gene enzyme is mediated by BER pathway of oxidative DNA damage, which is essential for HIV viral infection (Yoder et al. 2011). Ground-glass hepatocytes (GGHs) are a sign of the late stage of HBV infection, expressing the pre-S 1 and pre-S 2, two mutant types of large HBV surface antigens (HBsAg). The HBV carrying pre-S mutation aggravates the infectious process by induction of cellular oxidative stress and DNA damage and the expression of OGG1 in hepatocellular carcinoma. These results indicate that ROS-mediated DNA damage and OGG1-mediated repair are involved in the infection of HBV. Using SF91 lentiviral delivery, the overexpression of OGG1 ameliorates hematopoietic cell damage situation by repairing TEPA-induced DNA damage (Hsieh et al. 2004).

4.2.3 OGG1 Affects Fungus Infection

Fungal infections can also cause DNA damage similar to bacterial and viral infections. Proanthocyanidins can extenuate aflatoxin B1-induced oxidative DNA damage and carcinogenesis by regulation of the expression of *Ogg1* (Bakheet et al. 2016). OGG1 has a different role in maintaining the genetic stability of endogenous genes and coping with exogenous stress. OGG1 can protect genetic stability by reducing the accumulation of endogenous mutagenic lesions. However, OGG1 can incite toxicity and mutation when 4-nitroquinoline-1-oxide (4-NQO) treatment induces a potent intracellular oxidative stress in a ccc2-deficient strain of *S. cerevisiae* (da Silva et al. 2015). Aflatoxin B1, a carcinogenic molecule in the process of *Aspergillus* fungal infection, can accelerate cancer development by increasing base excision repair activity and OGG1 levels in mouse lungs (Guindon-Kezis et al. 2014).

4.3 OGG1 Is Linked to Inflammatory Lung Diseases

4.3.1 OGG1 Responses in Pulmonary Hyperoxia

Standard oxygen therapy functions as a supportive strategy for curing acute respiratory distress syndrome (ARDS) patients, which provides oxygen into the lungs to maintain the normal function while exchanging carbon dioxide in the blood at pulmonary alveoli (Ragaller and Richter 2010). Although there is no direct cure for ARDS, supportive therapies by providing sufficient oxygen for the patient are critical measure to heal the lungs from the injury (Bulger et al. 2000). However, it should be cautioned that hyperoxia is also toxic to the lung that receives oxygen therapy (Kallet and Matthay 2013), which is a proverbial cause antecedent of injuring alveolar epithelial cells (AECs) to developing lung disease (Li et al. 2018). The activation of p21- and p53-mediated signaling pathway produces similar results to those brought out by hyperoxia, resulting in extensive injury to AECs, especially

type II AECs, and an elevated risk of jeopardizing the lungs' functional integrity (O'Reilly et al. 1998; McGrath-Morrow et al. 2011; Yee et al. 2011). The injury or death of type II AECs is a devastating event for the alveoli because the type II cell is considered as the progenitor of alveolar epithelial cells, which may disrupt surface tension at the air/liquid interface and prevent the collapse of alveoli (Whitsett and Alenghat 2015). DNA damage derived from high concentrations of oxygen is one of the hyperoxia-derived pathogenic factors that is responsible for AEC injury in lung tissues by creating vast amounts of ROS, resulting in excessive inflammation (Ye et al. 2017). Fortunately, the damage done to DNA during hyperoxia can be repaired by OGG1, preventing programmed death of lung epithelial cells (Øvrevik et al. 2010). Overexpression of OGG1 alleviates the toxic effect of hyperoxia-derived injury in lung cells (Wu et al. 2002). Therefore, enhancing expression levels of OGG1 functions represents a potential approach to defend the lung against hyperoxia-induced lung injury.

OGG1 plays an important role in hyperoxia-induced inflammatory response. A recent study showed that high concentrations of oxygen could result in excessive proinflammatory cytokine, such as TNF-α, IL-6, and IFN-β, in *Ogg1*-deficient mice (Ye et al. 2017). Although not immediately evident, autophagy also plays a role in the regulation of inflammation associated with OGG1, along with a broad spectrum of DNA repair proteins (Ye et al. 2017; Van Houten et al. 2016). In cardiomyopathy, autophagy plays an important role in keeping a balance between ROS production and loss of OGG1(Ye et al. 2017; Ryter and Choi 2013). Moreover, pulmonary hyperoxia also stimulates autophagy, but OGG1-mediated control of autophagy results in reduced damage of lung tissues and inflammatory responses. The OGG1/Atg7 axis inhibits NF-κB phosphorylation process that exhibited attenuated inflammatory cytokines such as TNF-α, IL-6, and IFN-β, demonstrating that OGG1 associates with autophagy to regulate inflammatory responses to pulmonary hyperoxia. Taken together, in pulmonary hyperoxia, OGG1 is associated with autophagic pathway to repress the production of inflammatory cytokines, which alleviates hyperoxia-induced lung tissue injury (Ye et al. 2017). These findings imply that OGG1 plays a significant role in innate immunity against hyperoxia as well as novel targets for clinical therapy.

4.3.2 OGG1 Mediates Pulmonary Fibrosis

Pulmonary fibrosis is a chronic and progressive respiratory disease, in which a scar is formed in distal lung interstitial tissue over time (Richeldi et al. 2017). The scar tissue is formed by excessive extracellular matrix collagen deposition following the injury of overlying epithelium and activation of local myofibroblasts (Darby et al. 2014). The scarring and consequent stiffening of the lungs make breathing difficult and cannot afford for sufficient oxygen supply to the blood. Asbestosis is one of the widespread pulmonary fibroses that is caused by asbestos exposure (Walters et al. 2018). Thus far, asbestos-related pulmonary fibrosis remains a common public health issue around the world. The injured AECs are associated with pulmonary

fibrosis induced by asbestosis (Cheresh et al. 2015). Asbestos-associated AEC injury and inflammatory cells lead to ROS production to promote disease occurrence (Cheresh et al. 2015). In this process, mtDNA is more sensitive to crocidolite asbestos-induced DNA damage leading to the production of iron-derived free mitochondrial ROS and apoptosis (Kim et al. 2015). Moreover, the AEC-mediated apoptosis plays an important role in pulmonary fibrosis (Kim et al. 2015; Malsin and Kamp 2018). In addition, oxidative stress caused by asbestos exposure induces mitochondrial dysfunction by affecting mitochondrial ROS-mediated mtDNA damage, p53 activation, apoptosis, and inflammatory signaling (Shetty et al. 2017). It demonstrates that oxidative stress in asbestos fibrosis plays an important role for AEC-mediated mtDNA damage to promote the development of pulmonary fibrosis.

OGG1 is primarily responsible for mtDNA damage repair that facilitates ensuring of long-term cell survival, indicating that OGG1 as a bifunctional base excision repair protein shows importance for asbestos-induced pulmonary fibrosis (Cheresh et al. 2015). OGG1 mutant shows exacerbated lung fibrosis scores compared to WT mice. Moreover, ineffective base excision repair pathway results in increased mtDNA, and asbestos-induced AEC injury is strongly associated with apoptosis via p53 activation (Kim et al. 2015; Panduri et al. 2009, 2003, 2006). Furthermore, OGG1 deficiency augments caspase-3 activation in lung AEC cells to aggravate the pulmonary fibrosis (Cheresh et al. 2015). In addition, asbestos also induces an increased endoplasmic reticulum (ER) stress response in *Ogg1*$^{-/-}$ mice. Taken together, OGG1-mediated prevention of asbestos-induced AEC mtDNA damage and apoptosis is through p53 pathway to protect cell integrity and alleviate the extent of pulmonary fibrosis (Liu et al. 2013). This demonstrates that lung AEC integrity maintained by OGG1 plays an important role in pulmonary fibrosis disease, which represents a novel and useful therapeutic approach.

4.3.3 OGG1 Controls Allergic Airway Inflammatory Diseases

Asthma is a complex, chronic inflammatory lung disease and is thought to be heavily influenced by genetics as well as epigenetic changes induced by environmental factors (Ho 2010; Lambrecht and Hammad 2012). The genetics, development, immunopathogenesis, and ventilator/gas exchange impairment associated with the disease are dictated by multiple inflammatory mediators in addition to the oxidative stress induced by ROS (Zuo et al. 2013).

Environmental agents, acting individually or in combination, mainly affect airway epithelium and immune cells through increasing the generation of ROS levels, leading to the beginning and worsening of asthma (Birben et al. 2012). ROS generation significantly enhances genomic 8-oxo-dG levels that are connected with OGG1 protein. Moreover, OGG1-initiated repair of damaged DNA is an indispensable for neutrophil accumulation that is implicated in deregulation of the immune system and fundamental to histopathological changes in allergic airway inflammatory disease (Bacsi et al. 2013; Ba et al. 2015). OGG1-dependent KRAS activation

participates in the recruitment of airway allergic inflammatory cells and releases chemokine/cytokine Cxcl1, TNF-α, Ccl20, Ccl3, IL-1α, and IL-1β (Aguilera-Aguirre et al. 2014). In addition, OGG1-driven MAPK, PI3K, and MSK1 pathways on NF-κB/RelA activation could also be linked to airway allergic inflammatory disease. It activates IκB phosphorylation that becomes a target for ubiquitination and proteasome-mediated degradation leading to liberation of NF-κB/RelA activation. Moreover, OGG1 deficiency also modifies allergic airway inflammation by alleviating the expression and phosphorylation of STAT6 and IL-4, IL-5, IL-6, IL-10, IL-13, and IL-7 but increasing IFN-γ production in the lung tissue (Li et al. 2012). Taken together, OGG1 plays an important role in the regulation of allergic immune response via generating different endogenous signals for inflammation.

4.3.4 OGG1 Response Is Related to Other Diseases

OGG1 is etiologically linked with several other inflammatory diseases, including Alzheimer's disease, atherosclerosis, metabolic syndrome, rheumatoid arthritis, cancer, or diabetes. In the atherosclerosis disease, OGG1-mediated BER represses NLRP3 (NLR family pyrin domain containing 3) inflammasome activation and decreased apoptosis resulting in less IL-1β and IL-18 secretion to prevent the disease progression of atherosclerosis (Tumurkhuu et al. 2016; Bennett et al. 2018). It shows that OGG1 functions as a protective effector on atherosclerosis by restricting excessive inflammasome activation (Baldrighi et al. 2017). Alteration of OGG1-BER is an event preceding Alzheimer's disease so that the level of OGG1 as a potential diagnostic biomarker contributes to neuronal cell death in dementia (Sanyal 2007). On the other hand, single nucleotide polymorphism (SNP) of *Ogg1* gene associated with the control of the level of oxidative DNA damage has a notable role on the development of rheumatoid arthritis (Yosunkaya et al. 2012), cancer (Moghaddam et al. 2016), and Alzheimer's disease (Kwiatkowski et al. 2016). For example, increased rheumatoid arthritis risk is linked to *Ogg1* C285T and A5954G polymorphisms. Moreover, OGG1 is associated with low rates of obesity and Syndrome X (metabolic syndrome), due to its role in monitoring energy and fat balance, especially the alteration of lipid and mitochondrial metabolism (Vartanian et al. 2017). Lastly, in the type II diabetes disease, OGG1 is involved in AMPK-mediated NRF2 signals, which diminishes renal cell destruction by minimizing oxidative DNA damage (Habib et al. 2016). Overall, it is clear that the novel discovery of OGG1-mediated DNA repair has broad implications for the determination of multiple human disease mechanisms.

4.4 Conclusion and Perspective

Genomic instability is a hallmark of disease that increases due to the alteration of DNA damage response. OGG1 localizes in both nucleus and mitochondria and has been investigated for its role in DNA repair in many pathways modulating

pulmonary infection and other inflammatory responses. We highlighted OGG1-mediated DNA repair mechanisms that impact ROS production, DNA damage, p21/p53 activation, apoptosis, and autophagy. These researches provide insights into the molecular basis of OGG1-associated signaling in bacterial infection and inflammation, which may foster the development of novel therapeutic strategies for infection, degenerative disease, tumors, and aging. The OGG1/pulmonary disease paradigm will shed light into the molecular basis underlying diseases in other organs, such as the liver, heart, and kidney, which will provide an improved understanding of the pathogenesis of many common diseases, for which effective treatment regiments are urgently required. Furthermore, we may witness a flux of novel discoveries in elaborating basic principles of how OGG1 regulates inflammatory response and other cell signalings, such as the molecular binding partners, transcriptional control, promoter activation, and structural alterations in various disease conditions.

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The Dual Role of Oxidative Stress in Lung Cancer

5

Asmaa M. Ahmed

Abstract

Reactive oxygen species (ROS) are produced by several endogenous and exogenous sources, and their levels are controlled by several antioxidants. Oxidative stress results from an imbalance between ROS production and removal by antioxidant mechanisms. The risk factors which are commonly associated with chronic diseases (e.g., cancer) interact with the cells through the formation of ROS. These factors include stress, cigarette smoking, air pollutants, radiation, and infection. The lung is considered a target organ continuously affected by exogenous and endogenous ROS. There is now increasing evidence that there is association between oxidative stress and lung cancer. Interestingly, it was found that ROS could not only promote tumorigenesis, but also they have antitumorigenic effect. This dual role of ROS in cancers (e.g., lung cancer) relies on their amount, type, and the site of their production. For example, moderate amount of ROS was associated with tumor cell survival, proliferation, angiogenesis, and metastasis. On the contrary, excessive amount of ROS could induce tumor cell death. Thus, it is essential to investigate the dual role of ROS in cancers, for the development of novel therapeutic approaches targeting redox regulatory mechanisms.

Keywords

Oxidative stress · Lung cancer · Dual role · Reactive oxygen species

Abbreviations

ARE	Antioxidant response element
CAT	Catalase

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EGFR	Epidermal growth factor receptor
GPx	glutathione peroxidase
KEAP1	Kelch-like ECH-Associated Protein 1
MAPK	Mitogen-activated protein kinase
MPO	Myeloperoxidases
NOX	NADPH oxidase
NRF2	NFE2-related factor 2
NSCLC	Non-small cell lung cancer
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Trx	Thioredoxin

5.1 Introduction

Oxidative stress occurs as a result of an imbalance between the reactive oxygen species (ROS) production and elimination by effective antioxidant response. Thus, proper regulation of reduction-oxidation (redox) reactions maintains normal cellular growth and metabolic mechanisms (Brown and Griendling 2015).

Biological organs are continuously affected by variable endogenous and exogenous oxidants (Nohl et al. 2003). Of note, the lungs are exposed to excess oxygen and characterized by their large surface area and blood supply which increase their susceptibility to ROS-induced injury (Park et al. 2009). Accumulating evidences demonstrate that oxidative stress can play a crucial role in lung cancer initiation, promotion, and invasiveness (Filaire et al. 2013).

According to the Global cancer statistics 2018, lung cancer is considered the most common cancer worldwide and is the primary leading cause of cancer-related death (Fig. 5.1) (Bray et al. 2018). Two histologic types of lung cancer have been described: small-cell lung cancer and non-small cell lung cancer (NSCLC). The latter is further subdivided into three categories: adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma (Inamura 2017). A regional variation in the incidence of lung cancer was observed which mirrors the widespread of tobacco exposure (Bray et al. 2018). It was reported that the two main constituents of tobacco (nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and polyaromatic hydrocarbons) are considered the predominant risk factors for lung cancer (Shiels et al. 2013; Azad et al. 2008). In addition, other inhaled carcinogens as microorganisms and environmental pollutants can also induce lung cancer. These carcinogenic agents promote tumorigenesis through production of ROS with a resultant oxidative damage (Azad et al. 2008; Birben et al. 2012). Emphasis on the different molecular pathways involved in lung cancer pathogenesis is critical to the development of novel therapeutic modalities (Cooper et al. 2013). This review will focus on the possible mechanisms by which oxidative stress can induce lung cancer.

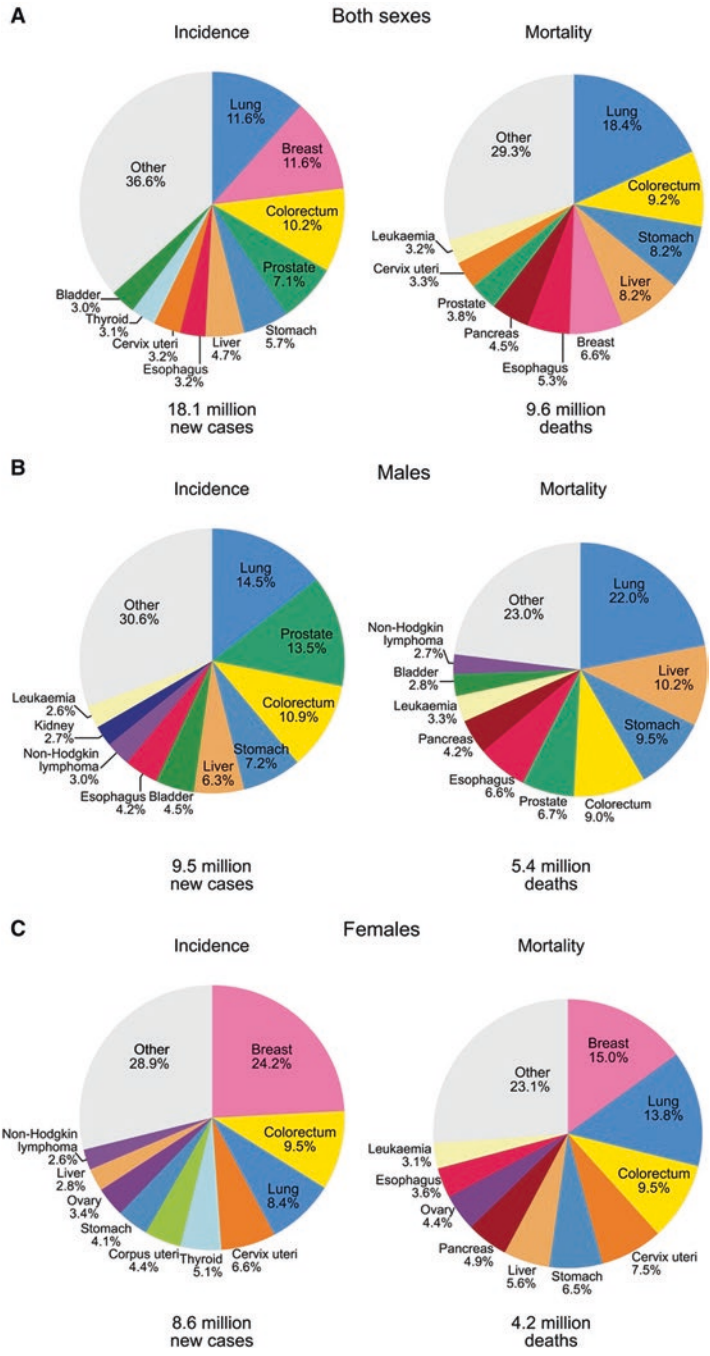

Fig. 5.1 The incidence and mortality rates of the most common cancers in 2018 for both sexes (**a**), males (**b**), and females (**c**). (Bray et al. 2018)

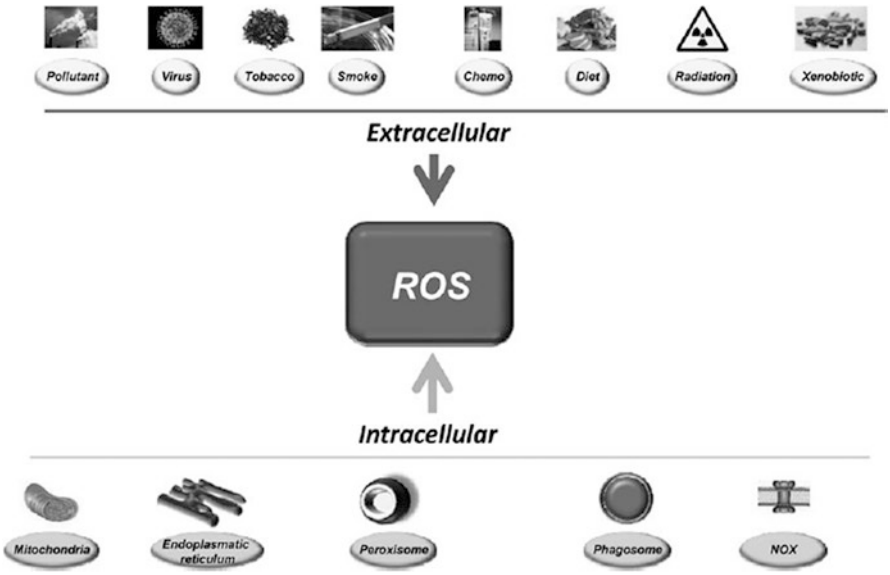

Fig. 5.2 Sources of reactive oxygen species (ROS). ROS can be generated by numerous extracellular and intracellular sources. (Gupta et al. 2012)

5.2 Overview of ROS

Reactive oxygen species are oxygen-reactive molecules involved in various physiologic and pathologic events (e.g., cancer, diabetes, and other age-related disorders) (Sena and Chandel 2012; Gill et al. 2016; Tafani et al. 2016). They can be grouped into two subtypes: (1) free radical ROS which have one or more unpaired electron(s) in the valence shell (i.e., superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$)) (Filaire et al. 2013), in which in attempting to pair up their own electrons, they extract electrons from a stable molecule and leave this original molecule in an unstable state with resultant cell damage (Finaud et al. 2006), and (2) non-radical ROS, in contrast to radical ROS, which lack the presence of unpaired electrons but are chemically reactive and have the ability to be converted into radical ROS (i.e., hydrogen peroxide (H_2O_2)) (Leone et al. 2017). ROS can be generated either by exogenous sources (Fig. 5.2) such as UV radiation, toxins, chemicals, drugs, aging, inflammatory cytokines and chemokines, growth factors, chemotherapeutics, and ionizing radiation (Richter et al. 2015) or by endogenous sources (Fig. 5.2) including NADPH oxidase (NOX) enzymes on the plasma membrane (Bedard and Krause 2007), and myeloperoxidases (MPO) in phagocytes (Robinson 2008) and as by-products of respiratory chain function in mitochondria (Murphy 2009).

Low levels of ROS can stimulate signaling pathways involved in cellular differentiation, proliferation and apoptosis (Gorrini et al. 2013; Waypa et al. 2010). However, excessive ROS levels can produce damage of important cellular structure such as DNA, proteins, and lipids. To counteract these effects, cells activate several

endogenous antioxidant ROS scavengers, as glutathione peroxidase (GPx), thioredoxin (Trx), catalase (CAT), superoxide dismutase (SOD), and the nuclear factor erythroid 2 (NRF2) pathway (Birben et al. 2012; Leone et al. 2015). If a further increase in ROS levels occurs, the cells will undergo apoptosis. Therefore, under physiological conditions, the balance between ROS formation and elimination is preserved through the help of the ROS scavengers/endogenous antioxidants. So the harmful effects of oxidative stress are avoided (Birben et al. 2012).

5.3 Possible Cellular Sources of ROS in the Lungs

It is evident that every cell type in the lung can produce some ROS. These cells include:

5.3.1 Alveolar Macrophages

They are phagocytic cells which are considered the first line of defense against infection in lung tissues. The main source of ROS in alveolar macrophages is the membrane NADPH oxidases which generate O^{-}_{2} under physiologic conditions (Thannickal and Fanburg 2000; Piotrowski and Marczak 2000).

5.3.2 Type II Pneumocytes

These cells form a part of the alveolar epithelium which are responsible for production of surfactant and act as precursor cells for type I pneumocytes after destruction of alveolar epithelium. Recently, it was described that type II epithelial cells have the ability to produce some ROS through their enzymatic properties (Kinnula et al. 1991; Tkaczyk and Vizek 2007).

5.3.3 Endothelial Cells

Under pathological conditions (e.g., hypoxia), endothelial cells can participate in oxidative stress and lung injury through stimulation of their xanthine oxidoreductase complex and their membrane-bound NADPH oxidase (Kelley et al. 2006; Souza et al. 2001; Jones et al. 1996).

5.3.4 Smooth Muscle Cells

Smooth muscle cells which present in either the airway or in the vessel walls have membrane-bound NADPH-like oxidase generating O^{-}_{2} which plays a major role in airway hyperreactivity (Li and Shah 2003; Thabut et al. 2002).

5.3.5 Lung Fibroblasts

After stimulation by inflammatory cytokines, lung fibroblasts may be a source of ROS through their two different enzymes including NADPH oxidase (phagocyte-like) which produces O^{-}_{2} intracellularly and NADH oxidase, which generates H_2O_2 into the extracellular space (Tkaczyk and Vizek 2007; Thannickal et al. 2000).

5.3.6 Peripheral Monocytes-Macrophages

After recruitment of the peripheral monocytes by different inflammatory cytokines, they differentiate into macrophages which are able to produce superoxide by xanthine oxidoreductase that plays a significant role in acute lung injury (Wright et al. 2004).

5.3.7 Inflammatory Cells

Large amount of inflammatory cells migrate into the pulmonary circulation during inflammation (e.g., neutrophils) and some allergic and infectious conditions (e.g., eosinophils) (Nagata 2005). They produce ROS as a result of their bactericidal activity by the action of their enzymes. For instance, the membrane-bound NADPH oxidase in both neutrophils and eosinophils generates large amounts of superoxide. Also, these cells produce cytoplasmic myeloperoxidase, which adds to their bactericidal action of producing mostly hypochlorous acid (Tkaczyk and Vizek 2007; Hammerschmidt et al. 2002).

5.4 ROS and Lung Cancer

A link between ROS and cancer dates back to 1981 when high H_2O_2 levels induced by insulin were shown to stimulate proliferation of tumor cells. Nearly three decades later, many researchers reported increased levels of oxidative damage products in the tumor specimens and plasma along with cancer cell lines (Trachootham et al. 2009). Based on these suggestions, altered redox balance was found to be involved in carcinogenesis (Hanahan and Weinberg 2011; Panieri and Santoro 2016).

As a result of the interface of the lung with the environment, it is considered a target organ continuously affected by exogenous oxidants and endogenous ROS. However, the exact mechanisms by which oxidants can cause lung cancer are still unclear (Ciencewicki et al. 2008).

Several studies have reported that ROS act as a double-edged sword in cancers (Fig. 5.3) and their role relies on the amount, type, duration, and the site of ROS production. For example, moderate amount of ROS was found to promote tumor survival, while excessive level serves to suppress tumors and enhance tumor cell death (Gupta et al. 2012; Halliwell 2007; Wu and Hua 2007). Also, NOX-derived

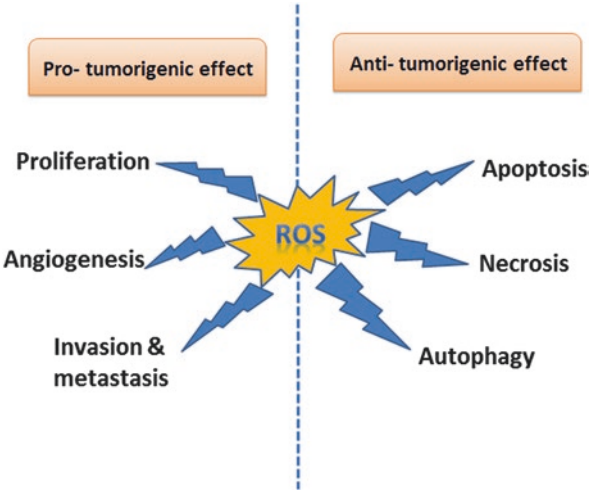

Fig. 5.3 The dual role of ROS in cancers

ROS in the cytoplasm in response to TNF-a promote tumor cell survival, whereas mitochondria-derived ROS stimulate apoptosis (Deshpande et al. 2000). In prostatic carcinoma, inhibition of ROS by antioxidants or NOX inhibitors was associated with an increase in apoptosis (Brar et al. 2003). This dual role of ROS in cancers provides a great challenge for the development of different targeting therapeutic modalities for cancers. The possible mechanisms by which ROS might exert their pro-tumorigenic and anti-tumorigenic action in lung cancer will be discussed below.

5.4.1 Pro-tumorigenic Role of ROS in Lung Cancer

Accumulating evidences suggested that ROS play a major role in cancer initiation, promotion, and progression through modulation of signaling molecules involved in cell proliferation, angiogenesis, as well as alteration of the migration/invasion program (Leone et al. 2015; Fiaschi and Chiarugi 2012). The roles of ROS in lung cancer can be described as follows.

5.4.1.1 ROS-Mediated Cellular Proliferation of Lung cancer

It was found that ROS and inhaled carcinogens (e.g., benzo[a]pyrene in both the environment and cigarette smoke) promote the uncontrolled cellular proliferation of lung adenocarcinoma by inducing overexpression of the phosphorylated epidermal growth factor receptor (EGFR) protein and its ligands such as amphiregulin and epiregulin (Kometani et al. 2009). Also, it has been described that mitochondrial ROS can control Kras-induced anchorage-independent lung cancer growth through the MAPK/ERK signaling pathway (Weinberg et al. 2010). Thus, disturbance of the mitochondrial respiratory chain was found to decrease tumor formation by reducing ROS (Weinberg et al. 2010).

5.4.1.2 ROS Promote Genomic Instability in Lung Cancer

ROS from different sources could lead to DNA damage in a wide range of cancers including lung cancer (Azad et al. 2008; Liu et al. 2008). This damage can be prevented by p53 "genome guardian," which has the ability to sense and remove oxidative damage to DNA (Lim et al. 2007). Unfortunately, in lung cancer cells, TP53 (gene which encodes p53) is commonly mutated (Gibbons et al. 2014), with inadequate DNA repair mechanisms, resulting in genomic instability with further stimulation of several oncogenes. These events will result in abnormal metabolic activity and reduced production of antioxidants which ultimately give rise to an increased level of intracellular ROS in a positive-feedback manner (Trachootham et al. 2009).

KEAP1-NRF2-ARE Signaling Pathway and Lung Cancer

Kelch-like ECH-Associated Protein 1 (KEAP1)-NFE2-related factor 2 (NRF2)-antioxidant response element (ARE) pathway is considered one of the main oxidative stress protective pathways which protects the cells from both endogenous and exogenous stresses (Kensler et al. 2007), particularly in the lung (Spiegelman 2007). Previous study reported downregulation of the oxidative stress sensor KEAP1 by DNA CpG methylation in NSCLC (Wang et al. 2008). Also, another study revealed loss of KEAP1 function activated NRF2 with consequent stimulation of NSCLC growth (Ohta et al. 2008). Thus, dysfunction of this oxidative stress protective pathway is associated with lung carcinogenesis (Ohta et al. 2008).

5.4.1.3 Role of ROS in Angiogenesis in Lung Cancer

High levels of ROS are observed in various cancer cells including lung cancer (Ushio-Fukai and Nakamura 2008). Accumulating evidences suggested a major role of ROS in angiogenesis and metastasis (Reczek and Chandel 2017). As tumors grow, their demand for nutrients and oxygen supply will increase. The resultant hypoxic tumor microenvironment stimulates ROS production and release from the mitochondrial electron transport chain (Chang et al. 2005; Srinivas et al. 2001). These ROS, in turn, will stabilize HIF-1α which stimulates the transcription of its target genes, such as vascular endothelial growth factor (VEGF), N-myc downstream-regulated gene (NDRG), and glucose transporter I (Liu et al. 2012). These genes are involved in glucose transport, glycolysis, and angiogenesis (Liu et al. 2012). In addition, HIF can potentiate the angiogenic potential of the tumors by its ability to induce the expression of several potent angiogenic factors, including VEGF receptors, matrix metalloproteinases, plasminogen activator inhibitor-1, platelet-derived growth factor B, angiopoietins, and the TIE-2 receptor (Hickey and Simon 2006).

5.4.1.4 ROS Promote Invasion and Metastasis of Lung Carcinoma Cells

Invasion of the malignant cells is considered the initial step in metastasis which is the primary cause of cancer-associated deaths (Luanpitpong et al. 2010). Increasing evidence suggests a strong association between increased ROS production and migration of lung cancer cells (Misthos et al. 2005; Chung-man Ho et al. 2001) possibly through alterations of some adhesion molecules and cellular cytoskeleton (Kopfstein and Christofori 2006). For example, ROS have been reported to regulate integrins (Chiarugi et al. 2003; Svineng et al. 2008), small GTPase Rho family

proteins (Tobar et al. 2008; Alexandrova et al. 2006), focal contact-forming proteins (Ben Mahdi et al. 2000), and extracellular matrix-degrading enzymes such as matrix metalloproteinases (Nelson and Melendez 2004; Lee et al. 2008). In addition, ROS have been described to control several transcription factors (e.g., Snail and HIF) and signaling pathways (e.g., PI3K/Akt and MAPK pathways) to enhance the invasion of cancer cells (Tochhawng et al. 2013). Furthermore, NOX-derived ROS promote tumor cell invasion through the formation of invadopodia (Diaz et al. 2009). These findings suggest that an increased ROS levels can modulate structural changes in tumor cells to enhance local invasion and metastasis.

5.4.2 The Anti-tumorigenic Effect of ROS in Lung Cancer

Although accumulated evidences have described an association between ROS and malignant cellular proliferation and metastasis, excessive ROS levels in the tumor microenvironment could induce cell cycle arrest, senescence, and cell death in several tumors (Moon et al. 2010; Hampton and Orrenius 1997). Cancer cells are able to die by either apoptosis, necrosis, or autophagy (Simon et al. 2000; Steller 1995; Wochna et al. 2007).

5.4.2.1 ROS and Apoptosis in Lung Cancer

Apoptosis is a form of programmed cell death which can be initiated by intrinsic (mitochondria) or extrinsic (death receptors) pathways. Activation of these two pathways results in opening of the permeability transition pore complex on the mitochondrial membrane with subsequent release of cytochrome c and activation of caspases (Elmore 2007). Opening of the mitochondrial pore occurs under the effect of ROS which activate pore-destabilizing proteins and inhibit pore-stabilizing proteins (Martindale and Holbrook 2002). Several studies on cell culture and animal models have demonstrated the potential role of ROS in inducing apoptosis in cancer cells (Redza-Dutordoir and Averill-Bates 2016; Price et al. 2009; Valdameri et al. 2011). For example, administration of exogenous H_2O_2 has been shown to induce lung cancer cell death through caspase-dependent apoptosis (Park 2018a) by regulating mitogen-activated protein kinase (MAPK) signaling pathways (Park 2018b). Interestingly, ROS can also induce apoptosis in lung cancer cells in a caspase-independent manner through mitochondrial-to-nuclear translocation of apoptosis-inducing factor and endonuclease G (Liu et al. 2005). Several signaling molecules have been shown to induce ROS and apoptosis in different cancers. The most important of which are kinases; pro-inflammatory transcription factors such as NFkB, caspases, cell survival proteins, pro-apoptotic proteins, and phosphatase; and tensin homolog deleted on chromosome 10 (PTEN) (Gupta et al. 2012).

5.4.2.2 ROS and Necrosis in Lung Cancer

Although an excess ROS levels can induce apoptosis, massive levels might lead to necrosis which is a “passive” form of cell death, initiated by stressful conditions and several toxic insults (Azad et al. 2009). In many cancers, apoptosis and necrosis

may occur sequentially as a result of switch from apoptotic to necrotic cell death due to a burst in intracellular ROS (Gupta et al. 2012). For example, a previous study investigated the effects of exogenous H_2O_2 on lung cancer cells. The authors reported that H_2O_2 induced lung cancer cell death through caspase-dependent apoptosis and necrosis (Park 2018a).

5.4.2.3 ROS and Autophagy in Lung Cancer

Autophagy is a self-digestive process by which the misfolded proteins and damaged organelles are sequestered within autophagosomes for further lysosomal degradation. Interestingly, autophagy was found to have a dichotomous effect on cancer cells as it can be involved in both cell survival and cell death pathways (Hippert et al. 2006; Marx 2006; Lerena et al. 2008; Altman and Rathmell 2009; Bhutia et al. 2013). Studies during the last few years have suggested a role for ROS as signaling molecules in stimulating autophagic death in cancers (Azad et al. 2009; Gibson 2010; Ishdorj et al. 2012) via JNK activation, which, in turn, increased the expression of autophagy protein Beclin-1. Examples of cancers in which ROS have been shown to effectively induce autophagic cell death include non-small cell lung cancer (NSCLC) (Li et al. 2010), breast cancer (Shrivastava et al. 2011), glioma (Park et al. 2011; Wang et al. 2010), glioblastoma (Chen et al. 2008), and cervical cancer (Guo et al. 2010).

5.5 Conclusion

Adequate homeostasis between ROS production and elimination via antioxidants should be maintained in order to prevent oxidative stress and oxidative damage. Oxidative stress has been contributed in the pathogenesis of several disorders, including cancer. Within the lung, ROS can be generated from different sources including alveolar macrophages, type II pneumocytes, endothelial cells, smooth muscle cells, lung fibroblasts, and inflammatory cells. Interestingly, ROS have both pro-tumorigenic and anti-tumorigenic effects on lung cancer. ROS play their tumorigenic effect through modulation of several signaling molecules which are involved in cell proliferation, angiogenesis, and migration. However, excessive production of ROS in the tumor microenvironment has been associated with lung cancer cell death. This dual role of ROS in lung cancer provides a great challenge for the development of different therapeutic modalities for lung cancer.

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Cigarette Smoke-Induced Oxidative Stress in Type I and Type II Lung Epithelial Cells

6

Subodh Kumar, Phulen Sarma, Bikash Medhi, and Krishan L. Khanduja

Abstract

Cigarette smoke (CS) is a complex combination of over 5000 compounds divided into two phases (tar and gas) whose exposure causes various lung pathological conditions especially COPD and cancer. In a single puff, tar phase basically contains extremely high concentrations of stable and long-lived radicals (~10^{17} radicals/gm), whereas gas phase contains ~10^{15} organic radicals. In contrast to stable life radicals (e.g., hydrogen peroxide, hypochlorous acid) present in tar phase, the radicals in gaseous phase are highly reactive nitrogen and oxygen-centered radicals (e.g., nitric oxide, reactive olefins, dienes) known to have lifetime of less than 1 s. Therefore, cigarette smoke constituents evoke the endogenous oxidants production and agitate normal functioning of tissues, especially type I and II pulmonary epithelium. As a result of smoke exposure, increased level of oxidants (exogenous or endogenous) leads to stress state termed as "oxidative stress," which arises due to commotion in prooxidant and antioxidant balance in favor of the prooxidant. The oxidative stress caused by the constituents of cigarette smoke in normal as well as in the mutated cells leads to activation of various upstream signaling events such as activation of lipid-specific enzymes especially phospholipases whose role is crucial in the regulation and remodeling of membrane lipids when subjected to hazardous and noxious environmental insults such as cigarette smoke-initiated tissue damage.

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6.1 Introduction

In the entire human body, the entire respiration process is done through the lungs and at the same time is vulnerable to all the environmental toxicants present in the air. The entire respiratory tract stretched from the nasal and oral cavity to alveoli is lined by highly branched and conducting airway epithelium with an abundant number of small airways to give rise the exchange of gases (Bastacky et al. 1995). This conductive branching pattern turns extra multifaceted around distal alveolar epithelium where terminal bronchioles give rise to a series of linked structures such as respiratory bronchioles, alveolar ducts, and alveoli (Hansen et al. 1975). More than 40 cell types exist, but alveolar epithelium is composed of only two types (type I and II alveolar cells). Type I epithelial cells are large and squamous in shape with surface area of about 5000 μm^2 per cell, spread over 95% of the alveolar surface, and contribute approximately 8% of total peripheral lung cells, whereas type II epithelial cells are small and cuboidal, characterized by unique intracellular storage organelles for pulmonary surfactants known as lamellar bodies (Dobbs and Johnson 2007). Type II cells contribute approximately 15% of peripheral lung cells covering roughly 5% of the alveolar plane with an apical face vicinity of concerning 250 μm^2 per cell (Stone et al. 1992). Alveolar type I cells contain abundant mitochondria, microvilli, and both rough and smooth endoplasmic reticulum, suggesting their role in active biosynthetic functions and regulation of alveolar fluid balance as well as exchange of gases (Johnson et al. 2002; Gumbleton 2001). Type II cells which situate between type I cells have many known functions. They produce, secrete, and reuptake immunomodulatory pulmonary surfactant proteins such as surfactant proteins A and D and regulate alveolar fluid in the lungs during the resolution of pulmonary edema (Mason 2006; Shannon and Hyatt 2004; Fehrenbach 2001). Apart from these vital functions, type II epithelial cells proliferate to re-epithelize the dissociated and injured layer by transforming into the type I epithelial cells and show a stem cell function for alveolar repair (Wright 2005). During the crucial process of inhalation, this alveolar epithelium plays central role in the regulation of host defense and intrinsic reparative capacity when subjected to hazardous and noxious environmental toxicants, i.e., cigarette smoke-initiated tissue damage.

6.2 Cigarette Smoke and its Composition and Effects on Alveolar Epithelium

Tobacco is the only legal product that kills at least half of its regular users. The mortality rate due to tobacco is more than 7 million people each year (approximately 10% of all deaths), accounting ≥6 million deaths through direct use of tobacco, while ≥1 million deaths happen in non-smokers due to passive smoking. Around the world's 1.1 billion smokers, approximately 80% are from low- and middle-income countries (WHO report on the global tobacco epidemic 2017).

6.2.1 Composition

Cigarette smoke is a complex combination of over 5000 compounds, and several of its components are well-known to be carcinogenic, cocarcinogenic, and mutagenic in nature. Over the passage of time as the studies came (1950s onwards) of involvement of cigarette smoke in the induction of lung cancer, the composition of cigarettes have changed a lot with new innovations like use of paper type, filter tips, processing of tobacco leading to changes in smoke constituents as well. When a cigarette burns, combustion zone temperature reaches up to 800 °C–950 °C giving rise to absolute pyrolysis of tobacco (Johnson 1977), and immediately downstream, temperature decreases approximately 200 °C–600 °C causing incomplete combustion of tobacco in lack of oxygen. Cigarette smoke (completely or partially burned tobacco) is a complex mixture of over 5000 chemicals including high concentration of oxidants and free radicals. It can be divided into two phases (tar and gas). In a single puff, tar phase basically contains extremely high concentrations of stable and long-lived radicals (~10^{17} radicals/gm), while gaseous phase contains ~10^{15} organic radicals. In contrast to stable life radicals (e.g., hydrogen peroxide, hypochlorous acid) present in tar phase, the radicals in gaseous phase are highly reactive nitrogen and oxygen-centered radicals (e.g., reactive oxygen species/reactive nitrogen species, nitric oxide, reactive olefins, dienes) known to have lifetime of less than 1 s (Diana 1993; Pryor and Stone 1993; Shishodia et al. 2003). Moreover, cigarette smoke consists of more than 100 known strong carcinogens and tumor initiators including polycyclic aromatic hydrocarbons (PAHs), benzo(a)pyrene, urethane, nitrosoamines, and nitrosonornicotine; weak carcinogens such as acetaldehydes and formaldehydes; and co-carcinogens such as catechol, phenol, and formaldehyde (Hecht 2003).

6.2.2 Effect on Alveolar Epithelium

The airway epithelium is continuously exposed to numerous cigarette smoke constituents who are known to damage the structural and functional components of the lungs (Hoyle and Brody 1996). All types of cells are vulnerable to damage due to these agents, but by virtue of its location, anatomy, and function, the alveolar epithelium is most susceptible. Carcinogens, cocarcinogens, and oxidants present in tar as well as gas forms of cigarette smoke along with endogenous free radicals injure the airways and cause structural and functional alterations in the natural and finely tuned pulmonary epithelium, thereby jeopardizing the primary lung function of gas exchange (Adler et al. 1994). For this reason, free radicals/oxidants are considered to be the main factor responsible for epithelial cell damage and death observed under these stress conditions. Endogenous oxidants are generated by oxidative phosphorylation, plasma membrane, NADPH oxidase, cytoplasmic xanthine oxidase, P450 metabolism, peroxisomes, and inflammatory cell activation. Therefore, cigarette smoke is a source of exogenous oxidants, and continuous exposure of these oxidants or constituents further enhances the endogenous oxidant production

drastically in these type I and type II epithelial cells (Yadav et al. 2016). Mechanistically, exposure to cigarette smoke constituents produces oxidative stress with a series of reactions of biochemical to molecular cascades including release of inflammatory mediators and apoptosis (Khanduja 1998).

6.2.3 Mediators of Oxidative Stress

"Oxidative stress" is defined as commotion in prooxidant and antioxidant balance in account of the prooxidants, ensuing in an overall raise in cellular oxidants, and is multi-stressor event (Klaunig and Kamendulis 2004). During cigarette burning, combustion of tobacco endorses the oxidation of various tobacco constituents and generates ROS/RNS and other free radicals. Tar phase stable radicals such as quinone and hydroquinone complexes reduce molecular oxygen to extremely reactive species such as superoxide radicals (SOR), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH). To thwart the effects of oxidative stress, both type I and type II epithelial cells show important defense systems that include enzymatic and nonenzymatic antioxidant components. The development of an oxidant/antioxidant imbalance activates or deactivates mitogen-activated protein kinases (MAPKs) and PLA_2s which are involved in many cellular programs such as proliferation, differentiation, and cell death by regulating the activation of various key regulators such as p53, Bcl-2, Bax, Bcl-xl, and c-myc (Miyashita and Reed 1995; Zha and Reed 1997; Kauffmann-Zeh et al. 1997; Chang and Karin 2001; el-Deiry 1998; Chang et al. 2000; Kumar et al. 2019). PLA_2s play a physiological role in cellular pathways and get activated during inflammation. But continuous exposure of cigarette smoke further leads to higher PLA_2s activity and stress (Yadav et al. 2016). Therefore once oxidant levels exceed the certain threshold value, oxidative stress-regulated gene network gets activated which in turn affects apoptosis, proliferation, and cell differentiation in both type of cells (Kumar et al. 2019). Moreover, cigarette smoke also induces damage to various cellular constituents. Thus, exposure and free radicals body burden is a lot higher in cigarette smokers than in non-smokers (Rahman and MacNee 2000), which thereby directly or indirectly induces the oxidative stress in cells.

6.2.4 Mode of Action

Free radicals present in CS constituents are extremely reactive in nature and, when exposed to cell membranes, tend to oxidize proteins and lipids (lipid peroxidation). This process continuously occurs in a chain reaction resulting to formation of many molecules of lipid hydroperoxide and oxidized proteins, hence severely disrupting their functions, and either leads to cell death or damage/alteration of cellular DNA (Brigham 1990). Therefore, free radical-mediated posttranslational modification of proteins (phosphorylation, nitrosation, nitration, acetylations, and polyADP-ribosylations), mutations in cancer-related genes, and lipid peroxidation

by-products such as reactive aldehydes, 4-hydroxynonenal, and malondialdehyde are a number of key actions who play major role in inflammation and cancer risk (Hussain et al. 2003).

6.2.4.1 Damage to Amino Acids and Proteins

Amino acids and polypeptides undergo extensive modifications during oxidative reactions and invariably lead to substantial changes in themselves (Table 6.1).

These substantial modifications are classified into three groups: aggregation, fragmentation, and vulnerability to proteolytic digestion (Forman and Azzi 1997). Protein aggregation may occur due to denaturation (Epe 1996) and is associated to the capability of ROS to form cross-linkages. Oxidation-induced protein conformational alterations make these proteins more prone to be the target of proteolytic enzyme-based digestion and degradation. Finally, oxidative susceptibility of amino acids and protein oxidation lead to impairment of functional protein involved in immune surveillance, antioxidant defense system, DNA repair mechanism, cell cycle regulation, and cell death.

6.2.4.2 Modification of Genomic and Cellular Structures

In a given cell, an approximately 10^5 oxidative lesions are created every day (Fraga et al. 1990). Cigarette smoke constituents can directly lead to single- or double-stranded DNA breaks and produce permanent alerted nucleotide sequences which may result either of cell cycle arrest, altered gene expression, and signal transduction pathways, hallmarks of carcinogenesis (Klaunig and Kamendulis 2004). In this regard, mitochondrial DNA is more susceptible to damage because of major source

Table 6.1 Effects of cigarette smoke oxidants and free radicals on cellular constituents

Target	Results
Proteins and DNA	Denaturation
	Phosphorylation
	Nitrosation
	Nitration
	Acetylations
	PolyADP-ribosylations
	Mutations
	Base modifications
	Cell cycle interruption
Antioxidants	Reduce availability of GSH, α-tocopherol, β-carotene, and ascorbate
Amino acids	Enzyme inhibition
	Cross-linking
Carbohydrates	Cell surface receptor changes
	Cell cycle changes
Unsaturated lipids	Organelle and cell membrane permeability changes
	Fatty acid oxidation
Cofactors	Reduced availability (nicotinamide and flavin)

of superoxide free radicals and due to inadequate capability of DNA repair processes (Backer and Weinstein 1980). Among all constituents of nucleic acid, cytosine and thymine are most susceptible to reactive oxygen species (ROS) damage, followed by adenine, guanine, and deoxyribose sugar moiety (Saul et al. 1987). The formation of free radical-mediated changes, such as DNA adduct, plays one of the major roles in gene modifications. Thus, the ultimate targets for free radicals are usually DNA and RNA molecules in both nuclear and mitochondrial organelles. The irreversible nature of these alterations ultimately leads to malignant and mutagenic states (Trush et al. 1982).

6.3 Repair Mechanism of Alveolar Epithelium

Tissue damage due to oxidative stress as a result of inhalation of cigarette smoke induces a cellular process, communally known as the inflammatory response in epithelial cell lining of the respiratory airways where they function both as "target" and "effector" cells (Pan et al. 2001). Target cells respond to various external/internal produced agents and mediators by alterations in their defensive role, such as secretion/absorption of transport-related molecules, secretion of mucus, and effective mucociliary clearance. As effector type, these cells respond to various exogenous/endogenous stimulus by synthesizing and/or releasing various inflammation mediators such as cytokines (Crestani et al. 1994; Fuchs et al. 2001), monocyte chemoattractant protein-1 (Paine et al. 1993), cytokine-induced neutrophil chemoattractant (Crippen et al. 1995), granulocyte macrophage colony-stimulating factor (GM-CSF) (Blau et al. 1994), prostaglandins (especially PGE2), eicosanoids, phospholipase A_2s (Yadav et al. 2016; Cheng et al. 2009), adhesion molecules, platelet-activating factor (PAF), and growth factors and can serve as paracrine regulatory factors (Pan et al. 2001). These inflammatory mediators either put in a local inflammatory response or diffuse away where they affect the functions of neighboring cells. Hence, a complex interrelationship develops between alveolar epithelial and other cells throughout the respiratory system in a complex endocrine and/or exocrine manner. Damage of epithelial cells linked with the initiation of repair processes started whether as part of an inflammatory response or as a response to injury is one of the main features of type I and II alveolar cells and is classically considered to make the physical and functional barrier between external and internal environment that leads to clearance of environmental agents (Thompson et al. 1995; Evans et al. 1975). Apart from vital functions in oxidative stress-induced conditions, type II epithelial cells proliferate and transform into type I epithelial cells and re-epithelialize the damaged alveolar surfaces through remodeling of these epithelial cells. In this special situation, type II alveolar epithelial cells behave like stem cells in alveolar repair (Crapo et al. 1980; Williams 2003). Therefore, alveolar type II epithelial cells are progenitor cells and are capable of proliferating and differentiating into type I cells (Beck-Schimmer et al. 2004). Reestablishment of the regular alveolar epithelium as a result of damage due to external toxicants also requires clearance of the excess hyperplasic type II cells through the process of apoptosis

(Fehrenbach et al. 2000). Therefore, in case of metabolic failure or noxious damage, alveolar epithelium becomes physiologically competent, and cells start the repair mechanism to overcome compromised performance of the lungs (Haddad 2002).

6.4 Conclusion

Type I and type II alveolar epithelial cells respond to a variety of exogenous and/or endogenous oxidative stimulus by generating additional mediators of inflammation (cytokines, eicosanoids, and oxidants); therefore they appear to play an important regulatory role in inflammatory and immune processes. Inflammation is a protective cellular response which destroys and removes the injurious agents and tissues, thereby promoting tissue repair. When this crucial process occurs in an uncontrolled manner, it results in chronic inflammation. Increased level of oxidants in cells (due to exogenous as well as endogenous oxidants) directs a stress state termed as "oxidative stress." Oxidative stress is associated with increased oxidant burden due to disturbance in pro- and antioxidant balance in favor of the prooxidant. Now, there is acceptability that effects of oxidants are not solely mediated through gross damage of the cellular components but also by their more discriminating role as a redox regulator of signal transduction and gene regulation. The control of signal transduction involves alteration in cellular redox status and posttranslational modifications of proteins at key amino acids. Oxidative stress caused by the constituents of cigarette smoke in normal as well as in mutated cells may lead to the remodeling of membrane lipids through activation of various upstream signaling events such as activation of lipid-specific enzymes especially phospholipases which play a crucial role during cigarette smoke-induced lung pathologies.

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Infectious Lung Diseases and Endogenous Oxidative Stress

7

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Abstract

Lower respiratory tract infections, according to the World Health Organization, account for nearly one third of all deaths from infectious diseases. They account for approximately 4 million deaths annually including children and adults and provide a greater disease burden than HIV and malaria. Among the common respiratory diseases, tuberculosis, influenza, and pneumonia are very common and can be life threatening if not treated properly. The causative agent of tuberculosis is the slow-growing bacilli *Mycobacterium tuberculosis*, while the causative agent of influenza is a segmented genome RNA virus. Pneumonia can be caused by a number of different microorganisms like bacteria, virus, and mycoplasma. In case of the entry of a pathogen in our body, the immune system gets activated, and the phagocytic cells try to eliminate it by generating reactive oxygen and nitrogen species (ROS and RNS) inside the phagosome. These reactive species or respiratory bursts are sufficient to eliminate most of the pathogens, except a few. *M. tuberculosis* is one such microorganism that has evolved mechanisms to escape this respiratory burst-mediated killing and thus survive and grow inside the macrophages. Infection with *M. tuberculosis* leads to the destruction of macrophages and release of cytokines, which lead to prolonged immune activation and oxidative stress. In some cases, the bacilli remain dormant inside macrophages for a long time. Flu viruses infect the epithelial cells present in respiratory tract, and the infection site is dependent on the hemagglutinin protein present on their capsid. Destruction of epithelial cells promotes secretion of mucus and activation of immune system leading to the oxidative damage. Community-acquired pneumonia is more serious and difficult to treat. In all

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these infections, ROS/RNS are developed as a defense mechanism against the pathogen. Persistence of the pathogen for a long time would lead to the uncontrolled production of ROS/RNS which will lead to oxidative stress and tissue damage to the host. Administration of antioxidants along with conventional treatments can be useful in the elimination of the reactive oxygen and nitrogen species.

Keywords
Tuberculosis · Influenza · Pneumonia · Macrophage · Neutrophil · Reactive oxygen species · Reactive nitrogen species · Respiratory burst · Oxidative stress · Antioxidants

7.1 Introduction

Respiratory infectious diseases are common problems among children and older people worldwide especially in developing countries. Nearly 20% of mortality in children under the age of 5 years is caused by these diseases. Most respiratory tract infections, especially the upper respiratory tract ones like rhinorrhea or pharyngitis, are mild and not incapacitating. However, lower respiratory tract infections like tuberculosis, flu, pneumonia, etc. can be more severe (Fitzpatrick et al. 2014). They are more likely to cause acute onset of fever, cough, dyspnea, or chest pain. Patients with respiratory infections should be diagnosed based on the disease features to expedite specific diagnosis and treatment.

This chapter is on three lower respiratory tract infections, viz., tuberculosis, influenza, and pneumonia, endogenous oxidative stress as a self-defense mechanism, and role of antioxidants on the infections.

Tuberculosis, an ancient infectious disease, can infect people of all age and almost every part of the human body. Tubercular lesions have been found on Neolithic skeletons (4500 BC) and on the bones of Egyptian mummies (1000 BC). The World Health Organization (WHO) declared that tuberculosis is among the top 10 causes of death worldwide, killing 1.6 million people in 2017 (Tuberculosis (TB) 2018). Among the worst affected countries are China, Southeast Asia, and the Indian subcontinent. The disease is most common in developing countries though the Centers for Disease Control and Prevention (CDC) reported over 9000 cases in the United States in 2016. Immunosuppression by stress or other illness, malnutrition, and poor hygienic condition activates the occurrence of tuberculosis (Sia et al. 2015).

Influenza, an acute respiratory infection, occurs in both pandemic and interpandemic forms. Over the past 100 years, global influenza pandemics have occurred approximately in every 10–30 years, whereas epidemic influenza occurs every winter in the temperate zones of both the hemispheres. Seasonal flu can be changed to acute respiratory illness with high mortality rate if converted to pandemic. The pathogenic H5N1 and H7N9 strains are currently in circulation and are a cause of great concern (Cline et al. 2017). Senior people, young children, and people with

long-term lung diseases are more prone to serious complications from the flu. Flu can make asthma symptoms worse and can increase the risk of COPD flare-up.

Pneumonia can be caused by bacteria, viruses, and fungi or from inhalation of a chemical. Pneumonia can be life threatening and a leading cause of death and hospitalization in elder people and in people with other chronic diseases. People older than 65 or younger than 2 years of age or already having health problems are susceptible to pneumonia (Metersky et al. 2012).

7.2 Cells in Alveoli

There are three major types of cells present in the alveoli, viz., alveolar cells or pneumocytes and type I and II and alveolar macrophages (Jones, radiopaedia.org).

Type I Alveolar Cells or Pneumocytes are thin and flat and form the structure of alveoli. Type I cells take a major role in the gas exchange process between the alveoli and blood circulation. They are unable to replicate and sensitive to toxins. *Type II alveolar cells* are few in number (< 5%), and they are mostly found at the blood-air barrier and secrete surfactants. Type II cells play a major role to protect the lung in case of damage. They undergo cellular division, giving rise to more type I and II alveolar cells, without which the alveoli would collapse. *Alveolar macrophages* or the dust cells are very important in providing the lungs protection against microorganisms. They not only engulf the microorganisms, but they also scavenge any foreign molecules like dust, carbon particles, and molecules developed from blood cell injuries. There are three types of macrophages found in lungs. Alveolar macrophages or AMΦs are found within the airways and guard the alveolar cells in bronchi, alveoli. The interstitial macrophages (IMΦs) are found in lung interstitium, while the exudate-derived macrophages, EMΦs, are recruited in response to an inflammation. These three populations are distinguishable from each other morphologically and functionally (https://epi.publichealth.nc.gov/cd/diseases/respiratory.html).

7.3 Role of Macrophages and Neutrophils in Combating Infections

The major phagocytic cells of our immune system are neutrophils, dendritic cells, and macrophages (Silva Manuel and Correia 2012) (Fig. 7.1). The phagocytic cells exhibit their microbicidal properties by generating huge amount of ROS and RNS within the phagosomes (Fang 2011). This metabolic process or respiratory burst is most prominent in neutrophils as compared to other phagocytic cells and causes oxidative stress. Respiratory burst initiates with the assembly of the components of an enzyme complex called NADPH oxidase (Groemping and Rittinger 2005) present on the plasma membrane and on the membranes of phagosomes in an inactive form. Upon phagocytosis of any molecule, assembly of the components leads to the formation of an active enzyme complex. This complex transfers one electron to O_2

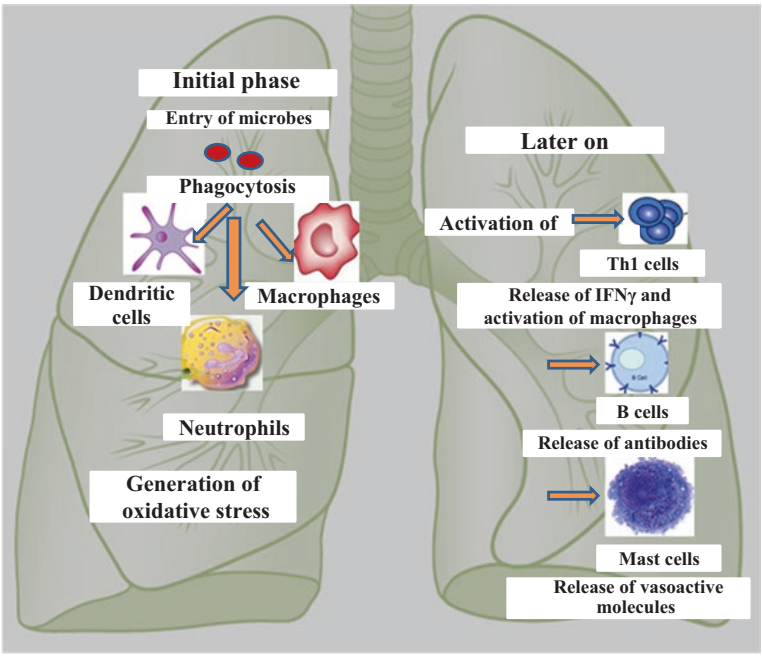

Fig. 7.1 Immune cells involved in fighting foreign invaders

from NADPH and forms superoxide radical ($O_2^{\cdot -}$) within the phagosomal lumen (Peterhans 1997). The superoxides can undergo spontaneous or enzyme-catalyzed dismutation to yield H_2O_2 and O_2. H_2O_2 combines with halides in presence of the enzyme myeloperoxidase and produces reactive hypohalous acid. These superoxide radicals may also interact with NO to produce peroxynitrite, ONOO-. These ROS/RNS can damage a variety of biomolecules and kill the ingested microorganism (Halliwell and Aruoma 1991). They form adducts with unsaturated bonds and clusters having less electrons. Thus they can damage enzymes and unsaturated fatty acids or can oxidize 4Fe-4S clusters present in enzymes or proteins resulting in metabolic defects. Damaged Fe-S clusters can release Fe, which will react with H_2O_2 (Fenton reaction) and yield $^{\cdot}OH$ leading to more damage. The cysteine residues present in proteins and enzymes can also interact with H_2O_2 directly. Superoxides do not damage membranes in bacteria, as they lack polyunsaturated fatty acids (Fig. 7.2).

Acidification of phagosomes is required for the optimal function of hydrolytic enzymes and thus for the degradation of the internalized pathogen. The acidification process is achieved by the recruitment of vacuolar proton-ATPase to the phagosomal membrane at the early phase of phagosome maturation. Recently it has been shown that TRPM2, the redox-sensitive transient receptor potential cation channel, expressed on the membrane of phagosome regulates the acidification and is essential for the efflux of Ca^{2+} during phagosome maturation (Di et al. 2017).

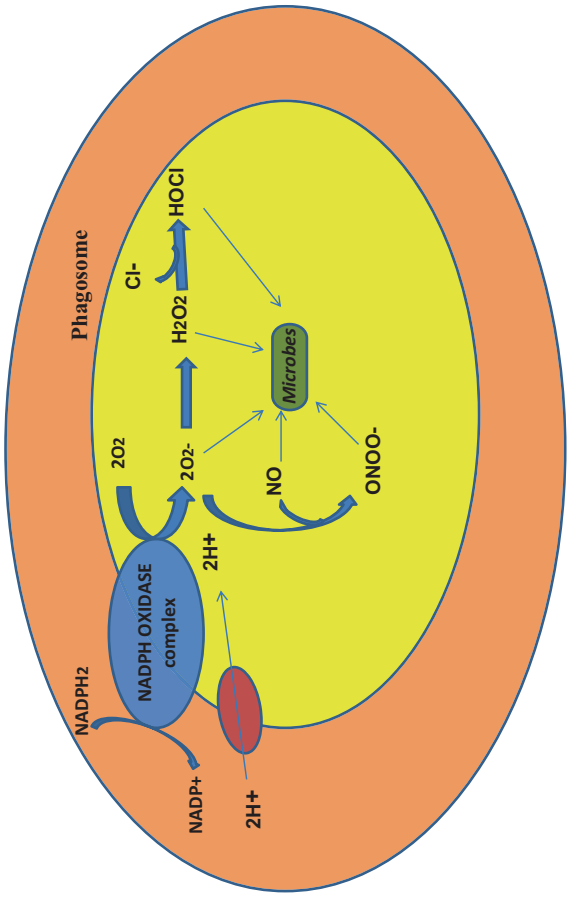

Fig. 7.2 Respiratory burst inside macrophage/Dendritic cell

The antimicrobial capacity of neutrophils is higher than that of macrophages as their cytoplasm contains granules full of multiple antimicrobial molecules and peptides like defensins, cathelicidins, and proteins like lactoferrin and bactericidal/permeability-increasing protein (Gudmundsson and Agerberth 1999).

7.4 Effect of Excess ROS/RNS on Human Health

The huge amounts of ROS generated as a defense mechanism against foreign molecules, if not removed adequately, will cause toxicity to all types of cells. It induces lipid peroxidation of host cell components, leading to the rise in intracellular calcium ions and DNA damage (Podinovskaia et al. 2013) (Fig. 7.3). Low levels of ROS influence the growth of some viruses like influenza, paramyxoviruses, HIV, etc. Chronic OS have been found to be associated with the development of cancer in case of some viral infections (Southwick 2007). Hence, antioxidants can play a major role in the treatment of viral diseases along by conventional drugs.

7.5 Tuberculosis

The highly aerobic and slow-growing microorganism *Mycobacterium tuberculosis* (Mtb) was discovered in 1882 by scientist Robert Koch. It can be identified with acid-fast stains or fluorescent stains under a microscope. The Mtb genome was sequenced in 1998 (Smith 2003) and has been found to have more than 99.9%

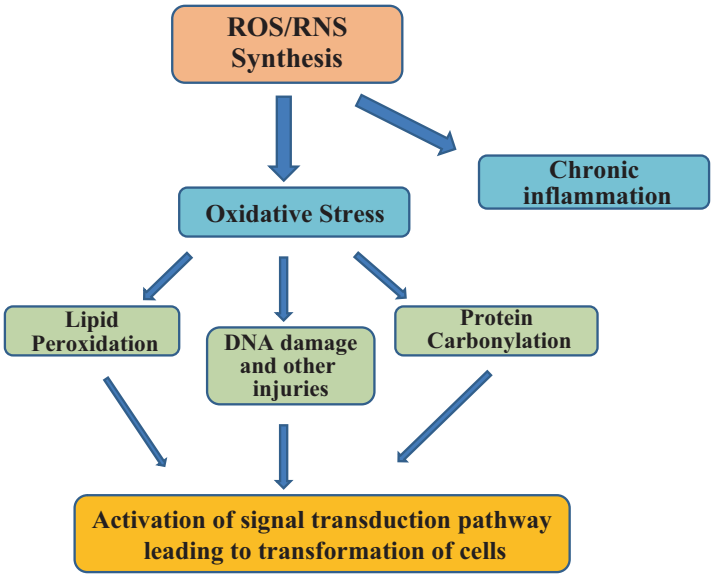

Fig. 7.3 Effect of oxidative stress on the host

similarity with many other strains of *Mycobacterium*. The ability of Mtb to remain dormant in the host with the capacity to revive at a later time is because of its anaerobic persistency (Rook and Hernandez-Pando 1996).

7.5.1 Symptoms of Tuberculosis

Symptoms of tuberculosis are characterized by cough for 3 weeks or more, coughing up cloudy mucus, sometimes with blood, loss of weight, loss of appetite, weakness, fever, night sweats, sometimes shortness of breath, and chest pain (Tuberculosis (TB) 2018).

7.5.2 Pathophysiology of Tuberculosis

As discussed in the general section, macrophages kill Mtb by producing ROS/RNS in an oxygen-dependent manner. However, the bacterium has evolved mechanisms to survive inside the macrophages (Rook and Hernandez-Pando 1996). Depending on the nature and site of occurrence, TB is named differently.

Pulmonary Tuberculosis is characterized by the infection of the lung tissue by Mtb. The ROS/RNS produced by phagocytic cells restrict mycobacterial dissemination. A tuberculous lesion, called granuloma, is formed to confine the organism at the center where macrophages infected with Mtb are surrounded with noninfected phagocytes and T lymphocytes (Gengenbacher and Kaufmann 2012). Release of cytokines attracts more cells in the region, and sometimes macrophages fuse together to form multinucleated cells. In severe cases, the cellular granulomas are converted to necrotizing granulomas and lead to pulmonary lesions with extensive tissue damage. The core of the granuloma, called caseum, contains dead host cells and the bacilli (Gutsmann 2016).

Extrapulmonary Tuberculosis occurs because of the spreading of the infection outside the lungs to pleura, central nervous system, lymphatic system, genitourinary system, bone, joints, and other tissues. Mostly young people and people with weakened immune system are susceptible to this kind of spreading (Lee 2015).

Active Tuberculosis is characterized by TB symptoms and highly contagious. Hence, proper treatment should be followed by people suffering from active tuberculosis.

Latent Tuberculosis is characterized by the latency of Mtb inside the host. Most of the infected individuals with Mtb inside their macrophages don't develop the

disease. Occurrence of the disease is dependent on two parameters, the virulence of the pathogen and the immune resistance of the host. The latent Mtb can become active at any time and needs proper treatment to cure the disease (Hauck et al. 2009).

7.5.3 Entry of *M. tuberculosis* into Macrophages

There are many molecules on the macrophage surface which act as receptors for Mtb like receptors for complement factors, carbohydrate moieties, Fc region of antibodies, etc. and intercellular adhesion molecules (Ernst 1998). The survival chance of Mtb depends on the receptor involved in phagocytosis. The entry of Mtb coated with specific antibodies and through receptors for antibody Fc regions would result in activation of respiratory burst in macrophages, whereas CR3 receptor-mediated entry doesn't lead to the activation of OS and thus increases the chance of survival of the bacterium (Caron and Hall 1998). Host plasma membrane cholesterol (Gatfield and Pieters 2000) has important roles to play in the entry of Mtb through phagocytosis and for the prevention of phagosome-lysosome fusion.

7.5.4 Mechanisms by Which *M. tuberculosis* Escape the Macrophage-Mediated Killing

- A macrophage membrane component, phosphatidylinositol 3-phosphate (PI3P), plays an important role in the phagosome maturation process. In Mtb-infected macrophages, Mtb glycolipids can block PI3P synthesis and thus interfere with phagosome-lysosome fusion and escape lysosome-mediated killing (Vergne et al. 2005).
- Another strategy is to prevent accumulation of PI3P on the membrane of phagosomes by degrading it by an acid phosphatase called SapM, a 28KDa protein, secreted by Mtb within the host cell cytosol (Saleh and Belisle 2000).
- Two protein phosphatases of Mtb, PtpA and B, interfere with host protein trafficking processes and increase the chance of survival for Mtb (Bach et al. 2008).
- PknG, another protein kinase of Mtb, prevents the degradation of Mtb in lysosomes (Scherr et al. 2007).
- The Mtb internalized phagosome is prevented from fusing with lysosomes by host protein coronin1/TACO and calcineurin and thereby helps in the survival of Mtb (Jayachandran et al. 2007).
- The proteasomes of Mtb neutralize the effect of NO and thus escape RNS-mediated killing. In addition, the induction of the antimicrobial peptide cathelicidin (LL-37) is done through TLR signaling, and the process is vitamin D dependent (Fabri et al. 2011).
- Mtb produces several proteins/enzymes that are responsible for removal or detoxification of ROS and RNS. One such protein, KatG, a catalase peroxidase, destroys hydrogen peroxide. An extracellular Mn-Fe SOD (SodA) converts superoxide radical to O_2 and H_2O_2. Another Cu-Zn SOD (SodC) remains bound

to the outer membrane of Mtb and possibly protects the surface against extracellular superoxides generated by host cells (Piddington et al. 2001).

- A genome-wide interaction study performed by Nambi et al. predicted the functional relationships among three proteins, the superoxide-detoxifying enzyme (SodA), an integral membrane protein (DoxX), and possibly a thiol oxidoreductase (SseA). This oxidoreductase complex helps Mtb to survive inside the host (Nambi et al. 2015).

7.5.5 Oxygen-Independent Clearance of *M. tuberculosis*

Upon entry into the system, Mtb are challenged to lung epithelial cells and macrophages (Rivas-Santiago et al. 2008). Infected bronchial epithelial cells produce defense molecules such as β-defensins, which directly kill the microbes by forming pores on their surface. In vitro infection of the lung epithelial cell line A549 (pneumocytes type II) with Mtb H37Rv induces the production of human beta defensin 2 (hBD-2) which is associated with mycobacterial lysis (Rivas-Santiago et al. 2005) Murine beta defensins 2 and 4 are expressed by the murine bronchial epithelium cells during the early phase of progressive pulmonary tuberculosis infection.

7.5.6 Role of Neutrophils in Combating Mycobacterial Infections

It has been found through studies that neutrophils are not very much engaged in combating Mtb but may play an important role by producing important cytokines and chemokines. Blomgran and Ernst showed that use of neutrophil-specific antibody (Ly6G-specific Ab 1A8) selectively depleted neutrophils and increased the frequency of dendritic cells (DC) in the lungs. They have concluded that neutrophils infected with Mtb secrete factors that attract DCs to facilitate interactions among these cells in the lungs (Blomgran and Ernst 2011).

7.5.7 Role of Dendritic Cells Against *M. tuberculosis*

There are controversies regarding the role of dendritic cells against Mtb. Some studies showed that dendritic cells, after phagocytosis of the pathogens, activate the T cells. Other reports showed that Mtb inhibits DC maturation and antigen presentation to T cells. Adane Mihret reported that infection of monocyte-derived dendritic cells (MDDCs) with Mtb leads to upregulation of major histocompatibility complex (MHC) proteins and other surface proteins like CD40, CD54, CD58, and CD80 which lead to effective presentation of the antigen to the T cells (Mihret 2012).

7.5.8 Tuberculosis and Oxidative Stress

It is evident from numerous studies that changes in redox status of cells result in host cellular responses that will lead to proliferation or apoptosis or cell necrosis or mutation (Choi et al. 2009). This results in the damage of cell membrane resulting in fibrosis and dysfunction of lungs in pulmonary TB. Vijayamalini M et al. reported significant increase in lipid peroxidation marker, thiobarbituric acid reactive substance (TBARS), in pulmonary tuberculosis patients, both newly diagnosed and untreated, as compared to controls. The levels of antioxidants in plasma were found to decrease leading to the pathogenesis and severity of the disease (Vijayamalini and Manoharan 2004).

Generation of ROS increases in TB patients coinfected with HIV. Rajopadhye et al. reported decreased NO levels in serum of TB patients, while no change was observed in patients having both HIV and TB (Rajopadhye et al. 2017). There were increased levels of TBARS and CRP in both groups though increase in superoxide anion was found in HIV-TB group. Increased ROS production by the tat protein of HIV might activate NF-κB (Fiume et al. 2012) and lead to the increased rate of transcription in HIV. The activities of catalase and SOD were found to decrease in TB group. Increased SOD activity and total serum protein concentration were found in both groups compared to control. Power et al. reported in their study that antioxidant supplementation along with antituberculosis treatment (ATT) for 6 months significantly decreased the MDA level and increased the levels of vitamin C and E and total antioxidant status. Hence, their study concludes that supplementation of micronutrients or antioxidants promotes recovery of patients from tuberculosis by reducing OS (Pawar et al. 2011). Another work conducted by Janiszewska and group also reported that MDA concentration in the plasma of pulmonary tuberculosis patients was significantly higher before and after 1 and 2 months of antioxidant treatment with ATT compared to the control group which received only ATT (Janiszewska-Drobinska et al. 2001). Wagh et al. found in their study that the levels of prooxidants were significantly increased, whereas antioxidant markers were significantly decreased in the TB population compared to healthy controls (Wagh et al. 2016). In another experiment, the authors measured the OS markers and antioxidant capacity in granulomatous lesions in BCG-vaccinated and non-vaccinated guinea pigs infected with Mtb (Palanisamy et al. 2011). They observed decreased serum total antioxidant capacity and increased MDA concentration within lesions in non-vaccinated guinea pigs within 2 weeks of infection which were partially restored by the treatment of N-acetyl cysteine. In addition, the antioxidant also decreased Mtb counts in the spleen and severity of lesion necrosis in the lung and spleen. Hence, proper antioxidants might be used as an adjunct therapy along with conventional ATT for better treatment and prevention of tuberculosis.

7.5.9 Diagnosis of TB

Diagnosis of TB includes detection of presence of Mtb like Mantoux test (Nayak and Acharjya 2012), sputum smear test (Desikan 2013), and chest X-ray. New sensitive and rapid techniques include microscopic examination, microcolony detection, lipid profile analysis by HPLC, SDS-PAGE profiling to identify different strains of Mtb (Fatima 2009), amplification of the 16S–23S rDNA spacer region, etc.

7.5.10 Antituberculosis Treatment (ATT)

7.5.10.1 Chemotherapy

ATT includes first line of drugs like isoniazid, pyrazinamide, and ethambutol and drugs like fluoroquinolones, capreomycin, kanamycin, and amikacin. The second-line drugs include thioamides, cycloserine, and aminosalicylic acid. Other second-line drugs are clofazimine, amoxicillin with clavulanate, linezolid, carbapenems, etc. (Guidelines for treatment of tuberculosis 2010). Mismanagement of TB treatment can lead to resistance against multiple drugs called multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB).

To eliminate tuberculosis, WHO had introduced the Directly Observed Treatment Short (DOTS) course strategy which involved health workers to keep in touch with the TB patients for proper consumption of ATT drugs. In a report published in 1996, WHO claimed that DOTS was remarkably effective in some places, whereas it failed in sub-Saharan Africa (http://www.searo.who.int/tb/topics/what_dots/en/).

7.5.10.2 Administration of Antioxidants Along with ATT

It has been found by many researchers that the levels of antioxidants significantly lowered down in TB compared to control. The levels again rise during and after treatment of TB (Wiid et al. 2004). Reddy et al. supported the use of antioxidant supplementation for better improvement as an adjuvant therapy along with ATT (Reddy et al. 2004). Lamsal et al. observed similar results while treated TB patients with micronutrients along with ATT. Micronutrient treatment reduced the oxidant levels and increased antioxidant status (Lamsal et al. 2007). The supplementation also increased the production of IL-2 leading to the increased proliferation of T cells and decreased production of prostaglandin-2, T-cell suppressor. Kowalski et al. observed similar results in patients taking vitamins C and E (after 1-month ATT) (Kowalski et al. 2004). Studies had showed that there exists a potential supplemental role for micronutrient and other antioxidants in management of both drug-sensitive and drug-resistant tuberculosis. The antioxidants may also have beneficial role in prevention and treatment of drug toxicity, particularly hepatotoxicity caused due to antitubercular drugs (Pawar et al. 2011; Verma et al. 2014).

7.5.10.3 Prevention

A live vaccine is used for a long time which was derived from an attenuated strain of *Mycobacterium bovis* called the bacille Calmette-Guérin (BCG) vaccine (www.who.int/tb/challenges/hiv/07_tb_prevention_diagnosis_and_treatment_eng.pdf).

7.6 Influenza

There are four subtypes of influenza viruses, A, B, C, and D, which are very similar in overall structure. The common type, influenza A virus (IAV), infects aquatic birds, poultry animals, and humans. Influenza viruses have seven or eight pieces of –ve sense RNA as their genome which code for total 11 proteins present in the virus. HA, NA, and M2 are present on the envelope. There are about 18 HA and 11 NA subtypes known, and all possible combinations are found in wild aquatic birds though HA 1, 2, and 3 and NA 1 and 2 are commonly found in humans.

7.6.1 Symptoms

Symptoms of flu include fever, cold sweats and shivers, headache, aching joints and limbs, fatigue, feeling of exhaustion, and gastrointestinal symptoms like nausea, vomiting, and diarrhea (http://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal)).

7.6.2 Pathophysiology of Influenza

Pathophysiology of influenza depends on the virulence of the flu virus and on host immune responses. Influenza virus can infect epithelial cells present both in upper and lower respiratory tract depending on the nature of the HA present on the capsid. In vivo studies showed that different IAV subtypes infect and replicate differently in the lung and thus result in different outcome (Taubenberger and Morens 2008). In most cases of mild and avirulent viruses, the HA is cleaved from the viral envelope by proteases in the upper respiratory tract, and these viruses mainly infect the nose, throat, and mouth. Hence, these strains are easily transmitted among people through sneezing and coughing. The HA of strains like H5N1 can be cleaved by a variety of proteases, allowing the virus to spread throughout the body. These strains cause severe flu but are not easily spread among people. The infected cells secrete huge amount of proinflammatory cytokines and chemokines that lead to inflammation. It is believed that the virus inhibits adrenocorticotropic hormone resulting in lowered cortisol levels (Taubenberger and Morens 2008). Coinfection of a susceptible cell by two IAVs at the same time can lead to antigenic shift where viral RNAs are mixed and a new shift virus strain can be generated.

7.6.3 Role of Macrophages in Removal of IAV

IAV infects mainly the epithelial cells and alveolar macrophages (MΦs) in the respiratory tract (Nicol and Dutia 2014). Tate et al. showed that viruses that readily infect MΦs in vitro were less pathogenic in vivo than viruses with a limited ability to infect MΦs (Tate Michelle et al. 2010). It has also been found that infection with IAV strains that replicate productively in MΦs can induce many changes in M2 MΦs, causing them to phenotypically resemble proinflammatory M1 MΦs. Expression rate of the antibody Fc receptors (CD16 and CD32) present on MΦs surface and responsible for opsonization has been found to decrease in IAV-infected cells (Cline et al. 2017).

Depletion of AMΦs in mice and pigs using clodronate (released inside the MΦs after phagocytosis and kills the cells)-loaded liposomes resulted in higher viral loads proving major role for MΦs in controlling disease severity (Duan et al. 2017). Recent studies have shown that commensal microflora play an important role in the activation of pulmonary dendritic cells and induce specific immune responses against influenza virus (Samuelson et al. 2015).

7.6.4 Role of Neutrophils on Influenza Virus

Presence and increase in number of neutrophils in the airways is correlated with suppressed virus replication after flu infection (Camp and Jonsson 2017). Tate et al. established the critical role of neutrophils in the clearance of influenza virus by the use of specific monoclonal antibody (RB6-8C5) against them. Neutrophil depletion leads to increased number of virus particles and increased morbidity and mortality compared to control mice (Tate Michelle et al. 2008). Tumpey et al. established the role of macrophages and neutrophils in early-phase protection against an infection with a recombinant human influenza virus (H1N1) (Tumpey 2005). It can be inferred that neutrophils contributed to early protection against low inoculum of the virus but not against the high inoculums indicating a limitation of PMN function. Haruo Fujisawa showed that neutrophils provide protection against influenza viruses in tumor-bearing mice with neutrophilic leukocytosis (Fujisawa 2008). In vitro multiplication of influenza virus was also prevented by neutrophils from both normal and tumor-bearing mice. Addition of *N*-formylmethionyl-leucyl-phenylalanine (fMLP), a chemotactic factor and macrophage activator, to the culture, increased the inhibitory effect of neutrophils. Virus infectivity was found to decrease in the initial phase after infection in ICR and BALB/c mice though no such decrease was observed in tumor-bearing C57BU6 mice. Administration of fMLP inhibited the virus propagation in the lungs of normal and tumor-bearing mice having intranasal IAV infection. Hence, neutrophils play a significant role against IAV infection in vivo.

7.6.5 Influenza and Oxidative Stress

High levels of proinflammatory cytokines, e.g., TNF-α, IFN-β, CCL5, MIP-1α (CCL3), MIP-1β (CCL4), MCP (CCL2), and IP-10 (CXCL10), have been found to be released by macrophages in humans infected with the highly pathogenic strain, H5N1 (Peschke et al. 1993). Infection with seasonal IAV also results in production of cytokines and chemokines from macrophages. The number of MΦs increases in the lung in case of an IAV infection and returns to regular numbers following the resolution of the infection. Yu-Hsiang Lee et al. compared the immune responses induced by two strains of influenza virus, A/WSN/33 (H1N1) and A/Panama-like (H3N2) in C57BL/6 mice. In contrast to A/Panama-like (H3N2), WSN virus induced severe lung pathology accompanied by massive Gr-1$^+$ and CD11b$^+$ cell infiltration and high levels of CXCL6/GCP-2, CCL2/MCP-1, and TIMP-1 production. Both the Gr-1$^+$ and CD11b$^+$ cells produced ROS and RNS in lungs (Lee and Huang 2017). The level of xanthine oxidase, an enzyme synthesizing O^{2-}, was found to increase in lung homogenates, and cells lavaged out of the lung showed a marked increase in O^{2-} production when stimulated with phorbol myristate acetate (PMA). In another model, Oda et al. showed that intravenously injected pyran copolymer-conjugated superoxide dismutase protected mice from the lethal effect of IAV (Oda et al. 1989). In addition to ROS, NO seems to play a role in the pathogenesis of IAV (Akaike et al. 2003).

Increased ROS production may contribute to an increase in the influenza virus titer (Belding et al. 1970). The reason lies on the effect of ROS on protease inhibitors present in the lung surfactant. The HA protein must be cleaved intracellularly into the dipeptides HA1 and HA2 for the propagation of the disease. Influenza virus strains that possess a HA that fits optimally with the intracellular protease is released into the extracellular space in the HA1/HA2 form, which is infectious. The HA that fits less optimally with the protease is released mostly in the noninfectious HA0 form (Kido et al. 1993). Cleavage of HA0 can still take place by the extracellular proteases present in pulmonary surfactant. As a protective mechanism, surface of alveoli carries antiproteases. These antiproteases can be inactivated by ROS. Hence, ROS generation can result into manyfold increase in infectious virus particles. A group of workers reported that intracellular ROS levels decrease in phagocytes after influenza infection. This decrease in ROS leads to increased susceptibility to secondary infections by bacteria like *S. aureus* (Sun et al. 2016a).

7.6.6 Diagnosis

Diagnosis of flu includes rapid influenza diagnostic test and rapid molecular assays (George 2012).

7.6.7 Treatment

The two types of anti-influenza drugs used are inhibitors of neuraminidase (oseltamivir, zanamivir, laninamivir, and peramivir) and M2 protein (adamantane derivatives) (Boltz et al. 2010).

7.6.8 Administration of Antioxidants Along with Conventional Treatment

In a study Xu and Liu showed that treatment with curcumin after IAV infection downregulated productions of cytokines in a dose-dependent manner and inhibiting NF-κB signaling pathway (Xu and Liu 2017).

7.6.9 Prevention

The available trivalent vaccine protects against H1N1 and H3N2 and an influenza B virus. Another quadrivalent vaccine, available in market, provides protection against four flu viruses including an additional B virus.

7.7 Pneumonia

Bacterial Pneumonia is the most occurring pneumonia in all age groups. The common causative agent of community-acquired pneumonia (CAP) is the Gram+ve bacterium *Streptococcus pneumoniae*. Other Gram+ve organisms that cause pneumonia are *Staphylococcus aureus* and *Bacillus anthracis*. Among Gram-ve bacteria, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bordetella pertussis*, and *Moraxella catarrhalis* are the frequent causative agents of pneumonia. Bacteria like *Coxiella burnetii*, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella pneumophila* also have been found to cause pneumonia. *Yersinia pestis*-caused pneumonia is usually called pneumonic plague. Nowadays drug-resistant *Streptococcus pneumoniae* (DRSP) and methicillin-resistant *Staphylococcus aureus* (MRSA) have become quite common (Nair and Niederman 2011).

Viral Pneumonia is caused by a number of viruses like rhinoviruses, coronaviruses, influenza viruses, respiratory syncytial viruses (RSVs), etc. Among them, RSVs are known to cause lung and airway infections in infants and young children more frequently. It is an enveloped virus with single-stranded RNA genome and belongs to the *Pneumoviridae* family of the *Mononegavirales* order (Dawson-Caswell and Muncie Jr. 2011).

Mycoplasma Pneumonia is the smallest and simplest self-limiting bacteria that cause mild upper respiratory tract infection to severe atypical pneumonia. *M. pneumonia* belongs to the class *Mollicutes* and family *Mycoplasmataceae*. They do not have cell wall and thus distinguished from bacteria. *M. pneumoniae* infections are transmitted via aerosols. Dorigo-Zetsma et al. genotyped and grouped clinical isolates of *M. pneumoniae* into eight subtypes based on P1 adhesin molecules (Dorigo-Zetsma et al. 2000).

7.7.1 Symptoms

include cough sometimes with greenish or yellow mucus or bloody mucus, fever, shortness of breath, chest pain, headache, sweating, loss of appetite, low energy, and fatigue.

7.7.2 Pathophysiology of Pneumonia

Neutrophils remove the microorganisms and release cytokines that result in the activation of the immune system resulting in common symptoms like fever, chills, and fatigue. Sometimes elevated immune response leads to leakage in the blood capillaries in the lungs leading to plasma seepage into the alveoli resulting in a less functional area for gas exchange. Mucus plugs are released from the leaky capillaries into blood. WBCs accumulate in the lungs to clear the plugs, and eventually cell debris accumulate in the alveoli and make it solid, called consolidation, a feature of bacterial pneumonia. Unremoved bacteria can travel to the pleural cavity and blood stream from lungs and can result in sepsis and eventually septic shock, leading to damage in multiple organs (Eddy 2005).

7.7.3 Macrophages Against Bacterial Pneumonia

AMΦs provide initial protection against bacterial pneumonia. However, when AMΦs fail, neutrophils are predominantly recruited to control the infection (Dockrell et al. 2003). Aberdein et al. have shown that situations which lead to generation of less numbers of AMΦs are major causes of development of pneumonia (Aberdein et al. 2013). *S. pneumoniae* is not killed by ROS as the bacterium has developed several mechanisms to escape OS-mediated killing. They are eliminated by NO and peroxynitrite, which are produced by macrophages in response to pneumococcal cell wall and the toxin pneumolysin (MacMicking et al. 1997).

7.7.4 Neutrophils Against Bacterial Pneumonia

It is known for a long time that lower respiratory tract bacterial infection causes appearance of morphologically immature neutrophils in the circulation resulting increased synthesis and release of neutrophils from the marrow. Experiments have proved that selective depletion of the neutrophils results in profound defects in the clearance of bacteria like *S. pneumoniae*, *K. pneumoniae*, and *L. pneumophila* from the lungs. Neutrophils secrete serine proteases like cathepsin G and elastase which mediate the effective killing of ingested *S. pneumoniae* (Marriott et al. 2008). Investigations have also shown that efficient neutrophil accumulation is important to induce a successful adaptive immune response in the host.

7.7.5 Macrophages Against RSV

The role of alveolar macrophages was studied by Philippa and coworkers in acute respiratory RSV infection by depleting macrophages by the intranasal administration of clodronate liposomes in an established mouse model (Pribul Philippa et al. 2008). The decreased concentrations of local inflammatory cytokines and chemokines, less number of macrophages and natural killer cells, and enhanced viral load in the lung proved that macrophages play a central role in the early responses to viral infection. However, macrophages have been found to have little effect on the adaptive response as there was no change in the number of T cells.

7.7.6 Role of Neutrophils in RSV Infection

Neutrophils play a major role in RSV infection and lead to a strong systemic immune response. They have been found to produce the enzyme elastase and express activation markers like CD11b, CD18, and CD54 on their surface in response to RSV. The amount of neutrophil response is dependent on the clinical severity and viral load. The presence of viral genome and mRNA inside neutrophils suggests phagocytosis or replication of virions within neutrophils (Stokes et al. 2013).

7.7.7 Immune Cells Against Mycoplasma

Neutrophils isolated from the airway infected with mycoplasma have been found to contain high amount of histamine compared to naive neutrophils. Mycoplasma has been found to directly stimulate the expression of mRNA encoding histidine decarboxylase, an enzyme required in histamine synthesis in vitro. Treatment with antihistamines in in vivo showed decreased severity of pneumonia and tracheobronchitis in infected mice (Xu et al. 2006). Mast cells also lead to allergic inflammation by the release of histamine in mycoplasma infection. A toxin called

community-acquired respiratory distress syndrome toxin (CARDS) produced by *M. pneumonia* promotes the generation of functional IgE in mice. Thus *M. pneumoniae*-induced infections are strongly associated with asthma and its exacerbations (Medina et al. 2017).

7.7.8 Community-Acquired Pneumonia (CAP) and Oxidative Stress

Oxidative stress plays a crucial role in the development and progression of community-acquired pneumonia, the most common infectious illness. It was revealed that five times higher H_2O_2 is released in exhaled air of CAP patients than control and the amount decreases with treatment. The authors suggested that the sources of H_2O_2 were activated leucocytes, monocytes, and macrophages, and development of OS leads to the activation of neutrophils and other effector cells with generation of excess active oxygen forms in the lungs of CAP patients (Majewska et al. 2004). These ROS migrate through the alveolar-capillary membrane in the process of gas exchange and are able to induce the OS development in the erythrocytes (Ugurlu 2016). Trefler in their work showed increased levels of TBARS in CAP caused by bacteria compared to control. However, they observed lower TBARS levels and increased glutathione redox system in viral CAP caused by H1N1 compared to normal (Trefler et al. 2014). In another study, Vilen et al. showed that there was significant increase in advanced oxidation protein products (AOPP) and MDA in blood plasma of CAP patients compared to control group though there was no change in the concentration of reactive protein carbonyl derivatives (Molotov-Luchanskiy et al. 2015). Muravlyova showed that parameters like erythrocyte aggregation, total oxidant status, and OS index increased in IIP patients than controls, whereas some parameters like erythrocyte deformability, PV, and total antioxidant status remain unaltered (Muravlyova et al. 2016). It has been found that postinfluenza *Staphylococcus aureus* pneumonia leads to extensive lung inflammation even after antibiotic treatment. The chronic granulomatous diseases in humans are linked to X chromosome-linked Nox2 expression (Sun et al. 2016b). Yuanyuan Chen et al. demonstrated increase in OS and cytokines like TNF-α and IL-6 in the lung and peripheral blood with increase in the severity of CAP. Vitamin C supplementation inhibited ROS, DNA damage, TNF-α, and IL-6 in LPS-stimulated macrophages. It also inhibited autophagy in MH-S cells exposed to LPS and H_2O_2 (Chen et al. 2014). Rodrigo et al. observed lower FRAP and the GSH/GSSG ratio and increased lipid peroxidation in both plasma and erythrocytes in CAP than control values. Thus the antioxidant status alterations are correlated with clinical severity (Castillo et al. 2013). Many studies have showed that administration of antioxidants such as Vitamins E and C have positive effects on oxidative biomarkers in in vivo and in vitro models of CAP (Siempos et al. 2008). There are controversies regarding the use of vitamins in CAP though (Merchant et al. 2004).

7.7.9 Diagnosis

Diagnosis of pneumonia is based on physical examinations, chest radiography, CT scan, and laboratory tests. Laboratory tests include leukocyte count, sputum Gram stain, blood cultures, pulse oximetry, and urine antigens. Recent studies open up the possibility of using OS indicators as diagnostic tools for CAP severity as well as for the estimation of effectiveness of CAP treatment (Pavlyshyn et al. 2014).

7.7.10 Treatment

Treatment of pneumonia includes antibiotics to treat bacterial pneumonia and with fever and/or pain relievers (Donovan 2018).

7.7.11 Prevention

Prevention of pneumonia is provided by pneumococcal conjugate vaccines against *Streptococcus pneumoniae* which are effective in children. Development of new and improved vaccines and awareness among people would lead to further improvement in strategies for the prevention of CAP.

7.8 Conclusion

The common lower respiratory tract infectious diseases like tuberculosis, influenza, and pneumonia can be life threatening if not treated properly. The host mechanism to fight with the microorganisms is mainly by generating ROS and RNS by the immune cells. Chronic infections and persistence of microorganisms inside the host lead to uncontrolled activation of immune system and generation of reactive species, which eventually cause enormous damage to the host system. Several studies have showed that the deleterious effects of the oxidative stress can be fought with the administration of antioxidants along with regular treatment regimen.

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Role of MMPs and Oxidants in Lung Diseases

8

Nilanjana Deb, Sudipta Mallick, Ashish Jaiswal, Anirban Manna, Ulaganathan Mabalirajan, and Snehasikta Swarnakar

Abstract

The lung matrix consists of numerous extracellular matrix (ECM) proteins and glycoproteins including collagens, elastin, fibronectin, laminin, heparin, and sulfated proteoglycans. Matrix metalloproteinases (MMPs) play a pivotal role in the remodeling of ECM and is central in lung organogenesis. Although not all MMPs are found in the lung matrix, there is considerable evidence that few MMPs are up- and/or downregulated during acute and chronic diseases of the lungs. The association of alveolar ECM is related in chronic inflammatory lung diseases like idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis (CF) which have been investigated extensively. IPF is associated with production, deposition, and remodeling of the ECM, whereas COPD is characterized by a loss of the same. MMPs target the structural ECM proteins, cell adhesion molecules, growth factors, cytokines, and chemokines that play role in the genesis and development of chronic lung diseases. The association between MMPs and lung cancer has been long documented although the precise role of MMPs in lung cancer remains unanswered. Herein, we have discussed the role of MMPs along with oxidants in the pathogenesis of chronic lung diseases, cancer, and their potential as targets for therapy.

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MMPs · Idiopathic pulmonary fibrosis · COPD · Asthma · Cystic fibrosis · Lung cancer · Oxidant in lung diseases

8.1 Introduction

The key role of proteases in myriads of tissue functions is well understood, and 569 human proteases have been found so far in humans. The existence of numerous "degradome" proteases itself indicates their importance in cellular and tissue homeostasis. Though there are five major classes of proteases, (a) metallo, (b) serine, (c) cysteine, (d) threonine, and (e) aspartic acid, the metalloproteinase (MMP) class is the largest with many members. MMPs are zinc-dependent endopeptidases of the superfamily metzincins that are critical components during the cell response to their microenvironment. They have specific domain structures, consisting of a propeptide followed by a catalytic domain along with the addition of a hemopexin domain connected by a hinge region (MMP-1, MMP-3, MMP-8, MMP-11, MMP-12, MMP-13, MMP-18, MMP-19, MMP-20, MMP-21, MMP-27, and MMP-28). However, MMP-2 and MMP-9 have all these above features along with a fibronectin-like domain in between catalytic and hemopexin domain. MMP-14, MMP-15, MMP-16, and MMP-24 consist of a transmembrane region and a short cytoplasmic "tail," while MMP-17 and MMP-25 have a glycosylphosphatidyl anchor. MMP-7 and MMP-26 are the smallest member and known as matrilysins. However, MMP-23 is exceptional and has a unique cysteine-rich, proline-rich, and IL-1 receptor type II-like domains and might initially be anchored by an N-terminal transmembrane domain prior to propeptide cleaving. The propeptide domain contains a "cysteine switch" that interacts with the catalytic zinc domain in order to maintain inactivity until the propeptide has been removed by proteolysis. The catalytic domains have the zinc-binding motif with three histidine residues ligated to the zinc ion. Activation of the MMPs by propeptide removal leads to the production of active MMP that controls its function as well. In addition MMP activity is regulated by the tissue inhibitors of MMPs (TIMPs), namely, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (Baker et al. 2002). The TIMPs consist of two domains with six loop disulfide-bonded proteins that interact via their N-terminal loops with the active site cleft of the catalytic domain. The balance between MMPs and its endogenous inhibitors like TIMPs has also been found to be perturbed in many disease conditions. In general, it is believed that MMPs degrade, and in contrast, TIMPs deposit the ECM proteins in the process of organ remodeling. Other MMP domains like the hemopexin-like domains of MMP-1, MMP-8, MMP-13, MMP-14, MMP-16 and fibronectin-like domains of MMP-2 and MMP-9 act as exosite for substrate interactions. The lung matrix is a complex network of proteins and glycoproteins which includes multiple types of collagens, elastin, fibronectin, laminin, and several heparin and sulfate proteoglycans. MMPs can both activate and deactivate effector molecules like cytokines and GFs. Likewise, cytokines can also activate or regulate the

secretion of MMPs. ADAMs (a disintegrin and metalloproteinases) and ADAMTS (ADAMs with thrombospondin motif type I) are closely related to SVMPs (snake venom metalloproteinases), and together they are grouped under adamalysins subfamily that belongs to a larger "metzincin" superfamily (Porter et al. 2005; Seals and Courtneidge 2003; Killar et al. 1999; Black and White 1998; Rocks et al. 2008). ADAMs and ADAMTS are structurally related to MMPs or matrixins, which belong to the metzincin superfamily. Till date, about 40 members of ADAM family have yet been discovered, and 25 are expressed in humans, and around 35 are found in house mouse (*Mus musculus*). ADAMs and ADAMTS have a catalytic site containing HEXXHXXGXXH consensus sequence, where the catalytic zinc ion coordinates with three histidine residues and is surrounded by a methionine residue known as "Met-turn" (Gomis-Ruth 2009; Stocker et al. 1995). The pathobiological roles of MMP are not only restricted to the degradation of ECM proteins but also involve the activation of growth factors (like NGF), receptors of these growth factors (like trkA), and activation of various cytokines (like TNF-α, IL-1β) and chemokines signifying its roles in various inflammatory diseases (Fig. 8.1).

8.2 MMPs in Lung Diseases

As the infiltration of immune cells and activation of cytokines and chemokines are crucial in inflammatory and immune responses, there is no wonder that MMPs are also involved in the pathogenetic role in various lung diseases like asthma, COPD, and idiopathic pulmonary fibrosis where lung inflammation, immune cell activation, and lung remodeling are crucial features. For example, most of the acute inflammatory cytokines like TNF-α, IL-1β, and IL-8 need the help of MMPs for their activation. A number of MMPs like MMP-1, MMP-2, MMP-3, MMP-9, and MMP-17 and ADAM17 are known for their role in TNF activation in vitro and in vivo conditions. In addition, it had been demonstrated that a number of other cytokines like interferon-γ and other growth factors such as VEGF, EGF, FGF, and TGF-β are relevant to lung diseases. The association between MMPs in lung cancer, and its progression has been long established, though the precise role of MMPs in lung cancer remains unanswered. One of the foremost causes of cancer-related deaths globally is attributed by lung cancer, along with long-term tobacco smoking. The expression of matrix metalloproteinases (MMPs) is extremely high in lung tumors compared with nonmalignant lung tissue according to recent studies. The most common and serious risk factor for lung cancer is tobacco use. Tobacco use and smoking attribute to 20% and 70% of global cancer deaths (Siegel et al. 2016). Smoking and environmental air pollution along with bacterial/viral infections also attribute to chronic obstructive pulmonary disorder of the lungs. COPD is characterized by restricted airflow in the lungs along with chronic inflammation. Though it is preventable and treatable, it estimated to become the third leading disease causing worldwide death (Kochanek et al. 2011; Sethi and Murphy 2008). Idiopathic pulmonary fibrosis is a restrictive lung disease with gradual and irreversible reduction in the lung function. The IPF patients will have a progressive difficulty in breathing,

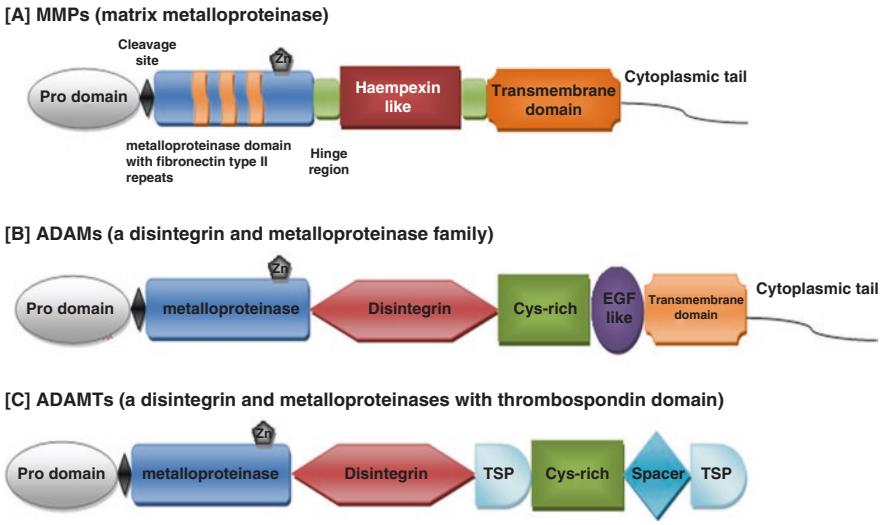

Fig. 8.1 Domain structure of (**a**) MMPs (matrix metalloproteinases), (**b**) ADAMs (a disintegrin and metalloproteinase family), and (**c**) ADAMTs (a disintegrin and metalloproteinase family with thrombospondin domain). MMPs have three major domains, namely, the propeptide, catalytic domain, and the hemopexin-like domain. The propeptide domain contains a conserved "cysteine switch" that interacts with the Zn ion present in the active site of the catalytic domain and allows binding and cleavage of the substrate. The catalytic domain is connected to the C-terminal domain of hemopexin by a hinge region. ADAMs are transmembrane metalloendopeptidases featuring a pro-domain, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor-like, and TM domain with a cytoplasmic tail. Not all ADAMs have functional protease domains indicating their biological efficacy depend upon protein-protein interaction. However, ADAMTs contain a disintegrin and metalloproteinase with thrombospondin motifs. [*TSP* thrombospondin type-I motif, *EGF-like* epidermal growth factor, *Cys-rich* a cysteine-rich region, *Zn* zinc atom]

and the mortality rate is very high. Asthma is characterized clinically by the difficulty in breathing, chest pain, increased sputum production, and pathologically airway hyperresponsiveness to nonspecific stimuli, airway inflammation including the recruitment of eosinophils, increased IgE production, goblet cell metaplasia, and structural changes/remodeling of airways referred to as "bronchial remodeling" (Lange et al. 1998). It is characterized by the basic symptoms of recurring periods of wheezing, shortness of breath, chest tightening, and coughing (in a long term). Airway remodeling in asthma is driven by secretion of few specific cytokines, IL-4, IL-5, IL-9, and IL-13 (Renauld 2001). IL-4 level was associated with decreasing of MMP-2 level but without haltering TIMP-2 production, and therefore TIMP-2/MMP-2 ratio was found increasing, thus IL-4 considered as a potential link between inflammation and collagen deposition in asthmatic patients (Bergeron et al. 2003). MMP-2 is known to play a very important part in tumor development and angiogenesis, hence suggesting that generating a potent MMP-2 inhibitor should be an important goal in lung cancer therapy (Togawa et al. 1999). Chronic progressive destruction of the lung is the major cause of death in patients with cystic fibrosis

Table 8.1 MMPs, ADAMs, and ADAMTS and their involvement in lung diseases

Lung diseases	MMPs, ADAMs, and ADAMTS
IPF	MMP-9, MMP-2 and TIMP-1, TIMP-2
COPD	MMP-9, MMP-12, ADAM-17, ADAM-33
Asthma	MMP-9, MMP-2, MMP-7, MMP-12, MMP-25
	ADAM-8, ADAM-9, ADAM-10, ADAM-12, ADAM-17, ADAM-28, ADAM-33
	ADAMTS-1, ADAMTS-12, ADAMTS-15
Cystic fibrosis	MMP-2, MMP-9, MMP-7, MMP-8, MMP-12
Lung cancer	MMP-2, MMP-7, MMP-9
	ADAM-8, ADAM-12, ADAM-15, ADAM-28
	ADAMTS-1, ADAM-2, ADAM-12

(CF) (Delacourt et al. 1995). CF airways are characterized by airway surface liquid (ASL) depletion producing mucus obstruction and chronic inflammation with persistent leukocyte accumulation, mainly of neutrophils and macrophages (Table 8.1).

8.2.1 Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease characterized by inflammation and fibrosis of the lung parenchymal cells with an estimated mortality of 50–70% at 5 years (Katzenstein and Myers 1998; Coultas et al. 1994). There is a progressive accumulation of ECM and connective tissue proteins, followed by formation of fibroblastic foci that are the hallmarks of IPF (Crouch 1990; Raghu et al. 1985). It is well reported that chronic inflammation leads to fibrosis; however, dysregulated fibrogenesis also attributes to the disease progression (Selman et al. 2001). This inflammatory process is characterized by infiltration of inflammatory cell such as neutrophils and macrophages, release of inflammatory cytokines, and secretion of several MMPs. The role of MMPs in the pathogenesis of IPF is relatively controversial though various clinical studies had demonstrated the increase of MMPs like MMP-9 and MMP-2 in IPF patients (Craig et al. 2015; Pardo et al. 2016). MMP-9 was found to be expressed in alveolar epithelia, fibrocytes, and alveolar macrophages, and secreted MMP-9 indeed activates TGF-β through proteolysis of latent TGF-β to generate active TGF-β and also facilitate the fibroblast migration. The MMP-2 promotes epithelial mesenchymal transition, lung fibroproliferative response and angiogenesis. Though all these studies indicate the positive pathogenetic roles of MMPs in lung fibrosis, their exact roles are in need of more investigation, because genetic knockdowns of most MMPs in mouse models of lung fibrosis indeed aggravate the lung fibrosis (Giannandrea and Parks 2014). Though mouse model of lung fibrosis is not the perfect model to mimic human IPF, the available two major drugs for human, pirfenidone and nintedanib, had been discovered using mouse models of fibrosis (Jenkins et al. 2017).

An imbalance of MMPS and TIMPS may play a pivotal role in fibrogenesis (Woessner Jr 1994). TIMPs form stable complexes with MMPs in a 1:1 ratio.

Profibrotic growth factors and GFs like IGFs, TGF-β1, and TNF- α play profound role in fibrosis (Black and White 1998). In animal models of bleomycin-induced pulmonary fibrosis, elevated levels of MMPs have been found in the bronchoalveolar lavage fluid of animals in chemotherapeutic agent bleomycin-induced PF. Batimastat, which acts by mimicking MMPIs, significantly reduced bleomycin-induced lung fibrosis indicating the role of MMPs in the disease progression (Corbel et al. 2001). Gelatin zymography using BAL fluid showed increased gelatinase activities attributable to MMP-2 and MMP-9 in IPF patients (Moises et al. 2000). ECM inducers cause changes in tissue structure during injury/repair of the lungs. Abnormal accumulation of extracellular matrix and fibroblast proliferation observed in damaged alveoli aid in abnormal lung remodeling with fibroblast foci. MMPs help in the proteolytic cleavage of key growth factors like IGFs, TGF-β, and TNF-α. The synthesis site of transforming growth factor (TGF-β1) and tumor necrosis factor (TNF-α) in fibrosis is build up by the alveolar epithelial cells. TGF-β1 expression is known to be upregulated in the fibrotic lung at sites of fibrotic foci in pulmonary fibrosis (Lasky and Brody 2000; Martinet et al. 1996). MMP-9 and MMP-2 are involved in proteolytic activation of latent TGF-β complexes. MMP-9 expression can be observed in fibroblasts from patients suffering from IPF. However, normal lung fibroblasts are incapable of expressing MMP-9 in vitro. Elevated levels of TIMP-2 can also be seen in fibroblasts from patients with IPF. In the alveolar space, fibroblasts migrate through gaps in the alveolar epithelial basement membranes and proliferate producing fibrosis (Selman et al. 2001). Gelatinases, namely, MMP-2 and MMP-9, in MMPs are pivotal in the migration of fibroblasts to the alveolar spaces. MMP-9 expression is significantly reduced in the bronchoalveolar fluid posttreatment. This pattern was associated with a consecutive decrease in TNF-α and TGF-β levels. This suggests a possible association between the MMP-9 activity and the release of these growth factors in lung injury (Corbel et al. 2001; Lasky and Brody 2000; Martinet et al. 1996) (Fig. 8.2).

8.2.2 Chronic Obstructive Pulmonary Disorder

Chronic obstructive pulmonary disease (COPD) is characterized clinically by excessive cough, sputum production, shortness of breath, and chest tightness and pathologically by two major features, chronic bronchitis and emphysema. It has been estimated that approximately 3% population is affected by COPD and importantly it is the fourth leading cause of death and further it has been estimated that it would be the third leading cause of death by 2030. The alveolar wall destruction is the key component in COPD pathogenesis, and on the other hand, the alveolar wall contains a number of substrates for a variety of MMPS like MMP-12, neutrophil elastase, etc. So MMPs are not only considered in COPD pathobiology, but they could be potent therapeutic targets (Churg et al. 2012; Houghton 2015). Further, MMPs also participate in mucus hypersecretion, another crucial component in COPD pathobiology (Houghton 2015). MMP-9 (gelatinase-b), MMP-12 (elastase), and MMP-8 (collagenase) are effectively involved in COPD by modulating airway

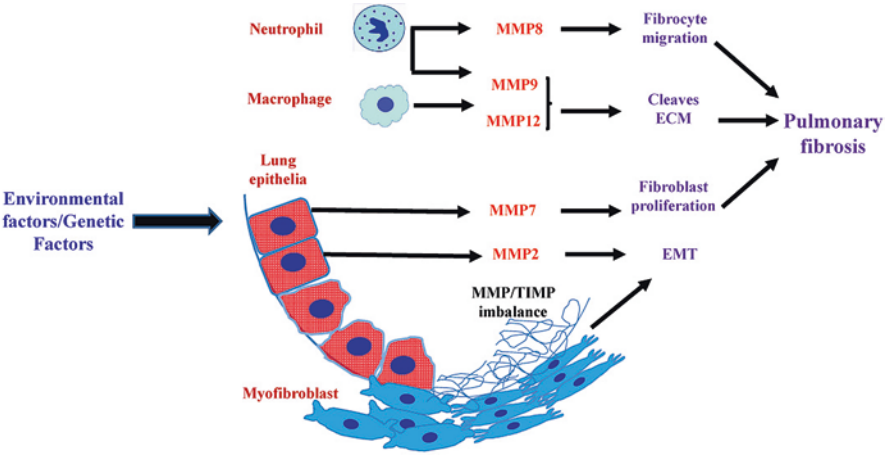

Fig. 8.2 Role of MMPs in idiopathic pulmonary fibrosis. Various environmental/genetic factors induce the secretion of MMPs by macrophages, neutrophils, and epithelial cells. These MMPs directly or indirectly cause fibrocyte migration, fibroblast proliferation, and EMT which ultimately lead to fibrosis. (*EMT* epithelial mesenchymal transition)

secretion from COPD patients that might trigger disease progression and exacerbation (Gomis-Ruth 2009), and together they can degrade all ECM (extra cellular matrix) and a actively destroy lung parenchyma actively and cause emphysema (Jeffery 2001; Davey et al. 2011). The chronic inflammation in airway epithelium in COPD patients is majorly due to cigarette smoking and initially can be repaired by MMP/TIMP and RECK ratio (Loffek et al. 2011) (RECK is a membrane-bound glycoprotein, and the KAZAL motif of RECK is found to play an inhibitory action on MMPs in lung carcinoma cells, in vitro and in vivo) balance repair system, but in severe cases the subsequent repair system causes excess collagen deposition that leads to thickening of bronchial wall and narrowing of small airways due to mucous hyperplasia and smooth muscle hypertrophy (Harju et al. 2010; Vestbo et al. 2012, 2013). The MMP-12 released by cigarette smoke-exposed macrophages further activates a number of chemoattractants to recruit more macrophages and neutrophil. These recruited cells further aggravate the situation due to neutrophil elastase to cause the emphysema condition thus forming a vicious cycle. In addition to the emphysema, MMP-12 also initiate the small airway remodeling in COPD. The small airway fibrosis in COPD leads to COPD complications like pulmonary arterial hypertension, cor pulmonale, etc. In addition to MMP-12, MMP-9 also had shown to be crucial in causing both emphysema and small airway fibrosis through neutrophil recruitment and conversion of latent TGF-β into active TGF-β, respectively. Neutrophil elastases (NE) along with MMPs are also involved in the pathogenesis of COPD. Epigenetic modifications like acetylation of histone protein due to reduction in the level of SIRT1 histone deacetylase linked to imbalance between MMP-9 and TIMP-1, abnormal DNA methylation at N-7 guanine, and miRNA

expression in COPD patients directly regulate the MMP expression in an unusual way (Nakamaru et al. 2009; Vucic et al. 2014; Yao et al. 2013).

MMP- 9 and MMP-12 are considered to play a decisive role in COPD (Nenan et al. 2005). It was reported that exposure of airway epithelium to cigarette smoking leads to upregulation of MMP-12 (Bezerra et al. 2011). MMP-12-/- knockout study on smoke-induced emphysema in animal models was found to play a protective role (Hautamaki et al. 1997). Excessive exposure to cigarette smoking leads to breakdown of collagen leading to the release of a tripeptide PGP (proline-glycine-proline) that acts as a neutrophil chemoattractant through CXCR2 binding and PGP deposition causing emphysema in mice. Release of PGP also promotes MMP-9 secretion that causes delayer of collagen (Weathington et al. 2006; Braber et al. 2011; Van Houwelingen et al. 2008; Malik et al. 2007). TGF-β released as a consequence of ECM degradation and its over-productivity stimulates signaling pathways that lead to fibrotic changes in the lungs and develop emphysema (Davey et al. 2011).

Animal experiments have reported the significant activity of leukotriene A4 hydrolase (LTR4H) in degrading PGP and controlling neutrophil influx, while smoking interferes with the action of LTR4H (Snelgrove 2011). The association between MMPs, NE, and their inhibitors is a complex one. MMP-12 degrades α1-antitrypsin (AAT), an inhibitor of NE and cathepsin G; on the other hand, NE degrades TIMPs, which are MMP inhibitors (Shapiro et al. 2003).

Exposure to smoking leads to secretion of proteins like prothrombin and plasminogen from the alveolar spaces and converts them to thrombin and plasmin, which are serine proteases (Churg et al. 2007a). Thrombin activates proteinase-activated receptor 1 (PAR-1) thereby promoting secretion of MMP-12 along with other inflammatory proteins (Raza et al. 2000). Surfactant protein D (SP-D) induces the secretion of MMP-12 and MMP-3 from human alveolar macrophages (Sin et al. 2008). On the contrary, SP-D−/− mice have been reported to develop emphysema with increased production of MMP-9 and MMP-12 (Yoshida and Whitsett 2006). Overexpression of IL-1β and TNF-α is also associated with emphysema development and increased MMP-12, MMP-9 and proinflammatory chemokine production (Lappalainen et al. 2005; Vuillemenot et al. 2004). In animal studies MMP-12 KO appears to be interesting in treating human diseases, but there are contrasting characteristics found between mouse and human MMP-12. Mouse MMP-12 acts as a proinflammatory molecule and activates neutrophil chemoattractants (Dean et al. 2008). However, human MMP-12 is non-proinflammatory, and its activity is found to be associated with switching off the neutrophil recruitment in site of inflammation. Small airways are vital site for air flow restriction in COPD. The cellular changes reported during airway remodeling include (a) obstruction in luminal airflow due to mucus plugging in, (b) PMN infiltration, and (c) submucosal fibrosis. Production of MMP-2, MMP-9, MMP-8, and MMP-12 in the airways has been reported during the disease (Lagente et al. 2009). Presence of MMP-10, TIMP-1, and ADAM-33 is found in human airways (Gosselink et al. 2010). Chronic bronchitis is associated with increased cough that often brings up mucus, but airflow obstruction is not as such related with this phenomena (Vignola et al. 1999). Sputum analysis in chronic bronchitis patients demonstrated the increased MMP-9 and

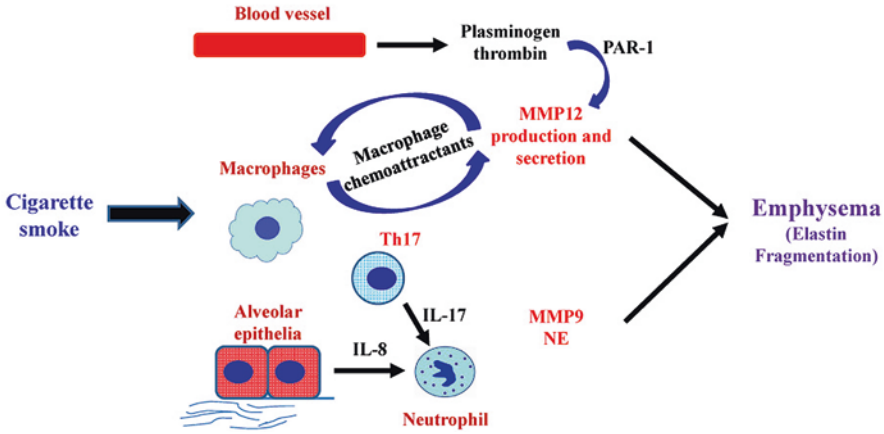

Fig. 8.3 Role of MMPs in chronic obstructive pulmonary disorder. MMP-9 (gelatinase-b), MMP-12 (elastase), and MMP-8 (collagenase) effectively modulate the airway secretions in COPD. Thrombin activates proteinase-activated receptor 1 (PAR-1) thereby promoting secretion of MMP-12 along with other inflammatory proteins. These MMPs degrade the extracellular matrix and destroy lung parenchyma actively and cause emphysema. (NE, neutrophil elastase; PAR-1, proteinase-activated receptor 1; Th, thrombin)

TIMP-1 level. Though MMP inhibition is considered as anti-COPD potential, the evidences are disappointing for the following reasons: (a) less benefits of broad-spectrum MMP inhibitor, (b) side effects of nonselective MMP inhibitors, and (c) hard to discover safe and selective MMP-9 inhibitors (Fujita 2014) (Fig. 8.3).

8.2.3 Asthma

Asthma is characterized by the development of bronchial hyperresponsiveness, dyspnea, chronic infiltration of the airway wall by inflammatory cells, and structural changes of airways. Structural changes in bronchial asthmatics are marked by thickening of epithelial cells and a subepithelial fibrosis, changes in ECM composition, hyperplasia of mucus-producing cells, increase in the surface of blood vessels in airway walls, and perichondral fibrosis (Cataldo et al. 2003; Bousquet et al. 1992; Vignola et al. 1998a). It is being believed that all of these features are mediated predominantly by Th2-dominant immune response. The inflammation of asthma is majorly triggered by overstimulation of allergen-specific CD4+ T cells by antigen-presenting cells like lung macrophages and dendritic cells (DCs). Dendritic cells are key players in asthma; they promote a T-helper cell type 2 (Th-2)-mediated immune response in the lungs. Various reports have demonstrated that acute inflammation leads to the degradation of ECM components, leading to the activation of TIMP-1; resulting in airway remodeling. In this context, MMP-9 is found to be increased in asthmatic airways (Ko et al. 2005). Thus, the delicate balance between MMPs and TIMPs is crucial for the acute exacerbations and resultant airway remodeling. In

this context, asthmatic patients with acute exacerbations have shown a significant increase of MMP-9 compared to stable asthmatics though there was no significant change in TIMP-1 levels (Ghada et al. 2012). Interestingly, both MMP-9 and MMP-12 are found to be upregulated in mice that were exposed to allergen for a long time, indicating the crucial importance of these two MMPs in the pathogenesis of chronic airway remodeling (Yu et al. 2012). In addition, a dual MMP-9/MMP-12 inhibitor had shown to ameliorate small airway remodeling caused by cigarette smoke, which was one of the pathobiological features of emphysema that lead deadly complications of COPD like pulmonary arterial hypertension and cor pulmonale eventually (Churg et al. 2007b). Further, montelukast therapy that reduced the asthmatic symptoms leads to reduce the levels of plasma MMP-9 (Chuang et al. 2007). Various studies have found not only the increased levels of MMP-9 in asthma but also the positive correlation between enhanced MMP-9 activity and asthma exacerbations (Oshita et al. 2003; Tanaka et al. 2000).

Airway remodeling in asthma is driven by secretion of few specific cytokines, IL-4, IL-5, IL-9, and IL-13 (Renauld 2001). IL-4 level was associated with decreasing of MMP-2 level but without haltering TIMP-2 production, and therefore TIMP-2/MMP-2 ratio was found increasing, thus IL-4 considered as a potential link between inflammation and collagen deposition in asthmatic patients (Bergeron et al. 2003). Overexpression of IL-13 in animal model study showed induction of MMP-2/MMP-9/MMP-12/MMP-13 and MMP-14 in asthmatic patients (Zheng et al. 2000). MCP-1 is also a contributor of asthma development by increasing the MMP-1/TIMP-1 ratio (Cataldo et al. 2002a).

Expression of several MMPs has been associated with asthma; increased levels of MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9 have been found in sputum and BAL from patients with asthma (Demedts et al. 2005). MMP-9 was the first MMP to be found in an in-depth study for its implication in pathology of asthma. Increased levels of MMP-9 were detected in bronchoalveolar lavage fluid (BAL) (Mautino et al. 1997), in sputum induced by the inhalation of hypertonic saline (Cataldo et al. 2000; Vignola et al. 1998b), and in serum of asthmatic patients (Bosse et al. 1999). Heightened MMP-9 activity was reported in the BAL of patients with severe asthma as compared to mild asthma or control patients in a clinical study. Higher TGF-β levels were found in the subepithelial basement membrane. Neutrophils expressed both MMP-9 and TGF-β, which were involved in breakdown and repairing of lung tissues (Wenzel et al. 2003). In asthma, the abundance of MMP-9 expression is correlated with tissue eosinophil numbers (Han et al. 2003). MMP-9 expressed by bronchial epithelium is an important factor promoting eosinophilic infiltration into the airways of patients suffering from asthma (Ohno et al. 1997; Ichiyasu et al. 2004). MMP-9 effectively recruits dendritic cells to the specific site and helps in initiation of asthma (Riese et al. 1996). MMP-9 recruits a cysteine protease cathepsin S (Riese et al. 1998) which also promotes DC trafficking, and cathepsin S attenuation in mouse model showed decreasing eosinophil and mast cell-mediated IgE generation in asthma (Boulay et al. 2004). In the sputum of asthma patients, the number of MMP-positive cells inversely correlates with forced expiratory volume (FEV1); this association may indicate that under acute exacerbations, the influx of

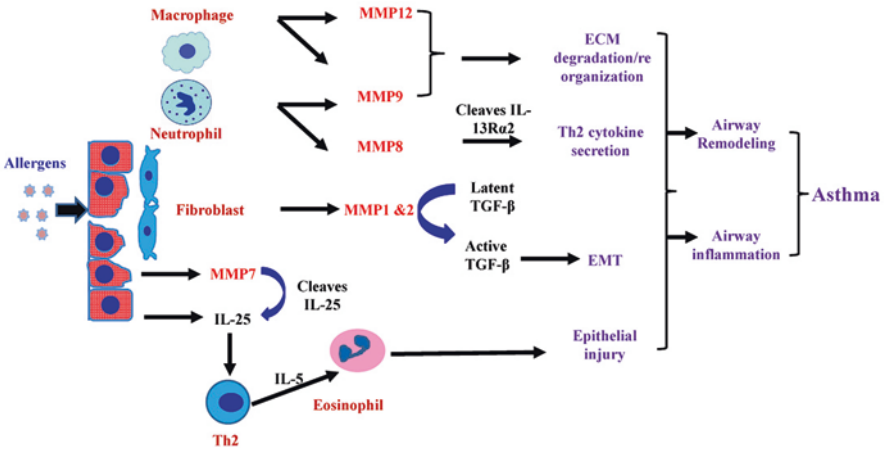

Fig. 8.4 Role of MMPs in allergen-induced airway remodeling in asthma. The allergen exposure leads to activate a number of proinflammatory mediators through innate and acquired immune responses, and these cause airway epithelial damage. The injured airway epithelia lead to IL-25 secretion. The MMPs secreted by airway epithelia, eosinophils, macrophages, neutrophils, and fibroblasts degrade and reorganize ECM to cause airway remodeling. Some MMPs also cleave latent TGF-β to make it active to induce EMT. (ECM, extracellular matrix; EMT, epithelial mesenchymal transition)

inflammatory cells interferes with normal gas exchange (Vliagoftis et al. 2000). Trypsin and mast cells stimulate the activation of "proteinase-activated receptor 2" (PAR-2) which promotes the release of MMP-9 that is involved in asthma remodeling (Cataldo et al. 2002b). MMP-9 KO study has shown the reduction of inflammatory cell infiltration in airways and BAL fluid in allergen-induced hyperresponsiveness (Cataldo et al. 2002a). Secretion of TIMP1 a major inhibitor of MMP-9 was found decreased in asthmatic patients (Cataldo et al. 2002a; Kelly et al. 2000).

Neutrophils are significant in asthma as they promote MMP-8 release, besides MMP-9 reported from BAL fluid analysis and biopsies from asthma patients (Corbel et al. 2001; Moises et al. 2000; Cataldo et al. 2001; Prikk et al. 2002). Excessive accumulation of smooth muscle cell is an important characteristic of asthma, and MMP knockdown prevents proliferation and accumulation of smooth muscle cells (Johnson and Knox 1999) (Fig. 8.4).

8.2.4 Cystic Fibrosis

Cystic fibrosis is characterized by the depletion of airway surface liquid leading to chronic inflammation with persistent accumulation of leukocytes. Although several studies of MMP-2 in CF have been reported, MMP-2 elevation has been observed in pulmonary specimens from individuals with CF (Gaggar et al. 2007).

MMP-7 has been shown to be involved in IPF and may play an important role in the injury/repair response in CF lung disease. MMP-7 is released apically from the

epithelial cells of the airways in CF patients (Zuo et al. 2002). In addition, MMP-8 or neutrophil collagenases highly expressed in neutrophils have common cleavage sites for type I, type II, and type III collagens, producing a three-fourth N-terminal fragment and a one-fourth C-terminal fragment (Williams and Olsen 2009). MMP-8 expression and activity are notably elevated in the airway secretions of patients with CF. A neutrophil chemokine, regulated by MMP-8, was found in the sputum of CF patients (Gaggar et al. 2008). However, the impact of MMP-8 dysregulation on CF pathogenesis has not been much explored. MMP-9 expression has been found to be elevated in both quantity and activity in the sputum and lower airway secretions of CF patients. Moreover, MMP-12 released from macrophages by a variety of inflammatory cytokines such as TNF-a and IL-1b is partially regulated by proteases such as thrombin and plasmin (Dasilva and Yong 2008). Overexpression of MMP-12 in the sputum of CF individuals has also been demonstrated (Gaggar et al. 2007). Detection of MMP-12 activity in leukocytes and biological fluids by FRET sensors can act as a potential biomarker of CF and other chronic inflammatory lung diseases (Correa et al. 2009). TIMP/MMP dysregulation has contributed to the pathology and disease severity in animal and human studies. The prospective of TIMP as a treatment for CF is being explored. TIMPs are naturally occurring inhibitors of MMPs in vivo that can be inactivated by proteolytic degradation or excessive MMP-12 activity (Jackson et al. 2010; Shapiro et al. 2003).

8.2.5 Lung Cancer

Lung malignancy is the foremost cause of cancer-related deaths globally and is frequently related to long-term tobacco smoking. Few of MMP expressions, namely, MMP-12, MMP-7, and MMP-9, increased at high level in lung tumor tissue compared with nonmalignant lung tumor tissue. Tobacco use and smoking are the most common factors that attribute to global lung cancer deaths (Siegel et al. 2016). MMP-2 is known to play a very important part in tumor development and angiogenesis (Jana et al. 2016); hence, targeting active MMP-2 is a rational approach for lung cancer treatment. Intravenous delivery of adenovirus-mediated *MMP-2 siRNA* gene into the malignant lung tissues prevented the metastasis of cancerous cells (Chetty et al. 2006). A high correlation between overexpression of MMP-7 and an aggressive phenotype in many malignant tumors has been supported by clinical studies. MMP-7-positive tumors showed a higher Ki-67 proliferation index compared with MMP-7-negative tumors (Thomas et al. 2000). In addition, increased MMP-9 is associated with the increase in lung tumor size. Higher levels of MMP-9 were found in advanced lung carcinoma stages as compared with primary stages (El-Badrawy et al. 2014). Moreover, MMP-12 has a strong correlation with recurrence and metastasis in lung cancer patients (Houghton et al. 2006). Furthermore, upregulation in the expression of MMP-13 via tumor necrosis factor-alpha pathway has been observed in cancer cell migration and increased metastasis (Thomas et al. 2000). The association of MMP-26 with carcinogenesis, lymph node metastasis, clinical stage, and the prognosis of lung cancer has also been well investigated. MMP-26

broadly expressed in epithelial cancer cells is used as a tumor marker for monitoring progression and to predict prognosis in lung cancer patients (Li et al. 2009).

8.3 Oxidants in Lung Diseases

The presence of high oxygen levels is not only crucial in the evolution of multicellular organisms on earth but also the key factor in the physiology of human body systems (Mannam et al. 2014). The metabolic usage of oxygen for cellular functions also leads to formation of reactive oxygen and free radicals as by-products. Very importantly, mild increase in oxidative stress or physiological levels of oxidative free radicals has been shown to be beneficial in a number of cellular events and signaling pathways. Thus, targeting the oxidative stress has not yielded any drastic benefit in reducing the diseases in general as excess use of antioxidants may also disturb normal homeostasis.

As the lungs are directly connected to the harsh environment that contains numerous oxidants, pollutants, allergens, and microbes, the lungs do have potent neutralizing mechanisms against all these exogenous environmental insults. Though innate and acquired immune mechanisms have been considered as central factors for these defense mechanisms, the role of mitochondria in protecting airway epithelial health in particular and lung health in general is the emerging research area in the respiratory research (Agrawal and Mabalirajan 2016). In addition to these environmental insults, endogenous sources of free radicals as well as mitochondrial NADPH oxidase and xanthine oxidase also participate in causing oxidative stress in the lung. While NADPH oxidase is considered as cytoplasmic source of reactive oxygen species, both mitochondria and NADPH oxidase systems cross talk each other for producing continuous oxidative stress (Mannam et al. 2014). To facilitate this, mitochondrial ROS indeed increases the expression of NADPH oxidase to create a vicious cycle. Though NADPH oxidase-mediated ROS is detrimental, the same ROS is needed by immune cells like macrophages and neutrophils which clear pathogens through NADPH oxidase-induced oxidative burst. Thus, it seems that therapeutic targets using NADPH oxidase system may compromise the defense mechanisms.

As mitochondria are traditionally considered as energy power houses, the exact role of mitochondrial pathology on neuromuscular diseases had been well explored as both neuron and muscle need to generate more energy through mitochondria. So it is not surprising that the role of mitochondria has not been thoroughly investigated in other organ-related diseases like lung diseases. However, multifaceted functions of mitochondria, like apoptosis and calcium buffering, have been gradually discovered. Various researchers had shown keen interest in dissecting the role of mitochondria in various chronic diseases including lung diseases (Agrawal and Mabalirajan 2016; Mabalirajan and Ghosh 2013). In this context, our lab had shown for the first time the mitochondrial dysfunction in airway epithelia of asthmatic mice (Mabalirajan et al. 2008, 2009). After the demonstration of mitochondrial dysfunction in asthmatic airway epithelia, another report emphasized the causative role

of mitochondria in the development of asthma (Aguilera-Aguirre et al. 2009). In this report, the authors have demonstrated the worsening of asthma features in ragweed-sensitized mice when they induced artificial mitochondrial dysfunction even before the initiation of asthma development. Similar dysfunctional mitochondria have been observed in human asthmatic patients (Reddy 2011). Indeed, mitochondrial haplogroups have been shown to be associated with asthma pathogenesis (Raby et al. 2007). The mesenchymal cell administration to asthmatic mice had alleviated asthma features by donating mitochondria to bronchial epithelia indicating the importance of healthy mitochondrial to maintain the healthy lungs (Ahmad et al. 2014).

Various reports have demonstrated the mitochondrial dysfunction in COPD (Kang and Shadel 2016). Very importantly, the oxidative stress caused by recruited neutrophils and macrophages upon cigarette smoke exposure is crucial in COPD pathogenesis. Further, it has been demonstrated that the skeletal muscle dysfunction in COPD is attributed to dysfunctional mitochondria in these cells (Gifford et al. 2018). All these indicate the important role of mitochondria in COPD pathogenesis. Though increased ROS generation could be the output of this mitochondrial dysfunction, the other mitochondrial functions in COPD pathogenesis are yet to be investigated. In addition to mitochondrial involvement, the involvement of NADPH oxidase in COPD pathogenesis is controversial as the deficiency of isoform 2 NAPDH oxidase indeed aggravated the COPD, whereas it is known that isoform 1 NAPDH oxidase can be induced by cigarette smoke (Mannam et al. 2014). Thus, more investigations are required to decipher the exact role of NADPH oxidase and its induced oxidative stress in COPD pathogenesis. However, a variety of antioxidant mechanisms like nuclear factor erythroid-2-related factor 2 pathway, glutathione, and superoxide dismutase have been shown to be involved in COPD pathogenesis, and activating these pathways has shown the improvement in lung function in COPD in many COPD mouse models. In any event, the therapeutic effects of antioxidants like N-acetyl cysteine and glutathione in COPD are disappointing (Mannam et al. 2014). Thus, further detailed investigations are required for effective therapy against COPD.

Acute lung injury and its mediated acute respiratory distress syndrome (ARDS) lead to clinically severe pulmonary failure with bilateral edema. The key pathological features are the accumulation of huge neutrophils in the lung parenchyma and consequent release of increased oxygen free radicals. It has been demonstrated that LPS (lipopolysaccharide) instillation causes mitochondrial dysfunction on airway epithelia. Also, increased expressions of NADPH oxidase isoforms 1, 2, and 4 along with the higher levels of oxidants in the lungs have been found in ARDS patients (Mannam et al. 2014). In this context, specific NADPH oxidase inhibitor and strategies to improve the mitochondrial biogenesis may be helpful in reducing the features of lung injury. Mitochondrial donation from mesenchymal stem cells to alveolar epithelial has been shown to be useful to reduce the features of acute lung injury (Islam et al. 2012).

Cystic fibrosis is the disease that affects the nature of mucus, and its symptoms are not only restricted to lung but also affect other organs like the gastrointestinal

system. The symptoms of cystic fibrosis due to the lung involvement are continuous cough with thick viscous sputum, breathlessness, and repeated pulmonary infections. The key molecular defect in this is the abnormal movement of sodium and chloride ions across the cells like epithelium. The investigations on the dissection of the role of mitochondria in cystic fibrosis are not much. However, collapsed mitochondria have been observed in mutated CFTR containing cells. Abnormal mitochondrial function along with increased apoptosis and oxygen free radicals have been observed in the airway epithelia of cystic fibrosis patients (Atlante et al. 2016). Similarly, oxidative stress due to NADPH oxidase system has been shown to be implicated in the pathogenesis of cystic fibrosis (Pongnimitprasert et al. 2008).

8.4 Future Prospective

As literature supports the important role of MMPs in ECM degradation, they do also have a role in the deposition of matrix protein. While ECM degradation role of MMPs is crucial in lung injury, ECM deposition plays a crucial role in lung repair. As both ECM deposition and degradation are dynamic processes, it is hard to judge the exact status in any given time in disease pathogenesis in clinics. This difficulty also reflects in the therapeutic as simple inhibition of particular MMP may disturb the MMP(s) mediated recovery from lung damage and thus sometimes may aggravate the disease pathology as reported in few cases. So stage-specific MMP modulation with specific inhibition of specific MMP may be helpful in targeting the lung inflammatory diseases. Detailed investigations are required to understand the exact role of MMP in lung diseases. In conclusion, pharmacological targeting of both MMPs and oxidative stress is challenging due to the dual nature of these endogenous phenomena in maintaining organ homeostasis. Thus, careful systematic investigations are required for effective therapeutics of lung diseases.

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Role of Chemical Exposure in Oxidant-Mediated Lung Diseases

9

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Abstract

The population-based epidemiological studies indicate a clear and close association between exposure to oxidant pollutants and commencement of cardiopulmonary diseases. The inhaled oxidants induce an avalanche of free radical production that affects several pathophysiological processes characterized by decrease in respiratory capacity, airway inflammation, etc. The pollutant exposure response shows variation in an age-dependent individualistic way. This review addresses different sources of pollutants, their contribution to reactive oxygen species (ROS) production, the cell- and tissue-specific effects of ROS, modulation of signalling cascades and onset of lung diseases.

9.1 Introduction

Oxidative stress (OS) primarily occurs due to the imbalance between oxidants and antioxidants and has an important role in inducing and progression of several lung diseases. In our modern-day lifestyle, our lungs get exposed to several exogenous

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and endogenous oxidants on a regular basis. The increasing rate of air pollution in developing and developed nations is one of the major reasons behind the death of children and adults due to acute and chronic lung diseases. Gaseous pollutants like nitrogen dioxide (NO_2), sulphur dioxide (SO_2), ozone (O_3), radiation, and particulate matters (PM) (e.g. cigarette smoke, mineral dust, quinones and transition metals resulted from diesel or gasoline exhaust, etc.) are the major reactive oxygen species (ROS)-forming agents. These air pollutants are deposited in the respiratory tract and induce sustainable chemical reactions producing ROS in the epithelial lining fluid covering the airways (Gurgueira et al. 2002).

Death from air pollution reflects a rise in mortality rates by 0.5% on an average with every 10 μgm^{-3} increase of fine PM, in a study conducted in ninety (90) largest cities of the United States of America (Kaiser 2000). In global stage as well, the number of premature victims of air pollution has surpassed the three (3) million mark in 2010, and with its ever-increasing trend under current scenario, the number of premature victims might be doubled by 2050 (Lelieveld et al. 2015). The pollution causes reversible loss of pulmonary function, immunodeficiency, hyperreactivity and inflammation of airways, increased vulnerability to respiratory infections, severe lung disease (e.g. asthma) and even death depending upon the exposure and response of the individuals. Susceptibility of an individual to the pollutant exposure depends on the idiosyncratic relationship with the risk factors to wit, pre-history of lung diseases, age, gender, genetic background, nutrition, etc. (Yin et al. 2004; Peden 2002; Dominici et al. 2006; Ciencewicki et al. 2007; Hollingsworth et al. 2007). This review gives a brief account of the various exogenous sources of oxidants, production of ROS/RNS in exposure response and their role in several lung diseases.

9.2 Exogenous Sources Causing Lung Diseases

Exogenous sources like environmental toxins and dietary resources may also cause lung injuries and boost ROS production. For example, the widely used herbicide paraquat is documented as a potent ROS inducer, causing alveolar epithelial cell injury through mitochondrial dysfunction, disruption of glutathione-dependent antioxidant defences and eventually cell death by inducing overexpression of cystine/glutamate transporter as well inflammation in lungs (Kobayashi et al. 2012; Chen et al. 2012; Toygar et al. 2015). Regular alcohol consumption causes ROS-mediated cysteine modification and upregulation of epithelial sodium channels that make the alcoholic lung more susceptible to acute respiratory distress (Downs et al. 2013). Another common avenue of lung injury involves OS-mediated perturbation of cytochrome P450 (CYP) metabolism triggered by different environmental toxins. Inhalation of sulphur mustard stimulates ROS formation and inhibits CYP activity leading to lung injury (Gray et al. 2010); the organic part of the diesel exhaust particles in contrary induces CYP, NADPH quinone oxidoreductase-1 expression and nuclear translocation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Baulig et al. 2003); tobacco carcinogens, arsenic and asbestos are also reported to increase

CYP expression and activity in a OS-mediated response (Anttila et al. 2011; Wu et al. 2009). All these changes cumulatively affect the lung immunity and stimulate the onset of lung cancer.

Among all the exogenous sources, cigarette smoking is one of the most important sources of ROS. The dense aerosol of tobacco smoke contains 10^{10} particles/ml, is characterized by 4000 different chemicals including highly porous carbonaceous polycyclic aromatic hydrocarbons (PAH), aza-arenes, polymeric material with adsorbed heavy metals, N-nitrosamines, etc. and on inhalation generates more than ten oxidants per puff through interplay of various pathways (Hecht 1999). The International Agency for Research on Cancer (IARC) has detected no less than 55 carcinogens in cigarette smoke with "sufficient evidence for carcinogenicity" (Hoffman and Hoffmann 1997; Hoffmann and Wynder 1986). Smoking is responsible for death from cardiovascular, chronic obstructive pulmonary degenerative diseases and accounts for one third of all cancer deaths in first-world countries (Vineis et al. 2004).

9.3 ROS/RNS Production from Different Exogenous Sources

After inhalation all the redox-active pollutant and ROS follows a series of radical and redox reaction cycles in the epithelial lining fluid (ELF). The preliminary step is the formation of reduced metal ions or semiquinones, which includes the transfer of electrons from antioxidants to transition metal ions or quinines (Pöschl and Shiraiwa 2015; Kumagai et al. 2012; Charrier et al. 2014). In the next step, the redox-active transition metal ions and quinines are regenerated in a reaction following the transformation of O_2 to superoxide ($O_2^{\bullet -}$) radical. Subsequent formation of hydrogen peroxide (H_2O_2) and the most toxic hydroxyl radicals ($OH^{\bullet}$) via Fenton-like reactions (Charrier and Anastasio 2011) is the primary event of oxidative stress in the respiratory tract. Various studies proved that formation of oxidative stress results in the destruction of cells and tissues in the respiratory tract (Pöschl and Shiraiwa 2015; Winterbourn 2008). Thus the formation of ROS in ELF is the initiation step of the diseases like asthma and allergies.

In phagocytic cells like neutrophils and macrophages, the overproduction of ROS was first observed and was named as "the respiratory burst" due to the transient consumption of oxygen. There are very few studies on the production of ROS induced by air pollutants. Lakey et al. (2016) exhibited in one of their studies that ROS concentration can be increased in highly polluted environment where fine particulate matter (PM2.5) containing redox-active transition metals, quinones and secondary organic aerosols is present. They had also proved that oxidative stress can also be enhanced by ambient ozone causing depletion of antioxidants and surfactants when it readily saturates the ELF (Lakey et al. 2016). On the other hand, a number of studies proved the central role of free radical mechanisms in tobacco smoke carcinogenesis and oxidative stress (Leaderson and Tagesson 1990; Asami et al. 1997; Huang et al. 2005). The cigarette tar, the particulate solid phase of tobacco smoke, consists of stable free radicals like semiquinone ($QH^{\bullet}$),

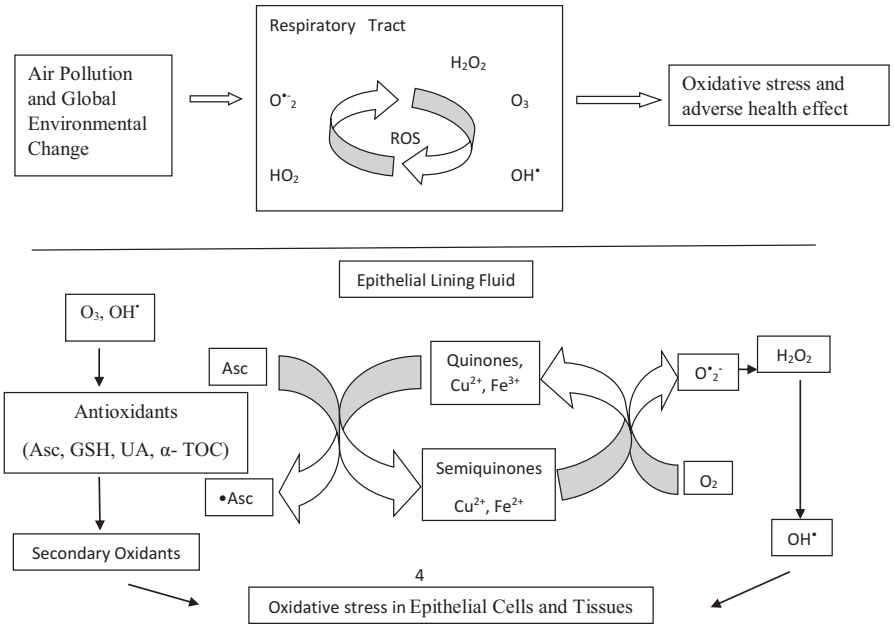

Fig. 9.1 Interaction of air pollutants and reactive oxygen species in the epithelial lining fluid of the human respiratory tract

carbon-centred radicals (C•) in high concentration (Pryor 1992) and a quinine/semiquinone/hydroquinone (Q/QH•/QH2) system in the tar polymeric matrix (Pryor et al. 1983). Bermudez et al. (1994) have identified the role of these stable free radicals in DNA damage through formation of OH•as detected by EPR spin-trapping. They also identified the free stable radicals as o- and p-benzosemiquinone radicals (Bermudez et al. 1994). The first step of the mechanism involves the reduction of O_2 into $O_2^{\bullet-}$ by the QH• radicals (Fig. 9.1). Then in the next step, hydrogen peroxide is formed from $O_2^{\bullet-}$ radical and then through the Fenton reaction highly oxidizing hydroxyl radicals are generated as cigarette tar itself contains high concentrations of iron (Fe^{2+}). In aqueous extracts, large amount of H_2O_2 can be produced from the cigarette tar (Nakayama et al. 1989). The oxidants present in the tar and gas phase implicate in the release of iron from the endogenous enzyme ferritin which alter iron metabolism in the lungs (Moreno et al. 1992; Lapenna et al. 1995). Smoking also increased the risk of cardiovascular diseases as oxidants and free radicals in cigarette smoke have been considered to promote lipid peroxidation of cellular membrane lipids, thus promoting atherosclerosis, endothelial dysfunction and onset of acute clinical events (Frei et al. 1991; Ambrose and Barua 2004). On the contrary ROS presence in the gaseous phase initiates the destruction of endogenous antioxidants (vitamins and enzymatic antioxidants) reducing the vital role of cellular antioxidant defences (Cross et al. 1999). Many studies show that the smokers lack adequate level of antioxidant vitamins which results in systematic oxidative stress

(Panta et al. 2000; Traber et al. 2000) and dietary antioxidant supplements failed to compensate the damage. Many studies also elucidate the increased risk factor from the synergistic effect of tobacco smoking with various respirable mineral fibres and fine particular matter (soot, fine dusts) in occupational environments. This also explains the increased risks of lung cancer and other occupational pulmonary diseases in industrial workers (Saracci 1987; Reif and Heeren 1999). The increase in lung cancer cases and documentation of DNA damage in bronchial epithelial cells among the workers of asbestos mine due to the synergistic effect of ROS formation from cigarette smoke and asbestos fibres has been demonstrated (Kamp et al. 1992; Jackson et al. 1987; Jung et al. 2000). Many studies reported similar synergistic effects of tobacco smoking and other environmental pollutants like radon (Band et al. 1980; Damber and Larsson 1982), coal dust (Dalal et al. 1995) and heavy metals like nickel, chromium and cadmium (Liu et al. 1999; Pershagen et al. 1981). In occupationally exposed workers, malignant neoplasms occurred from the synergetic effect of tobacco smoking and heavy metal (Costa et al. 1989; Landolph 1999). Other studies also interpreted that tobacco smoking when associated with alcohol consumption and exposure to mineral particle often leads to oral and pharyngeal cancers (Elwood et al. 1984; Franceschi et al. 1999). Moreover the gas phase of tobacco contains a large amount of free radicals including nitric oxide (NO), which participate in multiple physiological role, and also has toxic effects if generated in large amount. NO is already recognized as a carcinogen that reacts with $O_2^{\bullet-}$ to form peroxynitrite which causes oxidative damage to biomolecules (Beckman and Crow 1993; Ischiropoulos et al. 1992).

9.4 The Next Phase: Interactions of Oxidants with Molecule

There are many theories which describe the basic mechanism of lung diseases caused by exogenous sources. The most acceptable one voice for generation of oxidative stress and subsequently oxidative damage in the lung when air pollutant induced ROS formation exceeds the antioxidant defences. In this scenario, ROS reacts with proteins, lipids and DNA; the generated secondary metabolites cause diverse toxicological consequences that boost numerous pathophysiological conditions. The major effect of oxidative stress is lipid peroxidation of membrane polyunsaturated fatty acids caused by free radical chain reaction which has been exhibited in rodents and humans (de Burbure et al. 2007; Elsayed et al. 2002; Ergonul et al. 2007; Liu and Meng 2005; Yargicoglu et al. 2007). The end products of lipid peroxidation have also shown several pathological effects. For example, one of the end products 4-hydroxy-2-nonenal (HNE) is known for production of oxidative stress conditions through a depletion of intracellular glutathione and induction of peroxide production (Uchida et al. 1999), airway remodelling through activation of the epidermal growth factor receptor (Suc et al. 1998), induction of fibronectin production (Tsukagoshi et al. 2002) and induction of cell death in alveolar macrophages in mice (Li et al. 1996). Many studies also suggest that after ozone exposure, HNE-protein adducts have been found in the lungs of mice and humans (Hamilton

Jr et al. 1996; Kirichenko et al. 1996). Another secondary metabolites which have toxic effect are lipid ozonation products (LOP), formed by the interaction of ozone and unsaturated fatty acids in epithelial lining fluid and cell membrane (Pryor et al. 1995; Kafoury et al. 1998, 1999). LOP interaction with human epithelial cells initiates eicosanoid metabolism, and the generated reactive peroxides are capable to set off oxidative stress. The detrimental effects of LOPs include activation of phospholipases A2, C and D in bronchial epithelial cells and induction of inflammatory mediators such as platelet-activating factor, prostaglandin E2, interleukin (IL)-6 and IL-8 (Leikauf et al. 1993).

Various studies suggest that antioxidants and lipids present in the ELF accelerate the oxidant-induced membrane oxidation. Oxidation of the cell membrane proteins by O_3 is correlated with the presence of antioxidants like ascorbate and glutathione in the epithelial lining fluid (Ballinger et al. 2005). Other studies also seconded the increase in cell injury on O_3 and NO_2 exposure by the addition of ascorbate in ELF (Connor et al. 2004; Velsor and Postlethwait 1997).

Apart from this, the other primary detrimental effects of oxidative pollutants are modification of proteins by random peptide bond cleavage, cross-linking of proteins, oxidation of protein backbone or amino acid side chains, etc. (Kelly and Mudway 2003). The methionine and cysteine residues are more susceptible to oxidation, and methionine oxidation by ROS is reported to cause loss of anti-neutrophil elastase activity (Taggart et al. 2000). The neutrophil elastase has a harmful effect on alveolar matrix which leads to emphysema. ROS can also cause sodium channel inactivation by oxidation of multiple methionine residues (Kassmann et al. 2008). By oxidizing methionine residues in surfactant proteins, ROS can scale down the ability of the surfactant film to reduce the lung surface tension during breathing, resulting into respiratory distress. The oxidative modifications of surfactant proteins have other side effects like increase in lung vulnerability for lipid peroxidation, inflammation and oxidative damage (Bridges et al. 2000; Haque et al. 2007; Kierstein et al. 2006).

Exposures of air pollutants collectively enhance the risk of lung cancer. DNA damage is the primary cause behind cancer. Various studies exhibited that oxidative DNA damage in human airway epithelial cells is stimulated by particulate matter through free radical formation (Prahalad et al. 2001; Dellinger et al. 2001). It has also been proposed that $OH^{\bullet}$ generated from alveolar macrophages during particle phagocytosis may be the actual candidate causing DNA damage (Tokiwa et al. 1999). Other studies also exhibited the role of O_3 and SO_2 on DNA base modifications (Ito et al. 2005) and DNA-protein cross-linking in the mice lungs, respectively (Xie et al. 2007).

9.5 The Effect of ROS on Signal Transduction

Abrupt activation of signalling pathway is another detrimental effect of oxidant pollutants by which the risk of pathological response of lungs increased. The fluctuations in redox balance due to increase in ROS formation or decrease in antioxidant

activity affect the signalling pathways. ROS acts as a signalling molecule which helps to initiate and also to regulate the proper function of several signal transduction pathways. It has been reported that the non-phagocytic cells, a diverse group of cytokines and growth factors bind to the receptors of various classes and produce ROS (Valko et al. 2007). Other studies indicate generation of ROS by activation of signalling cascades through the epidermal growth factor (EGF), PDGF and vascular endothelial growth factor (VEGF) receptors (Neufeld et al. 1999). Another important effect of ROS and air pollutants is induction of mitogen-activated protein kinases (MAPK) signalling ensuing inflammation. MAPK transmitted signal into the cell by phosphorylation of proteins. The major functions of MAPK are regulating antimicrobial responses in neutrophils (Downey et al. 1998; Nick et al. 1999; Arndt et al. 2005) and helping the production of pro-inflammatory cytokines (Qi and Elion 2005). It has been proved that inhibition of c-Jun NH_2 terminal kinase (JNK) in mice decreases O_3-induced inflammation and hyperresponsiveness (Williams et al. 2007). The end product of lipid peroxidation activates signal-regulated kinase p44/42 (Erk1/2), JNK and p38MAPK (Uchida et al. 1999; Tsukagoshi et al. 2002). These kinases on activation increase the DNA binding activity of the transcription factor activator protein-1 (AP-1) and in turn initiate the transcription of stress response genes including phase II enzymes. Other studies confirmed that the NF-κB DNA binding increased due to air pollutant oxidants, and the release of the pro-inflammatory cytokine IL-8 in lung epithelial cells has been initiated (Dagher et al. 2007; Valacchi et al. 2004). Another study showed that when particulate matter activates NF-kB, it depends on EGFR activation and MAPK signalling pathway (Churg et al. 2005). Both pathways are associated with stress response. Nrf2 is another transcription factor which is affected by oxidative stress. Under oxidative stress Nrf2 binds to antioxidant response elements (AREs) which causes the induction of various genes associated with the mitigation of oxidative damage (Cuzzocrea et al. 1998). Activation of Nrf2 causes the transcription of the genes associated with DNA damage recognition, antioxidants, free radical metabolism and glutathione homeostasis including others which participated in oxidative stress response (Kensler et al. 2007). Mutations in Nrf2 will increase the risk of adverse effects of pollutant exposure, but more detailed studies are required to document the consequences of Nrf2 mutation and pollutant exposure.

9.6 Oxidative Stress and Lung Diseases

The reactive oxygen species and the consequent oxidative stress is the primary cause of various pulmonary diseases like asthma, cystic fibrosis, chronic obstructive pulmonary disease (COPD) and acute respiratory distress syndrome (ARDS). The oxidants are the major cause of airway inflammation and airway hyperresponsiveness (AHR), and both are designated as primary characteristics of asthma (Rahman et al. 1996). Asthmatics experience eosinophil-, neutrophil- and macrophage-mediated increased ROS production (Emelyanov et al. 2001). As a result elevated levels of eosinophil peroxidase and myeloperoxidase in the peripheral blood,

Bronchoalveolar lavage fluid (BALF) and sputum are observed among asthma patients and are used to monitor the prognosis of the disease (Monteseirin et al. 2001; Aldridge et al. 2002). External pollutants such as ozone inhalation have been documented to induce airway hyperresponsiveness (Kierstein et al. 2008). On the other hand, cigarette smoking is one of the major inducers of COPD characterized by progressive decrease of lung efficiency and shortage of airflow, causing slow but irreversible damage to the lung. Higher levels of chemokines, lipid peroxidation products, 8-isoprostanes and erythrocyte superoxide dismutase are illustrated in COPD patients in comparison to healthy non-smokers (Tomaki et al. 2007; Sahin et al. 2001; Corradi et al. 2003; Nowak et al. 1999; Morrow and Roberts 1997; Hanta et al. 2006). Although the mechanism of ARDS onset is still not clear, ROS is speculated to play an important role in it. In ARDS patients the increased ROS level is concurrent with reduced activity of antioxidant defence system characterized by drop in glutathione, vitamin E and C, superoxide dismutase and catalase level (Ware 2006).

9.7 Conclusion

It has been manifested that oxidative damage and enhanced ROS level induced oxidative stress are the primary causes of several chronic lung diseases. The lung is one of the most exposed organs to environmental pollutants. Lipid peroxidation, protein oxidation, carbonylation products and antioxidant levels have been shown to vary parallelly with the pathogenesis of the diseases but yet to be established as biomarker. Disease susceptibility is also heavily dependent on the health and nutrition level, age and genetic background of the population under consideration. Children under 8 years, aged people over 65 and maternal exposure during pregnancy are documented to exhibit the most adverse effects of pollutant exposure (Wang and Pinkerton 2007; Cardoso 2004; Pinkerton and Joad 2006; Anderson et al. 2003; Kelly 2005). Variation in pulmonary spirometric responses and polymorphisms of the genetic structure of inflammatory and antioxidative defence system in certain population are considered to be the reasons behind their increased vulnerability. But to link the genetic-pollutant interaction and pathogenesis of oxidant-mediated lung diseases, properly monitored clinical studies are required to be designed.

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Part II

Inflammatory Lung Diseases

Oxidative Stress and Immune Regulation During Chronic Respiratory Diseases

10

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Abstract

Chronic respiratory disease is one of the leading cause of death worldwide. A group of chronic diseases causing abnormalities in lung airway and other architectures of lungs can be defined as chronic respiratory diseases. Asthma, chronic obstructive pulmonary disease (COPD), lung fibrosis, and lung cancer are included in chronic respiratory diseases. The lungs contain different enzymatic and non-enzymatic anti-oxidants that buffer numerous pro-oxidant infiltrations or generations in lungs. Imbalance in pro- and anti-oxidants cause oxidative stress that is known to be associated with the pathogenesis of different chronic respiratory diseases. Both innate and adaptive immune components have positive and negative regulatory effects on different chronic lung diseases. Lung inflammation is an important phenomenon of all respiratory diseases. Oxidative stress has been found to propagate inflammation. Thus, oxidative stress is linked with immune regulation and significantly associated with the pathogenesis of chronic respiratory disease.

Keywords

Chronic respiratory disease · Asthma · Chronic obstructive pulmonary disease (COPD) · Pulmonary fibrosis · Oxidative stress · Immune regulation

10.1 Introduction

A group of chronic diseases affecting airway and other architectures of lung are called chronic respiratory diseases. The different adult respiratory diseases causing a high burden of morbidity and mortality worldwide are mostly chronic respiratory

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diseases (Speizer et al. 2006). It is a rising global health problem and was the second leading cause of death worldwide (Marsland et al. 2011). Diseases like chronic obstructive pulmonary disease (COPD), asthma, pneumoconiosis, interstitial lung diseases, and pulmonary sarcoidosis are chronic respiratory diseases (India State-Level Disease Burden Initiative CRD Collaborators 2018). It can develop at the interface between environmental factors and genetic susceptibilities. To date there are limited therapies available for chronic respiratory diseases. Ill defined lung injuries, failure in repair mechanisms, and prolonged exposure to different environmental pollutants are included as pathogenesis of chronic respiratory disease. Growing evidence has proved that events during the early ages of life may leads to chronic respiratory diseases.

Lung micro-environment contains numerous enzymatic and non-enzymatic anti-oxidants that keep buffering the prolonged exposure of different pro-oxidants in the respiratory airway and other parts of lung (Birben et al. 2012). This balancing of pro- and anti-oxidants allows lungs to perform properly and maintains their architecture. Imbalance in this pro- and anti-oxidant ratio promotes oxidative stress and influences chronic respiratory disease pathogenesis. Researchers have found influence of oxidative stress in nearly all chronic respiratory diseases. On the basis of these backgrounds, different therapeutic regimens have also been executed. Some of them have shown positive responses; some have not.

It will be incomplete if we do not include the influence of the immune system in any disease occurrence. Chronic respiratory disease is also not an exception. The immune system can broadly be divided into the innate and adaptive immune system (Chaplin 2010). The latter have been found to be highly associated with chronic respiratory diseases. Inflammation is a common fact with respiratory diseases (Lee and Yang 2013). Release of pro-inflammatory cytokines have been found to be associated with the inflammatory phase. Innate immune cells, such as dendritic cells, macrophages, and adaptive immune system key component T cells, have been found to be highly associated with chronic respiratory diseases.

There are a few connections between oxidative stress and immune regulation in chronic respiratory diseases. In this review, we have tried to understand how immune system and oxidative stress is associated in chronic respiratory diseases, such as asthma, COPD, and lung fibrosis.

10.2 Oxidative Stress in Chronic Respiratory Diseases

An imbalance between the pro-oxidant and anti-oxidant effects is defined as oxidative stress. There is a well-known relationship between oxidative stress and chronic respiratory diseases (Rahman and Adcock 2006). Although there are several mechanisms that have been proposed to be followed by the lung cells to maintain this pro- and anti-oxidant imbalance, failure of this process still leads to accumulation of different reactive oxygen species and other reagents having oxidative potentials in the lung for several years resulting in generation of different chronic respiratory

diseases, such as asthma, chronic obstructive respiratory disease (COPD), pulmonary fibrosis, pulmonary hypertension, and many others.

This oxidative stress is caused by several sources, such as exposure to cigarette smoke, exposure to different pro-oxidants, metabolic deregulation, and reduced levels of different anti-oxidants (Donohue 2006). Our lungs are exposed to all the components carried by the environment air, including different air pollutants, pollens, and particulate matter. These particles can easily enter into the lung and cause oxidative stress. Air pollutants contain different direct oxidative agents, such as ozone or nitrogen oxides.

Mitochondrial dysfunction in response to different environmental stimuli can also contribute to anion superoxide that may cause oxidative stress. These anion superoxides can form hydrogen peroxide by superoxide dismutase enzyme. These hydrogen peroxides can form hydroxyl radicals with the help of neutrophil peroxidase and form hypobromous acid or hypochlorous acid (MacPherson et al. 2001). Along with this, reactive oxygen species produce reactive nitrogen species from nitric oxide, which is also a potential source of oxidative burden in the lung micro-environment.

In asthma these pro-oxidative agents have been found to be prevalent and these are very difficult to control. Proteomic analysis has confirmed that oxidative modifications of nitro- and chloro-tyrosine has reduced catalase activity resulting in more hydrogen peroxide accumulation in the lung (Holguin 2013). This finding explains why asthma patients have greater systemic and airway burden of oxidative stress than healthy controls that is directly correlated with disease severity.

Our lungs contain different anti-oxidant enzymes, such as glutathione, superoxide dismutase, and catalase. They are also equipped with different non-enzymatic anti-oxidants, such as thionine, ascorbic acid, and ferritin (Kaarteenaho-Wiik and Kinnula 2004). These components always allow lungs to buffer different pro-oxidant exposures.

Like asthma, patients of COPD also show prevalence of airway oxidative and nitrosative stress. Most of the victims of this deadly disease have a high prevalence of nitrotyrosine in airway epithelium and sputum (Jin et al. 2011), though there is very little data showing the relationship of nitrotyrosine expression with disease severity. Significant imbalance in the airway thiol metabolism has been found in patients with COPD, and they also show a higher sputum glutathione level that has been found to be increased during exacerbations (Turgut et al. 2014). Tobacco smoke may also deplete GSH level by forming glutathione aldehyde derivatives, resulting in ill conversion of GSSG.

Patients with pulmonary fibrosis also follow similar oxidative burden in airway and lung micro-environments. Owing to the prevalent oxidative burden in the patients of pulmonary fibrosis, previously, an antioxidant such as N-acetyl cysteine was usually prescribed to the patients; later the drug failed to show enough success to cure fibrosis.

10.3 Immune Homeostasis Imbalance in Chronic Respiratory Diseases

Our very own immune system continuously protects us from exposure to various foreign dead or living components that can cause severe health hazards. We can broadly categorize our immune system as innate and adaptive immune systems. Both these systems have their own principles to protect our body. Mucociliary clearance, epithelial barrier, complement components, and surfactants are the innate immune defense components. Among immune cells, we can include macrophages, mast cells, natural killer cells, and neutrophils (Paul 2011).

The normal inflammation process induces immune responses that augment tissue immunity; though for COPD patients the process is abrogated. Here chronic pulmonary inflammation induces defective immune responses that worsen the inflammatory lung micro-environment and disease severity. The innate immune cells can sense tissue destructions and respiratory infections occurred during COPD. Host TLR-2 signaling axis was also found to be important in COPD (Pomerenke et al. 2016). Recent findings have shown damage associated molecular patterns (DAMPs) were found to activate inflammasome that mediate inflammation in COPD (Pouwels et al. 2016). During inflammation, phagocytes have been found to phagocytose neutrophils to regulate inflammation (Rosales 2018); though this mechanism has been found to be impaired in COPD patients, whereas scientists have found different expression of dendritic cells in COPD progression (Stoll et al. 2015; Demedts et al. 2007). Neutrophils are other important immune cells related to COPD pathogenesis (Hoenderdos and Condliffe 2013). Recent studies have shown that neutrophil elastase influences emphysema (Hoenderdos and Condliffe 2013). The latter also influences monocyte recruitment. Neutrophil and monocyte balance is believed to be a key factor for COPD pathogenesis.

Innate, non-antigen dependant immune systems have a huge impact in asthma pathogenesis (Finn and Bigby 2009). Bronchial epithelial cells and dendritic cells have been found to be one of the major regulators of asthma (Lambrecht and Hammad 2014). The bronchial epithelial cells express MHC class II antigens, and they act as professional antigen presenting cells. Environmental pollutants have been found to induce dendritic cell maturation from airway epithelium by releasing GM-CSF (Bleck et al. 2007). These finding prove their effect on progression of allergic asthma. Dendritic cells play a crucial role in regulating adaptive immune responses. They also act as professional antigen presenting cells. Substantial evidence supports that dendritic cells are increased in asthma proving its role in disease exacerbation. T cell activation needs to have an early signal from professional antigen presenting cells for its early activation and differentiation. In asthma, predominance of Th2 cytokines, such as IL-4, IL-13, and IL-5, has been found over Th1 cytokines, such as IFN-γ (Greenfeder et al. 2001). Asthma is also addressed as a Th2 type disorder.

Vast studies have been done on pulmonary fibrosis in both clinical and murine experimental studies. Our group has studied the effect of both innate and adaptive immune systems in experimental pulmonary fibrosis. The existence of

inflammation and dendritic cells has been reported in pulmonary fibrosis. We investigated the effect of cytokine TGF-β on dendritic cell maturation during experimental pulmonary fibrosis. Accumulation of CD11c + dendritic cells were prevalent in both the inflammatory and fibrotic phase of pulmonary fibrosis. Significant attenuation was observed in the CD11c + dendritic cell number in the absence of TGF-β in lung confirming the latter as a key regulator of CD11c + dendritic cell infiltration in the lung during experimental pulmonary fibrosis (Chakraborty et al. 2017). Recent studies have reported the importance of regulatory T cells (Treg) and Th17 cells in lung fibrosis. Our group depleted Treg cells in the lung in fibrotic condition to check how it regulates fibrosis and other T cell subsets. We found reduction in fibrosis score with Treg depletion and also reduction in Th17 and CD4 + CD28+ T cell subsets (Chakraborty et al. 2018). The results establish Treg cells as a key immune component regulating pulmonary fibrosis and other immune cells during fibrosis development.

10.4 Oxidative Stress and Immune System: Are They Connected?

It is usually believed that oxidative stress induces cellular damage, propagates inflammation, and is an injurious process, but even loss of anti-oxidants in our body is also harmful. Lack of supplementation of anti-oxidant loss contributes to the failure to successfully improve many diseases. It provides convincing evidence that pro- and anti-oxidant balance is a complex phenomenon. There are several research groups working worldwide to find a link between oxidative stress and immune responses. There are several nitrated fatty acids that have been reported to act as endogenous PPARγ ligands and inhibit proinflammatory cytokine production (Cui et al. 2006). Nitrated fatty acids were also found to interact with the p65 subunit of NF-κB, hence preventing its nuclear translocation and release of cytokine production (Delmastro-Greenwood et al. 2013). Autophagy is another cellular process executed by phagocytes. Reactive oxygen species were found to play an important role in regulating autophagy. In lungs, HO-1 modulates autophagic activation and regulates cell death (Ryter and Choi 2010). Thus, the HO-1 and autophagy connection can be addressed as a connection of immune system and oxidative stress.

10.5 Conclusion and Future Direction

Chronic respiratory disease is a rising global health hazard with limited effective therapeutic regimens available. Early age deformities or prolonged exposure may cause chronic respiratory diseases. It is currently the second leading cause of death worldwide. Injury, tissue damage, and exposure to different environmental factors may cause these diseases. Among the environmental factors, cigarette smoke was found to be a massive factor responsible for chronic respiratory diseases. Exposure to cigarette smoke was found to hamper the pro- and anti-oxidative components of

lungs leading to oxidative stress induced damage. Oxidative stress has a huge impact on the disease pathogenesis of asthma, COPD, and lung fibrosis. The lungs contain different anti-oxidant enzymes and non-enzymatic anti-oxidants, such as glutathione, superoxide dismutase, catalase, thionine, ascorbic acid, and ferritin. They buffer exposure of different anti-oxidants in lungs. Imbalance of these pro- and anti-oxidants cause oxidative stress.

Alteration of immune homeostasis was found to be significantly associated with chronic respiratory disease pathogenesis. Different innate and adaptive immune components do influence disease progression and suppression. Neutrophil elastase is known to promote COPD, whereas bronchial epithelial cells were found to show significant impact on asthma pathogenesis. Vast studies have shown the impact of innate and adaptive immune cells in lung fibrosis. Professional antigen presenting cells, such as dendritic cells, and adaptive immune cells, such as regulatory T cells and Th17 cells, are known to have a high impact upon lung fibrosis pathogenesis.

Autophagy is known to be another cellular defense mechanism executed by different immune cells. Researchers have found that reactive oxygen species are a key regulator of autophagy, and with it there are several nitrated fatty acids that have been reported to act as endogenous PPARγ ligands and inhibit pro-inflammatory cytokine production. Inflammation in the early phase of many chronic respiratory diseases is related to oxidative stress; hence, our study confirms that the immune system and oxidative stress is a linked process. Moreover, the latter has a positive impact on the pathogenesis of chronic respiratory diseases. More detailed studies are required for complete understanding of this scenario that may be helpful for some effective therapeutic strategies for combating against this deadly group of diseases.

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Declaration of Conflict of Interest The authors declare there is no conflict of interest.

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Immunological Basis of Oxidative Stress-Induced Lung Inflammation in Asthma and COPD

11

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Abstract

Oxidative stress is an outcome of imbalance in production vis-à-vis removal of reactive oxygen species (ROS). The critical role of oxidative stress in several chronic inflammatory diseases has been reported. In this chapter, we have discussed the participation of oxidative stress in the pathogenesis of chronic inflammatory disease of lungs: asthma and chronic obstructive pulmonary disease (COPD). Despite the differences in etiology, immunology, pathogenesis, and clinical symptoms, the involvement of oxidative stress in the manifestation of chronic airway inflammation is the most common feature of both respiratory disorders. First, we have discussed the role of various types of immune cell in the orchestration of oxidative stress-mediated lung inflammation in both asthma and COPD. Next, the contribution of cellular sources of ROS (mitochondria and NADPH oxidase) in activation of cellular signaling pathways, particularly nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nuclear erythroid 2-related factor 2 (Nrf2), is elaborated. Finally, we have highlighted the involvement of oxidative stress in the manifestation of steroid-stable conditions in patients with severe asthma and COPD. Unraveling the participation of ROS at cellular as well as intracellular events may enhance our understanding of the pathogenesis of both asthma and COPD for the development of effective treatment strategies in the area.

Keywords

Airway inflammation · Asthma · COPD · NF-κB · Nrf2 · Oxidative stress · ROS

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11.1 Introduction

Inflammation is a natural defense mechanism of the human body, activated upon exogenous and endogenous stimuli. Mainly, the purpose of this immune response is to protect our body from harmful agents (Biswas 2016a). Various types of inflammatory cells including macrophages, neutrophils, eosinophils, and lymphocytes actively perform their part in the manifestation of inflammation. Recruitment of inflammatory cells at the site of stimulus is one of the crucial steps of this process. Briefly, inflammatory cell infiltration is a sequential event through which inflammatory cells cross the endothelium lining for migration toward the target site (Biswas 2016a). During the inflammatory process, these immune cells produce various reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl-free radical, and nitric oxide (NO) for efficient destruction and removal of the invading agents (Nemat et al. 2009). Although ROS are continuously generated under normal conditions, they stay at a relatively low level due to fine balancing act exerted by the antioxidant defense system of the body (Schieber and Chandel 2014).

Recently, a positive reciprocal feedback loop between inflammation and oxidative stress has been reported (Biswas 2016b). Interestingly, the inflammation-induced oxidative stress further aggravates the inflammation through the activation of multiple pathways. Indeed, ROS particularly hydrogen peroxide (H_2O_2) can enhance the expression of various pro-inflammatory mediators through the activation of redox-sensitive master transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Oliveira-Marques et al. 2009). Inflammation and oxidative stress are two separate, but highly interdependent processes [comprehensively as reviewed in (Patrizia et al. 2014)] which collectively trigger a vicious oxidative cycle perpetuated by the release of pro-inflammatory mediators.

Under the normal physiological conditions, inflammation and oxidative stress can be resolved completely after the removal of stimuli. However, repeated and persistent exposures to stimuli can result in the chronic inflammation which fails to resolve completely at the target site. The accumulated phagocytic cells are mainly responsible for the overproduction of ROS which is not balanced by its counterpart, i.e., antioxidants, and thus cellular redox balance tilts toward exaggerated oxidative stress (Biswas 2016b; Ciencewicki et al. 2008). Further, exaggerated oxidative stress/inflammation can induce tissue damage and is the cause of many chronic inflammatory diseases (Biswas 2016a). Enhanced oxidant productions have been implicated in several lung inflammatory diseases including asthma and chronic obstructive pulmonary disease (COPD). Accordingly, we would discuss the cells of both innate and adaptive immune response as well as their relationship with the oxidative stress in the manifestation of chronic inflammation in asthma and COPD. Also, the various cellular events that result in the production of ROS and of associated signaling pathways would be elaborated.

11.2 Chronic Inflammatory Pulmonary Diseases: Asthma and COPD

Reports have shown the involvement of inflammation/oxidative stress in chronic inflammatory pulmonary diseases including asthma and COPD (MacNee 2001a). Briefly, asthma is a chronic inflammatory condition of lungs mainly characterized by inflammation, remodeling, and hyperresponsiveness of airways. It has been reported that enhanced levels of oxidants are responsible for the manifestation of airway inflammation and airway hyperresponsiveness (AHR) (Rahman et al. 1996). Indeed, the increased production of ROS by macrophages, eosinophils, and neutrophils has been reported in asthmatics (Emelyanov et al. 2001). Further, the enhanced ROS production leads to the increased vascular permeability to various inflammatory mediators via damaging epithelial and endothelial barrier leading to the perpetuation of oxidative stress cycle, continuously (Li et al. 2011). Animal studies have shown that intermittent exposure to the external oxidants complicates the allergic asthma symptoms further. Several oxidants such as ozone, sulfur dioxide (SO_2), nitrogen dioxide (NO_2), cigarette smoke, and smog are known to trigger the asthma exacerbation (Barnes 1994). It has been reported that the inhalation of ozone (a powerful oxidant) delays the apoptosis/clearance of eosinophils via promoting the expression of IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), the cytokines responsible for the survival of eosinophils (Kierstein et al. 2008), which suggest a key role of oxidants in the production of asthma-specific cytokines. In addition, the persistence of eosinophils in the airways may contribute to the consistent production of ROS results in chronic inflammation.

Likewise, COPD is slowly progressive, irreversible chronic inflammatory conditions of lung airways mainly characterized by emphysema and chronic bronchitis which lead to limited airflow with the gradual decline in lung function. Several preclinical as well as clinical studies have shown the critical role of oxidant in the pathogenesis of COPD (Ciencewicki et al. 2008). In addition to endogenous ROS source, cigarette smoke (CS) is one of the major exogenous sources of oxidants for COPD patients. Interestingly, the enhanced levels of oxidants have been reported in exhaled breath condensates, sputum, and BALF of COPD patients. Further, increased numbers of neutrophils and macrophages are believed to be responsible for generation of excessive ROS such as superoxide anion and hydrogen peroxide in COPD patients (Barnes 2004a; Saetta et al. 2001). Moreover, oxidative stress seems to cause systemic damage in COPD patients. Indeed, elevated levels of malondialdehyde in plasma, as well as 8-isoprostanes in the urine of COPD patients, have been observed (Sahin et al. 2001; Morrow and Roberts 1997). Overall, it is evident that inflammation and oxidative stress are the major hallmarks of asthma/COPD pathogenesis. As stated earlier, the immune responses elicited upon external stimuli are a set of sequential events developed by immune cells to protect the human body from additional damages. In the upcoming sections of this chapter, we will discuss first the activation/recruitment of various immune cells and then the underlying signaling responsible for redox imbalance and inflammation.

11.3 Implication of Oxidative Stress in Activation of Innate and Adaptive Immune Cells

In order to protect the internal milieu of body and to maintain their internal homeostasis, the human body has developed two types of protective system: the innate and adaptive immune systems.

The innate immune system is first to recognize and act against the external agents. The main components of innate immune response are macrophages, eosinophils, dendritic cell, and epithelial cells and that of adaptive immune response are T and B lymphocytes. The specificity of the innate immune system is relatively broad, while the adaptive immune system responds specifically to signals transferred by the innate immune system. In summary, the innate immune system stimulates the adaptive immune response to kill or neutralize the external harmful stimuli (Medzhitov and Janeway 1997). Collectively, innate as well as adaptive immune systems protect the host against multiple pathogens and other obnoxious agents in everyday life. Briefly, the phagocytosis of invading agents by professional phagocytes (macrophages and neutrophils) is the first step of innate immunity. Normally, these inflammatory cells die via apoptosis within a few days after the removal of stimuli. However, under the chronic exposure of stimuli, the process of phagocytosis is constitutively activated which requires the high consumption of molecular oxygen and in turn results in overproduction of ROS through a phenomenon commonly known as "respiratory/oxidative burst" (Dahlgren and Karlsson 1999a). Despite the eradication of these external agents, the product of oxidative burst, unfortunately, damage the healthy cells/tissues (Dahlgren and Karlsson 1999b) which ultimately leads to pathological events for respiratory complications associated with both asthma and COPD (Fig. 11.1). In the upcoming section, we elaborate on the role of innate as well as adaptive immune cells associated with asthma/COPD pathogenesis.

11.3.1 Epithelial Cells

Epithelial lining of the airways protects the internal milieu from the outer environment and is the first site of contact for various substances including pollutants, cigarette smoke, and allergens during respiration (Diamond et al. 2000). Classically, epithelial lining is considered as a physical barrier that participates in water/ion transport and mucociliary clearance of such inhaled substances. However, recently the innate immune functions of airway epithelium have been reported as it is actively engaged in a broad spectrum of activities induced by inflammation and tissue remodeling for host defense (Hiemstra et al. 2015). There are growing numbers of evidence which suggest the critical role of epithelial cells in the pathogenesis of chronic inflammatory pulmonary disease including asthma and COPD (Prefontaine and Hamid 2007; Gao et al. 2015). Mainly, ROS production due to the exposure of different stimuli results in activation of epithelial cells and subsequent airway inflammation. Interestingly, epithelial cells produce several chemoattractants to

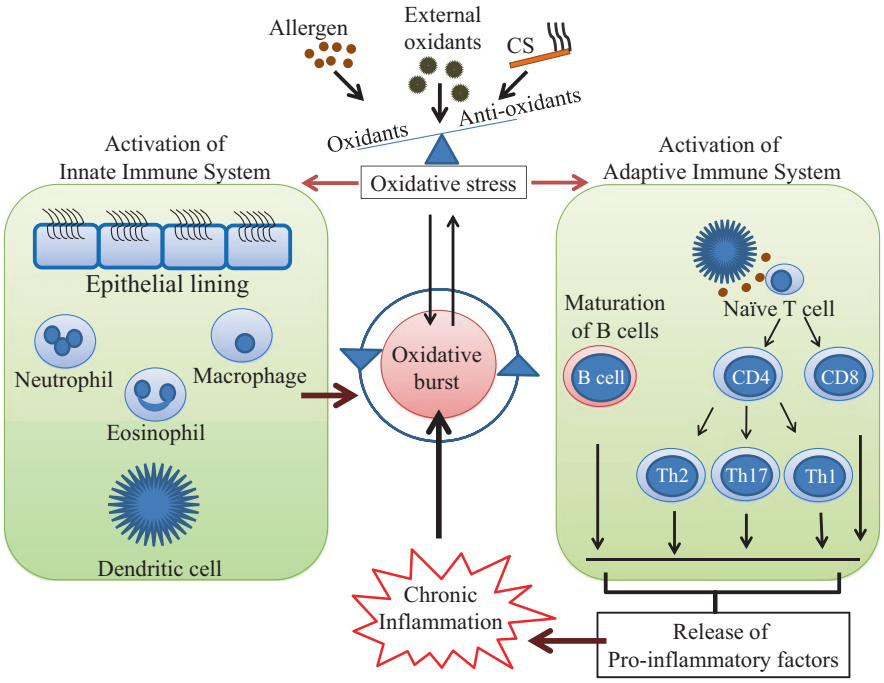

Fig. 11.1 Interplay between oxidative stress and activation of innate and adaptive immune response upon exposure to external agents. Various types of agents including allergen, cigarette smoke, and external oxidants can initiate a sequence of events leading to the oxidative stress-induced airway inflammation in asthma and COPD. Briefly, the generation of oxidative stress upon exposure to such external agents activates the innate/adaptive immune cells (epithelial cells, macrophages, neutrophils, eosinophils, dendritic cells, T cells, and B cells). Importantly, the dendritic cells act as a connecting link between the innate as well as adaptive immune system and thus direct the differentiation of naïve T cells into the different subtypes of mature T cell (T cytotoxic, T helper (Th)1, Th2, and Th17) and B cell in asthma. The persistent release of pro-inflammatory factors in lungs may result in the oxidative burst, which is ultimately responsible for the manifestation of chronic inflammation. Further, the mediators of inflammation regulate the orchestration of oxidative stress via a positive reciprocal feedback loop

facilitate the recruitment of other inflammatory cells including macrophages and neutrophils (Dent et al. 2014; Tanaka et al. 2006). Further, the alteration in the composition of airway epithelium lining has also been reported in asthma and COPD. Indeed, the neutrophil-derived proteins, S100A8 and S100A9, promote the mucin production from airway epithelial cells via redox-sensitive NF-κB signaling (Kang et al. 2015). It is well understood that an increase in the number of goblet cells and thus overproduction of mucus is one of the critical factors for clogging the airways during asthma and COPD (Hiemstra et al. 2015).

Further, the secretion of thymic stromal lymphopoietin (TSLP) from epithelial cells regulates the manifestation of inflammation at different levels. It has been reported that environmental triggers such as diesel exhaust particles (DEP) and CS

can increase the expression of TSLP in airway epithelial cells through activation of ROS-NF-κB axis (Takai 2012). Indeed, DEP and ambient particulate matter 2.5 (PM2.5) exposures increase the expression of TSLP by increasing the production of ROS in primary human bronchial epithelial cells (HBEC). Additionally, the disruption of the airway epithelium lining is another trigger for TSLP production (Lambrecht and Hammad 2012). It is important to mention here that the loss of epithelial integrity is an important pathological feature of asthma development. Oxidative stress has been shown to induce the phosphorylation of occludin, zonula occludens (ZO)-1, E-cadherin, and β-catenin and thus disrupts the tight junctions of epithelial cells (Rao et al. 2002). Moreover, it has been suggested that the TSLP produced from activated epithelial cells regulates the asthma pathogenesis during the sensitization phase. Indeed, TSLP promotes dendritic cells to carry out the differentiation of naïve $CD4^+$ T cells into T helper type 2 (Th2) cells (Prefontaine and Hamid 2007; Lee and Ziegler 2007).

In the case of COPD, CS is one of the major external risk factors associated with disease development (Salvi 2014). Importantly, CS contains thousands of harmful chemicals that include nicotine, tar, particulate phase components, gas phase constituents, and carcinogenic compound (Borgerding and Klus 2005). It is estimated that one cigarette contains around 1×10^{16} free radicals or 5×10^{14} per puff (Pryor et al. 1983). Studies have been shown that CS elicits the inflammatory response in epithelial cells by triggering the release of pro-inflammatory mediators and recruitment of immune cells at target site (Pace et al. 2008). Interestingly, the chronic presence of immune cells ultimately results in ROS-mediated damage to several cellular components such as proteins, lipids, and DNA. Indeed, the exposure of CS results in oxidative stress-induced DNA damage in epithelial cells leading to cell death (Zhao et al. 2009). Overall, epithelial cells being first to respond against external oxidants/allergen insults play a crucial role in pulmonary redox imbalance and manifestation of chronic inflammation associated with asthma and COPD.

11.3.2 Macrophages

Macrophages are ubiquitous innate immune cells. It constitutes the first line of defense against invading agents as they are the first to be recruited at the target site by activated epithelial cells. Importantly, alveolar macrophages uptake the foreign agents through phagocytosis to protect the local tissues from subsequent damages (Lambrecht 2006). Reports have shown the crucial role of macrophages in the pathogenesis of both asthma and COPD (Balhara and Gounni 2012; Barnes 2004b). Indeed, the elevated number of macrophages has been reported in BALF and sputum of patients with asthma (Yang et al. 2012). Activated alveolar macrophages are one of the important effector cells in the manifestation of airway inflammation in the chronic situation potentially through the repeated cycles of oxidative burst and consequent activation/expression of pro-inflammatory mediators (Iles and Forman

2002). Interestingly, macrophages produce various pro-inflammatory cytokines that contribute to cell proliferation, inflammation, airway damage, and airway remodeling. Indeed, macrophages produce IL-1β and IL-6, which directly regulates Th2 cell proliferation and accumulation/survival of eosinophils (Tang et al. 1998; Moon et al. 2007). Moreover, macrophages are responsible for producing nitric oxide (NO) which further converts into highly reactive peroxynitrite ($ONOO^-$) after combining with superoxide (MacMicking et al. 1997). Further, $ONOO^-$ participates in airway inflammation-associated tissue injury (MacNee 2001b). Interestingly, it has been suggested that the excessive generation of NO by macrophages might amplify the lung injury during asthma through enhanced oxidative DNA damage and inflammation in a murine model of disease (Su et al. 2008a; Naura et al. 2010).

The persistent inflammation further favors the orchestration of structural alteration in lung airways (Su et al. 2008b). Interestingly, macrophages regulate the orchestration of airway remodeling via modulating the production of Th2 cytokines mainly IL-13. Indeed, higher production of IL-13 from macrophages of asthmatic patients compared to that from healthy subjects has been reported upon repeated exposure to low-dose allergen (Prieto et al. 2000). Further, BALF samples from severe asthmatics were found to have enhanced numbers of IL-13-expressing macrophages (Kim et al. 2008a). Moreover, the role of macrophages during asthmatic exacerbation has been reported. Indeed, the enhanced production of cytokines including IL-1β, IL-6, and tumor necrosis factor (TNF)-α by macrophages plays a critical role in stimulating the Th2 cytokines in an animal model of acute exacerbation (Herbert et al. 2010).

Alveolar macrophages also play a key role in the manifestation of inflammation associated with COPD pathogenesis. Importantly, CS (well-known trigger of COPD) stimulates airway macrophages through the process of oxidative stress (Pappas et al. 2013). The molecular basis for CS-provoked amplified inflammatory responses within the macrophage involves activation of redox-sensitive NF-B signaling and consequent release of numerous cytokines including chemoattractants for neutrophils, a key event in the pathogenesis of COPD (Murugan and Peck 2009). Moreover, animal and clinical data suggest the role of macrophages in pulmonary emphysema, characteristic features of COPD (Barnes 2009). Indeed, CS induces macrophages to secrete potent proteinases especially matrix metalloproteinase (MMP)-12 (Wallace et al. 2008), which leads to parenchymal destruction (Abboud and Vimalanathan 2008). Further, MMP-12 generates elastin fragments and is considered to be responsible for the recruitment of monocytes upon CS exposure in the murine model of emphysema (Houghton et al. 2006). Therefore, the positive feedback loop created between accumulated macrophages and chemotactic elastin fragments creates conditions conductive to lung destruction. Additionally, macrophages regulate the proliferation of fibroblast via releasing transforming growth factor-beta (TGF-β) and connective tissue growth factor (CTGF), which ultimately cause fibrosis in the small airways (Hogg et al. 2004).

11.3.3 Neutrophils

Neutrophils are an important component of the innate immune system and constitute around 70% of the leukocytes in the blood. Interestingly, the exposure of different kinds of external agents stimulates the alveolar epithelial lining and macrophages to release various chemoattractants especially IL-8 which leads to the recruitment of neutrophils at the target site (Hellermann et al. 2002; Floreani et al. 2003). Numerous studies have shown the increased number of neutrophils in patients with COPD and severe asthma (Barnes 2004b; Moore et al. 2014; Hoenderdos and Condliffe 2013). Importantly, neutrophils are a prime source for ROS, cytokines, and a number of proteases (Barnes 2004b). Additionally, it has been reported that oxidative stress from external sources like cigarette smoke/environmental pollution results in aberrant neutrophil functions like prolonged survival, excessive ROS, and neutrophil elastase production (Vaidyanathan and Damodar 2015; Jaroenpool et al. 2016). Reports have shown that the oxidative stress conditions are responsible for the recruitment of neutrophils through NLRP3-mediated secretions of IL-1β. Additionally, it has been reported that NLRP3-mediated IL-18 production regulates the differentiation/secretions of Th17 cells (PMID-28979266; Lei-Leston et al. 2017). Indeed, increased expression of Th17 cytokines, namely, IL-17A and IL-17F, is found in BAL and sputum of patients with severe asthma which correlate strongly with lung neutrophilia (Ota et al. 2014; Pelaia et al. 2015a).

Further, neutrophilic elastase has the ability to degrade the elastin as well as to promote the production of mucus. Indeed, neutrophil elastase mediates the proteolytic cleavage of TGF-α, a ligand of epidermal growth factor receptor, and thus induces the mucin production (Shao and Nadel 2005; Bergin et al. 2008). Excessive production of mucus has been examined in patients with COPD (Fahy and Dickey 2010). Moreover, reports have shown that like macrophages, neutrophil activation also causes several deleterious effects in the lungs through the release of several matrix metalloproteinases, especially MMP-8 and MMP-9 (Ilumets et al. 2007), which may cause emphysema via degradation of elastin and collagen. In addition, neutrophils secrete some chemoattractants such as leukotriene B4 (LTB4) and IL-8 that recruit other neutrophils, thereby leading to chronic inflammation associated with COPD (Barnes 2007). Overall, it appears that ROS from external sources (environment, cigarette smoke) result in airway neutrophilia, which further increases ROS production and promotes tissue damage in patients with asthma and COPD.

11.3.4 Eosinophils

Eosinophils are bone marrow-derived circulating granulocytes that secure around 1–3% of total white blood cells (Possa et al. 2013a). Interestingly, the activated epithelial cells direct the recruitment of eosinophils at the target site via activating dendritic cells (Hammad and Lambrecht 2015). The crucial role of eosinophils in asthma pathogenesis is well evident. A large body of evidence is available showing that eosinophil counts are increased in airways, BAL, and sputum of patients with

asthma (Mehta et al. 2008; Possa et al. 2013b). Normally, the activated eosinophils undergo apoptosis after a few days of their recruitment in the absence of allergen. However, the blood eosinophils obtained from asthmatics show a delay in normal apoptosis compared to that of healthy subjects (Kankaanranta et al. 2000). Further, eosinophils are considered as one of the major sources of ROS. In fact, it has been reported that amount of ROS produced by eosinophils is related directly to the asthma severity (Ronchi et al. 1997). In addition, activated eosinophils are also responsible for the release of eosinophil peroxidase (EPO), which further magnify the oxidizing potential of H_2O_2 by using it as a substrate along with bromide (Br^-) and the thiocyanate (SCN^-) (Wu et al. 1999). Since EPO specifically uses bromide as a substrate, thus, the generation of bromotyrosine has used a marker for the presence of activated eosinophils (Erzurum 2016). Further, studies have confirmed the generation of bromotyrosine is absent or reduced to non-detectable levels in the EPO-knockout mouse (Brennan et al. 2002). Moreover, the high level of bromotyrosine is found in asthmatic airways which further increases with asthma exacerbations (Erzurum 2016; Aldridge et al. 2002). Statistically, the generation of bromotyrosine was found to be 3 times higher in the BALF of asthmatics compared with that in the control subject, whereas an increase of 100-fold was reported in severe asthmatics compared to individuals hospitalized for non-asthma causes (Sahiner et al. 2011).

Earlier, it was thought that eosinophils are exclusively involved in asthma pathogenesis; however, studies have shown their presence in a large subgroup of patients with COPD, particularly during exacerbation (Bafadhel et al. 2016; Gao et al. 2017). The presence of eosinophils in patients with COPD indicates the co-existence of asthma (Barnes 2016). Approximately, 15% of COPD patients also have the symptoms of asthma (Cosio et al. 2016). However, the underlying mechanism behind the presence of eosinophil in COPD patients is uncertain, but it is believed that the recruitment of eosinophils in COPD patients is an indicator of therapeutic response to steroid drugs (Barnes 2016).

11.3.5 Dendritic Cells

Dendritic cells are major antigen-presenting cells (APCs) of the immune system residing beneath the epithelial lining (Cook and MacDonald 2016). Dendritic cells are the connecting link between innate and adaptive immune systems as they play an important role to stimulate the adaptive immune response (Upham and Xi 2016). Interestingly, allergen-induced epithelial cells release several mediators particularly IL-25, IL-33, and TSLP that direct the activation of dendritic cells and subsequent differentiation of T cells (Cook and MacDonald 2016). Importantly, dendritic cells take up allergens, migrate to local lymph nodes, and regulate the differentiation of naïve T cells (Upham and Xi 2016). Animal as well as clinical data have shown the increased number of dendritic cells in airspaces, BALF, and sputum upon allergen exposure (Dua et al. 2010; Bratke et al. 2007; Todate et al. 2000). Interestingly, oxidative stress has been shown to influence the maturation of dendritic cells.

Csillag et al. have reported that pollen (ragweed) exposure-induced oxidative stress is responsible for the maturation of dendritic cells as reflected by the enhanced expression of surface markers, namely, CD40, CD80, and CD86, in peripheral blood mononuclear cells (PBMCs) (Csillag et al. 2010). Administration of antioxidant, namely, alpha-phenyl N-tertiary-butyl nitrone, blocks the oxidative stress-induced maturation of dendritic cells using in vitro and in vivo experiments which confirm the critical role of oxidative stress in dendritic cell maturation (Wang et al. 2011).

Very less information is available in the literature regarding the contribution of dendritic cells in COPD. However, the elevated level of dendritic cells is found in COPD patients especially under severe conditions (Van Pottelberge et al. 2010; Freeman et al. 2009). Interestingly, CS is responsible to modulate the number as well as the function of dendritic cells under both in vitro and in vivo conditions relevant with COPD (Van Pottelberge et al. 2009). Also, it has been suggested that CS induces the release of various chemokines from dendritic cells that play a role in the pathogenesis of COPD (comprehensively reviewed by (Givi et al. 2012)).

11.3.6 Lymphocytes

Lymphocytes, T as well as B cells, are the main component of the adaptive immune system which are activated by innate immune cells. The immune-regulatory role of lymphocytes in asthma/COPD pathogenesis is well documented. In eosinophilic asthma, naïve T cells differentiate into Th2 cells and secrete cytokines such as IL-4, IL-5, and IL-13 which further stimulate the production of immunoglobulin E (IgE) from B lymphocytes. This process further involves the degranulation of mast cell that leads to eosinophilic inflammation and tissue remodeling (Pelaia et al. 2015b; Larche et al. 2003). On the contrary, neutrophilic asthma and COPD involve the participation of Th1 and Th17 lymphocytes during disease manifestation. Particularly, Th17 plays a critical role in the recruitment of neutrophils. It appears that enhanced oxidative stress upon neutrophil recruitment in lungs results in excessive damage to the tissues (Halwani et al. 2013). It has been reported that Th17 contributes to structural changes in bronchial epithelial and airway smooth muscle (ASM) cells (Chesne et al. 2014). Indeed, elevated production of Th17 cytokines, viz., IL-17A, IL-17F, and IL-22, has been reported in severe asthma/COPD (Chien et al. 2013). Interestingly, Th17 cytokines induce the production of mucin as well as the proliferation of ASM via redox-sensitive NF-κB signaling (Fujisawa et al. 2011; Dragon et al. 2014; Chang et al. 2012).

Apart from an increase in Th2 cells and Th17 cells, reduced number of Treg cell has also been reported in patients with asthma (Huang et al. 2017). These cells play an important role to regulate T-cell-mediated immune responses. Studies have shown that the adoptive transfer of $CD4^{+}CD25^{+}$Treg cells ameliorates the allergen-induced activation of dendritic cells, airway inflammation, and remodeling (Xu

et al. 2012). Maj *et al.* observed that the increased mitochondrial activity and thus ROS overproduction are responsible for the reduced number of Treg (as it induces the apoptosis of Treg) (Maj et al. 2017). Thus, restorations of Treg number can be achieved potentially by lowering the level of oxidative stress for the amelioration of asthma-associated inflammation.

Importantly, $CD8^+$ T cells play an important role during the manifestation of COPD (Barnes 2004b; Maeno et al. 2007; Saetta et al. 1999). These subtypes of T cells have the potential to release TNF-α, perforins, and granzymes, thus performing cytotoxic functions (MacNee 2005) which further aggravate the oxidative stress (Martinvalet et al. 2005; Williams and Henkart 2005). Further, $CD8^+$ T can regulate the production of MMP via producing mediators such as INF-γ and is thus responsible for structural alteration, i.e., emphysema associated with disease progression (Freeman et al. 2010).

Additionally, B cells are associated with the development of inflammatory pulmonary diseases including asthma and COPD. Oxidative stress has shown to regulate the antibody production by B cells (Wan and Diaz-Sanchez 2006). During asthma pathogenesis, B cells regulate the allergen-induced innate immune response. Interestingly, B cells play a crucial role in IgE-mediated degranulation of mast cells (Oettgen and Geha 2001), thus further facilitating the development of allergic response as it regulates the processes such as antigen presentation by dendritic cells and differentiation of T cells (Sharquie et al. 2013). Interestingly, it has been reported that the administration of sulforaphane (an antioxidant compound derived from broccoli) effectively blocks diesel exhaust particle-induced generation of IgE from B cells (Wan and Diaz-Sanchez 2006). Likewise, activated epithelial cells, dendritic cells, and T cells collectively create an internal milieu for recruitment/expansion of B cells in COPD pathogenesis. Reports have shown the increased number of B-cell activating factor (BAFF) in patients with COPD. Recent studies have shown that targeting BAFF reduced the inflammation and alveolar destruction in CS-exposed mice, significantly (Seys et al. 2015; Morissette et al. 2016). Further studies are required to clarify the role of oxidative stress in B-cell activation/recruitment.

In summary, oxidative stress seems to play a critical role in mobilization and activation of both the innate and the adaptive immune systems upon exposure of external agents.

11.4 Intracellular Source of ROS in Chronic Lung Inflammatory Disease

Mitochondrial respiratory chain and the cytosolic nicotinamide adenine dinucleotide phosphate (NADP) oxidase-dependent complex are the main cellular sources of ROS (Sahiner et al. 2011), which are being discussed in details underneath.

11.4.1 Mitochondrial Source of ROS

Mitochondria are autonomous cellular organelles primarily involved in cellular metabolism and energy production. During aerobic respiration, mitochondria can generate ATP (oxidative phosphorylation) through electron transport chain (ETC) (Oyinloye et al. 2015). However, during the process, various kinds of ROS such as superoxide, hydrogen peroxide, and hydroxyl are produced as a by-product due to incomplete electron transfer. The mitochondrial ETC consists of five large protein complexes (complexes I–V), and most of the superoxide radicals are produced at complexes I and III during oxidative phosphorylation (Sureshbabu and Bhandari 2013; Yue and Yao 2016). Actually, ETC is a leaky process as some of the electrons from each cycle of ATP production are responsible for the generation of ROS (Cloonan and Choi 2012). Under normal physiological conditions, ROS are cleared by cellular antioxidant defense mechanisms, but under abnormal conditions, such homeostasis is disturbed (Iyer et al. 2017). Interestingly, overproduction of ROS stimulates the mitochondrial apoptotic pathway leading to release of complex IV also known as cytochrome c (COX) (Oyinloye et al. 2015).

In asthma, oxidative free radicals primarily affect COX, a key oxidative enzyme of ETC. Basically, it catalyzes the electron transfer to generate ATP and thus consumes most of the cellular oxygen (Capaldi 1990). Interestingly, the inhibition of COX has been reported to cause oxidative stress in an experimental model of allergic asthma (Mabalirajan et al. 2008). Accumulating evidence suggests that mitochondrial structural alterations along with its functional abnormalities are primarily associated with the development of asthma (Mabalirajan et al. 2008; Reddy 2011). Interestingly, the mitochondrial stability and function are both IL-4-dependent processes in experimental allergic asthma (Mabalirajan et al. 2008). Indeed, IL-4 is responsible for COX activity and inducing apoptosis in lung epithelial cells through upregulation of 15-lipoxygenase, an enzyme that causes mitochondrial degradation via oxidizing the mitochondrial membranes (Schewe 2002). Additionally, it has been shown that the oxidative stress-induced mitochondrial damage prior to allergen insult (ragweed exposure) significantly enhances the accumulation of eosinophils, mucin levels in the airways, and bronchial hyperresponsiveness (Aguilera-Aguirre et al. 2009). Moreover, studies have shown that pollutants and oxidants induce mitochondrial damage in human lung tissues which further amplify the ROS generation and cause exacerbation of disease (Fahn et al. 1998).

Likewise in patients with COPD, mitochondrial dysfunction has been reported in diaphragmatic and skeletal muscles, which result in loss of muscle strength and decline of physical activity (Lloreta et al. 1996; Meyer et al. 2013). Additionally, mitophagy is a highly conserved homeostatic process in which damaged mitochondria that are producing excess ROS continuously are selectively eliminated. Any disturbance in mitophagy can lead to the excessive release of ROS from mitochondria and consequent inflammation (Sureshbabu and Bhandari 2013). To the best of our knowledge, no information is available in the literature regarding the role of mitophagy in asthma pathogenesis. However, multiple studies have shown that CS

impairs mitophagy in epithelial cells through downregulation of parkin (an ubiquitin ligase associated with mitophagy), thus resulting in ROS-mediated damage and death, which further enhances inflammation (Ahmad et al. 2015; Ito et al. 2015). Indeed, overexpression of parkin reduced the cell death associated with CS (Ahmad et al. 2015). Furthermore, mitochondria changes such as fragmentation, swelling, and loss of cristae are reported in epithelial cells from patients with COPD upon CS exposure (Hara et al. 2013; Hoffmann et al. 2013). Taken together, it appears that mitochondrial ETC is a key contributor of intracellular ROS and plays an important role in the pathogenesis of pulmonary inflammatory disorders.

11.4.2 Cytosolic Source of ROS: Nicotinamide Adenine Dinucleotide Phosphate Oxidase (NADPH Oxidase) Signaling

NADPH oxidase (NOX), a membrane-bound enzyme complex, is one of the main sources of cellular ROS that helps to maintain a healthy level of oxidants in the cell. Initially, it was believed that it is a characteristic feature of phagocyte cells such as neutrophils and only involved in host defense. Later on, various reports indicate the presence of NOX in non-phagocytic cells and their involvement in other cellular functions also (comprehensively reviewed in (Lambeth 2004; Bokoch and Knaus 2003)). Several isoforms of this enzyme have been identified which are termed as NOX 1 to 5 and dual oxidase1 (DOUX1) and DOUX2. Structurally, NOX consists of regulatory subunits (p40phox, p22phox, p47phox, p67phox, and gp91phox) and the major binding partner Rac (Harijith et al. 2017). p22phox and gp91phox are integral membrane proteins which together constitute a heterodimeric unit termed as flavocytochrome b558. In resting cells, other components, namely, p40phox, p47phox, and p67phox, locate in the cytosol as a complex. Under pathological conditions, p47phox subunit gets phosphorylated and the entire cytoplasmic complex translocated to membrane and assembled with flavocytochrome b558 to generate active form of oxidase (Panday et al. 2015). Upon activation, NOX family (both NOX and DOUX proteins) catalyzes the transfer of the electron from NADPH to molecular oxygen with subsequent production of O_2^- that is converted to H_2O_2 by superoxide dismutase (Rada and Leto 2008).

Since asthma and COPD pathogenesis is associated with inflammatory cells' infiltration and lungs' airway epithelial activation, the NOX-induced oxidative stress is thought to play a critical role in their pathogenesis. Importantly, lung tissues express most of the isoforms of NOX proteins including NOX1 (mainly in epithelial and endothelial cells), NOX2 (mainly in alveolar macrophages and endothelial cells), NOX4 (mainly in smooth muscle cells, fibroblasts, and endothelial cells), as well as DOUX proteins, DOUX1 and DOUX2 (mainly in epithelial cells). Multiple studies have shown the contribution of NOX family-induced oxidative stress in asthma pathogenesis (Rada et al. 2014; Wan et al. 2016; Sutcliffe et al. 2012a). Enhanced expression of NOX4 and DOUX2 has been identified in bronchial biopsy samples (Sutcliffe et al. 2012a). Further, Habibovic *et al.* through their study utilizing nasal epithelial cells from asthmatic and non-asthmatic subjects found that DOUX1 expression is enhanced in asthmatic epithelia (Habibovic et al.

2016a). Various recent studies have shown the involvement of NOX4 and DUOX1 in the manifestation of several asthmatic features such as ciliary dysfunction, cytokine production, and development of Th2 immune response (Wan et al. 2016; Padgett and Tse 2016; Hristova et al. 2016). Earlier, Rada *et al.* reported that histamine-induced H_2O_2 production is mediated through DOUX1 and DUOX2 in human bronchial epithelial cells (Rada et al. 2014).

Moreover, the role of NOX members in the orchestration of airways remodeling has been reported (Harijith et al. 2017). The enhanced level of activated/phosphorylated epidermal growth factor receptors (EGFR), a critical factor in asthma pathogenesis, was observed in epithelial cells. Interestingly, the silencing of DUOX1 prevents the activation of EGFR and further ameliorates EGFR-dependent asthmatic features like inflammation, Th2 cytokine production, mucous metaplasia, and subepithelial fibrosis in house dust mite (HDM)-exposed mice (Habibovic et al. 2016b). Additionally, NOX4 plays a crucial role in smooth muscle hyper-contractility during asthma. Indeed, genetic and pharmacological inhibition of NOX4 results into the reversal of ASM agonist-induced contraction in primary airway smooth muscle cells isolated from individuals with asthma (Sutcliffe et al. 2012b). Moreover, NOX4 has been identified as a regulator in epithelial signaling pathways associated with the production of Muc5ac and MMP-1 (Amara et al. 2007; Kim et al. 2008b).

Likewise, the participation of NOX family has also been reported in COPD pathogenesis. Enhanced number of NOX2-positive cells was found in human emphysematous lungs compared to healthy controls (Trocme et al. 2015). In another study, Russell et al. (2016) reported that bronchial epithelial cells from the patients with COPD have increased expression of NOX2, NOX4, and DUOX2 when compared to healthy individuals (Richard Russell et al. 2016). Importantly, the enhanced expression of NOX4 positively correlated with the severity of COPD (Liu et al. 2016). Further, the critical role of NOX-induced ROS production in the development of characteristic features of COPD has also been highlighted. It was observed that intratracheal instillation of elastase resulted in elevated mRNA expression of NOX1 and NOX2; however, the genetic inhibition of NOX2, but not NOX1, prevented the elastase-induced emphysema in mice (Trocme et al. 2015). Further, it has been shown that ROS production in inflammatory cells was found to be dependent on NOX2 (Trocme et al. 2015).

Overall, the aforementioned studies pinpoint the critical role of NOX and associated oxidative stress in asthma and COPD.

11.5 Role of Redox Signaling in Pathogenesis of Chronic Lung Inflammatory Diseases

Studies have shown that various kinds of oxidants and antioxidants play a major role in aerobic organisms (ranging from prokaryotes to humans) to maintain the cellular homeostasis (Carvalho et al. 2015). In the previous sections, we have already discussed the crucial role of ROS activation and mobilization of innate as well as adaptive immune cells during the manifestation of asthma and COPD. Owing to more stability of H_2O_2 among various oxidant species and its capability to cross

the membranes, the major part of oxidant-associated signaling is mediated by H_2O_2 (Harijith et al. 2017). Indeed, a transient increase in H_2O_2 level has been evident after the exposure of various external stimuli such as cigarette smoke, antigens, and pollutants. Notably, the physiologic concentration of H_2O_2 in humans is increased up to 0.5–0.7 μM from 0.002 to 0.2 μM (under normal condition) during intracellular signaling (Stone and Yang 2006a). It is already discussed that such increase in H_2O_2 is mainly associated with dysfunctioning of mitochondrial as well as NOX signaling which is considered as a major source of cellular ROS (Brown and Griendling 2009; Carrasco et al. 2016). Additionally, it has been found that H_2O_2 acts as a critical intracellular secondary messenger and mediates various immune responses via regulating redox-sensitive transcription factors especially NF-κB (Stone and Yang 2006b). Moreover, studies have shown the crucial role of NF-κB-dependent mediators in the orchestration of oxidative stress. Thus, a creation of a vicious cycle between NF-κB signaling and oxidative stress results into a frequent execution of oxidative burst by over-activated phagocytic cells which may make the condition even more complicated (Saldanha et al. 2013).

To neutralize ROS-induced redox imbalance and to maintain the internal homeostasis, antioxidant system of the cell becomes activated. Antioxidants act as a natural defense system of cell which mainly consist of various cellular enzymes such as catalase, glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) (Marengo et al. 2016). Importantly, nuclear erythroid 2-related factor 2 (Nrf2) is a master transcription factor of antioxidant signaling. Moreover, studies have suggested that various cellular signaling pathways get activated depending upon the cellular levels of oxidants. For example, at lower levels, ROS perhaps initiate antioxidant defense system through the activation of Nrf2, whereas, at the higher level, it leads to inflammatory response through the activation of NF-κB (Riedl and Nel 2008; Li and Nel 2006). Overall, it seems that the level of oxidants and antioxidants regulates the activation of various intracellular signalings that play a crucial role in the orchestration of inflammatory disease (Fig. 11.2). Thus, maintaining the levels of intracellular oxidants in normal range can be considered as a potential approach to manage the various inflammatory diseases. Accordingly in this section, we will discuss the role of redox-sensitive NF-κB in oxidant signaling, as well as Nrf2 in antioxidant signaling in cells during asthma and COPD pathogenesis.

11.5.1 Role of NF-κB in Oxidant-Mediated Signaling

Reports have shown the involvement of various redox-sensitive transcription factors such as NF-κB, nuclear factor of activated T-cells (NFAT), and activator protein-1 (AP-1) in the manifestation of various inflammatory diseases (Rahman and MacNee 1998; Huang et al. 2001; Le Rossignol et al. 2018). Although the pro-inflammatory factors involved in the pathogenesis of asthma and COPD are entirely different, it appears that the inflammatory process in both the diseases has a strong connection with NF-κB activation, as explained below.

NF-κB is a redox-sensitive multiprotein complex. It is recognized as a master transcription factor that regulates the orchestration of inflammation (Liu et al. 2017).

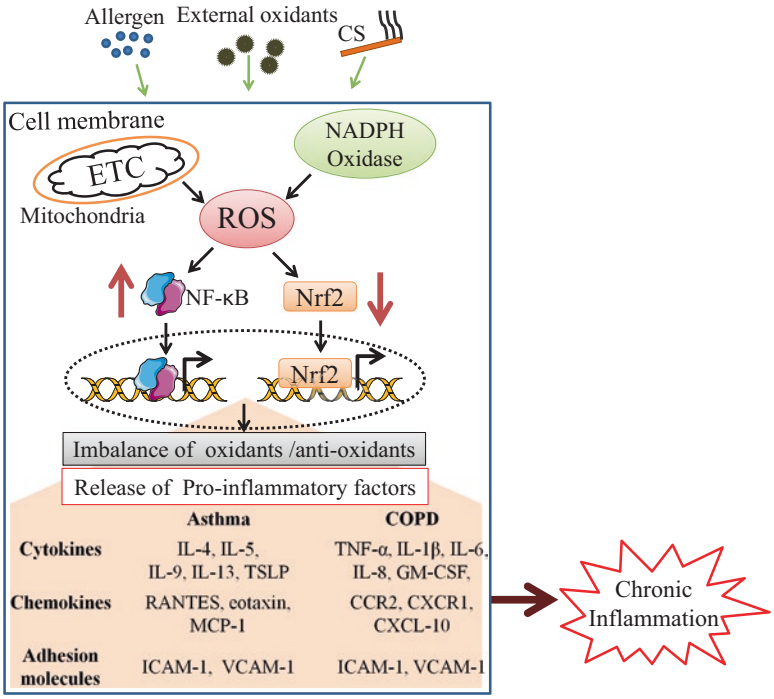

Fig. 11.2 Schematic presentation of oxidative stress-associated intracellular signaling responsible for the manifestation of asthma and COPD pathogenesis. Mitochondria as well as NADPH oxidases are the main sources of intracellular ROS production which get activated in response to external agents. Under the normal condition, the cellular homeostasis is maintained via a redox-sensitive antioxidant defense system. Importantly, Nrf2 is the master transcription factor for inducing antioxidant machinery. Under pathological conditions, redox balance tilts toward the enhanced production of oxidants *via* NF-κB signaling. Upregulation of NF-κB signaling but downregulation of Nrf2 signaling simultaneously exaggerate the oxidative stress conditions leading to the consistent activation and recruitment of inflammatory cells during asthma and COPD

It is a heterodimer complex made up of one subunit of 50 kDa (p50) and one of 65 kDa (p65). In normal cells, NF-κB remains inactive in the cytosol as it is associated with its inhibitory protein, inhibitory kappaBα (IκBα) (Baldwin 1996). Under oxidative stress condition, NF-κB becomes free as IκBα undergoes phosphorylation and ubiquitination catalyzed by IκB kinase (IKK) (Hinz and Scheidereit 2014). Subsequently, NF-κB translocates to the nucleus and regulates the expression of several pro-inflammatory factors by binding at their promoter sites. It has been suggested that NOX-derived ROS play an important role in the activation of NF-κB signaling and their dependent pro-inflammatory mediators (Janssen-Heininger et al. 2009).

NF-κB has been considered to be a critical transcription factor in the pathophysiology of asthma (Charokopos et al. 2009). Activation of NF-κB induces a variety of inflammatory genes that are abnormally expressed in asthma. These include the

genes for the cytokines (IL-4, IL-5, IL-9, IL-13, and TSLP), the chemokines (RANTES, eotaxin, and MCP-1), and the adhesion molecules (ICAM-1 and VCAM-1) (Schuliga 2015). Enhanced activation of NF-κB signaling was evidenced by increased expression of IKK, phosphorylation of IκBα followed by nuclear localization of NF-κB in the airways of asthmatics (Gagliardo et al. 2003; Hart et al. 1998). Further, the increased level of NF-κB nuclear binding was detected in sputum inflammatory cells of asthmatics (Hart et al. 1998). Additionally, animal model-based studies confirmed the role of NF-κB in asthma pathogenesis as the activation of NF-κB in inflammatory cells was increased upon allergen challenge in mice (e.g., ovalbumin (OVA) or HDM extract) (Tully et al. 2013; Poynter et al. 2004; Poynter et al. 2002). Interestingly, it has been shown that the level of NF-κB was increased in parallel with ROS in the murine model of asthma (Lee et al. 2004). Overall, it appears that the enhanced level of ROS upregulates the NF-κB activation in asthma.

As in asthma, the participation of NF-κB signaling in COPD pathogenesis has also been reported. NF-κB regulates the expression of several inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, and GM-CSF), chemokines (CCR2, CXCR1, and CXCL-10), and adhesion molecules (ICAM-1 and VCAM-1) that actively perform their part during the pathogenesis of COPD. Enhanced level of activated NF-κB is observed in the bronchial biopsies and inflammatory cells of patients with COPD (Di Stefano et al. 2002; Caramori et al. 2003). Further, the sputum neutrophils of COPD patients show an increase in NF-κB expression upon CS exposure (Brown et al. 2009). Interestingly, the activity of IKK in PBMCs obtained from COPD patients is higher compared to that of controls (Gagliardo et al. 2011). Additionally, the level of IκBα in lung tissue of smokers and COPD patients is comparatively lower to that in non-smoking healthy individuals (Szulakowski et al. 2006). Similarly, animal model-based experiments confirm the involvement of oxidative stress-activated NF-κB in COPD pathogenesis. An enhanced level of NF-κB and its recruitment to the promoter sites of number of inflammatory genes have been observed in the mice lungs upon CS exposure (Yang et al. 2009). Interestingly, the pharmacological or genetic inhibition of NF-κB signaling has been observed to alleviate CS/LPS-induced airway inflammation (Rastrick et al. 2013; Li et al. 2009; Rajendrasozhan et al. 2010).

Though entirely different network of inflammatory mediators is known to play a role in the pathogenesis of asthma and COPD, it appears that oxidative stress-NF-κB axis seems to be common and critical factor for inducing their expression for orchestration of several events required for inflammatory process in both the lung diseases. Delineation of critical factors in the process that tilts the inflammation toward Th1/Th2 may enhance our understanding for differential expression of inflammatory factors in asthma and COPD.

11.5.2 Role of Nrf2 in Antioxidant Signaling Under Oxidative Stress Conditions in Lungs

Nrf2 is a key regulator of cytoprotective proteins, especially antioxidants (Pall and Levine 2015). Normally, Nrf2 is continuously produced in the cytoplasm but presents at low levels as it is attached with its inhibitor, viz., Kelch ECH associating

protein 1 (Keap1), which mediates the degradation of Nrf2 via ubiquitination. Actually, Keap1 is a sensor molecule for oxidative stress. Under the stress conditions, Nrf2 becomes free from Keap1, translocates to the nucleus, occupies the antioxidant response elements (ARE) within the promoter regions of several genes, and ultimately exerts numerous immunomodulatory functions (Ahmed et al. 2017). Importantly, Nrf2 maintains the cellular ROS homeostasis by various ways such as promoting the glutathione synthesis, NADPH synthesis, and encoding the enzymes that directly inactivate oxidants (Moinova and Mulcahy 1999; Hayes et al. 2005; Thimmulappa et al. 2002).

Impaired response of Nrf2 has been reported in asthmatic patients. Indeed, the level of Nrf2 protein and their dependent responses are markedly reduced in ASM cells of severe asthmatics as compared to that of normal subjects (Michaeloudes et al. 2011). Additionally, it has been shown that Nrf2-deficient mice are more susceptible to exhibit increased oxidative stress, inflammation, Th2 cytokine secretion, mucus production, and AHR compared to wild-type controls upon OVA exposure (Rangasamy et al. 2005). Moreover, the role of Nrf2 during the sensitization phase of diseases has been explored. Interestingly, dendritic cells from Nrf2-deficient mice exhibit increased expression of surface activation markers, Th2 responses, and oxidative stress compared to that from wild-type mice upon ex vivo stimulation of allergen (Williams et al. 2008).

Further, the beneficial potential of various Nrf2 activators has been tested against asthmatic symptoms. Recently, it has been shown that the pharmacological activation of Nrf2 by 2-trifluoromethyl-2′-methoxychalone (TMC) enhances the epithelial barrier function as well as the level of tight junction proteins mainly zonula occludens-1 in lung epithelial cells. Further, TMC administration during the allergen challenge reduces the allergic inflammation and AHR in the animal model of disease (Sussan et al. 2015). Sulforaphane, a natural isothiocyanate, is another potent Nrf2 agonist (Dinkova-Kostova and Abramov 2015). It has been suggested that sulforaphane effectively suppresses the diesel exhaust particles-induced proinflammatory effects in human bronchial epithelial cells. Previously, it has been found that sulforaphane blocks the production of IL-8, GM-CSF, and IL-1β in the bronchial epithelial cells effectively (Mainardi et al. 2009). It is, therefore, possible that quenching of ROS through potentiation of Nrf2 signaling may alleviate pollutant-induced exaggeration in asthma. Additionally, bucillamine a thiol antioxidant acts as an Nrf2 activator (Mazor et al. 2006). It mediates the production of various precursors of glutathione synthesis via inducing the translocation of Nrf2 into the nucleus. Further, the contribution of Nrf2 signaling during airway remodeling, characteristics feature of chronic asthma, has also been explored. It has been found that polydatin (Nrf2 activator) suppresses the OVA-induced epithelial-mesenchymal transition (EMT) and lung fibroblast protein expression via inducing Nrf2-mediated antioxidation (Zeng et al. 2018). In addition, bixin, broccoli sprouts, curcumin, quercetin, and resveratrol are some other examples of natural Nrf2 activators, many of which have been reported for their beneficial effects in asthma.

The involvement of Nrf2 signaling in COPD pathogenesis has also been reported. Reduced expression of Nrf2, both at mRNA and protein levels, was found in COPD

subjects than that in control subjects (Suzuki et al. 2008; Mercado et al. 2011). Moreover, it has been suggested that Nrf2 plays a protective role against oxidative stress-induced cellular damages including apoptosis following the exposure of CS. Interestingly, Nrf2 knockout mice show a higher susceptibility to CS-induced pulmonary emphysema (Iizuka et al. 2005). Further, it has been shown that sulforaphane an Nrf2 activator reverses the disease manifestations via counteracting the oxidative stress-mediated alterations in mice exposed to CS and in macrophages isolated from COPD patients (Malhotra et al. 2011a). Further studies may be required to identify the key molecular factors that may be altered by Nrf2 signaling in both asthma and COPD.

11.6 Impact of Oxidative Stress on Steroid Refractory Nature of Chronic Inflammatory Pulmonary Diseases

Corticosteroids are the mainstay therapy for several inflammatory diseases including asthma and COPD (Barnes 2013). Most of the asthmatic symptoms are efficiently managed with the low doses of corticosteroids. However, a small proportion of asthmatics (5–10%) and the majority of COPD patients do not respond well even to the high dose of steroids; these are tagged as steroid-resistant (Luhadia 2014; Jiang and Zhu 2016). Reports have shown that the patients with severe asthma are less responsive to corticosteroids compared to that with mild asthma (Moore et al. 2007).

It has been found that the process of histone acetylation and deacetylation critically regulates the inflammatory action of steroids. Indeed, steroids direct the recruitment of HDAC2 to activated inflammatory genes and thus suppress their expression (Marwick et al. 2007). Interestingly, the reduced activity/expression of HDAC2 is correlated with the poor response of patients to steroids (Barnes et al. 2004). Reports have shown the reduced expression of HDAC2 in alveolar macrophages and airways in patients with asthma and COPD (Hew et al. 2006; Murahidy et al. 2005).

Further, studies have suggested the critical involvement of oxidative stress in the development of steroid-refractory severe asthma and COPD. It has been reported that enhanced oxidative stress suppresses the expression/activity of HDAC2. Cosio *et al.* have shown that redox imbalance mediates the nitration of tyrosine residues on HDAC2, thus resulting in its inactivation, ubiquitination, and degradation (Cosio et al. 2004a). Additionally, oxidative stress activates phosphoinositide 3-kinase (PI3K) δ, which further phosphorylates and inactivates HDAC2 (To et al. 2010).

It is noteworthy to mention here that the restoration of HDAC2 expression/activity via targeting the oxidative stress is the potential approach to enhance the responsiveness toward steroid therapy. Interestingly, administration of theophylline restores the HDAC2 activity in macrophages from COPD patients and consequently restores the steroid resistance (Cosio et al. 2004b). Further, it has been reported that theophylline reverses the steroid-stable conditions via inhibiting the oxidant stress-activated PI3Kδ signaling (To et al. 2010).

Moreover, the potential effect of several antioxidants in the context of steroid resistance chronic inflammatory disease has been examined. Nowadays, Nrf2-mediated signaling is the main focus of research. It has been reported that HDAC2 positively regulates the activity of Nrf2 (Malhotra et al. 2008). Interestingly, the reduced activity of Nrf2 correlates with the reduced expression of HDAC2 in COPD patients (Mercado et al. 2011). Further, sulforaphane has been shown to elevate the expression of HDAC2 and thus reverses the steroid resistance in mice exposed to CS and also in macrophages isolated from COPD patients (Malhotra et al. 2011b). Overall, it seems that targeting oxidative stress might be an important mechanism to reverse the steroid-refractory features of COPD and severe asthma.

11.7 Conclusion

Overall, studies strongly advocate the critical involvement of oxidative stress in the pathogenesis of asthma and COPD. Unfortunately, the outcomes of clinical trials targeting the redox imbalance to normalize the inflammatory diseases are not so encouraging. The exact reason behind the failure in the translation of oxidative stress-targeted therapy from preclinical to clinical level is not yet clear. There are some theoretical explanations of this failure: (1) It is possible that suppression of oxidative stress may also suppress other redox-sensitive factors which might otherwise be playing a protective role against chronic lung inflammation and hence neutralize the beneficial effects. (2) It is also possible that the persistent ROS production might trigger some other pathways which make the conditions more complex and resistant to antioxidant therapy at advanced stages. Overall, despite the intense research, the design of oxidative stress-based therapy at the clinical level remains a challenging task.

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Contribution of Aldose Reductase-Mediated Oxidative Stress Signaling in Inflammatory Lung Diseases

12

Himangshu Sonowal, Ashish Saxena, and Kota V. Ramana

Abstract

Asthma and chronic obstructive pulmonary disease (COPD) are the major lung inflammatory diseases that are characterized by expiratory airflow limitation, chronic inflammation, and structural and functional changes of airways. Although steroids are most commonly used to treat asthma and COPD, their compliance in adults and more often in children is poor, and a number of patients do not respond to steroids. We have recently demonstrated that inhibition of polyol pathway enzyme, aldose reductase (AR), is effective in the prevention of airway inflammation in rodent models of asthma and COPD. Specifically, our studies indicate that AR inhibitors prevent increase in NF-κB-dependent expression of inflammatory cytokines, eosinophil accumulation in airways and bronchial subepithelium, mucus production, airway epithelial cell death, and goblet cell metaplasia in cultured cell lines as well as in vivo acute and chronic mouse models of lung diseases. The AR inhibitors have already undergone phase-II and phase-III clinical trials in diabetic neuropathy and found to be safe in humans with no major irreversible side effects. In this chapter, we discuss the role of AR in oxidative stress-mediated lung inflammatory complications and potential significance of developing AR inhibitors to treat various lung inflammatory diseases.

Keywords

Aldose reductase · Asthma · COPD · Rhinitis · Oxidative stress · Allergy

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Abbreviations

4-HNE	4-Hydroxy-2-nonenal
8-OHG	8-Hydroxyguanine
AGE	Advanced glycation end product
ALI	Acute lung injury
AMPKα1	AMP-activated protein kinase α1
AP-1	Activator protein-1
AR	Aldose reductase, AKR1B1
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
BHT	Butylated hydroxytoluene
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
Cox-2	Cycloxygenase-2
CREB	cAMP response element-binding protein
DNA	Deoxyribonucleic acid
EMT	Epithelial mesenchymal transition
GS-DHN	Glutathionylated-1,4-dihydroxy-2-nonene
GSK-3β	Glycogen synthase kinase-3beta
HCC	Hepatocellular carcinoma
HMG-CoA	3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase
HO-1	Heme oxygenase-1
HOCl	Hypochlorous acid
IKK	IkB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MDA	Malondialdehyde
mTOR	Mammalian target of rapamycin
NADP	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NQO1	NADPH dehydrogenase [quinone]1
Nrf-2	Nuclear factor erythroid 2 (NFE2)-related factor 2
OVA	Ovalbumin
p38MAPK	p38 mitogen-activated protein kinase
PGE-2	Prostaglandin E-2
PGI2	Prostacyclin
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

RWE	Ragweed extract
SAEC	Small airway epithelial cells
SDR	Sorbitol dehydrogenase
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SPDEF	SAM pointed domain-containing Ets transcription factor
TGFβ1	Transforming growth factor β1
TNFα	Tumor necrosis factor alpha

12.1 Introduction

Aldose reductase (EC 1.1.1.21; AR; AKR1B1) is a member of the aldo-keto reductase superfamily of proteins. It is a cytosolic NADPH-dependent enzyme that catalyzes the first and rate-limiting step of the polyol pathway of glucose metabolism (Graham et al. 1991; Borhani et al. 1991). In this pathway, glucose is converted to sorbitol in the presence of NADPH, and the sorbitol thus generated is further converted by sorbitol dehydrogenase (SDR) to fructose in the presence of NAD^+. During normoglycemic conditions, about 3% of the available glucose enters the polyol pathway, whereas in hyperglycemia, around 1/3 (~30%) of the available glucose pool enters the polyol pathway (Morrison et al. 1970). During hyperglycemia, an excess of glucose flux through the polyol pathway leads to the generation of large amounts of sorbitol. Sorbitol is impermeable to the cell membrane and thus accumulates inside the cells and induces osmotic stress. Therefore, AR is responsible for excessive sorbitol formation during hyperglycemia leading to osmotic stress. The increased osmolality has been shown to be associated with secondary diabetic complications such as retinopathy, nephropathy, neuropathy, cataractogenesis, etc. (Oates and Mylari 1999; Chung et al. 2003; Ko et al. 1997; Yabe-Nishimura 1998). Besides osmotic stress, oxidative stress has also been shown to be involved in secondary diabetic complications (Giacco and Brownlee 2010; Srivastava et al. 2005). The glucose flux through the polyol pathway and activation of AR for conversion of glucose to sorbitol lead to oxidation of NADPH to $NADP^+$, and further downstream of the polyol pathway, sorbitol dehydrogenase (SDH)-mediated conversion of sorbitol to fructose lead to the generation of NADH from NAD^+ (Cheng and Gonzalez 1986; Lee and Chung 1999). This process leads to a decrease in NADPH and NAD^+ in the cells leading to oxidative stress (Giacco and Brownlee 2010; Niedowicz and Daleke 2005). Further, a decrease in NADPH levels could hamper the production of reduced glutathione and nitric oxide, which in turn could exacerbate the oxidative stress (Cheng and Gonzalez 1986; Tang et al. 2012). Thus, AR plays an important role in the hyperglycemia-induced osmotic and oxidative stresses (Fig. 12.1). Previous studies also suggest that oxidative stress but not osmotic stress is one of the major causes of secondary diabetic complications (Chung et al. 2003; Srivastava et al. 2005; Yan 2018; Zhang et al. 2012). For example, antioxidants such as trolox and BHT are able to prevent hyperglycemia-induced cataractogenesis without

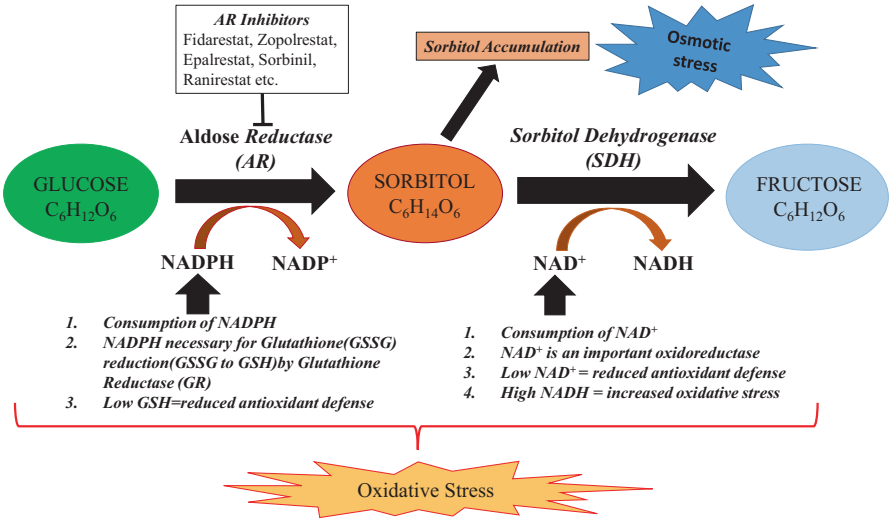

Fig. 12.1 The role of the polyol pathway enzyme, aldose reductase, in osmotic stress and oxidative stress

affecting the sorbitol levels (Ansari et al. 1994). These studies indicate the significance of oxidative stress in diabetic complications. Since active site of AR is lined with a number of hydrophobic amino acid residues, it was thought that polyols such as glucose might not be good substrates of AR (Km glucose = 30–50 mM) (Srivastava et al. 1985, 1999). In fact, various investigators have identified that aldehydes substrates such as saturated aldehydes, unsaturated aldehydes, phospholipids, and steroids are the major physiological substrates of AR with Km in micromolar range (Srivastava et al. 1985, 1999; Petrash et al. 1994; El-Kabbani et al. 2004; Vander Jagt et al. 1995; Ramana et al. 2000; Singh et al. 2006; Barski et al. 2008). These findings open up new avenues in identifying the novel role of AR in the mediation of various diseases apart from diabetic complications (Fig. 12.2). Studies on AR during the past decade or so have highlighted many important roles of this enzyme in diabetes, myocardial infarction, cardiac injury, ocular and systemic inflammation, sepsis, cancer, etc. (Yabe-Nishimura 1998; Tammali et al. 2011a; Chen and Zhang 2012; Ramana and Srivastava 2010; Laffin and Petrash 2012). Experimental evidence obtained from in vitro and in vivo studies suggest that AR regulates important transcription factors such as NF-κB, AP-1, Nrf2, etc. (Tammali et al. 2011a; Ramana 2011). Thus, these studies indicate the significance of AR in various complications associated with inflammation and oxidative stress. Various studies have reported that inhibition of AR protects the cells from oxidative stress-induced damage in vitro and in vivo (Yabe-Nishimura 1998; Srivastava et al. 2005; Tammali et al. 2011a; Ramana and Srivastava 2010). Specifically, AR inhibitors have shown to prevent growth factor-induced proliferation of colon cancer cells as well as prevent in vivo tumor growth and metastasis in mouse models of colon cancer (Tammali et al. 2011a, b; Saxena et al. 2013). AR has also been reported to play an important

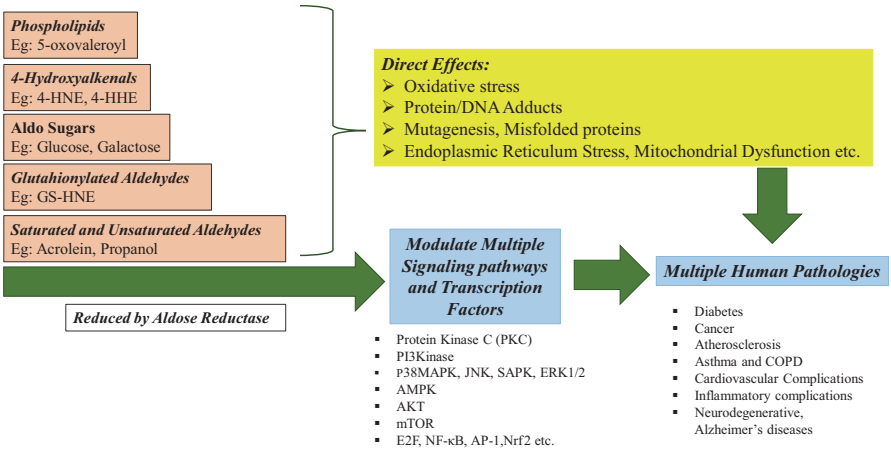

Fig. 12.2 Some physiological substrates of aldose reductase and the role of AR in human health and disease

role in hepatocellular carcinoma (HCC) progression and development (Wang et al. 2017; Zhao et al. 2017; Torres-Mena et al. 2018). Specifically, expression of many aldo-keto reductase isoforms has been found to be upregulated in HCC, and AR has been reported to regulate HCC growth by regulating AR-AKT interactions via AKT/mTOR signaling pathway (Zhao et al. 2017; Torres-Mena et al. 2018). Recent studies have also shown that AR plays an important role in chemotherapeutic drug metabolism and that combination therapy for cancer with AR inhibitors showed a better therapeutic index in in vivo and in vitro models (Lee et al. 2011; Sonowal et al. 2017; Lee et al. 2002; Sonowal et al. 2018). Aldo-keto reductases have also been shown to play an important role in doxorubicin metabolism in adipose tissues (Sheng et al. 2017). These studies highlight the important role played by this enzyme in chemotherapeutic drug metabolism. AR also plays an important role in cardiac function, atherosclerosis, and atherosclerotic plaque formation (Vedantham et al. 2011). AR has also been reported to modulate myocardial ischemia/hyperfusion (I/R) injury, and AR inhibition prevents I/R injury in mice (Ananthakrishnan et al. 2009). AR has also been reported to affect cellular bioenergetics and affect mitochondrial biogenesis in different cell types by activating AMPK and initiating mitochondrial biogenesis (Shukla et al. 2017a; Shi et al. 2017; Yeung et al. 2017). Further, various studies have also shown the potential role of AR in neurodegenerative disorders including significant protection against diabetic polyneuropathy-induced reduction in nerve conduction velocities (Yeung et al. 2017; Sekiguchi et al. 2018; Ho et al. 2006). AR inhibitors such as epalrestat and fidarestat have already undergone phase-II and phase-III clinical trials for diabetic neuropathy and have been found to be safe for use in humans without any major side effects. Thus, AR could be a potential therapeutic target for inflammatory complications. In this chapter, we discuss how AR could be involved in various lung inflammatory complications and how inhibition of this enzyme could prevent asthma, COPD, and other lung pathologies.

12.2 Oxidative Stress

Oxidative stress is defined as the imbalance between the production of reactive oxygen species (ROS) and antioxidative defense machinery (Davies 1995). Catabolic and anabolic activities in the cells generate a reductive environment that leads to the production of reactive superoxide radicals ($^{\circ}O_2$) from oxygen, which acts as the precursors for most of the other free radical species such as peroxides (H_2O_2) and hydroxyl radicals (OH°) in cells. Short-lived superoxide radicals are highly reactive and unstable and can interact with cellular macromolecules spontaneously. Superoxide dismutase (SOD) forms hydrogen peroxide (H_2O_2), which is highly stable than $^{\circ}O_2^-$ and is cell membrane permeable. The H_2O_2 is also an important signaling molecule generated in oxidative stress. The reaction of superoxide with nitric oxide leads to the production of peroxynitrite ($ONOO^-$). Among other species of ROS generated in cells, catalytic activity by peroxidases leads to the generation of hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$) which are also among the other prominent ROS species reported in cells. Hydrogen peroxide (H_2O_2) is finally reduced to hydroxyl radical or water molecules either by electrophilic addition or by Haber-Weiss reaction, catalyzed by iron (Kehrer 2000; Schieber and Chandel 2014). ROS are important signaling intermediates in cells, and these either activate or inhibit a variety of signaling pathways (Table 12.1). Studies on signaling mechanisms activated by ROS have indeed shed light on the important roles mediated by ROS during normal cellular homeostasis and also during pathological complications such as cardiovascular complications, diabetes, cancer, and aging. (Davies 1995; Brieger et al. 2012).

Oxidative stress generated by ROS has been reported to be pro-tumorigenic in nature and induces aberrant cellular proliferation or mutations in DNA, which may lead to malignant transformations in cells leading to abnormal cell growth (Schieber and Chandel 2014). ROS induces oxidative damage in nucleic acids and lipids. Guanine bases in DNA and RNA are readily oxidized by ROS to generate 8-hydroxyguanine (8-OHG). 8-OHG can pair with adenine, and resulting G to T and C to A substitution during DNA replication introduces missense mutations. 8-OHG is readily accepted as a marker for oxidative damage to DNA and RNA in various pathologies including cancer (Valavanidis et al. 2009; Cadet and Wagner 2013). Elevated levels of 8-OHG have been reported in the urine of patients with various cancers, atherosclerosis, and diabetes (Valavanidis et al. 2009; Wu et al. 2004). Thus, implicating the critical role of 8-OHG in oxidative stress-induced DNA and RNA damage in multiple diseases. Further, increased oxidative stress causes the peroxidation of the lipids and generates various toxic lipid aldehydes such as 4-hydroxynonenal (HNE) (Ayala et al. 2014). Increased lipid peroxidation-derived aldehydes have also been shown to be involved in various inflammatory complications (Yadav and Ramana 2013; Barrera 2012). Most of the lipid peroxidation-derived aldehydes are the catalytic substrates of AR, thus providing substantial evidence to the hypothesis that AR plays an essential role during different pathological complications associated with oxidative stress (Srivastava et al. 2005; Yadav and Ramana 2013). Indeed, AR expression has been shown to be

Table 12.1 Some important reactive oxygen species (ROS) and reactive nitrogen species (RNS) and their role in cellular signaling

Reactive oxygen species	Symbol	Roles
Superoxide anion	$O_2^{\bullet-}$	Most widely formed ROS species in the cells. Reacts with another superoxide radical catalyzed by superoxide dismutase (SOD) to form H_2O_2 and O_2. They affect multiple signaling pathways and transcription factors (PI3K, p38MAPK, NF-κB, Nrf2), regulate protein-protein and protein-DNA interactions, DNA damage, cytokine and chemokine secretion, aging, etc.
Singlet oxygen	$^1O_2$	Highly reactive oxygen species. Induces oxidative damage to major cellular macromolecules and acts as a signaling intermediate in various cellular signaling pathways
Hydroxyl radical	$OH^{\bullet}$	Highly reactive and most widely generated during immune response to cope with pathogenic infections. Induces damage to a variety of macromolecules including DNA and RNA
Hydrogen peroxide	H_2O_2	Relative stable product with high oxidant capacity and reactivity with the majority of the biomolecules. Induces oxidative damage and acts a second messenger in various oxidative stress-mediated cellular signaling pathways
Nitric oxide	$NO^{\bullet}$	Highly reactive, vascular, and neuronal signaling molecule. Important for cardiovascular physiology. Reacts with cellular iron to form cGMP which mediates multiple signaling pathways in the cells
Peroxynitrite	$ONOO^-$	Oxidizing properties, induces oxidative damage to DNA, RNA, proteins, etc. Activates multiple signaling pathways like PI3K, MAPK, etc.

Table showing a list of some important ROS and RNS generated in cells during cellular metabolic processes and their roles in cellular signaling. For a detailed description on the various ROS and RNS species and their roles in cellular signaling, readers are requested to refer the cited references (Schieber and Chandel 2014; Ray et al. 2012; Kim and Park 2003; Forman et al. 2010; Finkel 2011; Franchina et al. 2018; Apel and Hirt 2004; Lane and Gross 1999; Liaudet et al. 2009)

upregulated during various oxidative stress conditions indicating the association between oxidative stress and AR in mediating several disease pathologies (Srivastava et al. 2005; Tammali et al. 2011a; Ramana and Srivastava 2010; Ramana 2011; Saraswat et al. 2006; Ravindranath et al. 2009).

12.3 Aldose Reductase in Oxidative Stress Signaling

Hyperglycemia is a major inducer of ROS. Apart from ROS generation, hyperglycemia also affects several biochemical pathways such as activation of protein kinases, transcription factors, cellular growth, and metabolism in the cells (Busik et al. 2008; Fiorentino et al. 2013; Volpe et al. 2018). Hyperglycemia-induced increase in glucose flux leads to enhanced mitochondrial oxidative phosphorylation and is the primary source of ROS in cells (Sivitz and Yorek 2010). Another major pathway that is activated during hyperglycemia is the polyol pathway. As discussed earlier, activation of the polyol pathway during hyperglycemia interferes with the cellular

antioxidant defense capacity by reducing the glutathione (GSH) generation (Lee and Chung 1999). The decrease in the ratio of NAPDH and NAD^+ in hyperglycemia due to increased polyol pathway activity could also lead to increased oxidative stress, as these cofactors are responsible for maintaining cellular redox balance (Ying 2008). Further, NADPH-like enzymes (NOX) and NADH-oxidases have been identified as inducers of ROS in vitro and in vivo during hyperglycemia (Lassegue et al. 2001; Ellis et al. 1998). Further, metabolites of the polyol pathway have been reported to initiate the generation of non-enzymatic advanced glycation end products (AGEs), which are inducers of various signaling pathways and are toxic second messengers (Hamada et al. 1996; Ramasamy and Goldberg 2010). AGEs play an important role in oxidant-induced damage to cells. Fructose from the polyol pathway can be metabolized to fructose-3-phosphate and 3-deoxyglucosone, which are extremely potent non-enzymatic AGEs (Ramasamy and Goldberg 2010). The increase in AGEs leads to elevated oxidative stress in cells. AGEs have been reported to be either directly or indirectly involved in the pathogenesis of many metabolic diseases and pathological symptoms like type 2 diabetes, cardiovascular and neurological complications, etc. (Ramasamy and Goldberg 2010; Yao and Brownlee 2010). Thus, activation of the polyol pathway during hyperglycemia leads to a decrease in the antioxidant defense capacity along with an increase in ROS generation.

In addition, AR reduces a number of lipid aldehydes generated during oxidative stress conditions. AR has been shown to reduce not only lipid aldehydes but also glutathione conjugates of lipid aldehydes (GS-LDAs) (Srivastava et al. 2001). Further, the catalytic activity of AR is significantly high for the reduction of glutathionylated aldehydes as compared to the corresponding parent aldehydes (Ramana et al. 2000; Srivastava et al. 2001). Glutathione conjugates of acrolein, trans-2 hexenal, trans-2-nonenal, and trans-2,4-decadienal were more efficiently catalyzed by AR (4–1000-fold efficiently) when compared to the unconjugated or free alkanals (Vander Jagt et al. 1995; Srivastava et al. 2001; Dixit et al. 2000). Subsequent to oxidative stress-induced lipid peroxidation of polyunsaturated fatty acids in cells, various reactive aldehydes such as 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA), and acrolein are formed, which are highly toxic and act as secondary signaling messengers (Pizzimenti et al. 2013). Reactive aldehydes, particularly HNE, have the ability to induce multiple signaling pathways along with formation of DNA adducts, which regulate important signaling cascades in the body (Zhong and Yin 2015). HNE, depending on its intracellular concentration, can either elicit proliferation or induce apoptosis in various cells such as macrophages, vascular smooth muscle cells, vascular endothelial cells, and cancer cells (Zhong and Yin 2015; Ramana et al. 2006a; Chapple et al. 2013). In fact, metabolic pathways involved in the detoxification of HNE and lipid peroxidation products have been suggested to be important targets for alleviating symptoms associated with various inflammatory diseases (Zhong and Yin 2015; Gasparovic et al. 2017). Specifically, lipid peroxidation product 4-HNE has been shown to play an important role in cardiovascular complications, neurodegenerative disease, COPD, lung cancer, and other inflammatory complications (Csala et al. 2015; Shoeb et al. 2014). Since HNE has been involved in many cellular signaling pathways, and AR being the major metabolizing enzyme

of HNE, AR could also regulate cellular signaling pathways. Indeed, recent studies indicate that AR mediates cell signaling pathways initiated by cytokines, growth factors, bacterial endotoxins, allergens, and chemotherapeutic drugs (Tammali et al. 2011a; Ramana and Srivastava 2010; Sonowal et al. 2018). Specifically, AR inhibitors have been shown to prevent the activation of redox-sensitive transcription factors such as NF-κB, AP-1, E2F1, CREB, and Nrf2 as well as the signaling pathways and expression of inflammatory cytokines, chemokines, and growth factors mediated by these transcription factors (Tammali et al. 2011a; Ramana and Srivastava 2010). Small molecular AR inhibitors such as sorbinil, tolrestat, zopolrestat, fidarestat, and epalrestat have been shown to prevent activation of various protein kinases such as PKC, PLC, MAPK, IKK, and JNK which are upstream to the transcription factors and responsible for their activation (Tammali et al. 2011a; Ramana and Srivastava 2010; Ramana 2011; Chatzopoulou et al. 2013). Further, AR inhibitors have been shown to prevent the formation of ROS in oxidant-treated cells (Srivastava et al. 2011). Thus, by reducing cellular-free radical content and inhibiting the activation of various protein kinases, AR inhibition could regulate cellular signaling pathways. Further, AR-catalyzed reduced products such as GS-DHN but not substrate GS-HNE have been shown to play a major role in the cell signaling process. Recent evidence suggests that GS-DHN could be a major signaling intermediate in the activation of redox-sensitive transcription factors (Ramana et al. 2006a). GS-DHN but not GS-HNE has been shown to activate PKC and NF-κB in vascular cells and cancer cells (Ramana et al. 2006a; Tammali et al. 2006). AR inhibition has been shown to increase the activity of Nrf2 and thereby increases the expression of anti-inflammatory proteins such as HO-1 and NQO1 (Shukla et al. 2017a, b). On the other hand, AR inhibition has been shown to prevent NF-κB-mediated pro-inflammatory activities (Ramana 2011; Yadav and Ramana 2013). Thus, the use of AR inhibitors has dual benefit as they block pro-inflammatory responses and activate anti-inflammatory responses. This property of AR inhibition is very significant in controlling a number of inflammatory complications including various lung diseases.

12.4 Aldose Reductase in Asthma

Asthma is a chronic lung disease that affects approximately 25 million people in the United States (CDC.gov, 2009). Among the 25 million individuals estimated to be affected by asthma, 7 million are children and have been reported to be a huge financial burden (Nurmagambetov et al. 2018). Asthma is a chronic inflammatory pathological condition, where severe airway inflammation along with mucous deposition is observed leading to difficulties in breathing and gas exchange (Ullmann et al. 2018; Papi et al. 2018). The condition is further aggravated in patients who are prone to allergies or have a weakened or altered immune system. Asthma attacks are termed flare-ups or exacerbations and are manageable with medications such as inhaled corticosteroids, anti-inflammatory drugs like cromolyn, aspirin, and various immunomodulators (Papi et al. 2018). The situation is further complicated in children as the diagnosis of this disease in children is tough, and the use of inhalers is a

daunting task in younger age groups (<5 years) (van Aalderen 2012; Papadopoulos et al. 2018). Further, steroid therapy has major side effects and hampers patient's quality of life (Hanania et al. 1995; Dahl 2006). Thus, there is a dire need for alternate and novel therapeutic strategies for asthma. Among the precautionary measures to cope with asthma, continued education and management of the disease could help in preventing the disease (Papi et al. 2018). Immunotherapy with monoclonal IgE antibodies and antibodies to block cytokines such as IL-5 has been very successful as alternate strategies for the treatment and management of asthma (Papadopoulos et al. 2018; O'Byrne 2011). Asthma being a chronic inflammatory disorder, the use of anti-inflammatory agents greatly relieves symptoms, and many new studies are currently underway to develop novel anti-inflammatory agents for the treatment, cure, and management of asthma. The use of AR inhibitors such as fidarestat has shown immense promise for alleviating symptoms associated with asthma (Srivastava and Ramana 2010; Ramana et al. 2011). AR inhibitors have been shown to prevent cytokine-, LPS-, and Ragweed extract (RWE)-induced cell death in small airway epithelial cells (SAECs). AR inhibition prevents oxidants and allergen-induced ROS generation and activation of NF-κB in SAECs (Yadav et al. 2011a, 2013a). Further, AR inhibition also prevented the expression of various inflammatory markers such as iNOS, Cox-2, PGE-2, IL-6, and IL-8 in SAECs (Yadav et al. 2011a, b). Further, inhibition of AR has been shown to prevent the RWE-induced airway inflammatory response in a mouse model of asthma. AR inhibition prevents RWE-induced mucin production, eosinophil infiltration, perivascular and peribronchial inflammation, and airway hyper-responsiveness (Fig. 12.3) (Yadav et al. 2009a, 2011b). Similarly, AR inhibition has been shown to prevent ovalbumin (OVA)-induced asthma in a mouse model (Yadav et al. 2013b). AR inhibitor, fidarestat, has been shown to prevent airway inflammation and hyper-responsiveness in OVA-challenged mice. Further, AR inhibition decreases the levels of IgE, eosinophil infiltration, and release of Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) in the BAL fluid of OVA-challenged mice (Yadav et al. 2009b). Most significantly, AR-deficient mice have been shown to be resistant to RWE-induced asthma complications. As compared to wild-type mice, AR-null mice challenged with RWE showed significantly decreased airway hyper-responsiveness, mucus secretion, infiltration of eosinophils, and metaplasia of epithelial cells (Yadav et al. 2013b). Further, AR-null mice challenged with RWE showed significantly lowered Th2 cytokines when compared to WT mice. AR inhibition prevents airway remodeling in chronic asthma model using extended multiple challenged OVA in mice (Yadav et al. 2009b). In these mice, AR inhibitor, fidarestat, prevents goblet cell metaplasia, collagen deposition, airway thickening, and airway hyper-responsiveness. Further, AR inhibition prevents the TGFβ1-induced expression of epithelial-mesenchymal transition (EMT) markers such as E-cadherin, vimentin, occludin, and MMP-2 in SAECs. AR inhibition has been shown to prevent TGFβ1-induced EMT by regulating the PI3k/AKT/GSK3β pathway independent of the Smad2/Smad3 pathway. AR inhibition also prevented the expression of alpha-smooth muscle actin and fibronectin in mouse lung fibroblasts (Yadav et al. 2013b). Thus, these studies suggest that AR mediates allergen-induced airway inflammatory response and inhibition of AR could be a novel therapeutic strategy for the prevention of asthma.

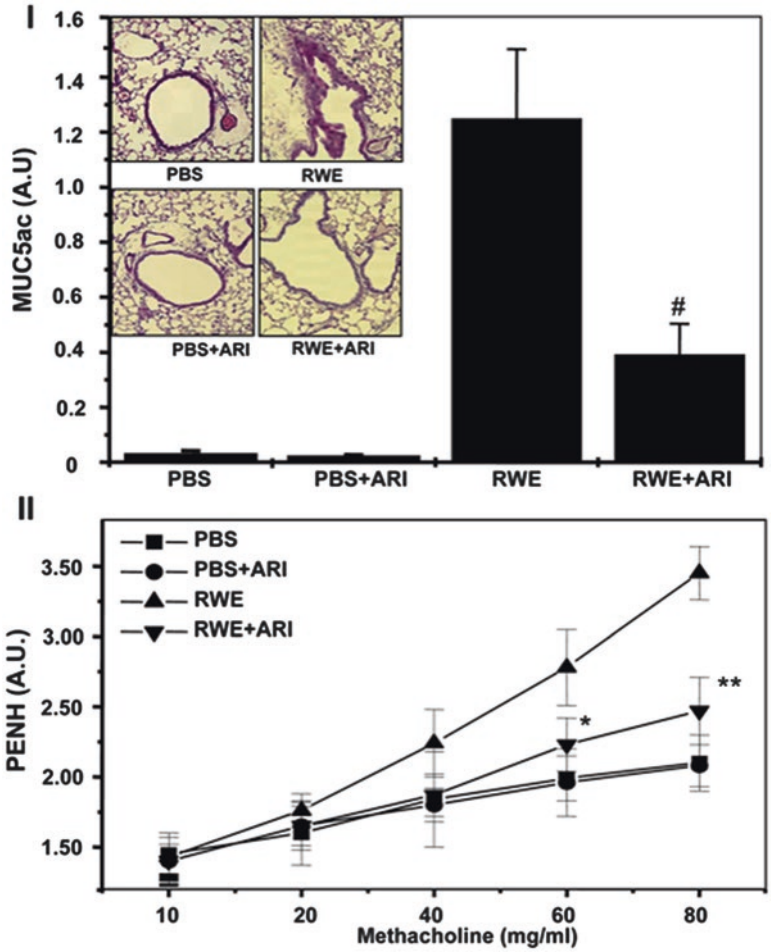

Fig. 12.3 AR inhibition prevents RWE-induced mucin production and airway hyperresponsiveness in mice. (**I**) MUC5ac levels in BAL fluid and inset showing PAS staining of lung sections to visualize mucin-producing cells after 72 h RWE challenge without or with ARI. (**II**) Whole-body plethysmography of mice showing changes in pause of breathing "enhanced pause" (PENH), an index of airway obstruction. Please see PLOS ONE 4 (8):e6535 for further details. (This figure was reproduced from Yadav et al. (2009a) (Creative Commons Attribution License))

12.5 Aldose Reductase in Rhinitis

Rhinitis is associated with severe inflammation in the nasal mucous membranes. It is also referred to as *coryza* and is characterized by sneezing, nasal congestion, itching, and rhinorrhea. Rhinitis is divided into two broad categories: *allergic rhinitis and non-allergic rhinitis* (Small et al. 2018). In non-allergic rhinitis, immune system is not involved, and it is generally characterized by year-round symptoms of a runny nose

and nasal congestion, which is generally observed in adults. Various environmental and lifestyle factors have been attributed to be causative factors for non-allergic rhinitis. *Allergic rhinitis or hay fever*, on the other hand, is characterized by an IgE-mediated hypersensitive immune response which may be triggered by a variety of environmental allergens such as seasonal pollen, dust particles, fungi, and various indoor allergens like dust mites, pets, etc. (Tran et al. 2011; Sin and Togias 2011; Wang et al. 2018). A sustained and profoundly activated inflammatory response is observed during allergic rhinitis with the reported increase in inflammatory and activated monocytes, granulocytes, and $CD4^{+}$T cells in the nasal mucosa (Ling and Luster 2016). The cytokine milieu is prominently a Th2 type (IL-4, IL-5, IL-13), and uncontrolled pro-inflammatory response may ultimately lead to tissue damage (Christodoulopoulos et al. 2000; Bachert et al. 1998). The allergic rhinitis-induced inflammatory response is not only limited to the nasal mucosa and inflammation. Tissue damage and discomfort can be observed in the throat, eyes, ears, skin, palate, etc., which further aggravates the disease pathology (Borish 2003). Therapy for allergic rhinitis includes the prescription of antihistamines and corticosteroids to relieve symptoms (Borish 2003; Solelhac and Charpin 2014). Apart from the prescribed drugs, various immunotherapeutic approaches have also been postulated in the recent past as effective treatment strategies for allergic rhinitis and asthma. But most of these treatment strategies have not been found to be as effective and have also been reported to induce noticeable side effects (Solelhac and Charpin 2014; Oktemer et al. 2016; Calderón et al. 2013). Nevertheless, attaining a reversal of the Th2 pro-inflammatory response to a Th1 phenotype during allergic rhinitis is one of the primary objectives of these treatment strategies (Kirmaz et al. 2005; Elenkov 2004). Inhibition of AR has shown promising evidence in suppressing airway inflammatory response. AR inhibitor, fidarestat, has been shown to suppress the expression of Th2 cytokine and airway inflammation in RWE-induced allergic rhinitis symptoms in mice (Yadav et al. 2009b). Mice treated with AR inhibitor, fidarestat, showed a reduced early phase response to allergen exposure along with a reduction in the levels of inflammatory Th2 cytokine release and infiltration of inflammatory cells into the nasal passage. The histological analysis also showed that the AR inhibitor prevented allergen-induced epithelial cell remodeling in the nasal passage (Yadav et al. 2009b). Similarly, AR-knockout mice showed resistance to RWE-induced rhinitis (Yadav et al. 2013c). A recent study by Marti et al. (2018) analyzed single nucleotide polymorphisms (SNP) in patients diagnosed with asthma and rhinitis. This study demonstrates a significant correlation between AR expression and a missense mutation, which induces susceptibility of cells to an allergen or cytokine-induced inflammatory response (Garcia-Martin et al. 2018). Thus, these studies show that AR plays an important role in nasal inflammation and could be a potential therapeutic target for allergic rhinitis.

12.6 Aldose Reductase in COPD

Chronic obstructive pulmonary (COPD) is characterized by inflammation in the airways, which is typically a sustained inflammatory response that ultimately leads to lung tissue damage and loss of pulmonary function. Although the number of

cigarette smokers declined in the United States from 20.9% in 2005 to 15.5% in 2016 (Source: CDC.gov), cigarette smoking-related deaths remain among the leading cause of preventable deaths in the United States. Cigarette smoke is a major risk factor for COPD pathogenesis. Apart from cigarette smoke, toxins from the environment are among the causative factors of COPD. If preventive steps are not taken, COPD progressively leads to irreversible loss of lung function. Apart from the loss of pulmonary activity, COPD also induces a loss of immune function in patients, which increases the susceptibility of the patients to secondary infections and has been termed as a risk factor for comorbidity in COPD (Sethi 2010; Sethi and Murphy 2001). Bronchodilators containing albuterol, levalbuterol, tiotropium, etc. are commonly prescribed to deal with COPD-associated complications. Among others, steroids, phosphodiesterase-4 inhibitors, theophylline, and antibiotics have been shown to be effective for relieving symptoms of COPD (Stolz et al. 2018). These drugs have potent anti-inflammatory properties and provide substantial relief by relaxing the airways and facilitating the exchange of air easily. Apart from the existing drugs for relieving symptoms of COPD, several alternative new therapeutic strategies for the treatment of COPD have been postulated and have attained considerable attention from the scientific community, with many of these undergoing clinical trials with promising results. Strategies to modulate immune cell function, inflammatory macrophages, and antioxidants to prevent oxidative damage have been in the forefront as alternative approaches for the treatment against COPD (Babu and Morjaria 2015; Ross and Hansel 2014; Patrick and Johnston 2013; Barnes and Stockley 2005; Calverley 2001). Among the many new agents to treat COPD and alleviate COPD-associated symptoms, inhibition of the polyol pathway enzyme AR has emerged as a potential new target. Inhibition of AR exerts anti-inflammatory roles in different pathologies. Proteomic analysis of BAL fluids of lung cancer and COPD patients showed upregulation of aldo-keto reductase isoforms (AKR1B10 and AKR1C3) (Pastor et al. 2013). Yadav et al. (2013a, b, c) have shown that inhibition of AR by fidarestat is effective against acrolein-induced cytotoxicity in human small airway epithelial cells (Yadav et al. 2013a). Acrolein is the principal component of cigarette smoke and environmental pollutants and is one of the primary risk factors of air pollutant-induced lung damage (Bein and Leikauf 2011). Mucus hypersecretion that leads to airway lumen obstruction is the major complication in patients with COPD. Goblet cell metaplasia has been shown to cause excess mucus secretion. Several studies have shown that pro-inflammatory cytokine IL-13 is a key in mediating the goblet cell metaplasia (Tukler Henriksson et al. 2015; Kondo et al. 2006). Yadav et al. (2010) have shown that AR inhibition prevents IL-13-induced goblet cell metaplasia in SAECs. They have shown that the AR inhibitor prevents IL-13-induced expression of Muc5AC, Muc5B, and SPDEF in SAECs. Further, AR inhibition prevents IL-13-induced goblet cell metaplasia by regulating the phosphorylation of JAK-1, ERK1/2, and STAT6 (Yadav et al. 2010). In the same study, Yadav et al., 2010, have also shown that fidarestat prevents RWE-induced goblet cell metaplasia in a mice model. Further, they have shown that AR-null mice are resistant to RWE-induced goblet cell metaplasia (Fig. 12.4). Similarly, Jiang et al. (2012) have shown that inhibition of AR by zopolrestat and AR-siRNA inhibits IL-13-induced mucus production in human bronchial epithelial

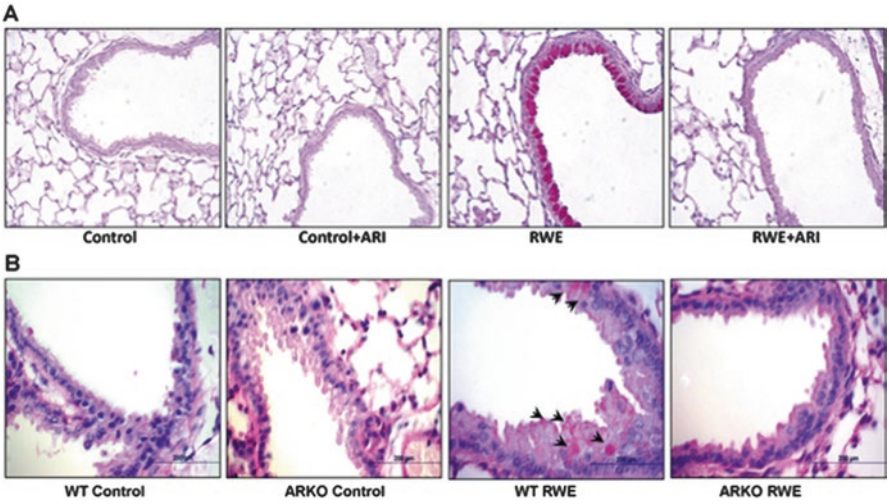

Fig. 12.4 Inhibition or deficiency of AR prevents RWE-induced goblet cell metaplasia in mice lungs. Photomicrographs showing PAS-stained lung sections isolated from 72 h RWE-challenged mice without or with AR inhibitor (**a**) or AR-null mice (**b**). Magnification 200× (**a**); 400× (**b**). Please see PLoS ONE 5(12): e14440 for further details. (This figure was reproduced from Yadav et al. (2010) (Creative Commons Attribution License))

cells (Jiang et al. 2012). Thus, these studies provide evidence that AR inhibitors have the potential for use as effective drugs for COPD.

12.7 Aldose Reductase in Acute Lung Injury

Acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) are characterized by an inflammatory response of the lung and clinically described as acute onset of diffuse bilateral pulmonary infiltrates with hypoxemia (PaO2/FiO2≤300 mmHg for ALI and ≤200 mmHg regardless of the level of PEEP) without evidence of hydrostatic pulmonary edema with wedge pressure ≤18 mmHg (Ragaller and Richter 2010). The incidence of ALI and ARDS is substantially high in the United States with about 18–79 ALI and 13–59 ARDS diagnosis per million individuals each year and a mortality rate of 35–40% with the highest observed in risk group patients further diagnosed or parallelly diagnosed with pneumonia, sepsis, or asphyxia (Rubenfeld and Herridge 2007). Treatment strategies for ALI or ARDS include treatment of the underlying disease (trauma, shock, or sepsis), mechanical ventilation to relieve hypoxia, and adjunctive therapeutic options like inhalation of nitric oxide to relieve or relax the endothelium or administration of aerosolized prostacyclin (PGI2), inhaled corticosteroids, or perfluorocarbons, and these have been reported to be helpful in relieving the symptoms (Johnson and Matthay 2010).

Although pharmacological agents have not been recommended as standard management for ALI, clinical trials with aspirin, statins, β-2 adrenergic agonists, and inhaled corticosteroids have shown promising results in clinical trials (Boyle et al. 2013). These pharmacological agents have been shown to be effective in targeting platelet and neutrophil interactions (Erlich et al. 2011; Looney et al. 2009), leucocyte adhesion, and inhibition of proinflammatory cytokines and chemokines (Levitt and Matthay 2012). HMG-CoA inhibitor, simvastatin, has been found to be effective in reducing pulmonary and systemic inflammation and improvement in organ dysfunction in ALI (Craig et al. 2011). AR inhibitors have been shown to prevent the endotoxin-induced inflammatory complications in a mice model of cardiomyopathy (Ramana et al. 2006b). Further, studies using AR transgenic mice have also highlighted the importance of AR in inflammatory lung disease. AR-overexpressing transgenic mice have shown increased inflammatory response and neutrophil accumulation in the lungs (Ravindranath et al. 2009). Further, lung cells isolated from AR transgenic mice have shown increased production of IL-6 in response to LPS exposure. AR activity is required for the TNFα-induced regulation of Rho kinase/MKK4/JNK/IL-6 pathway in human pulmonary microvascular cells (Ravindranath et al. 2009). These studies suggest an important role of AR in pulmonary function and lung pathophysiology.

12.8 Conclusions and Future Perspectives

Thus, it is evident that AR plays an important role in the pathophysiology of various lung inflammatory complications. Specifically, AR inhibition has been shown to prevent allergens, cytokines, and endotoxin-induced airway inflammatory response in cell culture and mouse models of asthma, COPD, and rhinitis. Current therapies for various lung inflammatory complications include bronchodilators and corticosteroids, which have been shown to exert severe side effects, and prolonged use could significantly alter the patient's quality of life. Further, some subsets of lung pathologies develop resistance to steroids and are not responsive to the standard steroid therapy. Therefore, novel therapeutic strategies are required to control lung inflammatory pathologies. Small molecule AR inhibitors such as epalrestat and fidarestat which have gone to phase-III clinical trials for various diabetic complications, especially diabetic neuropathy, and found to be safe for human use could be developed as anti-asthmatic drugs. However, additional preclinical studies using different animal models such as guinea pig or higher mammals may be required prior to clinical studies. Ex vivo studies using clinical samples from patients with lung inflammatory complications are required to examine the potential of AR inhibitors. Overall, since the inhibitors of AR have been already tested in the humans for diabetes complications, they could also be tested in the clinical studies for asthma and COPD.

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13

Oxidative Stress and Pulmonary Carcinogenesis Through Mechanisms of Reactive Oxygen Species. How Respirable Particulate Matter, Fibrous Dusts, and Ozone Cause Pulmonary Inflammation and Initiate Lung Carcinogenesis

Athanasios Valavanidis

Abstract

The majority of new cases of cancer worldwide in 2012 were lung cancers (1.8 million). Epidemiologic studies established that the most important risk factor for lung cancer is tobacco smoking (active and passive). Other factors which contribute substantially are occupational exposures to carcinogenic chemicals; vehicular air pollution in urban and industrial areas; environmental exposure to inorganic dusts and asbestos fibers; certain carcinogenic metals Cd, As, and Cr; and indoor air pollution. Lungs are exposed to air oxidants generated either endogenously or exogenously, but aerobic organisms are protected against oxidative damage by enzymatic and low molecular weight nonenzymatic antioxidants. Lung cancer mechanisms are promoted through the generation of reactive oxygen and nitrogen species (ROS/RNS) as a result of oxidative stress and exposure to external air pollutants, leading to oxidative stress and inflammation. Their role in the initiation and progression of cellular and mitochondrial DNA damage (cDNA and mtDNA), membrane lipid peroxidation, and oxidative damage to proteins and enzymes is crucial. Also, airborne particulate matter (PM_{10} and $PM_{2.5}$) are microparticles or nanoparticles which can penetrate the respiratory system, entering deep into the lung alveoli and trapped in the interior. Physical and chemical characteristics of particles (size, transition metal content, speciation, stable free radicals, etc.) play an important role in oxidative stress. Chronic exposures to these air pollutants initiate the synthesis of mediators of pulmonary inflammation in lung epithelial cells and promote the initiation of carcinogenic

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mechanisms. Inhalable quartz, metal powders, mineral asbestos fibers, ozone, soot from gasoline and diesel exhausts, tobacco smoke, and PM_{10} and $PM_{2.5}$ are involved in various oxidative stress mechanisms. Pulmonary cancer initiation and promotion have been linked to a series of biochemical pathways of oxidative stress, DNA oxidative damage, macrophage stimulation, telomere shortening, modulation of gene expression, and activation of transcription factors with important role in carcinogenesis. This review presents the role of ROS and oxidative stress in the production of mediators of pulmonary inflammation and the promotion of mechanisms of carcinogenesis.

Keywords
Reactive oxygen species · Oxidative stress · Inflammation · Mechanisms of carcinogenesis · Tobacco smoke · Respirable particulate matter · Asbestos fibers

13.1 Introduction

Cancer is the second most important cause of human morbidity and mortality worldwide, and lung cancer for several decades, according to the International Agency for Research on Cancer (WHO, Lyon, France), is the most deadly. It is estimated that lung cancer caused 1.8 million new cases worldwide in 2012 (or ~13% of the total cancer incidence), and the majority (1.2 million) of cases were in men. Lung cancer is the most common cause of mortality from cancer worldwide (nearly 20% of the total). Worldwide the most diagnosed cancers were lung (1.82 million), breast (1.67 million), and colorectal (1.36 million). Statistical evidence showed that in 2012 the most common causes of cancer death were lung (1.6 million deaths), liver (745,000 deaths), and stomach (723,000 deaths) (International Agency for Research on Cancer (WHO) 2012; Ferlay et al. 2015).

The majority of epidemiologic studies have concluded that the most important risk factor for lung cancer is tobacco smoking, active and passive smoking. Research has established without doubt that there is a clear connection between any type of smoking and the array respiratory system cancers. Also, epidemiologic evidence showed that smoking accounts for more than 25% of cancer deaths and 20% of all new cancer cases. Years of promoting antismoking campaigns in most developed countries convinced millions of smokers to kick the habit and stop this deadly habit. The majority of high-income countries witnessed considerable decrease in smoking prevalence, and as a result, lung cancer incidence and mortality declined to a great extent. But despite major changes in tobacco control, with current smoking patterns, cancer of the lung and the respiratory system will remain a major cause of death for several decades (Doll and Hill 1950; Doll et al. 2004; Islami et al. 2015).

Other important contributory factors to lung cancer are occupational acute and prolonged exposures to carcinogenic substances; ambient air pollution (especially inhalable particulate matter, diesel exhausts, industrial fumes); indoor air pollutants from coal, wood, and solid waste burning; and environmental exposure to radon

(radioactive element), ionizing radiation (α-, γ-rays), asbestos fibers (occupational exposure), and carcinogenic metals (miners) such as chromium (Cr), cadmium (Cd), and arsenic (As). Exposures to organic carcinogenic chemicals, such as polychlorinated compounds, dioxins, coal gasification products, coal-tar pitch, iron and steel founding, rubber production, diesel fumes, and silica dusts are also able to cause extensive damage to the lungs and promote lung canrcinogenesis (Wong et al. 2017; Field and Withers 2012).

13.2 Oxygen Reactive Species, Oxidative Stress, and Respiratory Diseases

The diseases of the human respiratory system are inevitably linked to air pollutants with oxidative capacity, which in the beginning promote oxidation in tissues and cells by oxygen-free radicals and reduction in antioxidant enzymes. The result is oxidative stress in biological systems and in the case of long-term exposures to chronic inflammatory processes. Many studies showed that smoking is responsible for pulmonary inflammatory processes which in turn increase macrophage recruitment, delayed neutrophil clearance, and oxidative stress in the lung tissues. The pulmonary diseases that are associated with the greatest risk for lung cancer are characterized by abundant and deregulated inflammation. For example, chronic obstructive pulmonary disease and emphysema are characterized by profound abnormalities in inflammatory and fibrotic pathways. Cytokines, growth factors, and the developing tumor microenvironment have been recognized as paving the way for both epithelial-mesenchymal transition and destruction of specific host cell-mediated immune responses (Walser et al. 2008; Rosanna and Salvatore 2012).

In addition to smoking, respiratory inflammation in humans can be initiated from exposure to various inhalable dusts, particles containing redox metals, and mineral fibers (such as asbestos and quartz). Also, ambient airborne particulate matter of fine and superfine size (PM_{10}, $PM_{2.5}$, PM_{1}), ozone (O_3), and vehicular diesel fumes have been implicated with lung carcinogenesis. In recent years, ambient air pollution, especially smaller in size (aerodynamic diameter) of 2.5 μm, has gained particular attention by toxicologists as a causative factor in the incidence of respiratory diseases and lung cancer. Reviews that collected numerous experimental and clinical studies in the last decades provided evidence for the association of PM exposure and lung cancer. Particulate matter (PM) of ambient air pollution and motorcar diesel exhausts was designated long time ago by International Agency for Research on Cancer (belongs to WHO) as human carcinogens in Group 1 (Valavanidis et al. 2008; Strak et al. 2012; Hamra et al. 2014; Huang et al. 2017; Loomis et al. 2013).

It must be emphasized that exposure to a multiple factors can be the result of synergistically acting chemicals with tobacco smoke, thus increasing substantially the risk for epithelial inflammation and subsequent pulmonary diseases including lung cancer. Most research until now indicate that carcinogenic potential of airborne particles, fibers, and dusts increases substantially due to the synergistic effects with tobacco smoke carcinogens. They generate large amounts of free radicals and ROS

and catalyze redox reactions in human lung epithelial cells, leading to oxidative stress and increased production of mediators of pulmonary inflammation (Valavanidis et al. 1996; Sangani and Ghio 2011; Turner et al. 2014a; Yuwadee et al. 2015).

Size and composition of respirable particles play an important role in the penetration, retention, and clearance inside the respiratory system. Fine and superfine particles can penetrate deeper into the respiratory airways. The majority are deposited in the respiratory bronchioles and alveoli unable to come out again. The longer a particle is retained, the greater the potential to cause harm, such as fibrosis, emphysema, or tumor initiation. Durability, solubility, and reactivity of inhalable particles are very important characteristics. Some particles can be broken down and transported out of the lung by both mucociliary clearance and macrophage transport. Superfine particles (<1 μm) or fibrous mineral particles are more hazardous due to their ability to penetrate deeper into the lung and resist clearance from the lung interstitium (Lippmann et al. 1980; Valavanidis et al. 2008).

Inhalable particles from combustion sources contain chemical constituents generating ROS by a variety of redox reactions. The most important are transition metals with redox properties (e.g., Fe^{2+}), persistent free carbonaceous radicals, redox-cycling quinones, polycyclic aromatic hydrocarbons (PAHs), and volatile organic compounds (VOCs) which may be metabolically activated to ROS. These substances are unstable and highly reactive, attacking the nucleolus forming bulky adducts or strand breaks on cellular DNA (Squadrito et al. 2001; Risom et al. 2005; Riva et al. 2011; Chen et al. 2016). A great number of research projects have linked air pollution particle exposure to oxidation of DNA in cells, tissues, or their metabolites in urine of rodents and humans. Studies have investigated the effect of diesel exhaust particles (DEP) or ambient air pollutants in terms of oxidized DNA nucleobases (Valavanidis et al. 2008; Douki et al. 2018).

In the last decade, a prominent free radical-induced oxidative lesion [in nuclear DNA and mitochondrial DNA (mtDNA)] has been used widely as a predominant biomarker for oxidative stress and carcinogenesis in experimental animals and humans. The 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG) and methylguanine (N7-MeG) can be measured quantitatively by High Performance Liquid Chromatography and Gas Chromatography-Mass Spectrometry in animal and in human biomonitoring studies. These biomarkers are correlated with oxidative and methylated DNA damage by many carcinogenic factors (Pilger and Rüdiger 2006; Valavanidis et al. 2009a; Lai et al. 2017).

Air pollution by ozone (O_3) has been added to the factors of pulmonary irritants leading to oxidative stress, inflammation, and tissue injury. Human bronchiolar epithelium is highly susceptible to injury and oxidative stress induced by acute exposure to O_3, accompanied by altered lung functioning. Clinical studies investigated exposures to combined air pollutants, such as ozone and cigarette smoke or ozone and ambient particulate matter. The results showed increased pulmonary oxidative stress, associated inflammation, and pulmonary mortality (Gardi and Valacchi 2012; Sunil et al. 2013; Valavanidis et al. 2013).

13.3 Reactive Oxygen Species, Aerobic Organisms, and Oxidative Stress

The appearance of eukaryotic cells, approximately 2 billion years ago on Earth, has been linked to the increased concentration of oxygen (O_2) in the atmosphere leading to the initiation of aerobic metabolism in biological systems. Mitochondria became powerhouses of the eukaryotic cell with energy-releasing activities, and aerobic biological systems use O_2 as an essential part of their physiological processes. Most of these ROS and RNS are physiological and beneficial and necessary for subcellular events, signal transduction, enzyme activation, gene expression, etc. But a small percentage, ~5%, of ROS escape metabolic processes and can be toxic because of their high oxidant capacity and reactivity (Wiegman et al. 2014; Cadenas and Davies 2000; Droge 2002). Under physiological conditions, there is a constant endogenous production of reactive intermediates of radical species of oxygen and nitrogen that interact as regulatory mediators of signaling processes for metabolism, cell cycle, intercellular transduction pathways, cellular redox systems, and mechanisms of apoptosis (Inoue et al. 2003; Brookes et al. 2002; Circu and Aw 2010; Ray et al. 2012; Valko et al. 2007).

Also, exogenous sources, such as tobacco smoke, carcinogenic metals, toxic compounds, etc., increase the ROS levels in biological systems, but healthy levels of antioxidant defenses protect aerobic organisms. Cellular ROS sensing and metabolism are tightly regulated by a variety of proteins involved in the redox (reduction/oxidation) mechanism. Oxidative stress is connected with perturbation or imbalance of cell redox homeostasis, playing a pivotal role in the development of human degenerative diseases and aging. Human lungs are exposed daily to air oxidants generated either endogenously or exogenously (Sena and Chandel 2012; Lin and Beal 2006; Sies 2015). Excessive or sustained increase in ROS production, not balanced by antioxidant enzymes and low molecular weight antioxidants, has been implicated in chronic inflammatory conditions, malignant neoplasms, diabetes mellitus, atherosclerosis, and various neurodegenerative diseases. Inflammation and lung cancer are associated (Pisoschi and Pop 2015; Valko et al. 2004, 2007; Van Eeden and Sin 2013; Gomes et al. 2016).

Enzymatic antioxidants in aerobic organisms can intercept, scavenge, neutralize radicals and free radicals and ROS, and can reactivate intermediates generated in excess under physiological conditions. The most important antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), and the glutathione redox system (glutathione peroxidase and glutathione-S-transferase). Also, low molecular weight antioxidant compounds are ascorbic acid (vitamin C), vitamin E (tocopherol), uric acid, bilirubin, glucose, ceruloplasmin, etc. and proteins that bind metal ions in forms unable to accelerate free radical reactions (Valko et al. 2007).

The balance between oxidants and antioxidants in biological systems is called "redox homeostasis." It has been established that it is a crucial event in living aerobic organisms for health and longevity. Subjecting cells to oxidative stress (pro-oxidative/pro-inflammatory pathways) can result in severe metabolic dysfunction and multiple oxidative damages to basic biomolecules, such as proteins (enzymes),

carbohydrates, DNA, RNA, mtDNA, and membrane lipids (Halliwell and Gutteridge 1990; Ottavio et al. 2008; Ursini et al. 2016).

13.4 Oxidative Stress, Inflammation, and Pathways to Carcinogenesis

Inflammation has been recognized as the predisposing conditions for neoplasm development. Although it is now accepted from the majority of scientists that chronic inflammation plays an essential role in tumorigenesis, the underlying molecular mechanisms have not been explored. Oxidative acute and chronic inflammation has been correlated with increased risk for the initiation and progression of various malignant neoplasms. The possible mechanisms by which inflammation can contribute to carcinogenesis include genomic instability, alterations in epigenetic events, inappropriate gene expression, enhanced proliferation of initiated cells, resistance to apoptosis, aggressive tumor neovascularization, invasion through tumor-associated basement membrane, angiogenesis, and metastasis. ROS act as effector molecules which participate in host defense or as chemoattractants recruiting leukocytes to wounds, thereby influencing the inflammatory reaction in damaged tissues (He et al. 2017; Azad et al. 2008a; Ziech et al. 2011; Wu et al. 2014). Inflammatory cells are particularly effective in generating most of the ROS, and the redox metabolism of the inflammatory cells generates a highly oxidative environment (King 2015; Federico et al. 2007; Lazennes and Richmond 2010).

Clinical investigation observed that chronic inflammation promoted tumor development, progression, and metastatic dissemination, affect negatively treatment by anticancer drugs. Cancer development and malignant progression are also associated with accumulation of genetic alterations and loss of normal regulatory processes, which cause expression of tumor-specific antigens that can activate antitumor immune responses (Crusz and Balkwill 2015).

Oxidative stress can activate a variety of transcription factors including:

(a) NF-κB (nuclear factor, induces the expression of antiapoptotic genes)
(b) AP-1 (activator protein**,** controls a number of cellular processes including differentiation, proliferation, and apoptosis)
(c) p53 (is a tumor-suppressor gene)
(d) HIF-1α (hypoxia-inducible factor 1-alpha)
(e) PPAR-γ (peroxisome proliferator-activated receptor gamma)
(f) Wbt/β-catenin (signaling pathway, essential role during development and adult tissue homeostasis)
(g) Nrf2 (nuclear factor erythroid 2-related factor 2, an emerging regulator of cellular resistance to oxidants)

Until now most experimental results suggest that oxidative stress, chronic inflammation, and cancer are closely linked. Activated transcription factors lead to the expression of over 500 different genes, growth factors, inflammatory cytokines,

specific chemokines, regulatory molecules of the cell cycle, and anti-inflammatory molecules. Oxidative stress is promoting inflammatory pathways, transformation to tumor cell, tumor cell survival, proliferation, chemoresistance, radioresistance, invasion, angiogenesis, and stem cell survival (Shalapour and Karin 2015; Reuter et al. 2010).

A range of inflammation mediators, including cytokines, chemokines, free radicals, prostaglandins, growth and transcription factors, microRNAs, and enzymes, collectively act to create a favorable microenvironment for the development of tumors. The possible mechanisms by which inflammation can contribute to carcinogenesis include genomic instability, alterations in epigenetic events and subsequent inappropriate gene expression, enhanced proliferation of initiated cells, resistance to apoptosis, aggressive tumor neovascularization, and invasion through tumor-associated basement membrane and metastasis (Fernandes et al. 2015; Coussens and Werb 2002). In addition, tumor cells have co-opted some of the signaling molecules of the innate immune system (selectins, chemokines) and their receptors for invasion, migration, and metastasis. These findings are promoting new anti-inflammatory therapeutic approaches to cancer development (Federico et al. 2007; Kundu and Surh 2008; Mantovani et al. 2008).

Angiogenesis helps tumor growth and metastasis. Pathological angiogenesis (hallmark of cancer progression) and various ischemic diseases are associated with chronic inflammation. Both inflammation and angiogenesis are exacerbated by increased production of chemokines/cytokines, growth factors, proteolytic enzymes, proteoglycans, lipid mediators, and prostaglandins. The leading stages of initiation and progression of cancer are also closely linked to angiogenesis. Infiltration of macrophages is a common feature of inflammation, angiogenesis, and cancer and has been recently highlighted (Landskron et al. 2014; Ono 2008; Rivas-Fuentes et al. 2015; Lazennes and Richmond 2010).

13.5 Exposure to Airborne Particulate Matter, ROS, and Oxidative Stress

Airborne respirable particular matter (PM) was carrying for years a prominent position in toxicology and lately was associated with the concept of oxidative stress through the generation of ROS. The ability of respirable particles or fibrous dusts to enter the respiratory system (because of their small size) and penetrate deep into the lung's alveoli in order to generate free radicals and ROS is suggested to be the main factor involved in their pathogenic potential. There is abundance of scientific evidence that PM through the production of ROS is involved in cellular damage, lipid peroxidation, DNA double breaks or mutations, and protein oxidative damage. Size and chemical composition of PM are fundamental in their toxicological potential. Particles with aerodynamic diameter less than 10 and 2.5 μm generate a bigger amount of hydroxyl radical ($HO^{\bullet}$) through redox reactions, due to the heavy metals adsorbed on their porous surfaces, whereas particles of larger size (PM_{10}) deposit mainly in the upper airways and can be cleared by the mucociliary system. Recently,

however, interest has also focused on the ultrafine particles (UFPs) with less than 100 nm diameter because of their very high alveolar deposition fraction, large surface area, chemical composition (carbonaceous stable radicals), and ability to enter into the blood circulation to induce inflammatory processes. In addition, experimental results indicate that PM-mediated ROS production is involved in the generation of prolonged inflammation, and activated inflammatory cells can increase substantially further the ROS production (De Palma et al. 2017; Gurgueira et al. 2002; Møller et al. 2014; Li et al. 2015).

The net result is lowering the antioxidant defenses, leading to oxidative stress which stimulates respiratory adverse effects through airway inflammation. This is particularly acute when the human respiratory system is exposed to respirable airborne particles (occupational and urban ambient air pollution), exacerbated by high concentrations of petrol and diesel fumes in motorways (Pardo et al. 2016).

A study by Chinese scientists investigated the in vitro toxicity of $PM_{2.5}$ collected at six urban sites in China and how PM composition affects their cytotoxic mechanisms through the production of ROS. They used in their experiments human bronchial epithelial (BEAS-2B) cell lines as model in vitro to expose to $PM_{2.5}$ and analyzed the production of ROS, superoxide dismutase activity, and total antioxidant capacity. The results revealed that high concentrations of polycyclic aromatic hydrocarbons (PAHs) and elemental Nickel (Ni) were strongly associated with high apoptosis rates and high expression of IL-1β. They noticed that addition of Fe element was associated with the increased ROS level, and furthermore, Fe and Cr elements in PM were associated with DNA strand breaks and other type of damage (Ghio et al. 2012).

Another recent study examined the impact of repeated exposures to air pollution by using urban particulate matter (PM) on mouse lungs with focus on inflammatory and oxidative stress parameters. Aqueous extracts of urban PM were administered to mice by five repeated intratracheal instillations. Repeated exposure to PM caused systemic inflammation and oxidative damage (from ROS) to lung tissue lipids and proteins. Multiple exposures led to an increase in cytokine levels in both bronchoalveolar lavage fluid and in the blood serum, indicating a systemic reaction. Lung mRNA levels of antioxidant/phase II detoxifying enzymes decreased by exposure to the PM extract, but not when metals were removed by chelation. Finally, disruption of lung tissue oxidant-inflammatory/defense balance was exacerbated by increased levels of lipid peroxidation and oxidative protein damage (Yang et al. 2016).

A number of experimental studies established that airborne PM, like tobacco smoke, are responsible for producing ROS that have been implicated in the activation of mitogen-activated protein kinase (MAPK) family members and activation of transcription factors such as NF-κB and AP-1 (the activator protein- 1). These signaling pathways have been implicated in processes of inflammation, apoptosis, proliferation, transformation, and differentiation (Pardop et al. 2016; Gurgueira et al. 2002). Airborne PM (mainly exhaust fumes of diesel and petrol cars) represent a mixture of many different chemical components consisting of a variable carbonaceous particle core (with pores absorbing metallic elements) and a large array of surface-bound constituents including PAHs, redox heavy metals, and stable quinoid

free radicals. These components continuously release highly reactive hydroxyl radical (HO$^{\bullet}$) (Di Terzano et al. 2010; Knaapen et al. 2002; Shi et al. 2003).

Synergistic mechanisms of respirable PM (trapped into the lung's alveoli) and other components such as O_3, NO_x, soot, heavy metals, PAHs, and tobacco smoke have been studied extensively to investigate their potential for increased toxicity and carcinogenicity. The porous surfaces of airborne particles provide a fertile ground for catalyzing the increased generation of ROS or other damaging oxidants which are potential initiators of pulmonary carcinogenesis. Synergistic effects for increased ROS formation have been experimentally verified. Also, the alteration of intracellular calcium homeostasis induced by $PM_{2.5}$ is closely correlated to an increase of oxidative stress (Valavanidis et al. 2010; Dellinger et al. 2001).

Increasing ROS generation has been observed from synergistic mechanisms between ambient transition metals and PM quinoid stable radicals, PAHs of airborne particles and tobacco smoke, ambient NO_X, and cigarette tar which is similar to PM composition (Deweirdt et al. 2017; Penning et al. 1999; Park et al. 2006). Synergy for increasing ROS production occurs between coarse carbon particles (soot) and iron particles, PM_{10} and O_3, mineral fibers, and tobacco smoke. Other experimental investigations found synergistic actions for increased oxidative potential between PM and wood smoke (Valavanidis et al. 2005, 2009b; Zhou et al. 2003)

Aside from direct generation of ROS by the airborne PM and other respirable materials, ROS can be generated by target cells such as lung epithelial cells and pulmonary macrophages upon interaction with and/or uptake of particulate materials.

Alveolar macrophages have an important role in clearing xenobiotic particles from the lungs, and in response, they can release ROS. Phagocytic cells of the innate immune system such as alveolar macrophages and polymorphonuclear neutrophils are highly proficient producers of ROS to enhance microbicidal conditions in phagocytic vacuoles and eliminate pathogenic bacteria or potentially harmful particles. Alveolar macrophages are directly activated by PM of ambient air pollution, and the oxidative stress produced depends strictly on the availability of antioxidant defenses (Valavanidis et al. 2009c; Danielsen et al. 2011; Aam and Fonnum 2007).

Resident macrophages in the airways and the alveolar spaces can release ROS/RNS after phagocytosis of inhaled particles. These macrophages also release large amounts of TNF-alpha (tumor necrosis factor-alpha), a cytokine that can generate responses within the airway epithelium dependent upon intracellular generation of ROS/RNS. As a result, signal transduction pathways are set in motion contributing to inflammation in the lung airways. In respiratory diseases, chemokines have been shown to regulate inflammation and immune cell differentiation. Such effects include increased expression of intercellular adhesion molecule 1, interleukin-6, cytosolic and inducible nitric oxide synthase, manganese superoxide dismutase (MnSOD), cytosolic phospholipase A2, and hypersecretion of mucus. Ultimately, ROS/RNS may play a role in the global response of the airway epithelium to particulate pollutants via activation of kinases and transcription factors common to many response genes. In this respect, antioxidant defense mechanisms respond to

highly oxidative particulates by a complex cascade of events which can contribute to airway pathology (Lakey et al. 2016; Lee and Yang 2013; Turner et al. 2014b).

ROS/RNS can damage the respiratory system tissues in a chronic pulmonary inflammatory condition. People with chronic inflammatory diseases are more susceptible to adverse health effects in the lungs by exposure to airborne PM in urban settings. Oxidative stress and inflammation in the pulmonary tissues are crucial factors for DNA strand breaks and DNA oxidation, factors that are implicated in the initiation stage of carcinogenesis. A substantial number of toxicological studies consider that the generation of ROS in pulmonary cells is considered as one of the most important mechanism for lung carcinogenesis (Wong et al. 2016; Azad et al. 2008b).

Studies revealed that cancer cells increase ROS levels, in comparison to their normal counterparts, due to an enhanced metabolism and mitochondrial dysfunction that contribute to the biochemical changes necessary for the tumor initiation, promotion, and progression. This role of ROS is being delineated continuously and becoming pronounced, adding complexity to these radicals in understanding of their pathophysiology. DNA damage, leading to activation of oncogenes or inactivation of tumor-suppressor genes, is one of the plausible mechanisms by which ROS can promote carcinogenesis (Storr et al. 2013; Galadan et al. 2017).

13.6 Fibrous and Non-fibrous Dusts, Ozone, and Oxidative Stress

Respirable inorganic particulates, silica dusts, quartz dust, coal mine dusts, asbestos, and other fibrous minerals are some of the etiological agents for the production of ROS in the respiratory tract, contributing to oxidative stress and inflammation. Exposure of the respiratory system to these agents stimulates alveolar macrophages or bronchial epithelial cells and releases chemotactic factors that recruit inflammatory cells to the lung (Donaldson and Tran 2002; Rom 2011; Otsuki et al. 2016).

The human respiratory system has developed effective antioxidant defenses to be able to deal with xenobiotic particles. The mucociliary escalator in the airways traps and sweeps particles out of the lungs and the alveolar macrophages phagocytose particles and then migrate for removal to the gut. Problems develop when excessive oxidative stress damages macrophages and stimulates the release of inflammatory mediators, signaling the production of ROS. Cellular signaling induced by inhalable particles and O_3 exposure varies with cell type and physiochemical properties of these pollutants. Cellular signaling plays a critical role in the regulation of inflammatory pathogenesis (Yan et al. 2016).

In the last decades, numerous in vivo studies were performed with respirable particles, dusts, and fibrous inorganic materials in experimental animals. Persistent inflammation and associated excessive oxidative stress in the rat lungs have been crucially implicated in quartz-induced pulmonary diseases, including fibrosis and cancer (Albrecht et al. 2005). At intermediate levels, oxidative stress activates mitogen-activated protein kinases (MAPKs) and central pro-inflammatory transcription factors such as NF-κB and AP-1 leading to upregulation of

pro-inflammatory genes (Ovrevik et al. 2015). Similar experiments with rat alveolar macrophages showed that exposure to coarse chalk dust particles resulted in respiratory burst and oxidative stress. The net result was the increased generation of ROS/RNS in the alveolar mactophages (Zhang et al. 2015).

Although there has been considerable progress in the production of engineered nanomaterials (ENMs) and substantial advances in nanotechnology and its applications, understanding the health and safety aspects of engineered nanoparticles (ENPs) is still in its formative stage. Respiratory system toxicity from inhalable ENMs is the most important concern to health specialists. Scientific evidence has shown that there is close connection between respirable ENMs and pulmonary oxidative stress through the generation of ROS/RNS, leading to oxidative stress with elicitation of an inflammatory response via pro-inflammatory gene transcription. Studies in vitro and in vivo have shown that exposure to ENMs can cause ROS generation in the respiratory system, oxidative stress, and pro-inflammatory gene expression (Yoker and McPhail 2011; Vlachogianni et al. 2013).

Exposure of human lung epithelial cells to ENPs showed that there was an additional role of engineered nanoparticles (ENPs) as carriers of heavy metals. Toxicity results indicated that the ENPs could efficiently enter the cells by a Trojan-horse type mechanism which provoked up to 8 times higher oxidative stress in the case of cobalt or manganese if compared to reference cultures exposed to aqueous solutions of the same metals. The presence of redox metallic components in ENPs could strongly alter the extent of their oxidative potential in tissues (Limbach et al. 2007).

Nitrogen oxides (NO, NO_2) and ozone (O_3) are ubiquitous air oxidants capable to induce damage to the respiratory epithelium. In an experimental setting, primary human bronchial epithelial cells were exposed for 2 h at an air-liquid interface of NO_2, and O_3, or filtered air (control test). Gene expression was measured using PCR arrays for toxicity and oxidative stress. The results showed that genes related to oxidative stress were highly induced by NO_2 (Mirowsky et al. 2016).

13.7 Pulmonary Oxidative Stress, Inflammation, and Oxidative DNA Damage

The evolutioanry process on Earth and the subsequent appearance of eukariotic cells (~2 billion years ago) has been linked to the appearance of higher concentrations of O_2 in the Earth's atmosphere, transforming into a more efficient energy source of aerobic organisms. It is important to know that aerobic metabolism produces 16–18 times more adenosine triphosphate (ATP) per hexose sugar than anaerobic metabolism (Jiang et al. 2010; Dismukes et al. 2001). But aerobic organisms are exposed continuously on the oxidative cellular damage by oxygen. Multicellular eukaryotic life forms evolved the ability to extract up to 18 times more energy from food sources via oxygen-dependent complete oxidation in mitochondria (Cadet and Davies 2017; Cadet et al. 2017).

Endogenous and exogenous oxidative damage (from ROS) to cellular and mitochondrial DNA in aerobic organisms is an everyday occurrence. It has been

estimated that one human cell is exposed daily to approximate 10^3–10^5 hits by the hydroxyl radical (HO$^\bullet$) alone (Beckman and Ames 1997; Valavanidis et al. 2009d; Jena 2012). Additionally, the accumulation of oxidative damage to cellular and mitochondrial DNA in aerobic organisms has been established as an important causative factor of human diseases and aging. These oxidative lesions were investigated in the past several decades, and the results suggested that the oxidatively induced DNA adducts, especially bulky DNA lesions, may serve as biomarkers for exploring the role of oxidative stress in human diseases (Yu et al. 2016).

Mutations of mtDNA cause a variety of human mitochondrial diseases and are also heavily implicated in age-associated diseases, such as cancer, diabetes, cardiovascular, neurodegenerative, and aging. There has been considerable progress in understanding the role of mtDNA mutations in human pathology during the last two decades (Dizdaroglu 2012). Studies have shown that the mitochondrion is a sensitive target of both oxidative stress and environmental pollutants like ultrafine particulate matter (Li et al. 2003). Exposure to airborne PM is associated with mitochondrial DNA damage during pregnancy in both mothers and their newborn. The study observed that PM air pollution exposure in early life plays a role in increasing systemic oxidative stress, at the level of the mitochondria, both in the mother and fetus (Grevendonk et al. 2016).

According to epidemiological and toxicological evidence, PM acts as a carcinogenic factor inducing high rates of genomic alterations or damages. The respiratory system inevitably is in the frontline of exposure to airborne PM. These genomic damages are capable of acting as a driving force of the carcinogenic process. The frequency of these alterations is related to the loss of fidelity in mechanisms such as DNA replication, chromosomal segregation, DNA repair, and cell-cycle progression (Santibáñez-Andrade et al. 2017).

Biomonitoring studies in humans have shown associations between exposure to air pollution particles and high rates of oxidative damage to DNA. The elucidation of carcinogenic mechanisms by oxidative DNA damage indicates at least two different pathways thought to play an important role. The first mechanism acts through modulation of gene expression affecting intracellular signaling pathways, whereas the second proceed through the induction of genetic damage (mutations, strand breaks, chromosomal rearrangements) and blockage to the DNA replication (Hou et al. 2010; Ralph et al. 2010; Møller et al. 2010).

One of the most important and widely studied biomarkers of oxidative DNA damage caused by reactive ROS/RNS is the adduct of hydroxyl radicals (HO$^\bullet$) on DNA nucleobases, especially deoxynucleosides. The 8-hydroxy-2′-deoxyguanosine (8-OHdG) and/or its tautomeric 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) has been proved to be an important mutagenic adduct to DNA and therefore a potential biomarker of carcinogenesis. Mutations of 8-oxodG involve a GC → TA transversion.

In nuclear and mitochondrial DNA, 8-hydroxy-2′-deoxyguanosine (8-OHdG) is the most frequently detected and studied DNA lesion which is excreted in the urine. The 8-OHdG is not only a biomarker of oxidative stress but also can be used as a risk factor for cancer, atherosclerosis, and diabetes. Elevated level of urinary

8-OHdG has been detected in patients with various cancers. In the last decade, 8-hydroxydeoxyguanosine (8-OHdG) is a commonly used marker of DNA oxidative stress in epidemiological studies (Wu et al. 2004; Chen et al. 2015; Kasai and Kawai 2016).

Various biomarkers in urine samples of oxidative stress, such as 8-hydroxy-2′-deoxyguanosine (8-OHdG), malondialdehyde (MDA, a biomarker of lipid peroxidation), 8-isoprostane (8-IsoP, a bioactive metabolite resulting from the peroxidation of arachidonic acid), and vascular endothelial growth factor (VEGF), are used regularly in many studies for quantitative measurements of exposure ambient air pollutant levels (Zanolin et al. 2018; Pelletier et al. 2017). A recent study found a statistically significant correlation between the levels of 8-OHdG and smoking index in humans. The quantitative determination of 8-OhdG is very sensitive and can identify a patient with lung cancer. A high number of analytical and clinical studies indicate that 8-OHdG is a sensitive diagnostic biomarker for lung cancer (Cao et al. 2016).

13.8 Conclusions

Recent epidemiologic investigations have shown associations between increased incidence of respiratory diseases and lung cancer from exposure to tobacco smoke and various forms of respirable fibers and particulate matter (PM), at occupational or urban air polluting environments. Substantial number of studies showed that the generation of reactive oxygen species and nitrogen species (ROS/RNS), leading to oxidative stress and inflammation with high DNA damage, plays an important role in initiation and progression mechanisms of lung carcinogenesis. Although tobacco smoke, active and passive smoking, is a major factor of lung cancer, inhalable particles, inorganic dusts, asbestos fibers, and vehicular exhausts have a substantial contribution to respiratory diseases and in particular lung cancer. These factors can act synergistically in the respiratory system and increase substantially the production of ROS causing an imbalance between oxidants and antioxidants in the respiratory tissues. In turn, oxidative stress can damage cellular proteins, membrane lipids, and DNA, leading to genomic instability and activation of various inflammatory signaling cascades related to lung tumorigenesis.

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Part III

Other Lung Diseases

The Redoxomics of Bronchopulmonary Dysplasia

14

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Abstract

Bronchopulmonary dysplasia (BPD) is a chronic inflammatory lung disease affecting all lung tissues, primarily in premature and very-low-birth-weight (VLBW) infants needing oxygen therapy. Despite many decades of therapeutic advances, BPD remains a major clinical and costly complication in premature infants. Oxidative stress, inflammation, and prematurity are major interrelated contributing factors to BPD. This chapter will focus on the systems medicine of BPD with particular emphasis on the application of redoxomics for obtaining biomarkers that could prove useful in predicting susceptibility and prognosis as well as helping to formulate personalized and precise treatment strategies. Molecular insights gained from a systems medicine/redoxomics approach could help in developing new treatment modalities. Redoxomics is a subset of "omics" focusing on the genomics, epigenomics, proteomics, and metabolomics of oxidative stress, reactive oxygen species (ROS), reactive nitrogen species (RNS), and antioxidants. There is a considerable body of evidence connecting ROS and RNS to BPD risk within the framework of "oxidative damage." Nevertheless, it is now recognized that ROS, RNS, and antioxidants can also modulate many signal transduction pathways. This chapter will also review the gaps and opportunities in the application of systems medicine to BPD as well as practical issues such as sample acquisition, optimal assays, cost, and availability of state-of-the-art data analysis software.

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14.1 Introduction

Bronchopulmonary dysplasia (BPD) is a chronic inflammatory lung disease affecting all lung tissues, primarily in premature and/or very-low-birth-weight (VLBW) infants needing oxygen therapy. VLBW infants are those with a birth weight less than 1500 g. Premature infants are those born before 37 weeks of gestation, while extremely premature infants are those born before 30 weeks of gestation. Most infants developing BPD are born with respiratory distress syndrome (RSD), also called hyaline membrane disease, which, in turn, is the result of insufficient production or functioning of pulmonary surfactant (see www.nhlbi.nih.gov/health-topics/bronchopulmonary-dysplasia) (Clements and Avery 1998). Pulmonary surfactant is a lipid-protein complex (lipoprotein) that facilitates gas exchange in the lungs and also prevents pulmonary fluid accumulation. In the absence of sufficient and/or functioning surfactant, there is inadequate blood oxygenation with low hemoglobin saturation levels (e.g., <85%) (Group BIUKC 2013). BPD is associated with long-term impairment of both pulmonary function and neurodevelopment (Landry et al. 2011; Malavolti et al. 2018).

Despite the enormous progress in treating of BPD since it was first described in 1967 by Northway et al. (1967), BDP remains a major complication of prematurity with about 10,000 infants being affected annually in North America. Among the various medical conditions affecting premature/LBW infants, BPD is the costliest with an average direct cost of $116,000 (in 2007) per discharge in the USA (Russell et al. 2007). Lung diseases, in general, are a major cause of illness in the pediatric population (Pereira-Fantini and Tingay 2016).

14.1.1 Bronchopulmonary Dysplasia (BPD), a Disease Often Defined by Treatment Rather than Pathophysiology

As recently noted (Day and Ryan 2017; Merritt et al. 2009), the current clinical definition of BPD is peculiar because it depends upon treatment modality rather than pathophysiology. Since the treatment modalities for BPD have changed over time (see below), this has led to a somewhat confusing division of BPD into an "old BPD" and a "new BPD." As initially described by the pioneering work of Northway et al. (1967), the "old BPD" was characterized by the persistence of RDS, the need for continuing oxygen therapy, and abnormal chest X-rays. Oxygen therapy for the old BPD consisted of invasive mechanical ventilation at high (e.g., 100%) oxygen

levels. The key diagnostic criteria for the old BPD was the requirement for oxygen therapy at 28 days after birth. Oxygen therapy enabled many premature infants with gestation periods as low as 34 weeks and birth weights as low as 2200 g to survive but was limited by the invasiveness of mechanical ventilation, severe pulmonary tissue immaturity, acute lung injury, poor repair of lung injury, respiratory infections, and oxygen toxicity (Northway et al. 1967). Early work using a mouse model strongly supported the notion that oxygen toxicity was a major contributor to BPD and an inhibitor of normal lung growth and development (Bonikos et al. 1976). The initial work of Northway et al. (1967) was of tremendous significance in pediatrics by providing the initial impetus for the establishment of neonatal intensive care units. Moreover, it firmly established the view that neonates were not just "small adults." The central role of oxygen therapy/toxicity also supports the potential relevance of redoxomics to BPD.

14.1.2 The New Bronchopulmonary Dysplasia and the New Challenges

Major advances were eventually made in BPD treatment modalities, including the use of antenatal corticosteroids, surfactant instilled into the airways, and noninvasive continuous positive airway pressure (CPAP) ventilation. These advances again pushed the survival envelope toward extremely premature infants (e.g., 24 weeks' gestation) and extremely low-birth-weight infants (e.g., 700 g) giving rise to a "new" BPD (also called chronic lung disease of infancy), with a new set of signs and symptoms. With these new treatment modalities, the classic radiographic stages of the old BPD were no longer generally observed. Pulmonary hypertension emerged, however, as a sign observed in about one-third of the new BPD cases. Moreover, the work of Shennan et al. in 1988 showed that the requirement for oxygen therapy at 36 weeks' postmenstrual age was a superior predictor of abnormal pulmonary outcomes than the requirement for oxygen therapy at 28th day of life. The O_2 requirement at 36 weeks' postmenstrual age became a new defining parameter for BPD.

14.2 The Need for a BPD Definition Based on Pathophysiology and the Utility of a Systems Medicine Approach

The immature state of lung development in extremely premature infants is now considered a new barrier to survival in infants with BPD (Day and Ryan 2017; Merritt et al. 2009). A key pathophysiological mechanism in the new BPD is thought to be the impaired development of neonatal pulmonary vasculature (Day and Ryan 2017; Merritt et al. 2009). The lung is unique among organs with development occurring just prior to birth, as well as in the immediate postnatal period. This postnatal development process is thought to be particularly sensitive to a variety of

environmental factors that can disrupt the angiogenesis required for alveolarization. Day and Ryan (2017) stress the importance of reframing BPD into a set of pathophysiological categories or clinical phenotypes such as in utero inflammation, infection, ventilation parameters, and genetic factors. In this chapter, we posit that a systems medicine approach would be optimal in helping to define a set of molecular factors (molecular phenotypes) relevant to BPD.

A complete clinical review of BPD is beyond the scope of this review, but an outstanding series of video recordings of the recent Stanford Children's Health Bronchopulmonary Dysplasia Symposium is available online (med.stanford.edu/alviralab/center-of-excellence-events/bpd-symposium-new/video-highlights-from-the-stanford-bpd-symposium.html), as well as excellent reviews (Davidson and Berkelhamer 2017). This symposium came to a number of powerful conclusions about how progress in treating BPD could proceed. The first consensus was that BPD treatment must be individualized rather than a "one-size-fits-all" approach, i.e., a precision health approach. The second conclusion was that "progress occurs at the margins of disciplines previously considered distinct." The third interrelated assertion was that "discovery drives care and care drives discovery."

It is now recognized that advances in treating BPD will hinge on developing new pathophysiological biomarkers that provide clinically relevant information about disease mechanism, severity, prognosis, treatment, and disease/risk stratification. As detailed below, this is a goal for which a systems medicine (or "omics") approach is particularly well-suited.

14.3 Systems Medicine, Mono-omic, and Multi-omic

Systems medicine utilizes a multidisciplinary approach to clinical problems that relies on "omics" information primarily from genomics, epigenomics, proteomics, and metabolomics, as well as inputs from conventional pathophysiology and biochemistry (see Fig. 14.1). One aspect of systems medicine is the "bench-to-bedside"

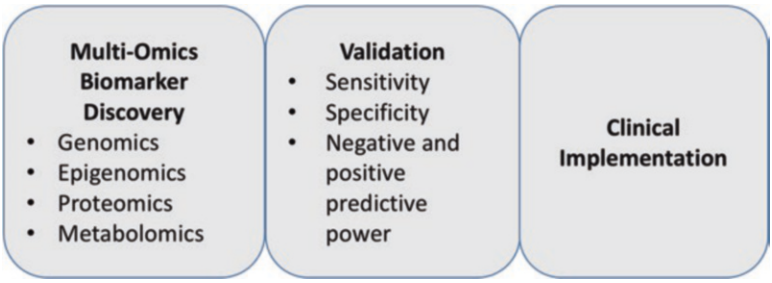

Fig. 14.1 Work flow for multi-omic studies. A set of biomarkers from genomic, epigenomic, proteomic, and metabolomic platforms are sought to stratify, diagnose, and prognose a disease state as well as guide individualized treatment therapy. After statistical validation, assays for selected multi-omic biomarkers are optimized for rapid turnaround and the type of sample, e.g., BAL, plasma, and urine

path which emphasizes the application of basic science to solve clinical problems, i.e., translational medicine. Basic science, in the form "omics" technology, is undergoing a continual revolution that is now poised to have far-reaching clinical repercussions. Omics technology provides clinically useful information from genomics (full genome genotyping or DNA sequence), epigenomics (genome-wide alterations in DNA expression without alterations in DNA sequence), proteomics (all the alterations in protein expression in a biospecimen), and metabolomics (all alterations of small molecules in a biospecimen). The overall goal is to utilize omics data to discover a set of biomarkers useful in diagnoses, in prognoses, and in formulating optimal and individualized patient care. This integrative approach is essentially that proposed by the contributors to the recent Stanford Children's Health Bronchopulmonary Dysplasia Symposium. There are only a few publications detailing the role of omics technology in BPD (Piersigilli and Bhandari 2016) and none with an emphasis on redoxomics.

The complexities and pitfalls in the design of clinical omics biomarker discovery experiments have recently been reviewed by Forshed (2017) who emphasizes the need to formulate a coherent "molecular hypothesis." Moreover, most omic biomarker studies have been limited to a single omic platform (i.e., "mono-omic") such as genomics. It is very likely that utilizing biomarkers from more than one omic platform (i.e., multi-omics) would be particularly effective, and a freely available software platform has been developed for just this specific purpose (mixomics.org/mixdiablo/).

14.4 Candidate Genes Based on the Pathophysiological and Molecular Mechanisms Underlying BPD

The first step in a systems medicine approach is to identify the pathophysiological and molecular mechanisms that underlie BPD and formulate a "molecular hypothesis." The initial goal is to identify possible candidate genes for further genomic/epigenomic/proteomic analyses. Formulating an initial molecular hypothesis for BPD involves identifying the relevant etiological factors. For BPD these would include oxidative stress, inflammation, and preterm birth (PTB). These three broad factors are, therefore, a reasonable starting point in seeking candidate genes relevant to BPD (Joung et al. 2011). As detailed below, these factors are interrelated since they all have reactive oxygen species (ROS), reactive nitrogen species (RNS), and antioxidant mechanisms as common underlying molecular factors. Moreover, inflammation is always accompanied by varying degrees of oxidative stress (cf. Salzano et al. 2014). As detailed below, oxidative stress is also known to accompany a wide range of infections (Ivanov et al. 2017). Both prenatal and postnatal infections and the accompanying inflammation are contributing factors to BPD pathogenesis (Balany and Bhandari 2015).

14.5 Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), Oxidative Stress, and BPD

The role of oxidative stress in BPD is most often framed in the context of molecular and cellular damage due to increased production of ROS and RNS. Oxidative stress, although a much-used phrase, does not have a clear functional definition and, therefore, has some ambiguity (Rahal et al. 2014). It is most often defined as an "imbalance" in which levels of oxidant species "outweigh" antioxidant mechanisms. Oxidative stress could be at a high, yet physiological level, with minimal molecular damage and cell death. Alternatively, oxidative stress could be above physiological levels resulting in molecular and/cellular damage sufficient to cause cell death. The term ROS is preferred over "oxygen free radicals" since not all ROS are free radicals and not all oxygen free radicals are particularly reactive. Molecular oxygen exhibits the properties of a diradical yet does not directly damage macromolecules or biomembranes. Similarly, the superoxide radical ($^{*}O_2^-$), formed by the addition (reaction 14.1) of one electron ($*$) to O_2, is a mild reducing agent but is the precursor of most ROS and RNS. Superoxide dismutase (SOD) catalyzes (reaction 14.2) the conversion of $*O_2^-$ into hydrogen peroxide (H_2O_2) and oxygen. Catalase (CAT), glutathione peroxidase (GPX), and peroxiredoxin (PRX) can all remove H_2O_2 (see Table 14.1).

$$O_2 + e- \rightarrow *O_2^- \tag{14.1}$$

$$2*O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \tag{14.2}$$

$$2H_2O_2 \xrightarrow{CAT} O_2 + 2H_2O \tag{14.3}$$

One well-known mechanism for generating the highly reactive hydroxyl radical ($*$OH) lies in the redox properties of some metal ions (e.g., Fe^{2+}/Fe^{3+}) via the Haber-Weiss reaction (reaction 14.4).

$$*O_2^- + H_2O_2 \rightarrow O_2 + *OH + OH^- \tag{14.4}$$

Lipid peroxidation is another source of free radicals, and this process can damage/alter biomembranes and/or lipid-protein complexes. Vitamin E (tocopherols and tocotrienols) acts as lipophilic chemical antioxidants by quenching the lipid radicals produced during lipid peroxidation.

$*O_2^-$ can also rapidly react with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$) which is very reactive but not a free radical.

$$*O_2^- + NO \rightarrow ONOO^-$$

$ONOO^-$ can react with the tyrosine residues in proteins to form 3-nitrotyrosine which is considered a biomarker for $ONOO^-$-mediated protein damage (Ahsan 2013). It is relevant, therefore, that premature infants that develop BPD have elevated plasma levels of 3-nitrotyrosine (Banks et al. 1998).

Table 14.1 Genes, proteins, and protein functions relevant to bronchopulmonary dysplasia

Gene name	Enzyme name	Function
NFE2L2 (all called Nrf2)	Nuclear factor (erythroid-derived 2)-like 2, also called Nrf2	A transcription factor regulating responses (see the genes 2–6 below) to oxidative stress
SOD1	Superoxide dismutase 1	Cytoplasmic form of copper/zinc-dependent superoxide dismutase
SOD2	Superoxide dismutase 2	Mitochondrial form of manganese-dependent superoxide dismutase
SOD3	Superoxide dismutase 3	Extracellular from of copper/zinc-dependent superoxide dismutase
GCLC	Glutamate-cysteine ligase or gamma-glutamylcysteine synthetase	The rate-limiting enzyme for glutathione (GSH) synthesis
GSTP1	Glutathione S-transferase Pi 1	A detoxification enzyme that conjugates GSH with numerous hydrophobic and electrophilic compounds
HMOX1	Heme oxygenase 1	Cleaves a heme ring to form biliverdin
NQ01	NAD(P)H quinone dehydrogenase 1	A cytoplasmic 2-electron reductase. This FAD-binding protein reduces quinones to hydroquinones
FOXO1	Forkhead box O1	A transcription factor regulating oxidative stress
FOXO3	Forkhead box O3	A transcription factor regulating oxidative stress
CAT	Catalase	Converts hydrogen peroxide to water and oxygen
PRDX3	Peroxiredoxin III	A thioredoxin-dependent mitochondrion-specific peroxidase
GPX-1	Glutathione peroxidase 1	A selenoprotein that reduces hydrogen peroxide and lipid hydroperoxides with glutathione (GSH)
NOX1	NADPH oxidase 1	Catalyzes the one-electron transfer to oxygen generating superoxide
TRN	Thioredoxin	Small (12 kDa) thiol-active proteins that participate in various redox reaction and responses to nitric oxide
CRP	C-reactive protein	

14.5.1 ROS, RNS, and Antioxidants May Affect Signal Transduction Mechanisms Relevant to BPD and Preterm Birth (PTB)

ROS and RNS at high levels can result in oxidative damage to biomolecules as well as cell death. The pioneering work of Irani et al. (1997), followed by much additional evidence (Hensley et al. 2000), showed that ROS could also act as second messengers capable of modulating many signal transduction pathways, particularly those involved in inflammation and cell growth. RNS can also affect signal

transduction pathways. In vitro work by Gow et al. (1996) demonstrated that the ability of protein tyrosine kinases to phosphorylate tyrosine residues on a substrate protein could be inhibited if the protein substrate was modified by exposure to peroxynitrite. The ability of kinases to alter protein functions by tyrosine phosphorylation is a major mechanism for modulating many signal transduction pathways.

Similarly, antioxidants (both enzymatic and chemical) were traditionally viewed as simply reducing the levels of damaging ROS (Hensley et al. 2000). Nevertheless, antioxidants can also affect many signal transduction pathways either by modulating ROS levels and/or by their intrinsic biochemical properties distinct from their antioxidant capacity. Vitamin E compounds (tocopherols and tocotrienols) and their metabolites, for example, are chemical antioxidants that can modulate some signal transduction pathways in a manner not corresponding to their antioxidant abilities (Hensley et al. 2004).

The ability of ROS, RNS, and antioxidants to modulate signal transduction pathways raises the question of whether or not BPD pathophysiology is solely due to cellular damage caused by increased ROS production. The early work of Northway et al. (Bonikos et al. 1976) is illuminating in this regard. These researchers found that newborn mice exposed to 100% oxygen developed lung injuries phenotypically similar to those observed in BPD, but unexpectedly found that newborn mice exposed to 100% oxygen could survive for weeks, in marked contrast to adult mice, that died in days. If oxygen toxicity and the resulting ROS-induced cellular damage were the sole mechanism for BPD, it is not obvious why newborn mice would be more resistant than adult mice, particularly since many lung antioxidant mechanisms are generally lower in newborns compared to adults (Poggi and Dani 2014; Berkelhamer and Farrow 2014). Collectively, the results above suggest that the quest for candidate genes relevant to BDP includes not only genes coding for enzymatic antioxidants but also for genes coding for signal transduction proteins affected by oxygen, ROS, RNS, and antioxidants.

14.6 Evidence for Oxidative Stress in BPD Is Difficult to Interpret

In clinical studies, oxidative stress (OS) is most often assessed by measuring biomarker levels in a "systemic" biofluid such as plasma or urine. It should be noted, however, that very localized (e.g., subcellular) OS stress/damage could also occur which might not be evident by assaying a systemic biofluid. Moreover, localized oxidative stress could modulate local signal transduction events. Evidence supporting the etiological role of OS in BPD has been reviewed and collectively is very persuasive (Joung et al. 2011; Poggi and Dani 2014; Perrone et al. 2012; Saugstad 2010).

As mentioned above, both the therapeutic strategies and the definitions of BPD have changed over time, and therefore, the "historical" evidence for OS in BPD must be carefully evaluated for its current clinical relevance. A pivotal therapy for treating RDS was the use of surfactant, which reduces the need for prolonged

oxygen therapy and its attendant potential for OS. Moreover, the type of surfactant preparation and its composition has changed since its widespread use in the early 1990s (Halliday 2005; Niemarkt et al. 2017). Surfactants derived from natural sources (porcine or bovine) also contain varying amounts of antioxidant enzymes (e.g., SOD, CAT) (Poggi and Dani 2014). Classically, mechanical ventilation for oxygen therapy utilized invasive endotracheal intubation, which also provided a convenient means for surfactant delivery. A number of clinical trials showed, however, that positive continuous airway pressure was superior to mechanical ventilation since it did not require intubation but also negated the convenient delivery of therapeutic surfactant. Although beyond the scope of this chapter, a number of strategies are being developed and tested to effectively deliver surfactant to preterm infants without intubation (Niemarkt et al. 2017; Shim 2017).

14.7 Oxidative Stress, Oxygen Therapy, and BPD

There is considerable support for the notion that oxygen therapy and its potential toxicity play key etiological roles in BPD, yet gaps remain in our knowledge and understanding (Jobe and Kallapur 2010; Menon 2014). Oxygen therapy is monitored by noninvasive pulse oximetry, which provides an estimate of arterial oxygen saturation, or SaO_2. Multiple studies have looked at high (90–95%) and low (85–89%) SaO_2 targets in treating BPD. The low oxygen saturation target is often chosen in an attempt to reduce the incidence of retinopathy of prematurity (ROP) in premature infants. ROP is an oxidative stress neonatal disease caused by abnormal retinal blood vessel growth and proliferation (Stone et al. 2016). The retina is particularly sensitive to oxygen toxicity since it has a uniquely high rate of oxygen consumption, as well as lipids with a very high content of polyunsaturated saturated fatty acids, which are susceptible to lipid peroxidation (Stone et al. 1979a, b). Nevertheless, multiple studies have shown that a low SaO_2 target results in increased mortality and that a 90–95% SaO_2 might be a better choice (Group BIUKC 2013; Darlow and Morley 2015). A systems medicine approach might help to determine the optimal therapeutic oxygen level for an individual neonate.

14.7.1 Intra-amniotic Infection and Oxidative Stress

One likely source of enhanced OS during pregnancy is inflammation arising from intra-amniotic infection (IAI), which is common in preterm births (PTBs) (Menon 2014). IAI includes any combination of infections in the amniotic fluid, amnion, placenta, and chorion. Macrophages and neutrophils are major sources of immune-modulated OS, which originates from an "oxidative burst" caused by the generation of $*O_2^-$ via NADPH oxidase (NOX) (Hampton et al. 1998; Slauch 2011). SOD, in turn, generates H_2O_2 from $*O_2^-$ (see reaction 14.2 above). Most of the H_2O_2 in neutrophils is utilized by myeloperoxidase (MPO) to produce hypochlorous acid, which is a potent bactericidal agent. Until fairly recently, glutathione peroxidases (GPXs)

were thought to be the main enzymes for rapidly degrading H_2O_2, but accumulating evidence now suggests that peroxiredoxins (PRXs) are also quite important, with some estimates suggesting that PRXs reduce more than 90% of cellular H_2O_2 (Knoops et al. 2016). In animal models at term gestation, oxygen exposure has been found to increase levels of SOD3, thioredoxin (TRN), and PRXs (Berkelhamer and Farrow 2014). Furthermore, the TRN system is now recognized as being important not only for its antioxidant role but also for being a redox regulator of signal transduction pathways involved in normal and pathological lung development (Tipple 2014). For the TRN system, redox regulation occurs by thiol-disulfide exchange reactions.

14.7.2 Smoking During Pregnancy, Oxidative Stress, PBD, and PTB

Maternal cigarette smoking during pregnancy (and smoking in general) is a major cause of severe in vivo oxidative stress (Stone et al. 2014) and is thought to be a BPD risk factor (www.lung.org/lung-health-and-diseases/lung-disease-lookup/bronchopulmonary-dysplasia/bpd-symptoms-causes-risk.html). Nevertheless, research connecting maternal smoking to BPD is very limited and often complicated by assessing maternal smoking solely by the mother's self-declaration rather than a definitive laboratory test. For infants with BPD, exposure to second-hand smoke is associated with a trend toward increased inhaled steroids use, as well as increased duration of supplemental oxygen therapy (Collaco et al. 2014). The evidence connecting smoking to PTB is both abundant and very persuasive (Ion and Bernal 2015).

14.7.3 Prematurity, Oxidative Stress, and BPB

Inflammation/OS is important in both PTBs and BPD, and it is reasonable to suggest that etiological insights could be gained by studying the molecular pathways modulated or damaged by these factors. Menon (2014) has reviewed the role of OS in PTB pathology with an emphasis on OS damage, rather than the potential roles of ROS as second messengers. Nevertheless, Menon cites a considerable body of evidence supporting the notion that OS plays a key role in initiating normal labor by triggering signals that cause senescence of the fetal cells of the placenta membranes: a process primarily modulated by p38 mitogen-activated kinase (p38MAPK). Moreover, Menon suggests that abnormalities in OS are responsible for initiating premature senescence of the placental membranes resulting in PTB. A better understanding of the pathophysiology of PTB is critical to the future development of therapies aimed at preventing both PTB and BPD. At present, the primary therapy (albeit with limited success) for preventing PTB is by inhibiting uterine contractions.

14.8 The Genomics of BPD and Evidence for Its Inheritability

Genomics has many advantages in systems medicine since it is high-throughput and an individual's DNA sequence is time- and tissue-independent. Maternal and neonatal DNA for genomic analyses is easily obtained from blood samples. However, for genomic data to guide very early clinical BPD decisions, obtaining fetal DNA as soon as possible would be optimal. Prenatal noninvasive cell-free fetal DNA (cffDNA) holds great potential in this regard and is a rapidly developing technology (Norton 2016; Chan et al. 2016). Although the cost for whole-genome sequencing has significantly decreased (now about $1000–$1600), it remains more expensive than whole-genome single-nucleotide polymorphism (SNP) genotyping (about $100–$200).

Moreover, an increasing number of people have already had SNP genotyping done by commercial labs (e.g., 23andMe). SNPs are a frequent (about one in every 300 nucleotides) type of genetic variation, and a typical human genome has about ten million SNPs (ghr.nlm.nih.gov/primer/genomicresearch/snp). Most SNPs do not occur in expressed genes and do not influence health or disease susceptibility. Those SNPs occurring in the gene body have the potential of being clinically important markers for disease or disease susceptibility. SNP association studies look at the correlation between disease incidence/susceptibility and a set of specific single-nucleotide polymorphisms in candidate genes.

The case for genomics in BPD would be bolstered by evidence showing an inheritable component as determined by classic genetic studies. Such studies were reviewed in 2006 by Bhandari and Gruen (2006), and more recently by Lal and Ambalavanan (Lal and Ambalavanan 2015), with the conclusion that genetics accounts for about 53–79% of the observed variance in the susceptibility to BDP. These studies did not, however, identify any specific genes. Nevertheless, the robust contribution of genetics to BPD strongly supports efforts to identify relevant candidate genes and test for genetic associations.

14.8.1 The Association of Single-Nucleotide Polymorphisms (SNPs) in Oxidative Stress Genes and BPD

Poggi et al. (2012) found that neonatal SNPs in mitochondrial SOD2 and extracellular SOD3 (see Table 14.1) were associated with BPD risks. These authors found that SOD2 haplotype GT (tag SNPs) increases the BPD risk while SOD3 haplotype TGC (tag SNPs) decreased BPD risk. SODs play pivotal roles in redox signaling, angiogenesis, and vascular function (Fukai and Ushio-Fukai 2011). Extracellular SOD3 is a Nrf2-independent antioxidant enzyme that could be important in the pathogenesis of BPD (Poonyagariyagorn et al. 2014). SOD3 is, in fact, the primary source of SOD activity in the airways and lung vasculature. Reduced expression of SOD3 in a mouse model correlates with alveolar hyperoxic injury (Poonyagariyagorn et al. 2014).

More recently, Sampath et al. (2015) explored the potential association of antioxidant response genes (see Table 14.1) with susceptibility to BDP in VLBW infants. In particular, they studied the antioxidants and detoxification genes regulated by nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) gene (also called Nrf2) which is a transcription factor (see www.selfhacked.com/blog/about-nrf2-and-natural-ways-to-increase-it/#Introduction_to_NRF2). A fairly large cohort (n = 659) of infants was utilized: 284 had BPD out of whom 135 developed severe BPD. The presence of SNP rs1800566 (homozygous) in the NAD(P)H quinone dehydrogenase 1 (NQ01) gene was associated with increased BPD incidence. SNP rs1800566 results in a decrease in NQ01 functioning. In contrast, the presence of SNP rs6721961 in the Nrf2 gene was associated with a decreased severity of BPD. In this study, common SNPs in SOD2, HMOX1, GSTP1, and GCLC (see Table 14.1) were not found to be associated with BDP or its severity. The authors emphasize that their results need to be replicated in an independent cohort (Sampath et al. 2015).

Surprisingly, there have not been many additional SNP association studies looking at other enzymatic antioxidants. The class O of forkhead box (FoxO) transcription factors, for instance, are well-known regulators of OS and multiple antioxidant enzymes (Wang et al. 2014; Akasaki et al. 2014). FOXO3, for example, increases levels of SOD2, catalase, and peroxiredoxin III (Wang et al. 2014). Moreover, peroxiredoxins and the thioredoxin system are highly expressed in the pulmonary epithelia of newborns, where they play a key role in preventing OS as well as modulating signal transduction pathways important in lung development (Tipple 2014).

14.8.2 Genome-Wide Association Studies (GWAS) and BPD

Genome-wide association studies (GWAS) are a second style of genomic analyses. In these studies, no assumptions are made about a set of candidate genes. In typical GWAS studies, a very large set of SNPs (over a million) are compared between a large number of cases and controls. The vast majority of GWAS studies have focused on the pulmonary diseases of adults, with only a handful dealing with BPD (Pouladi et al. 2016). The few BPD GWAS studies to date have provided limited information, and larger numbers of infants (e.g., >1000) may be required in future studies. In general, these GWAS studies have not affirmed the results of SNP-BPD association studies, possibly due to the higher statistical standard of GWAS studies ($P < 10^{-8}$) compared to SNP association studies ($P < 0.05$). Ambalavanan et al. (2015) studied a total of 751 infants out of which 428 developed BPD or died. No SNPs achieved genome-wide significance, but a number of significant pathways were identified that could be of pathophysiological significance. The "phosphorus-oxygen lyase" pathway was one such pathway and is defined as the collective set of enzymes catalyzing the cleavage of a phosphorus-oxygen bond by mechanisms other than hydrolysis or oxidation. A second implicated pathway was miR-219 which is a microRNA important in epigenetic regulation (see below). A more recent GWAS by Mahlman et al. (2017) was published in 2017 with a Finnish population

(60 cases of BPD and 114 controls). This study also resulted in no SNPs being statistically associated with BPD but did "implicate" one SNP (rs11265269) near the C-reactive protein (CRP) gene. Moreover, plasma levels of CRP in the first week of life were predictive of BPD. Plasma CRP levels are considered a measure of inflammation. Moreover, in adults CRP levels have been found to correlate with plasma levels of 8-iso-PGF_{2alpha} (an oxidative stress biomarker) as well as vascular endothelial cell dysfunction (Cottone et al. 2006).

14.8.3 The Genomics of Prematurity and the Importance of Both Fetal and Maternal DNA Variants

Prematurity is a major determinant of BDP, and therefore, a systems medicine approach would be incomplete without at least a brief discussion of studies focused on the general role of genetic factors contributing to prematurity. Spontaneous preterm delivery has a robust inherited predisposition(Bhattacharya et al. 2010). A number of candidate genes and associated SNPs (from maternal DNA samples) have been documented (Sheikh et al. 2016). The SNPs found to be associated with PTB cover a wide range of physiological functions, e.g., endocrine, remodeling of tissue and angiogenesis, but the majority are related to inflammation, which is clearly consistent with a predisposition for BPD.

There is an important experimental design factor to be learned from the genetic studies of PTBs, i.e., the primary focus is on the genetics of the mothers and not the newborns. This strongly suggests that the genomics of both the mother and newborn would be important determinants of BPD. Nevertheless, the BPD-SNP association and GWAS studies detailed above focused only on genomic data obtained from the newborn.

14.8.4 The Limitations of Genomics

Genomics is the most "static" of the "omics" (see Fig. 14.2), and both BPD and PTB are both processes involving dynamic tissue injury, remodeling, infection, exposure to environmental stressors, and inflammation. Omic biomarkers responsive to these dynamic processes are likely to be more sensitive to the risks of developing PTB and BPD. These dynamic omics would include epigenomics, proteomics, and metabolomics which will be discussed below (Kitano 2002). The cost of a DNA methylation array assay, which includes quantifying 850,000 methylation sites across the genome at single-nucleotide resolution, is about $50–$150. Epigenomic studies, like proteomics and metabolomics, are both tissue/biofluid-dependent and time-dependent. The time-dependent nature of epigenomics, proteomics, and metabolomics supports the requirement for obtaining serial samples, e.g., before BPD is manifest and when initial signs are present.

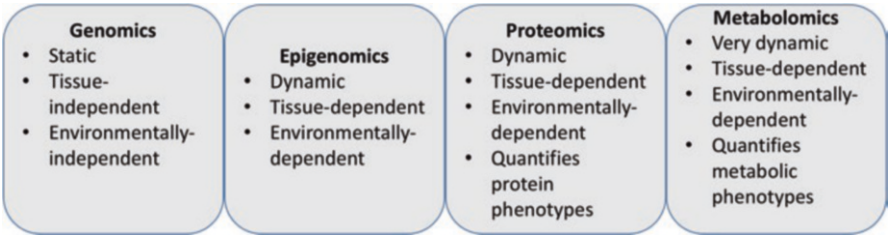

Fig. 14.2 Omic platforms. Each omic platform has varying degrees of (Clements and Avery 1998) disease dynamics, (Group BIUKC 2013) disease tissue-specificity/relevance, and (Landry et al. 2011) sensitivity to environmental influences. Dynamic omics are those where the parameters being measured change over the disease course and obtaining serial samples for analyses would be important. Both proteomics and metabolomics can provide a comprehensive set of dynamic molecular phenotypes for a disease

14.9 Epigenomics of BPD, a Major Gap in a Systems Medicine Approach

Hagood (2014) has reviewed the overall involvement and central importance of epigenetics in lung remodeling and its modulation by OS and inflammation. The major epigenetic mechanisms include methylation of DNA and covalent modifications of histone and noncoding RNA such as miRNA. Despite its clinical importance, there is very limited information on the epigenomics of BPD, and this is a major gap in a systems medicine approach (Piersigilli and Bhandari 2016; Hagood 2014; McEvoy et al. 2014). NIH is, however, currently devoting resources to this gap (grantome.com/grant/NIH/K08-HL140198-01). The GWAS studies (mentioned above) by Ambalavanan et al. (2015) also provide a strong rationale for future epigenetic studies since these investigators found evidence supporting the role of the miR-219 pathway in BPD. Other investigators have found the miR-219 pathway to be important in regulating neurobehavioral functions (Kocerha et al. 2009).

Although not explicitly focused on BPD, the work of Knight and Smith (2016) is relevant, since it does review the epigenetic factors related to PTB in the hopes of finding useful biomarkers. DNA methylation is the most studied epigenetic mechanism linked to BPD. In most cases, DNA methylation in a gene promotor will decrease the expression of that gene. Multiple studies have assessed CpG methylation sites in both neonatal and maternal DNA samples. Parets et al. (2015) looked at paired maternal and neonatal DNA methylation patterns in peripheral leukocytes related to PTB in African Americans. Surprisingly, a significant correlation was observed between the DNA methylation patterns in the mothers and her neonate. African Americans infants exhibit a 1.5 higher rate of spontaneous PTB than white infants, and this difference could, in part, be attributed to the influence of environmental (e.g., smoking) and social stressors (e.g., socioeconomic stressors) on epigenetic mechanisms (Burris et al. 2016). The reader is referred to the excellent review of Knight and Smith (2016) for more details. Suffice it to say that Knight

asserts that "due to the paucity of research in this area," more studies are required to unravel any connections between DNA methylation patterns and PTB.

14.10 The Proteomics of BPD and PTB

Of all the omic technologies, proteomics is likely to yield the most useful clinical information on the molecular mechanisms and alterations giving rise to both BPD and PTB. The general role of proteomics in neonatology has been reviewed (Young and Stone 2012), as well as the more specific application of proteomics to lung injury in childhood (Pereira-Fantini and Tingay 2016). The general application and importance of proteomics in redox biology has also been reviewed (Go et al. 2013; Go and Jones 2013a, b). Nevertheless, there has been no systematic application of redox proteomics to BPD, despite the central etiological role of OS. It is clear that proteomics is being underutilized in studies of pediatric injuries of any kind (Pereira-Fantini and Tingay 2016).

Proteins are the "nanomachines" performing the overwhelming majority of the body's functions, and almost all disease states are accompanied by alterations in either the expression or posttranslational modifications of a set of proteins. Neither proteins levels nor their posttranslational modifications can be confidently predicted based only on genomic/epigenetic data. Moreover, proteomics, like epigenomics, is dynamic and sensitive to transient stressors and environmental factors.

In the "discovery" phase, reverse-phase liquid chromatography-nanospray ionization-tandem mass spectrometry (RP-LC-NSI-MS/MS)-based proteomics is useful for identifying unbiased candidate proteins differentially expressed in cases versus controls. Targeted proteomics is now emerging as a powerful tool for rapidly quantifying a set of candidate proteins established by discovery proteomics, genomics, or conventional molecular biology (Song et al. 2017). LC-MS/MS technology suffers from being expensive but is becoming as "high-throughput" as genomics (Garbis et al. 2005; Mann and Kelleher 2008). There have been rapid advances in both LC-MS-proteomics hardware and software (Mann and Kelleher 2008). Outstanding (and free) software is now available for the detailed quantitative analysis of label-free targeted proteomic data obtained from high-resolution LC-MS instrumentation (e.g., skyline.ms/).

Once a set of proteins has been found (e.g., by LC-MS) to serve as appropriate disease biomarkers, a protein microarray might be the optimal method for the rapid detection and quantification required for informed "bedside" clinical decisions (c.f. Duarte and Blackburn 2017). Multiplex immunoassays are another viable platform (Tighe et al. 2015).

14.11 Neonatal Bronchoalveolar Lavage (BAL), Surfactant Proteins, and Inflammation

Proteomics, like epigenomics, is tissue/biofluid specific and best done with a disease-relevant sample. For BPD, neonatal bronchoalveolar lavage (BAL) is ideal and serially obtainable (Kotecha 1999; Magi et al. 2006; Chen et al. 2008; Carvalho and Matthiesen 2017). BAL is known to contain surfactant proteins A (SP-A) and D (SP-D), which are powerful and clinically useful lung disease biomarkers, e.g., RDS (Kuroki et al. 1998). Both SP-A and SP-D are collagen-containing C-type lectins or collectins, which play important roles in innate immune responses (Sorensen 2018). Sorensen (2018) has reviewed the literature supporting the view that SP-D is a promising disease biomarker for both BPD and RDS. Both SP-A and SP-D are hydrophilic proteins that are lacking in therapeutic surfactants isolated from organic solvent extracts of natural surfactants.

SP-D plays a key role in modulating inflammation and regulating the oxidative burst of phagocytic cells (Mahajan et al. 2013). Moreover, both in vivo and in vitro experiments have demonstrated that the SP-D protein can be damaged by oxidative stress with resulting impairment of its antibacterial functioning (Starosta and Griese 2006a, b). Significantly, SP-D from BPD subjects showed evidence of oxidative modification suggesting a potential impairment of host defense (Starosta and Griese 2006a). In addition to the antioxidant proteins list in Table 14.1, surfactant proteins would be excellent proteins to quantify by targeted proteomics in BAL from BPD subjects and controls.

Despite its clinical importance, there are very few proteomic studies of BPD with BAL samples. The first such study by Magagnotti et al. (2013) utilized two-dimensional gel electrophoresis and gel imaging software followed by reverse-phase liquid chromatography with tandem mass spectrometry (RP-LC-MS/MS) to identify differentially expressed proteins. This discovery phase study identified a number of Ca-signaling proteins that were altered in infants with gestational ages between 23 and 25 weeks compared with infants in the 26–29 gestational age group: these included calcyphosine, calcium and integrin-binding protein 1, chloride channel protein 1, and annexin-3. Other differentially expressed proteins were leukocyte elastase inhibitor (SERPINB1) and pulmonary surfactant-associated protein-A2 (SFTP-A2). Moreover, downregulated calcium signaling-related proteins were correlated with BPD progression. None of the dysregulated proteins found by Magagnotti et al. (2013) were obviously related to oxidative stress or inflammation.

14.11.1 Placenta and Umbilical Cord

The placenta contains both fetal (villous chorion) and maternal tissues (decidua basalis) which are anatomically distinct. Like BAL, placental tissues are directly relevant to BPD. Torchin et al. (2016) found that placenta-mediated pregnancy complications were related to moderate to severe BPD in very preterm infants. Moreover,

inflammation of the placenta and the accompanying OS was associated with the development of BPD (Perrone et al. 2012). An exhaustive proteomic study of placental tissue was recently published with an emphasis on gestational diabetes (Roverso et al. 2016). Unfortunately, this technology has not yet been applied to BPD and represents yet another large "gap" in a systems medicine approach.

The umbilical cord is another tissue relevant to BPD but has been underutilized in omics studies. Cohen et al. (2007) studied perturbations in RNA expression in premature infants with (n = 20) or without (n = 34) BPD. Infants who developed BPD had distinctive RNA microarray profiles involving chromatin remodeling and histone acetylation pathways (important in epigenetics). The authors emphasize the need to repeat this study with a much larger population size.

14.11.2 Plasma

Maternal and/or newborn plasma samples are conveniently obtainable, but any differentially expressed protein might reflect a systemic effect of BPD rather than being tissue specific. Nevertheless, the convenience and ability to collect serial samples over a long time period could override this objection. A very recent paper by Förster et al. (2018) utilized the SOMAscan® assay to look at the differentially expressed protein in neonatal plasma samples obtained in the first week of life. SOMAscan is a protein array platform based on modified nucleic acid aptamer ligands (Rohloff et al. 2014). The assay is capable of simultaneously measuring about 1300 different proteins over a wide dynamic range (micromolar to femtomolar) and has been optimized for protein biomarker discovery (Candia et al. 2017). Although plasma has been the primary biofluid tested, BAL and urine samples (see below) should also work with this assay but have not yet been tested. Förster et al. (2018) studied a relatively small population of 35 premature infants out of which 4 had no BPD, 8 had mild BPD, 2 had moderate BDP, and 4 had severe BPD. Despite this small population, a set of three plasma proteins were found (and validated) that could identify BPD shortly after birth with a high degree of sensitivity and specificity. The three proteins are sialic acid-binding Ig-like lectin 14 (SIGLEC14), basal cell adhesion molecule (BCAM), and angiopoietin-like 3 (ANGPTL3). ANGPTL3 is important in angiogenesis and inflammation (Santulli 2014), SIGLEC14 in the innate immune system, and BCAM functions as a receptor for laminin (an extracellular matrix protein). The SOMAscan assay holds great promise in the discovery phase of proteomics.

14.11.3 Urine

Obtaining serial urine samples is noninvasive and quick. For neonates, cotton balls can be inserted into a diaper, and only about 150 microliters of urine are needed for LC-MS/MS proteomics. Although not yet peer-reviewed, the thesis of Saima Ahmed at Harvard (Ahmed 2016) presents an excellent preliminary study of biomarker

discovery for BPD with LC-MS/MS proteomics using urine samples from 96 premature infants. Many technical issues with the processing of urine samples and the software used for subsequent proteomic analyses are covered in this excellent thesis. Obtaining serial urine samples before, and after BPD signs are manifest, appears to be critically important.

14.12 Metabolomics, BPD, and PTB

The application of metabolomics in neonatology is relatively new and limited to only a few publications (Hanna and Brophy 2015). Metabolomics is the most dynamic of the omic technologies and gives a downstream small molecule profile resulting from the collective inputs of the genome, the transcriptome, proteome, and environmental influences, e.g., nutrition and oxygen therapy. High-throughput technologies (NMR and gas or liquid chromatography) make metabolomics a viable yet an underused platform for biomarker discovery. Metabolomics, utilizing amniotic fluid and mass spectrometry analyses, can distinguish PTB infants from term infants, as well as PTB infants that develop BPD from those that do not (Baraldi et al. 2016). Moreover, 4-hydroxynonenal alkyne, which is produced during lipid peroxidation, was a key metabolite increased in PTB infants. This result supports the role of oxidative stress in prematurity. Amniotic fluid sampling is invasive, rarely obtained on a serial basis and not routinely available for all newborns. Amniotic fluid is, however, gradually replaced by fetal urine. By week 20 this process is complete, suggesting that results obtained with amniotic fluid could parallel those obtained with newborn urine.

Urinary biomarkers have recently been identified in newborn urine samples using ^{1}H-NMR which is nondestructive but less sensitive than MS (Fanos et al. 2014; Pintus et al. 2018). Quite encouragingly, a number of metabolites (alanine, betaine, trimethylamine N-oxide, lactate, and glycine) were found whose concentrations could predict BPD diagnosis. Furthermore, the authors link many of these metabolites to oxidative stress.

14.13 Conclusions

The overall hypothesis that oxidative stress plays a pathoetiological role in BPD and PTB remains worthy of future testing. Omic technology is particularly promising at identifying biomarkers relevant to complex multifactorial diseases with environmental components, e.g., BPD. In the near future, performing SNP genotyping and genome-wide DNA methylation assays (for both maternal and fetal/newborn DNA) on a very large case-controlled cohort should be a major goal. Both these assays are now so inexpensive that there is a good rationale for eventually integrating the resulting information into the healthcare management of all pregnant women at risk for PTB and/or for having infants with BPD. Moreover, SNP genotyping is a logical first step in the path toward individualized medicine in general, and this assay could

be cost-effective in the long run. Proteomic and metabolomic studies on serially obtained plasma, BAL, and urine samples also need to be performed on much larger, multi-site populations. Despite being a systemic biofluid, urine appears to be quite optimal for BPD biomarker discovery since it can be obtained serially, conveniently, and noninvasively.

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Oxidative Stress in Environmental Lung Diseases

15

Hsiao-Chi Chuang

Abstract

The lung is the first target organ to interact with harmful particles, mists, vapors, or gases. Inhalation of the environmental pollutants is able to induce environmental lung diseases. Reactive oxygen species (ROS) are usually produced from pollutant itself (from the physicochemical characteristic) or disrupt the oxidative stress in normal biological system. The overload ROS is able to attack DNA and the surrounding protein, leading to DNA damage and protein adduct. Particulate matter (PM)-containing ROS interacts with sulfur-containing methionine (Met) to produce oxidized forms of methionine sulfoxide (MetO). An overload of ROS causes the MetO reductase (MSR) repair system to malfunction, leading to MetO accumulation in cells. Apoptosis pathways are activated due to self-defense mechanisms when oxidized proteins accumulate. Disruption of protein repairing system caused by air pollution may be a potential role of the pathogenesis of environmental lung disease. In this review of the literature on environmental lung diseases, the focus is on environmental oxidants and protein oxidation.

Keywords

Air pollution · Lung disease · Oxidative stress

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15.1 Introduction: Lungs and the Environment

15.1.1 Lungs Are the First Target Organ to Interact with Environmental Stressors

The lungs are the main organ which interacts with numerous inhaled and ingested xenobiotics on a daily basis. Physiologically, the major function of the lungs is to maintain gaseous exchange, whereby oxygen (O_2) from the atmosphere is transported to respiratory tissues, and carbon dioxide (CO_2) made by tissues is expelled to the atmosphere. On the other hand, the lungs also play an essential role in preventing damage/injury caused by the inhaled xenobiotics. Generally, a healthy adult human breathes more than 7000 L of air each day (Rogers 1999). Gas exchange in the lungs requires exposure of a surface area of approximately 80 m^2 during inhalation. There are three lobes, i.e., the superior, middle, and inferior lobes, on the right and two lobes, i.e., the superior and inferior lobes, on the left lung (Gilroy et al. 2008).

The respiratory tract is a complex system, consisting of the nasopharynx, tracheal and bronchial tracts, respiratory bronchioles, alveolar ducts, and alveoli (Rogers 1999; Gilroy et al. 2008). Airway patterns in the human lungs are regulated by a dichotomy of branching from the trachea (generation 0) to the alveolar ducts and sacs (generations 20–23) (Hasleton 1996). In terms of functionality, the respiratory system is divided into two regions, the conducting (generations 1–16) and respiratory zones (generations 17–23). The conducting zone includes all anatomical structures without alveoli that the air passes through to arrive at the respiratory zone. The respiratory zone is the region within the lung that participates in gas exchange, via the alveoli. The main function of alveoli is gaseous exchange, which is provided by a network of capillaries. The dominant cell types present within the alveolar units include type I and II pneumocytes, endothelial cells, fibroblasts, and alveolar macrophages. Due the complex functions and structure of the lungs, it is important to understand the effects of environmental stressors on the lung environment.

15.1.2 Environmental Lung Diseases

Increasing numbers of reports have indicated that environmental stressors such as air pollution are risk factors for the development of respiratory diseases. Environmental stressors include particles, mists, vapors, or gases that cause harmful effects after inhalation, leading to environmental lung diseases. Environmental lung diseases are associated with exposure to pollutants from occupational (so-called occupational lung diseases) and environmental sources. For occupational lung diseases, pneumoconiosis is used to describe a lung disease that occurs after inhaling dust during work. However, environmental lung diseases do not only occur due to occupational exposure to relatively high levels of pollutants. Notably, recent reports also observed significant adverse effects on the pulmonary system after chronic

exposure to low levels of air pollution (DeVries et al. 2016; Olmo et al. 2011). Therefore, the potential deleterious effects of ambient air pollution on human health raised by epidemiological and clinical evidence have led to heightened concerns.

A report from the World Health Organization (WHO) entitled "Reducing Risks, Promoting Healthy Life" emphasized that environmental factors play important roles in initiating lung diseases and stated that controlling air pollution and tobacco consumption would be among the most critical/essential interventions to promote human health. Therefore, it is important to understand the effects of occupational and environmental stressors on the development of pulmonary diseases. However, occupational and environmental lung diseases are difficult to distinguish from those of non-environmental origin. Generally, pulmonary diseases can occur due to environmental pollution and environmentally related diseases clinically present in an indistinguishable manner from that of diseases not caused by such pollution. Furthermore, the etiology of lung diseases can be multifactorial. Occupational and environmental stressors can interact with other factors such as smoking and genetic risks. Thus, a careful investigation of the exposure history is essential for understanding environmental lung diseases. Herein, we used chronic obstructive pulmonary disease (COPD) as an example that is commonly recognized as an environmental lung disease.

15.1.3 Environmental Lung Disease: Chronic Obstructive Pulmonary Disease (COPD)

COPD is the fourth leading cause of death worldwide (Vestbo et al. 2013), and it is estimated to become the third leading cause of death worldwide by 2020. COPD is characterized by progressive and irreversible airflow limitations (Barnes 2000). Pathological changes occur in the proximal and peripheral airways, lung parenchyma, and pulmonary vasculature (Vestbo et al. 2013). There are some risk factors that are recognized as being associated with the development of COPD. For example, approximately 10% of the population over 45 years of age is associated with COPD, and that rises to 50% among heavy smokers (Kirkham and Barnes 2013). Notably, environmental stressors such as air pollution have been linked to the development of COPD. Several air pollutants such as particulate matter (PM) of <10 μm in aerodynamic diameter (PM_{10}) at levels typically found in urban areas are considered to have adverse health effects, including exacerbation of preexisting respiratory conditions such as COPD (Kumar et al. 2013). Chronic exposure to PM_{10} is associated with increases in the prevalence and incidence of COPD (Schikowski et al. 2014). Our previous study further indicated that exposure to PM_{10} caused impairment of the diffusion capacity of the lung, oxygen desaturation, and oxidative-inflammatory responses in patients with severe COPD (GOLD stages III and IV) (Lee et al. 2016).

Pulmonary inflammation caused by repeated injury and repair from inhaled particles is usually observed in COPD. Resultant inflammatory cell types are commonly observed in the airways, lung parenchyma, and pulmonary vasculature. Abnormal inflammatory responses in the lung to noxious particles and gases play an

important role in the related pathogenesis, in which neutrophils, alveolar macrophages, and T lymphocytes, predominately $CD8^+$ T cells, are recruited by macrophages and epithelial cells (Barnes 2000). Lung inflammation leads to a second wave of systemic inflammatory responses in the lungs.

15.2 Sources of Environmental Oxidative Stress

15.2.1 Particulate Matter (PM)

Airborne PM usually presents in solid or liquid phases with variable and composite sizes and multiple chemical compositions in the atmosphere. Because of the heterogeneous nature of PM, it is commonly measured and regulated based on its mass within defined size ranges. The aerodynamic diameter is defined as the diameter of a unit density sphere with the same aerodynamic properties, and this is usually used to describe the size of a particle (Brook et al. 2004; Brook 2008). To understand the effects of PM on the respiratory tract, PM size is categorized according its ability to penetrate into the human respiratory system when inhaled. First, PM_{10} is recognized as thoracic particles that are able to readily penetrate and deposit in the tracheobronchial tree (BeruBe et al. 2007). Second, $PM_{2.5}$ (<2.5 μm in aerodynamic diameter, i.e., fine particles) can bypass the upper airways and deposit in the lower and distal lung environments (Bai et al. 2001; Monn et al. 2003). Third, ultrafine particles (UFPs) (<50 nm in aerodynamic diameter) tend to be short-lived in the ambient atmosphere but are able to reach the alveolar region and interact with alveolar cells. However, UFPs agglomerate and coalesce into larger particles with variable morphologies (e.g., spheres and aggregates) (BéruBé et al. 1999), leading to an increase in particle size. As a particle decreases in size, the surface area (SA) per unit mass increases, and additional compounds (e.g., metals and organics) may also be absorbed by the same mass (Oberdörster et al. 2005). Different types of particles have characteristic shapes and sizes (Shi et al. 2003); under ambient conditions, fine particle fractions containing individual particles (singlets) have a propensity to form chains that grow into small clusters (aggregates) and eventual agglomerates (large clusters) (BéruBé et al. 1999). Particulate structures also offer a platform or nucleation site for chemicals (e.g., polycyclic aromatic hydrocarbons, PAHs) (Murr 2008; Sander et al. 2011; Lee et al. 2014). Therefore, surfaces of PM provide a platform to interact with chemicals, leading to increases in the oxidative potential after inhalation.

Sulfate and nitrate, together, comprise the main inorganic fraction of PM and are generally present as ammonium sulfate ($(NH_4)_2SO_4$) or neutralized salts, such as ammonium bisulfate and letovicite ($H(NH_4)_3(SO_4)_2$), along with ammonium nitrate (NH_4NO_3) (Schlesinger 2007; Reiss et al. 2007). Most sulfate is derived as a secondary source from the oxidation of SO_2. The gas-phase SO_2 is converted into PM-phase sulfuric acid or the aqueous-phase SO_2 reacts with hydrogen peroxide (H_2O_2), O_3, or oxygen (catalyzed by transition metals) into PM-phase sulfuric acid (Schlesinger 2007). Similar to SO_2, NO_2 is converted into nitric acid vapor by

reacting with hydroxyl radicals via sunlight (UV), whereas it is oxidized by O_3 to nitric acid at night (Reiss et al. 2007). In addition, nitric acid vapor reacts with alkaline PM, resulting in secondary nitrate-containing PM. Some sulfate- and nitrate-containing PM (e.g., sulfate acid or ammonium bisulfate) are strongly acidic, as observed in acid fog droplets. Inorganic fractions of PM may increase its oxidative potential during transformation processes.

In addition to sulfate and nitrate, PM also contains other inorganic and carbon-based compounds. Crustal-associated PM is predominantly derived from soil dust and wind-blown PM from rocks and road traffic. Some crustal-associated PM can be aerosolized from combustion processes, including aluminum (Al), calcium (Ca), magnesium (Mg), potassium (K), and silicon (Si). This type of particle composition is normally observed as an oxide containing carbonate and sulfate in the coarse size mode (Schlesinger 2007), whereas Al and Si are mainly observed in fine size fractions. Some metals and inorganic compounds were identified as highly reactive species that are able to increase oxidative stress after inhalation (Chuang et al. 2011; Chuang et al. 2013a).

15.2.2 Gaseous Pollutants

Carbon monoxide (CO) is a colorless, odorless, and poisonous gas, which is usually produced during the incomplete combustion of organic substances such as hydrocarbons. The solubility of CO in water at 1 atm and 25 °C is 2.14 ml/100 ml, thus permitting vigorous reactions with oxygen, acetylene, chlorine, fluorine, and nitrous in the air. Natural CO emissions account for one-third of the CO in the atmosphere, whereas the remaining portion (i.e., two-thirds) is mainly attributed to anthropogenic sources. The indoor environment is the main location for human exposure to CO, due to limited ventilation.

Seven oxides of nitrogen have been identified in the atmosphere (WHO 2010). Nitrogen oxides (NOx), for example, often refer to nitrogen monoxide (NO) and NO_2 which are highly reactive gases. NOx are generated by a reaction of oxygen and nitrogen at high temperatures during combustion processes (WHO 2010). Generally, NO is rapidly oxidized to form NO_2 by other oxidants (e.g., oxygen, O_3, and volatile organic compounds (VOCs)) in ambient conditions. NO_2 is a strong oxidant that can react with water to form nitrous acid (HNO_2) and is soluble in sulfuric and nitric acids. Traffic emissions are the principal outdoor source of NOx, whereas combustion-related activities are the most important indoor sources. Such highly oxidant NOx have been linked to numerous adverse effects to human health (Leikauf et al. 1995; Liu et al. 2018). Indoor exposure poses higher risks for human respiratory health compared to outdoor exposures, due to limited ventilation rates (WHO 2010).

Sulfur dioxide (SO_2) is a highly reactive gas, which is produced from combustion involving sulfur. SO_2 readily reacts with water to form sulfuric acid (H_2SO_4) which produces acid rain in the atmosphere. SO_2 is also converted to sulfate in ambient air – an important inorganic component of PM. The main emission source of SO_2 in

the atmosphere in the USA is from coal-fired power plants, which accounts for 73% of SO_2, while the remaining portion (27%) is released from industrial sources. It is also a common indoor pollutant during combustion processes (Jetter et al. 2002; Kulshreshtha et al. 2008).

15.2.3 Organic Pollutants

Organic carbon (OC) compounds are the principal species emitted following the combustion of biomass, produced either as primary emissions or through chemical reactions with gaseous OC species (Kim et al. 1999). Studies of the composition of PM from organic matter combustion showed that a significant fraction of total particulate emissions could be attributed to organic constituents (Wang et al. 2006, 2007). VOCs are released from certain solid and liquid phases and during specific human activities, especially combustion. Indoor environments are principal areas where human exposure to VOCs may be 2–5 times higher than outdoor levels. This increased level of exposure has been attributed to combustion-related activities (e.g., cooking and heating) (Liao et al. 2006; Lung et al. 2007).

Polar organic compounds, including acids, alkanols, aldehydes, and sugar derivatives, have been identified in fine-sized PM from various combustion sources (Oliveira et al. 2007; Kourtchev et al. 2008). Polar organic compounds are also produced by the formation of secondary organic aerosols, as a function of photochemical reactions with O_3, and hydroxyl and nitrate radicals, as well as via volatile hydrocarbons from anthropogenic and biogenic sources (Cheng et al. 2004). Carbonyls are a functional group composed of a carbon atom double-bonded with an oxygen atom. This ambient chemical is often formed in the troposphere because carbonyls are stable intermediates of photochemical reactions of gas-phase hydrocarbons and free radicals (Lü et al. 2010; Possanzini et al. 1996).

Quinones are often emitted from combustion-derived aromatic compounds (e.g., benzene and naphthalene) following gasoline, diesel, and wood combustion (Allen et al. 1997; Cho et al. 2004). In addition, photochemical oxidation of PAHs was shown to produce atmospheric quinones (Barbas et al. 1996). Quinones are predominantly present in the particle phase (Allen et al. 1997). An association between the molecular weights (MWs) of quinones and PM size fractions was reported (Allen et al. 1997). For example, coarse PM normally consists of 168–208-MW quinones, and those with MWs of >248 are often found in fine-sized PM.

15.3 Oxidative Stress

15.3.1 Reactive Oxygen Species (ROS)

Lung defense involves a wide array of mechanisms, from the nostrils to the alveoli, which remove inhaled materials without undue inflammation in order to maintain normal gas exchange in the pulmonary regions. When PM is inhaled into the lungs,

alveolar epithelial cells actively participate in host defense. Cells protect underlying tissues from desiccation, toxic challenge, and physical trauma caused by environmental stressors. Also, alveolar macrophages perform multiple functions by releasing numerous mediators for host defenses in alveolar spaces (Nicod 1999). These cells produce eicosanoids, cytokines, and growth factors that form a complex network regulating oxidative and inflammatory responses and initiate numerous cell signaling pathways (Levine 1995; Eaton et al. 2009). It was noted that a number of air pollutants are directly linked to lung inflammation, specifically the particle burden and ROS (BéruBé et al. 2009; Chen and Lippmann 2009; Lai et al. 2016).

The generic term, ROS, indicates a metabolic reaction involving the partial reduction of O_2, such as $O_2^{\cdot-}$ (the superoxide anion, an oxygen-centered radical) and OH. (hydroxyl, a highly reactive oxygen-centered radical). Both free radicals are reactive molecular species with an unpaired electron in their outer orbit (Novo and Parola 2008; Halliwell and Gutteridge 1999). Due to the instability of this unpaired electron, free radicals are paramagnetic and reactive chemicals which can interact with surrounding molecules by redox reactions to become more stable non-radicals/water (Eqs. 15.1, 15.2, 15.3, 15.4 and 15.5) (Novo and Parola 2008):

$$O_2 + e^- + H^+ \rightarrow \mathbf{HO_2^{\cdot}}\left(\text{hydroperoxyl radical}\right) \tag{15.1}$$

$$HO_2^{\cdot} \rightarrow H^+ + \mathbf{O_2^{\cdot-}}\left(\text{superoxide radical}\right) \tag{15.2}$$

$$O_2^{\cdot-} + 2H^+ + e^- \rightarrow \mathbf{H_2O_2}\left(\text{hydrogen peroxide}\right) \tag{15.3}$$

$$H_2O_2 + e^- \rightarrow OH^- + \mathbf{OH^{\cdot}}\left(\text{hydroxyl radical}\right) \tag{15.4}$$

$$OH^{\cdot} + e^- + H^+ \rightarrow H_2O \tag{15.5}$$

Hydroperoxyl radicals ($HO_2^{\cdot}$) dissociate to provide superoxide anion radicals at physiological pH 7.4 (Eq. 15.2) (Flora 2009). Superoxide anions are then rapidly generated by other ROS either directly (redox reaction) (Eq. 15.3) or through enzyme- or metal-catalyzed mechanisms (e.g., NADPH oxidase) (Aam and Fonnum 2007). The latter, for example, can convert superoxide anions into H_2O_2 by isoforms of superoxide dismutase (SOD) (Eq. 15.6) (Flora 2009):

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{SOD}} O_2 + \mathbf{H_2O_2} \tag{15.6}$$

H_2O_2, which can easily diffuse across cell membranes, interacts with superoxide anions to generate a highly reactive OH· via the Haber-Weiss reaction (Eq. 15.7) (Flora 2009). Notably, the presence of transition metals, such as iron (Fe), can convert H_2O_2 to OH· by the Fenton reaction (Eq. 15.8) (Squadrito et al. 2001; Vidrio et al. 2008):

$$H_2O_2 + O_2^{\cdot-} \rightarrow O_2 + OH^+ + \mathbf{OH^{\cdot}}\left(\text{Haber} - \text{Weiss reaction}\right) \tag{15.7}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + \mathbf{OH^{\bullet}}\left(\text{Fenton reaction}\right) \tag{15.8}$$

The product of H_2O_2 reactions is $OH^{\cdot}$ which has high reactivity and a very short half-life. Therefore, OH rapidly reacts with any surrounding molecules (Novo and Parola 2008). Also, the interaction of $OH^{\cdot}$, which possesses an organic carbon-hydrogen bond, generates free radicals, including organic and peroxyl radicals, organic peroxide, and the alkoxyl radical (Novo and Parola 2008).

15.3.2 Oxidative Imbalance

Many human respiratory diseases have their origins as a result of oxidative imbalances (Lawless et al. 2010; Lawless et al. 2009; Loukides et al. 2011). ROS are continually produced from various intracellular sources under normal biological conditions, especially mitochondria. Mitochondria leak electrons out to generate $O_2^{\cdot -}$ followed by conversion by mitochondrial SOD to H_2O_2 (Li et al. 2003). NADPH oxidase and 5-lipoxygenase are able to produce ROS in normal biological environments (Risom et al. 2005). Antioxidant like SOD can protect against such ROS. Antioxidants are classified into two groups, enzymatic and nonenzymatic, and they are diffuse free radicals which, through their ability to inhibit/delay oxidative processes, reduce damage at cellular/molecular levels caused by oxidative stress (Flora 2009).

Environmental stressors such as air pollution are extracellular sources that can directly or indirectly provoke oxidative stress in the lung environment. Air pollutants are physicochemically characterized as free radicals which have the ability to drive free radical reactions. Oxidative stress may occur due to the physicochemistry of PM, as described below (Risom et al. 2005; Tian et al. 2009; Chuang et al. 2012). First, ROS are directly generated from the surfaces of particles or particles themselves. Soluble organic and inorganic compounds are able to produce extracellular and intracellular ROS. Inhaled PM disrupts mitochondria or NADPH oxidase functions. Finally, ROS and reactive nitrogen species (RNS) are generated by inflammatory cells after inhalation of PM. In normal biological systems, extracellularly induced ROS can be mitigated or delayed by antioxidants. However, a biological defense mechanism is initiated in the form of an inflammatory response when the antioxidant network cannot cope with excess ROS, and the physiological effects of an ROS imbalance cause oxidative stress. This defense response of oxidative stress entails an influx of inflammatory cells such as macrophages and neutrophils into the lungs. ROS act as intracellular second messengers as inflammatory stimuli which induce micro-oxidative imbalances essential for cellular activation (Park et al. 2009). Infiltration of inflammatory cells into the lungs can lead to a second wave of oxidative stress due to activation of inflammatory cells which generate large quantities of free radicals (Kelly 2003).

15.4 Mechanisms of Damage to Cellular Targets by Oxidative Stress

Oxidative stress is recognized as a major predisposing factor in the pathogenesis of chronic lung diseases. In terms of chronic lung diseases, the antioxidant capacity is substantially decreased which results from endogenous ROS that persist after cessation of air pollution or the occurrence of exacerbation. Oxidative stress results in inflammation and the phenotype of a rapidly ageing lung in chronic lung diseases with increased risk of lung injury. Environmental stressors such as air pollutants are either free radicals or promote the synthesis of free radicals, which attack tissues and cause cell injury (e.g., mitochondrial and DNA damage), cell death (e.g., necrosis and apoptosis), and protein damage (Lai et al. 2016; Yin et al. 2004).

15.4.1 DNA Damage

Nuclear DNA is protected by histones and nuclear membranes. However, the presence of several nucleophilic sites on a molecule shows that DNA is susceptible to attack from free radicals at double-bonds, such as oxygen and nitrogen atoms (Yu and Anderson 1997). Previous studies indicated that PM-containing ROS are able to cause bulky DNA damage, including single- and double-strand DNA breaks (Risom et al. 2005; Danielsen et al. 2009; Chuang et al. 2013b). In particular, single-strand breaks usually occur due to ROS resulting in a sequence of radical reactions within the DNA backbone and subsequent breakage of the molecule (Bertram and Hass 2008). Single-strand breaks also cause severe obstacles to appropriate transcription and replication (Bertram and Hass 2008). Furthermore, a sequence of reactions that triggers intra- and extracellular oxidative stress leading to accumulation of ROS (i.e., an ROS imbalance) in macromolecules (such as DNA) may eventually initiate carcinogenesis (Vallyathan et al. 1998). The development of cancer is regulated by DNA and includes genetic alterations involving oncogenes, aberrant controls in the cell cycle, impaired DNA repair processes, and cell death (Schins and Knaapen 2007; Sedelnikova et al. 2010).

15.4.2 Protein Damage

ROS are reactive molecular species with an unpaired electron in their outer orbit (Novo and Parola 2008), so they can easily extract a second electron from a neighboring molecule leading to injury of surrounding tissues and result in the formation of highly reactive organic molecules. These reactions are able to nonenzymatically modify proteins, and they target specific peptide residues (Kirkham and Barnes 2013). Oxidative damage to proteins is a critical mechanism in the regulation of aging and multiple diseases (Berlett and Stadtman 1997). Because of the important roles of proteins in cellular homeostasis, protein oxidation is able to alter cell structure regulation, cell signaling, and various cellular enzymatic processes.

ROS are able to damage surrounding cells and tissues, which leads to the formation of highly reactive organic molecules. These organic molecules can nonenzymatically modify proteins and interact with specific peptide residues such as lysines, arginines, cysteines, and histidines (Kirkham and Barnes 2013). Such interactions cause the formation of reactive carbonyls and protein carbonylation. The accumulation of reactive carbonyls and the subsequent protein carbonylation have been linked to chronic diseases and ageing (Kirkham and Barnes 2013). Carbonyl stress is able to regulate/modify protein functions and leads to disruption of normal cell functions and physiological mechanisms (Berlett and Stadtman 1997). When inhaled PM interacts with proteins, it is associated with some degree of protein unfolding. Unfolding affects the biological functions of the protein (Roach et al. 2005). The surface chemistry of PM is known to greatly influence the amount of protein binding to distinct ligands (Chen et al. 2014), which may be associated with alterations in oxidative stress and inflammation (Gasser et al. 2012). Furthermore, ROS are able to modify protein structures after interaction (Chiang et al. 2013). ROS on PM surfaces can interact with nearby protein molecules to produce stable protein-particle conjugates (Chiang et al. 2013). Transitions of entropy can lead to reversible or irreversible states of proteins (Basharov 2012).

15.5 Methionine Oxidation and Repair

Sulfur-containing methionine and cysteine residues are particularly sensitive to interacting with more than 70% of ROS (Johannes and Majcherczyk 2000; Barreiro et al. 2013), which convert them to disulfides and methionine sulfoxide residues (Berlett and Stadtman 1997). For example, ROS are most reactive in attacking thiol-containing side chains of cysteine. Peptides containing a single cysteine residue can form dimers by oxidation of cysteine side chain thiols, linking two chains together by a disulfide bridge. Peptides containing two or more cysteines can form intramolecular disulfide bridges yielding cyclic peptides. Notably, methionine plays an important role in response to PM exposure in the lungs (Lee et al. 2014; Pardo et al. 2015). When methionine is oxidized by ROS, the oxidized form, methionine sulfoxide (MetO; oxidized methionine), is produced with two enantiomers: *S*-MetO and *R*-MetO.

MetO reductase A (MSRA) and MSRB, MetO peptide enzymes, play critical roles in repairing proteins damaged by MetO. They are able to catalytically reverse *S*-MetO and *R*-MetO, respectively, allowing maintenance of the protein's function in some cases (Gilmour et al. 2003). For example, ROS-induced methionine oxidation can be reversed by MSRA which reduces *S*-MetO and MSRB which reduces *R*-MetO (Ghesquiere et al. 2011). The repair system attempts to maintain protein functions and reduce the accumulation of damaged proteins in cells (Feng et al. 2015). Notably, environmental stressors such as PM are able to disrupt the repair function of MSRA and MSRB3 in biological systems as shown in Fig. 15.1. The protein repair mechanism is activated by ROS produced from PM, which converts oxidized methionine to methionine by MSRA and MSRB3. However, an overload

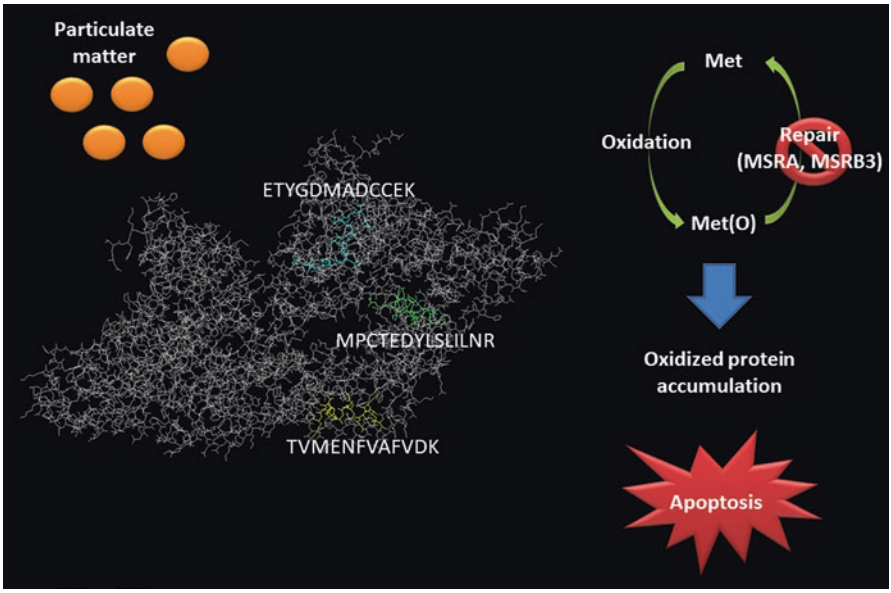

Fig. 15.1 Particulate matter (PM)-containing reactive oxygen species (ROS) interact with sulfur-containing methionine (Met) to produce oxidized forms of methionine sulfoxide (MetO). An overload of ROS causes the MetO reductase (MSR) repair system to malfunction, leading to MetO accumulation in cells. Apoptosis pathways are activated due to self-defense mechanisms when oxidized proteins accumulate

of ROS from a PM source disrupts the MSR repair system, leading to cellular MSRA and MSRB3 deficiencies (Lai et al. 2016; Feng et al. 2015). Such reductions in MSRA and MSRB3 levels in cells may cause oxidized proteins to accumulate in cells. The overload of oxidized proteins is able to activate apoptosis pathways, leading to cell death.

15.6 Conclusions

Environmental lung diseases are caused by harmful particles, mists, vapors, and gases that are inhaled into the lungs. Oxidative stress can produce cell injury by multiple pathways. ROS production directly and indirectly damages tissues and cells. Failure to repair proteins because of oxidative stress causes the accumulation of reactive molecules and MetO, and it is predominantly associated with chronic diseases and ageing. For example, a major etiologic factor driving environmental lung diseases such as COPD is likely oxidized proteins in the lungs following long-term exposure to environmental stressors such as air pollution. The imbalance of oxidant-antioxidant systems and the resultant protein oxidation are hallmarks of the development of environmental lung diseases.

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Bronchopulmonary Dysplasia and Oxidative Stress in the Newborn

16

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Abstract

Bronchopulmonary dysplasia (BPD) is a major cause of respiratory morbidity in preterm infants. The "new BPD" is the form currently observed in very preterm infants, related to disrupted and impaired lung development, and characterized by decreased alveolarization and impaired capillary development. Prematurity, oxygen toxicity, inflammation, mechanical ventilation, and surfactant deficiency are major determinants for BPD pathogenesis. Oxidative stress (OS) and inflammation are strictly interrelated originating a vicious circle that self-amplifies and ultimately leads to the damage of the immature lung. This chapter focuses on the role of OS on the lung injury and analyzes the most recent biomarkers in clinical studies. The comprehension of molecular basis of BPD is crucial for expanding the current possibilities for prevention and treatment and discovering new strategies for this condition.

Keywords

Bronchopulmonary dysplasia · Oxidative stress · Inflammation · Lung

16.1 Introduction

Bronchopulmonary dysplasia (BPD) is the major cause of chronic lung disease and pulmonary morbidity in preterm infants. In 1967, Northway first defined bronchopulmonary dysplasia as the presence of persistent respiratory signs and symptoms, the need for supplemental oxygen to treat hypoxemia associated with an abnormal chest radiograph at 36 weeks postmenstrual age (Northway et al. 1967; Kinsella et al. 2006).

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During the last decades, the advances in perinatal care, the prophylactic use of antenatal steroids, and surfactant replacement therapy for respiratory distress syndrome (RDS) improved the rate of mortality due to this condition but not the overall incidence of BPD and modified its pathophysiology and its clinical presentation.

The "old BPD" or "classic BPD" was considered a lung injury occurring in modestly preterm babies, born between 29 and 32 weeks of postmenstrual age, and related to surfactant deficiency and the need for oxygen supplementation and invasive ventilation; classic BPD was mainly characterized by inflammation, fibrosis, and scarring. In contrast, the "new BPD" currently occurs in younger preterm babies below 29 weeks PMA and can be considered a consequence of disrupted and impaired lung development; new BPD is mainly characterized by decreased alveolarization and impaired capillary development (Perrone et al. 2018; McEvoy and Aschner 2015).

Consequently, the definition of BPD has been very challenging over the years, and despite extensive studies, it remains extremely heterogeneous up to now. In 2000, a workshop organized by the National Institute of Child Health and Human Development (NICHD), the Nation Heart, Lung and Blood Institute (NHLBI), and the Office of Rare Disease (ORD) reviewed the definition of BPD and detailed diagnostic criteria for this condition based on gestational age and severity (Jobe and Bancalari 2001). In particular, it scored the severity of the disorder according to the gestational age less or more than 32 weeks PMA and to the level of respiratory support needed at 28–56 days of life and near term at 36 weeks PMA (Table 16.1).

During the last decade, despite the efforts for a consensus definition, published reports are still highly variable in terms of BPD definition with marked difference in identifying the risk of poor lung and neurodevelopmental outcome (Hines et al. 2017) and wide a variation in reporting incidence of BPD. In addition, the current definitions of the disease do not take into account the recent changes in respiratory management (such as the use of high flow nasal cannula), leading to an

Table 16.1 Definition of BPD, modified from (Jobe and Bancalari 2001)

Gestational age	< 32 weeks	≥ 32 weeks
Time point of assessment	36 weeks PMA or discharge to home (whichever comes first)	28 days but <56 days postnatal age or discharge to home (whichever comes first)
Definition	Treatment with oxygen >21% for at least 28 days +	
Mild BPD	Breathing room air at 36 weeks PMA or discharge (whichever comes first)	Breathing room air by 56 days postnatal age or discharge (whichever comes first)
Moderate BPD	Need for <30% oxygen at 36 weeks PMA or discharge (whichever comes first)	Need for <30% oxygen by 56 days postnatal age or discharge (whichever comes first)
Severe BPD	Need for ≥30% oxygen and/or positive pressure (PPV or NCPAP) at 36 weeks PMA or discharge (whichever comes first)	Need for ≥30% oxygen and/or positive pressure (PPV or NCPAP) by 56 days postnatal age or discharge (whichever comes first)

PMA: postmenstrual age

underestimation and a misclassification of the pathology (Kalikkot Thekkeveedu et al. 2017).

16.2 Epidemiology

It is difficult to establish accurately the incidence of BPD that change according to the clinical practice, to the definition used and to the ethnicity. Overall BPD affects almost 10,000–15,000 infants annually in the United States (Jensen and Wright 2018)**.**

Furthermore, the risk of BPD is inversely related to gestational age and to the birth weight. According to the data from the Vermont Oxford Network, the rates of BPD vary from 12% to 32% among infants born less than 32 weeks gestation. Infants with birth weight less than 1000 g are the most susceptible to develop this condition: previous studies demonstrated that 75% of affected babies weighed less than 1000 gr at birth, but only 5% weighed over 1500 gr at birth (Jensen and Schmidt 2014).

16.3 Pathogenesis

The pathogenesis of BPD is complex and multifactorial. Lung injury in the neonatal period has multiple etiologic factors (genetic, metabolic, hemodynamic, nutritional, mechanical, and infectious) that act in a synergic way (Perrone et al. 2012). Prematurity, oxygen toxicity, inflammation, mechanical ventilation, and surfactant deficiency are all major determinants for the disease (Perrone et al. 2018). Recent hypothesis postulated a two-hit model that could lead to the appearance of BPD: the interaction between inadequate lung development due to several antenatal factors (genetic susceptibility, intrauterine growth restriction, maternal infections, or cigarette smoking) and postnatal damage (related to infections and sepsis, mechanical ventilation, and oxidative stress due to the exposure to a huge amount of free radicals, FRs). According to this model, the severity of the clinical picture depends on the efficacy of repair and antioxidant mechanisms: the airway remodeling is linked to a suboptimal repair and to the subsequent development of chronic lung disease (Kalikkot Thekkeveedu et al. 2017). Inflammation and oxidative stress are strictly interrelated in the pathogenesis of BPD, and they originate a vicious circle that self-amplifies and leads to the damage of the immature lung (Fig. 16.1).

16.3.1 Oxidative Stress in the Perinatal Period: A Role for BPD

Free radical reactions are a normal occurrence in living organisms, and free radicals (FRs) are deeply involved in signaling molecules to regulate a wide variety of physiological events (Perrone et al. 2018). The production of superoxide anion is the first stage of a physiological mechanism of host defense, followed by the production of

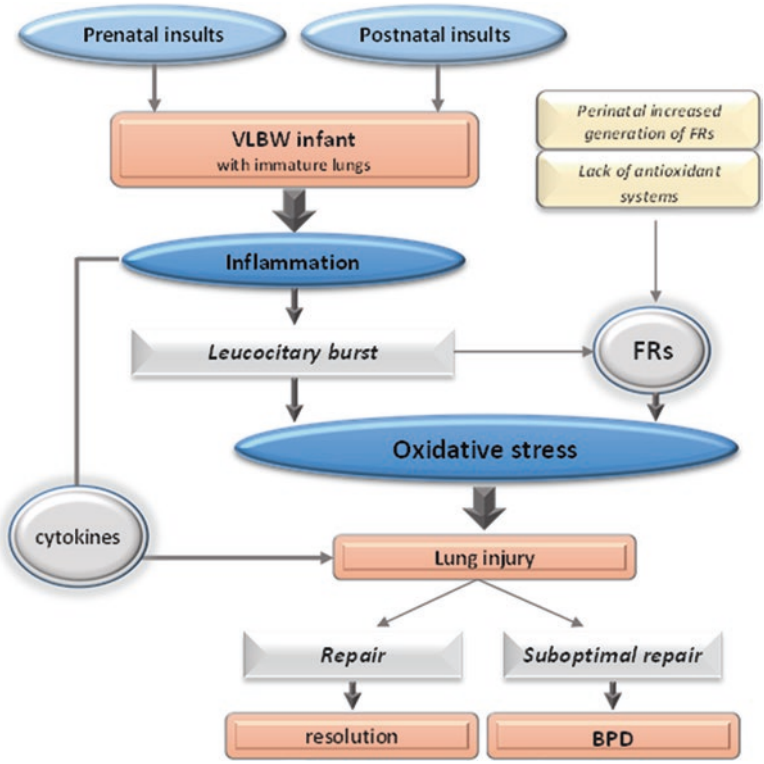

Fig. 16.1 Physiopathological mechanisms of BPD: role of inflammation and oxidative stress VLBW, very-low-birth-weight; FRs, free radicals

other reactive species, such as hydrogen peroxide (H_2O_2) by superoxide H_2O_2 dismutase (SOD), hydroxyl radicals (OH-) catalyzed by transition metals, and hypochlorous acid (HOCl-) by myeloperoxidase. These substances contribute normally to killing bacteria but, on the other side, favor tissue damage and increase capillary permeability, contributing to the passage of pro-inflammatory cytokines. Many of those increase the expression of inducible nitric oxide synthase (NOS), which forms nitric oxide (NO); NO combine with superoxide radicals to produce peroxynitrite that can form other potentially damaging metabolites such as hydroxyl radicals, nitrogen dioxide (NO_2), and nitrogen dioxide radical (NO_2+) (Perrone et al. 2012)**.** Other sources of increased FRs generation are hyperoxia, hypoxia, ischemia, blood transfusion increased levels of nonprotein-bound iron, xenobiotics, and drugs. The antioxidant enzymes like SOD, catalase, and glutathione synthetase (GSH) have usually the capacity to scavenge the levels of FRs produced in physiological conditions, but under ischemic conditions and especially in preterm infants they fail to protect tissues from oxidative damage because of the overproduction of oxygen radicals and consumptions of antioxidant defenses (Perrone et al. 2018). The imbalance between oxidant and antioxidants with predominance of one or the other has

been called oxidative stress (OS). Over the last decades, emerging data have suggested that OS is involved in the development of BPD and that the lung injury process leading to BPD occurs within hours to days from delivery with the oxidation representing a major contributor to the process (Perrone et al. 2012).

Since 1996, several studies on murine model detected an inhibition of fetal lung growth after exposure to high oxygen concentrations, partially reversible using low-molecular-weight SOD mimetic; these results suggested that superoxide anion and possibly hydroxyl radical are the oxygen-centered FRs most likely responsible for the growth effects of hyperoxia on mouse fetal lung morphogenesis (Wilborn et al. 1996)**.** More recently, Datta et al. showed that early (day 0) but not late (day 4) postnatal hyperoxia compromises the lung development decreasing the alveolar count and septal count, increasing distal artery muscularization, and inducing right ventricular hypertrophy. All these alterations were attenuated by the use of antioxidants during the early hyperoxia. In addition, the study group demonstrated that hyperoxia-induced NOX1 enzyme could amplify the FRs signaling contributing to the observed defects in lung and pulmonary vascular development (Datta et al. 2015)**.** NOX1-deficient mice presented hyperoxia-induced lung injury due to cell death of the alveolo-capillary barrier via c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways, two mitogen-activated protein kinases involved in cell death signaling (Carnesecchi et al. 2009).

Hyperoxia also induces the fibrotic process in newborn with BPD (Perrone et al. 2012)**.** Hyperoxia-induced FRs could interfere with surfactant production inducing an intracellular accumulation of surfactant proteins SP-A, SP-B, and SP-C_{85} (SPC) (Perrone et al. 2012; Zhang et al. 2015) and reducing surfactant phospholipid production (Perrone et al. 2012). FRs could also inhibit the trans-differentiation of alveolar epithelial cells II (AEC II) into alveolar epithelial cells I (AEC I) (Zhang et al. 2016). AEC II are the most important stem cells in lung tissue and play a crucial role in the regulation of lung tissue growth, maturation, wound-repair process, and lung fluid homeostasis; proliferation and differentiation of these cells are the determinant to regulate the post-injury repair of alveolar epithelial structures and functions. AEC II are physiologically converted to AEC I that secrete bioactive substances to restore alveolo-capillary barrier integrity and maintain alveolar function: hyperoxia-induced OS determines excessive apoptosis of AECII and inhibits their proliferation (Wang and Dong 2018)**.**

Pulmonary remodeling but also vascular remodeling induced by hyperoxia represents the histopathological basis of new BPD (Carnesecchi et al. 2009). Delaney et al. postulated that OS and the consumption of antioxidant enzymes as SOD could be responsible for the appearance of pulmonary hypertension disrupting the VEGF/VEGFR2/nitric oxide (NO) pathway.

VEGF (*vascular endothelial growth factor)* signaling is crucial for normal alveolar and vascular growth; previous studies have shown that VEGF and VEGFR2 (*vascular endothelial growth factor receptor type 2)* are decreased in infants who died with BPD, and in models of BPD and pulmonary hypertension, disruption of VEGF signaling contributes to pathogenesis. In the longer period, FRs could reduce

the levels of VEGFR2 indispensable for angiogenesis in the fetal and neonatal lung by phosphorylation (Delaney et al. 2015).

Other studies have found that increased FRs production in the pulmonary vasculature influences redox-sensitive signaling pathways in pulmonary vascular cells, leading to activation of redox-sensitive transcription factors and increasing the expression of many proteins involved in pulmonary vascular remodeling, inflammation, and altered bronchial reactivity (serine/threonine kinases MAPK, ROCK, Akt) (Freund-Michel et al. 2013).

Emerging theories indicate also that endoplasmic reticulum stress could be crucial for the cell survival. At the same time, FRs cause protein misfolding and activate the transcription of factors as GRP78 and CHOP that are involved in the apoptosis pathway (Lu et al. 2015).

16.3.2 Oxidative Stress and Inflammation

An important common denominator in the pathogenesis of BPD is inflammation. Both antenatal and postnatal causative factors activate several inflammatory cascades, determining on an immature immune system an impairment in cytokines' production and lung uptake of inflammatory cells. Cytokines mediate acute lung injury, exacerbate ventilator-associated lung injury, modulate host defense, and contribute to normal lung development. In a study conducted on over 1000 extremely low-birth-weight infants, those who developed BPD or died had elevated IL-8 accompanied by a relative predominance of T-helper2 cytokines (IL-10, IL-6) in comparison to T-helper1 cytokines (TNF-β) or T-cell products (RANTES), although some Th1 cytokines (IL-1 β, IFN-γ) were also elevated and associated with BPD or death. This pattern of cytokines reveals an increased early neutrophil influx and a relative decrease in effector T-cells and confirms the hypothesis that BPD may be associated with an impairment in the transition from the innate immune response mediated by neutrophils to the adaptive immune response mediated by T-lymphocytes (Ambalavanan et al. 2009). Higher levels of IL-1β, IL-8, and IL-6 in tracheal aspirates at birth are predictive for later BPD, suggesting that early exposure of the premature lung to inflammation is important to the development of BPD and that pro-inflammatory cytokines appear to mediate this process (Ryan et al. 2008). In an early stage of development, cytokines stimulate the infiltration of lung interstitial space by polymorphonucleated cells (PMN) and macrophages (MAC) and then lead to the overproduction of metalloproteinases (MMPs) that play a key role in disruptive processes with simplification of alveolar tree and activation of fibrotic processes. Furthermore, the persistence of lung inflammation and release of inflammatory mediators contribute to the overexpression of adhesion molecules by endothelial cells resulting in trans-endothelial cytokine migration (Perrone et al. 2018) and by smooth muscle cells with consequent appearance of pulmonary hypertension and bronchial over-reactivity (Ryan et al. 2008). The entire molecular pathway is not fully understood, but recent advances suggest that oxidative stress is the

final common endpoint for a complex convergence of events mediated by FRs generation (Wang and Dong 2018).

This process involves the activation of inflammatory cells ("leukocitary burst") triggered by antenatal or postnatal infections or by aspecific stimuli like lung stretching during mechanical ventilation, with subsequent release of a large amount of oxygen radicals and proteases (Perrone et al. 2012; 2018). Indeed, the inflammatory cascade is associated with the production of FRs, with neutrophil generating superoxide anions, hydrogen peroxide, and hydroxyl anions. The physiologic deficiency of antioxidant systems contributes to oxidative stress (Wang and Dong 2018). FRs can directly damage all biological molecules but especially proteins, lipids, and nucleic acid and cause cell death through a variety of patterns. They increase the amount of ceramides to induce apoptosis by lipid peroxidation, affect the activity of various enzymes by processes of protein oxidation and nytrosilation, can modify the DNA structure, and activate multiple signal transduction pathways in cells, promoting the transmission of cytokines, growth factors, and calcium signals. All of these mechanisms contribute to maintain the previously activated damaging processes (Saugstad 2003).

16.4 Biomarkers of Oxidative Stress in Clinical Study

Despite recent advances on the role of FRs in murine models of BPD, our knowledge about their action on fetal and newborn lungs is less strong. Newborns and especially preterm infants are particularly vulnerable to the oxidative damage because of their major production of FRs and their weak antioxidant systems with lower possibility to scavenge FRs in excess.

Physiologically, a complex relationship between oxidant and antioxidant systems exists, and the imbalance between these two components with predominance of the first one could activate several processes – epigenetic and enzymatic – that mediate a remodeling of the small airways and the lung vascular bed of the newborn.

However, a useful recognized biomarker (on bronchoalveolar lavage (BAL), plasma, or urine) that can correlate with the risk of developing BPD or that can predict the severity of lung injury and the long-term respiratory impairment is not yet know (Perrone et al. 2018). During the last years, several metabolites have been studied as indicators of OS in respiratory distress syndrome and chronic lung disease.

In 2015, Negi et al. detected higher levels of plasma *malondialdehyde (MDA)*, *protein carbonyl,* and *8-hydroxy-2-deoxyguanosine (8-OHdG)* in the umbilical cord blood of a population of low-birth-weight preterm babies who developed respiratory distress syndrome compared to those who did not develop the disease, with a significant difference between the groups. MDA is a marker of lipid peroxidation as final product of polyunsaturated fatty acids peroxidation; protein carbonyls are markers of early protein oxidation; 8-OHdG can be considered a marker of oxidative DNA damage. (Negi et al. 2014)**.** FRs could lead to an increased permeability

of vascular bed, with shift of proteins in the interstitial spaces and consequent storage of oxidized proteins and fat; at the same time the cellular damage may promote DNA modification and activation of cell death pathways. How the acute lung injury could be related to the chronic modifications that underpin the structural and functional changes in BPD is up to date ongoing on study.

Studies on short-term measurements demonstrated increased levels of OS products in infants with BPD. Joung et al. measured the urinary levels of *8-hydroxydeoxyguanosine (8-OHdG)* and *leukotriene E4* (*LTE4*, product of degradation of the phospholipid bilayer of cell membrane), oxidative and inflammatory stress markers, respectively, in a group of very-low-birth-weight (≤1250 g) infants ≤30 weeks GA. The authors reported that the urinary excretion of 8-OHdG and LTE4 in newborns with BPD was higher respect to the ones without disease. The first biomarker was associated to the clinical phenotype of the old BPD, while the second one was associated with the phenotype of the new or atypical BPD, characterized by a more marked bronchial reactivity. Collaterally, they showed that the level of urinary LTE4 on day 1 was greater than the normal adult value in both the no/mild BPD and moderate/severe BPD groups, supporting the hypothesis that prenatal inflammatory insults may play a role in the pathogenesis of the disease. Furthermore, the persistence of high excretion of 8-OHdG on the 3th day of life was related to the duration of mechanical ventilation (Joung et al. 2011)**.** Conversely, Moore et al. in 2016 demonstrated that the infants with lower urinary 8-OHdG levels on day 7 of life required higher levels of supplemental oxygen during all the study period. This difference could be partially attributable to the lower gestational age of newborns that developed BPD, but the authors supposed that it could be explained by the immaturity of the enzymes that recognize and remove the oxidized lesions (Moore et al. 2016)**.** The results of this study documented the extreme complexity of the role of oxidative stress in the pathogenesis of disease.

A recent study measured and compared *glutathione (GSH)* and *lipid peroxidation products (LOOH)* concentrations in the bronchoalveolar lavage fluid (BALF) of a group of very-low-birth-weight newborns with and without BPD. The first group showed significantly lower antioxidant defense (GSH levels) and higher levels of LOOH than the second one. Furthermore, LOOH were associated with respiratory support parameters such as the length of mechanical ventilation, supporting the role of volu/barotrauma in the activation of inflammatory cascade with consequent amplification of lung damage (Fabiano et al. 2016)**.**

In 2015, Kuligowski et al. measured the levels of lipid peroxidation metabolites isoprostanes (IsoPs), isofurans (IsoFs), neuroprostanes (NeuroPs), and neurofurans (NeuroFs) on serial urinary samples in a population of preterm infants ≤32 weeks gestational age and compared the levels between babies who later developed BPD and controls. IsoPs and IsoFs are reliable and stable compounds originating from oxidation of arachidonic acid, in condition of normoxia or hyperoxia, respectively; in a similar manner, NeuroPs and NeuroFs originate from lipid peroxidation of docosahexaenoic acid (DHA) in brain tissue. The authors detected that increased urinary elimination of isofurans in the early perinatal period (in the first 4 days after birth) was related to the development of BPD **(Kuligowski et al.** 2015**).**

Matthews et al. have shown that increased plasma concentrations of isoprostanes (F2-IsoPs) in the first month of life are associated with an increase in the need of respiratory support at term equivalent age of preterm infants. The authors also reported a worse neurodevelopmental outcome at 12 months corrected age in extremely preterm infants with high isoprostanes levels (Matthews et al. 2016).

Various pro-inflammatory cytokines like IL-1β, IL-6, IL-8, IL-10, IL-18, TNF-α, and IFN-γ have been shown to correlate with an increased risk of BPD in preterm babies (Ambalavanan et al. 2009; Ryan et al. 2008; D'Angio et al. 2016; Jónsson et al. 1997)**.** A longitudinal analysis studied the main pro-inflammatory cytokines (IL-6, IL-8, and *granulocyte-colony stimulating factor*, G-CSF) in different times point between birth and 42 days of age. In infants with BPD, inflammation occurred shortly after birth with gradual but not complete attenuation in the first neonatal period, and corticosteroids seemed to be able to modulate the process (Leroy et al. 2018)**.**

The link between interleukin pathway and OS biomarkers has been recently reported. IL-6 and 8-OHdG from serum and tracheal aspirate were higher in very-low-birth-weight (VLBW) infants with BPD than in babies without BPD on the first day after birth. The IL-6 and 8-OHdG levels in tracheal aspirate fluid were also persistently increased on postnatal day 28th in the BPD group, confirming the hypothesis that the persistence of the inflammation can be an important mechanism in the pathogenesis of BPD (Hsiao et al. 2017).

16.5 Antioxidant Drugs and BPD Prevention

Another important pathogenic factor of BPD is the peculiar deficiency of antioxidant systems in preterm newborns (Perrone et al. 2010)**.** Giusti et al. identified in the association between single-nucleotide polymorphisms (SNP) of genes encoding for superoxide dismutase SOD1, SOD2, SOD3, and CAT a protective or risk factor for developing prematurity complications, including BPD (Giusti et al. 2012). Moreover, an increased rate of BPD was demonstrated in infants with SNP of NAD(P)H: quinone oxidoreductase 1 (NQO1), a flavoprotein enzyme that catalyzes two electrons reduction of a variety of substrates including quinones (Sampath et al. 2015).

The beneficial effects of antioxidant therapies in the prevention of FRs-mediated lung injury are still controversial, because the consequences of prematurity are multifactorial and many prenatal and perinatal insults could influence their clinical course. Therefore, data from clinical trials are up to date insufficient.

The main data available in literature concern melatonin (MT), antioxidant enzymes supplementation (AOEs), and vitamins.

Melatonin is the main product of the pineal gland and has antioxidant, antiapoptotic, and anti-inflammatory properties; it inhibits the NOS and lipid peroxidation and favors the transcription of antioxidant enzymes. In particular, MT exerts a double antioxidant activity: it scavenges directly ROS and induces the expression on glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase

(Poggi and Dani 2014). Previous studies demonstrated its role in preventing brain injury during hypoxic-ischemic encephalopathy and the possibility to influence positively the clinical course in septic newborns reducing the concentrations of lipid peroxidation products (Gitto et al. 2001), but there are fewer studies considering its efficacy in preventing chronic lung disease of prematurity. Melatonin seems to be able to reduce IL-6, IL-8, and TNF-α concentrations and nitrite/nitrate levels in serum in the first hours of life and in the early postnatal period of neonates with respiratory distress (Gitto et al. 2004). Newborns mechanically ventilated with pressure support ventilation mode with guarantee volume and receiving melatonin presented a greater reduction of serum levels of inflammatory cytokines than did newborns ventilated in other modes or not receiving melatonin. Furthermore, MT seems to reduce the accumulation of leukocytes into the lung manifested by the reduction in myeloperoxidase activity, and this other effect contributes to limit the oxidative damage predisposing to BPD (Gitto et al. 2013). In recent animal studies, melatonin seems to have positive effects even upon the vascular side: postnatal administration of MT blunts the cardiopulmonary response to hypoxia, reduces the pathological vascular remodeling, and increases angiogenesis in pulmonary hypertensive neonatal lambs, improving the pulmonary vascular structure and function in the neonatal period under chronic hypoxia (Astorga et al. 2018).

In animal models the lack of SOD leads to impaired alveolar development, pulmonary hypertension, and disrupted pulmonary vascularization (Delaney et al. 2015), while transgenic expression of SOD preserves the AEC II proliferation and attenuate hyperoxic induction of vascular remodeling (Auten et al. 2006; Nozik-Grayck et al. 2008). Moreover, exogenous supplementation of SOD could increase in lung tissue the bioavailability of NO and downregulate the NF-KB pathway, exerting a protective role on lung development. Actually, there is not a clear evidence of efficacy of SOD supplementation for the newborns. Endotracheal administration of recombinant human SOD (rhSOD) has been proved to reduce lung injury in preterm newborns receiving mechanical ventilation for RDS (Poggi and Dani 2014): its recombinant form has a short half-life; consequently it seems to be able to reduce lung inflammation and the oxygen requirement in respiratory distress syndrome if administered in single doses. Therefore, repeated doses do not seem to modify the oxygen dependency and the duration of mechanical ventilation but to exert a positive influence on the frequency of respiratory problems after the discharge (Suresh et al. 2001).

A possible application vitamin A in preventing BPD was argued observing that vitamin A deficiency in animal model linked to changes in the respiratory tract epithelium including necrotizing tracheobronchiolitis and squamous metaplasia; all these modifications were reversible by restoring the vitamin storage (Wardle et al. 2001).

In animal models exposed to hyperoxia, vitamin A and its metabolites lowered lung injury (considered as epithelial injury, hemorrhage, and macrophages infiltrate), and retinoic acid supplementation prevented the hyperoxia-induced increase of IL-1β, IL-6, and TNF-α (James et al. 2010). In 2016, a Cochrane review of the studies in preterm infants unlighted the efficacy of this vitamin in reducing the need

of oxygen therapy at terms and 28 days after the discharge, without significantly modifying the neurodevelopmental outcome of the examined population at 18 and 22 months of corrected age (Darlow et al. 2016).

16.6 BPD Treatment

Considering its multifactorial pathogenesis, BPD requires a multidisciplinary approach for an effective treatment.

Despite the several therapeutic strategies proposed over the time (bronchodilators, diuretics, pulmonary vasodilators), the use of systemic corticosteroids is the only one related to a significant reduction of the incidence of BPD. However, this treatment prolonged on the time is associated with an impairment of brain maturation and worse developmental outcome (Iyengar and Davis 2015). In a meta analysis, Shah et collegues demonstrated that early and late administration of systemic corticosteroids reduce the risk of BPD, facilitate extubation, and reduce the incidence of patent ductus arteriosus but at the same time increase the risk of gastrointestinal bleeding, growth failure, and neurological impairment in preschooler age. (Shah et al. 2017a).

More debated is the usefulness of airway-administered corticosteroids, in particular budesonide.

According to the last Cochrane review about the topic, the use of nebulized budesonide does not modify significantly the risk of chronic lung disease and the oxygen dependency (Shah et al. 2017b).

The Neonatal European Study of Inhaled Steroids (NEUROSIS) Trial Group have recently reported a significant reduction of incidence of BPD in infants treated by inhaled budesonide respect the ones who received placebo (Bassler et al. 2015). The subsequent analysis on long-term follow-up did not show a significantly difference in neurodevelopmental disability between the groups of study, but the mortality rate was higher among preterm infants who received budesonide (Bassler et al. 2018).

The mechanism by which the steroid therapy reduces lung inflammation modulating the immune response has been extensively studied, while the relationship between the use of corticosteroids and the oxidative damage is less known. In a cohort of extremely premature newborns, Vento et al. detected an association between the antenatal steroids supplementation and reduction of postnatal OS conditions (Vento et al. 2009). In a more recent study, Sandal et al. detected a significantly reduction in total oxidant status in infants with BPD after the treatment with hydrocortisone given orally. The authors measured the total oxidant status (TOS) and total antioxidant capacity (TAC) levels of a group of preterm infants before and 1 week after the hydrocortisone therapy and calculated oxidative stress index (OSI) levels. Following the treatment with hydrocortisone, they observed a statistically significant decrease in TOS and OSI index and an increase in TAC levels in comparison with pre-treatment levels (Sandal et al. 2013). This is the only study in literature considering specifically the effects of steroid on oxidative stress, and then

we can only hypothesize that steroids could block a cascade of events (which include cellular recruitment, cytokines production, and enzymatic activation) having as common final endpoint the FRs, the true effectors of tissue damage.

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17

Pulmonary Oxidative Stress and Antioxidant Defence System in the Lung Ageing and Fibrotic and Diabetic Lungs

Fusun Oztay, Ozgecan Kayalar, and Merve Yildirim

Abstract

Increased pulmonary oxidative stress is accompanied by the exceeding of antioxidant capacity and imbalances in the antioxidant system and redox signalling. Increased oxidants damage membrane lipids, proteins, enzymes and DNA and lead to the development of inflammation in the lungs. Chronic oxidative stress contributes to the ageing of the lung and chronic lung diseases such as chronic obstructive lung disease, asthma, pulmonary emphysema, pulmonary hypertension and idiopathic pulmonary fibrosis. The present chapter explains the generation of pulmonary oxidative stress, its harmful effects on the lung structure and functions, and pulmonary antioxidant system. Additionally, it discusses the mutual relations between pulmonary oxidative stress and the lung ageing, pulmonary fibrosis and hyperglycaemia-induced lung injury. The contribution of increased pulmonary oxidative stress to the processes of the lung ageing, pulmonary fibrosis and diabetic lung injury and also antioxidant therapies for these processes are explained in the present chapter. It appears that using antioxidant therapy combined with various effective molecules on the regression of the disease pathogenesis, instead of antioxidant therapy alone, can be more helpful in the treatment of lung diseases related to pulmonary oxidative stress.

Keywords

Pulmonary oxidative stress · Ageing of lung · Pulmonary fibrosis · Diabetic lung · Antioxidant therapy

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17.1 Introduction

17.1.1 Generation of Oxidative Stress in the Lung and Pulmonary Antioxidant System

All aerobic organisms use oxygen to provide the energy required for the physiological and metabolic processes in their cells. However, oxygen may cause several harmful effects on cells and tissues due to its chemical activity. Oxygen performs these harmful effects through both enzymatic and non-enzymatic reactions, by the production of reactive oxygen species (ROS) and nitrogen species (RNS), which lead to protein, lipid and DNA damage. More or less, aerobic organisms can form two different reactive products in addition to ROS and RNS: the reactive sulphur and chlorine species. The most important ROS are superoxide anions (O_2^{-}•), hydrogen peroxide (H_2O_2), hydroxyl radical (• OH), singlet oxygen ($^1O_2$) and ozone (O_3) (Larosa and Remacle 2018). O_2^{-}• is the first formed free radical and transforms into H_2O_2 by an enzymatic reaction. H_2O_2 can turn into the more reactive • OH by either Fenton reaction in the presence of ferro or an enzymatic reaction. It can also produce hypochlorous acid (HOCl) in the presence of chloride and bromide ions (Wang et al. 2015). • OH can generate O_2^{-}• via Haber–Weiss reaction. Nitric oxide (NO•), which is produced at the highest amount among all RNS, can react with O_2^{-}• to form the peroxynitrite anion ($ONOO^{-}$). And then, it can be converted into • OH and nitrite anion (NO_2^{-}) (Lewandowski and Gwozdzinski 2017).

Pulmonary ROS/RNS originate from exogenous and endogenous sources. Exogenous ROS sources include environmental gases such as aldehyde carbonyl, nitrogen dioxide, sulphur dioxide and carbon monoxide, automobile exhaust, industrial chimney fumes, volatile organic compounds, O_3 generated by a series of chemical reactions containing nitrogen oxides, particulate matters in various pollutants, tobacco smoke, pollens produced especially in spring and autumn and viral, bacterial and fungal toxins (Chen et al. 2007; Białas et al. 2016). Endogenous ROS can be produced in pulmonary epithelial, endothelial and mesenchymal cells, macrophages and neutrophil and eosinophil leucocytes. In these cells, mitochondrial electron transport system, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, lipoxygenases, peroxisomal oxidases (glycolate oxidase, urate oxidase, D-amino acid oxidase, etc.), nitric oxide synthase (NOS), myeloperoxidase (MPO), xanthine oxidase (XO) and angiotensin II contribute to endogenous ROS production (Białas et al. 2016; Global Initiative for Chronic Obstructive Pulmonary Disease 2016; Hancock et al. 2010). There is a constant production of endogenous ROS/RNS under physiological conditions in the cells. The produced ROS/RNS are neutralized by the pulmonary antioxidant system. Redox signalling mediated by ROS/RNS regulates the important cellular events such as apoptosis, cell survival, the control of cell cycle, inflammation and several intracellular signal transductions. Low ROS concentration increases the cell proliferation by altering the expression of proto-oncogene and growth factors, while high ROS concentration induces apoptosis and necrosis (Rharass et al. 2014; Redza-Dutordoir and Averill-Bates 2016; Kutuk et al. 2017). ROS may alter the expression of the genes in many signalling

pathways such as cyclic adenosine monophosphate cascade, calcium/calmodulin-mediated signal networks and NO signalling (Rharass et al. 2014).

Oxidative stress refers to pathologic conditions that occur as a result of imbalance between oxidants and enzymatic [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and thioredoxin (Trx)] or non-enzymatic antioxidants [mucin, urate, glutathione (GSH), ascorbate, ceruloplasmin, transferrin, vitamin E and beta-carotene] in pulmonary cells and epithelial lining fluids. ROS/RNS cause toxic effects on the cell, when their concentrations exceed 5%. The high ROS/RNS concentration damages organic molecules, such as membrane lipids, proteins, enzymes and DNA, and leads to the development of inflammation in the lungs (Fig. 17.1). Several transcription factors including nuclear factor-kappa B (NF-κB), activator protein 1 (AP-1) and hypoxia-induced factor (HIF-1) trigger pulmonary inflammation by the regulation of their target genes as a response to redox signalling (Madamanchi and Runge 2013). Antioxidant capacity is exceeded, the antioxidant system and redox signalling are imbalanced, and increased oxidants damage membrane lipid, protein and DNA damage under induced oxidative stress (Fig. 17.2). In the following process, cell/tissue damage and inflammation can be seen in the lung. Moreover, chronic oxidative stress provides a basis for various lung diseases such as chronic obstructive lung disease (COPD), asthma, pulmonary emphysema, pulmonary hypertension and idiopathic pulmonary fibrosis (IPF). In many chronic lung diseases such as COPD, asthma and IPF, it has been described undesired changes mediated by increased oxidative stress, such as the decreased and inadequate antioxidant response, protease/antiprotease imbalance, induced pulmonary inflammation, cell death and tissue damage which generate the pathogenesis of diseases (Fig. 17.2). Experimental and clinical studies indicate that the use of various antioxidants protects lung function (forced

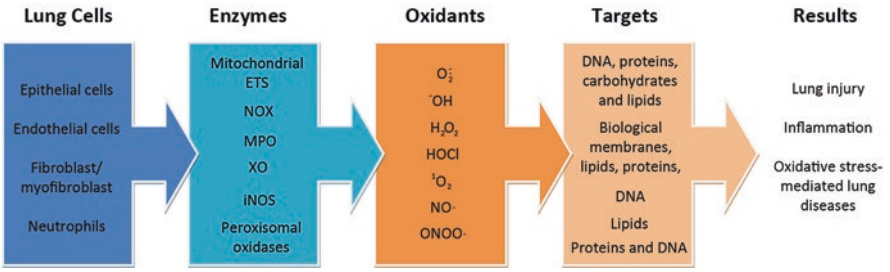

Fig. 17.1 Pulmonary cells produce reactive oxygen and nitrogen species through cellular enzymes localized in the subcellular compartments of the cell. ROS/RNS damages membrane lipids, proteins, enzymes and DNA and lead to the development of inflammation in the lungs. Chronic oxidative stress causes chronic lung diseases as a result of the imbalance of the antioxidant system and redox signalling, the structural and functional dysfunction of molecules, the induction of pulmonary inflammation and cell and tissue damage. *ETS* electron transport system, *NOX* NADPH oxidase, *MPO* myeloperoxidase, *XO* xanthine oxidase, *iNOS* inducible nitric oxide synthase, $O_2^{-}\bullet$ superoxide anion, $\bullet$ *OH* hydroxyl radical, H_2O_2 hydrogen peroxide, *HOCl* hypochlorous acid, $^1O_2$ singlet oxygen, *NO*• nitric oxide, $ONOO^-$ peroxynitrite anion

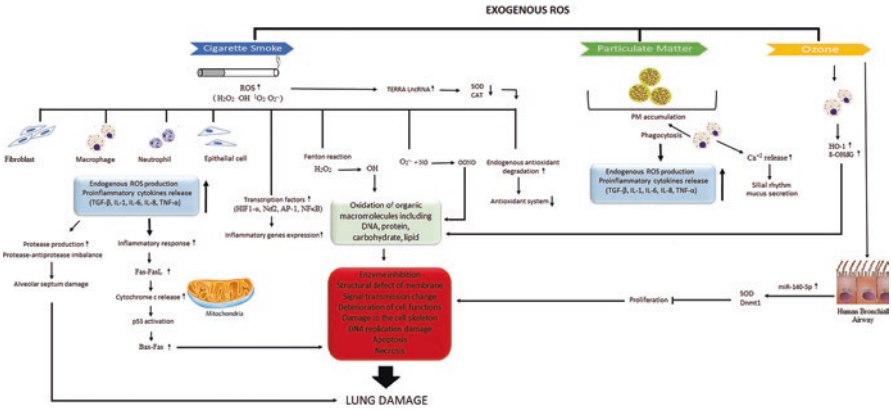

Fig. 17.2 The effects of exogenous ROS sources including cigarette smoking, particulate matter and ozone on lung injury. *ROS* reactive oxygen species, *PM* particulate matter, *TGF-β* transforming growth factor-β, *IL-1* interleukin-1, *IL-6* interleukin-6, *IL-8* interleukin-8, *TNF-α* tumour necrosis factor-α, *NF-κB* nuclear factor-kappa B, *AP-1* activator protein-1, *HIF1-α* hypoxia-inducible factor 1-α, *Nrf2* nuclear factor-erythroid-related factor 2, *SOD* superoxide dismutase, *CAT* catalase, *HO-1* heme oxygenase-1, *8-OHdG* 8-hydroxy-2′ -deoxyguanosine, *Dnmt1* DNA methyltransferase 1

expiratory volume in 1 second and forced vital capacity) and reduces the risk of disease in patients and experimental animal models (Grievink et al. 1998; Baccarelli et al. 2014; Sener et al. 2007).

It has been recently reported that oxidative stress can stimulate the expressions of various microRNA (miRNA) and long-non-coding RNA (LncRNA). The mutual relation between oxidative stress and miRNA/LncRNA has been known. Lungs exposed to O_3 show transcriptional alterations of miRNAs related to immune and inflammatory response such as miR-132, miR-143, miR-145, miR-199a, miR-222, miR-424 and miR-582-5p (Fry et al. 2013). MiR140-5p can regulate oxidative stress via SOD (Zhang and Xu 2014). Recently, the contribution of oxidative stress-mediated miRNAs to the pathogenesis of lung diseases has been reported by several studies. H_2O_2-induced miR34a regulates the oxidative stress-dependent ageing responses in patients with COPD. The expression of sirtuin-1 deacetylase, an antiageing enzyme related to oxidative stress, is reduced by the increased miR34a in COPD patients (Baker et al. 2016). The human lung adenocarcinoma cell line (A549 cells) treated with H_2O_2 exhibits decreased SOD and CAT levels, increased oxidative stress, and induced TERRA LncRNA expression. An improvement in the functions of oxidative stress-associated genes, such as SOD and CAT, and apoptosis-related genes, such as cytochrome c, caspase-3, and caspase-9, has been found when the TERRA LncRNAs are silenced by siRNA applications (Gao et al. 2017).

17.2 Pulmonary Oxidative Stress Damages to Organic Macromolecules

Lipid peroxidation (LPO) occurs as a result of the interaction of polyunsaturated fatty acids of cell membrane with free radicals. This reaction damaging the cellular membranes causes cell death in the next step. Malondialdehyde (MDA), the secondary end product of LPO, may inactivate many cellular proteins and cause the development of pulmonary emphysema by regulating the destruction of the alveolar wall and the induction of pulmonary inflammation (Van Eeden and Sin 2013). Other LPO products, such as ethane, pentane, and 8-isoprostane, are found highly elevated in the breath and serums of patients with COPD (Rahman and Kinnula 2012). Another toxic product of LPO is 4-hydroxy-2-nonenal. It induces the NF-κB expression, production of pro-inflammatory cytokines, mitochondrial dysfunction, and apoptosis by the accumulation of cytoplasmic calcium in human and mice lung exposed to O_3 (Kirichenko et al. 1996; Li et al. 1996). O_3 directly interacts with unsaturated fatty acids in the epithelial lining fluid to form the ozonized lipid products of the cell membrane. Experimental studies have shown the induction of inflammatory mediators such as interleukin (IL)-6 and IL-8, platelet-activating factor, prostaglandin E2 and the phospholipases A2, C and D activation after ozonized lipid products exposure in bronchial epithelial cells (Kafoury et al. 1999).

ROS mostly target monosaccharides. Oxidation of monosaccharides results in the production of peroxides and oxaldehydes. Oxaldehydes bind to DNA, RNA and proteins and exhibit antimitotic effects by forming cross-linking bonds among molecules. Thus, they play an important role in cell ageing by the prevention of cell proliferation (Schauenstein et al. 1977; Baraibar et al. 2012). A number of oxaldehydes are found in cigarette smoke. However, the unwanted effect of oxidative stress on carbohydrates and the contribution of this effect to the pathogenesis of chronic lung diseases are not well known.

ROS modulates the structure and function of proteins, by allowing the protein dimerization, protein interaction with Fe–S or other metal complexes and the modification of critical amino acid residues in proteins. The tertiary and quaternary structures of proteins are altered by ROS-induced chemical changes in the amino acid residues (Ramsey and Sharpless 2006). H_2O_2-induced oxidative stress develops the formation of neo-antigenic epitopes by the configuration-changing proteins in human A549 cells (Upadhyay et al. 2018). The side chains of amino acid residues, particularly methionine and cysteine residues, are susceptible to ROS attacks. The proteins most sensitive to oxidation are albumin, α1-antitrypsin and surfactant proteins. For example, excessive oxidative stress results in the carbonylation of albumin protein in bronchoalveolar lavage fluids of long-time smokers with age (Nagai et al. 2006). In addition, H_2O_2-induced oxidation of the methionine residue in α1-antitrypsin results in loss of anti-elastase activity in vitro (Taggart et al. 2000). When α1-antitrypsin is not protected from ROS attacks, the alveolar areas are susceptible to destruction by neutrophil elastase, and emphysema can develop (Kelly et al. 2010; Palmgren et al. 1992). Deficiency of protease inhibitor such as the α1-antitrypsin inhibitor is a common condition in COPD patients. Additionally,

oxidation of methionine residues in the surfactant protein-B causes the inactivation of this protein. Inactivated surfactant protein-B loses its ability to form a surfactant film that lowers the surface pressure of the lung during breathing (Manzanares et al. 2007). Similarly, acute exposure to O_3 in guinea pigs alters the surfactant protein-A function and triggers the inflammatory response in the lungs. Oxidative modification of surfactant proteins makes the lungs more sensitive to LPO, inflammation and oxidative damage (Devendra and Spragg 2002).

ROS lead to oxidative DNA damages. The oxidative DNA damages include strand breakage, deoxyribose oxidation, organic base modifications in the nucleotides and DNA-protein cross-links. The 8-hydroxy-2′-deoxyguanosine (8-OH-dG) is an oxidized base derivative and commonly known as one of the major products of oxidative DNA damage. The peripheral lungs of smokers with and without COPD possess significantly the increased expression of 8-OHdG (Caramori et al. 2011; Igishi et al. 2003) and in the bronchiolar epithelial cells and type 2 pneumocytes of animal models exposed to cigarette smoke (Aoshiba et al. 2003; Deslee et al. 2009). The mitochondrial DNA is more sensitive to oxidative damage than nuclear DNA, because of its vicinity to electron transport system, the absence of a histone protective shield and the presence of a limited DNA repair mechanisms (Fathi et al. 2015). The mitochondrial DNA damage can result in mitochondrial dysfunction and mitochondrial dysfunction-mediated cell death that are commonly seen in the oxidative stress-induced alveolar epithelial cell damage in the pathogenesis of COPD and IPF. Additionally, it has been shown that oxidative stress induces chromatin modifications through alterations of histone acetylation/deacetylation and methylation/demethylation enhancing pro-inflammatory gene expression in lungs of patients with COPD (Sundar et al. 2013). In addition, oxidative stress reduces histone deacetylase protein expression and activity in the alveolar epithelial cells of IPF patients. The deficiency of histone deacetylase activity promotes pulmonary fibrosis by increasing acetylation of mitochondrial proteins including SOD and oxidant-dependent mitochondrial DNA damage and cell apoptosis (Jablonski et al. 2017).

17.3 Pulmonary Oxidative Stress Related Directly to the Ageing and Injury of the Lung and the Pathogenesis of Several Lung Diseases

17.3.1 Ageing of the Lung

The cells of the body age over time and begin progressively to lose the functional and proliferative abilities as they age during adulthood. Ageing changes occurred in cells result also in the ageing of tissues and organs. The human lung grows until the age of 10–12 years and matures further until it reaches its maximum function at the age of ~20 years for females and ~25 years for males. Thereafter, lung function progressively declines with increasing age as a consequence of structural and physiological changes occurring in the lung (Rojas et al. 2014). The pulmonary cellular and metabolic alterations depending on ageing influence the structure and function

of the lung. Age-associated changes in the lung include reduced lung volume, decreases in the performance of the inspiratory and expiratory muscles and airway clearance due to increased mucociliary dysfunction (Culham et al. 1994; Proença de Oliveira-Maul et al. 2013; Lombardi Jr et al. 2005). Reduced respiratory functions and pulmonary circulation result in reduction of expiration flows and decreased gas exchange (Vaz Fragoso et al. 2013; Taylor and Johnson 2010). Additionally, dilated alveolar ducts, enlargements of alveoli and loss of elastic recoil of the lungs occur in the setting of the normal ageing process. For example, the emphysematous alterations are accompanied with the destruction of alveolar walls and the inflammatory cell infiltration in the aged adult in patients with emphysema. Exposure to oxygen radicals from cigarette smoke and neutrophils and proteases from macrophages and neutrophils is effective on the destruction of alveolar walls in pulmonary emphysema (Sharafkhaneh et al. 2008).

Telomere shorting, DNA damage, decreases in repair system of DNA, alterations in the chromatin remodelling, mitochondrial and lysosomal dysfunctions and induced oxidative stress are the general hallmarks of ageing in cell, tissue and organs. The lung is one of the vulnerable internal organs constantly exposed to the outside environment. Moreover, it has over 40 morphologically distinct cell phenotypes (Ochs and Weibel 2008). Because of the external environmental and organic exposures, and the large variety of cell types in the lung, the causes and consequences of ageing display a broad spectrum of phenotypes. In addition to the causes of ageing mentioned above, the extra ageing hallmarks include the loss of proteostasis, genomic instability, cellular and immuno-senescence, impaired regenerative capacity, altered cellular communication and the dysregulation of extracellular matrix (ECM) (Aunan et al. 2017; Brandenberger and Mühlfeld 2016; Meiners et al. 2015). The increased production of ROS plays a critical role in the cell ageing. Increased oxidative stress-induced ageing is based on the fact that age-associated functional losses are due to the accumulation of oxidative damage to lipids, nucleic acids, proteins and carbohydrates. Finally, the dysfunction of these macromolecules leads to damage in the cell ultrastructure, single-strand breaks and chemical modification of bases in the DNA and alterations in protein structure or the dysfunction of specific enzymes. These harmful alterations mediated by excessive oxidative stress are the well-known reasons of cell ageing (Fig. 17.3). An important mechanism linking oxidative stress and ageing is the inhibition of mitochondrial functions enhanced by oxidative stress. The dysfunction of mitochondria contributes to much more ROS production and ROS-mediated structural/functional loses and stress response to age-dependent damage (Hekimi et al. 2011). Additionally, increased ROS promote cell senescence referred to as a state of irreversible growth cycle arrest due to O_2^{-}•-induced oxidative protein or DNA damage. Cell senescence is accompanied by the impaired cellular function, the increased production of ROS, the induced pro-inflammatory signalling and the expression of senescence-associated molecules such as β-galactosidase, p53, p16 and p21 proteins (Campisi 2013). Aged lung has reduced repair and regeneration capacity due to cell senescence (Fig. 17.3).

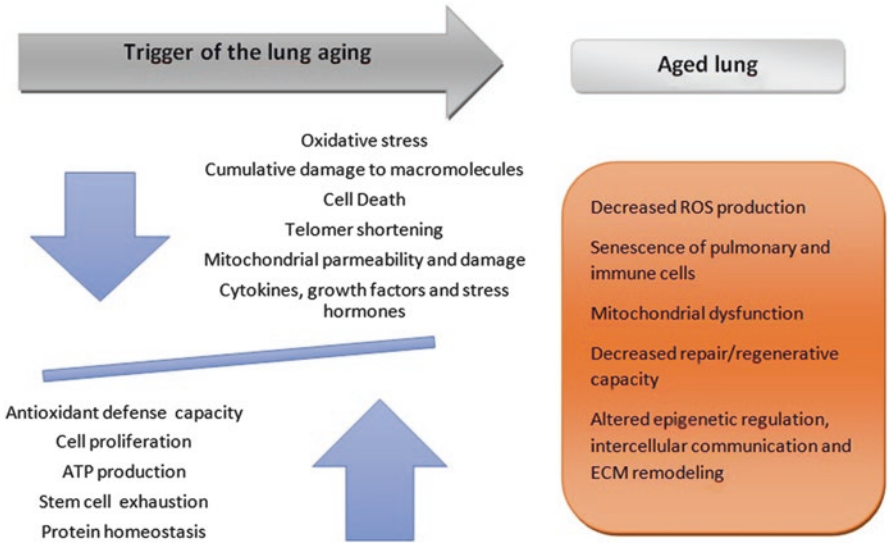

Fig. 17.3 Molecular and cellular alterations activated by pulmonary oxidative stress trigger the lung ageing. Increased pulmonary oxidative stress and decreased antioxidant capacity contribute to the processes of the lung ageing, whereas the lungs of the elderly humans are mainly characterized by the decreased ROS production due to reduced metabolic demand

The development of pulmonary antioxidant enzyme system in mammals including human, mice, rat and rabbits is the last 10–15% of gestation (Fanucchi 2004). The mature lung protects itself from the detoxification of endogenous and exogenous compounds through well-developed antioxidant system against any cellular injury. However, pulmonary antioxidant capacity, which is exceded by the increased oxidant production and the deterioration of antioxidants response with advancing ageing, decrease the lung ability to manage oxidative stress in the lung. The reduced GSH levels in the epithelial lining fluid and mitochondria diminish in the aged human and mice lung (Ramesh et al. 2012; Gould et al. 2015). The mitochondria cannot protect itself from ROS attacks due to the mitochondrial GSH oxidized during ageing (Rebrin and Sohal 2008). Although there are discordant opinions, generally, SOD and CAT protein expressions and activities decrease in the lung tissue of aged murin rodents (Ramesh et al. 2012). Lower level of GPx enzyme and higher level of MDA are found in the group of older adults who are physically active (Mergener et al. 2009). Dietary supplementation *of Panax ginseng* extracts containing rich antioxidant content could conceivably defend the lung against the effects of LPO, free radicals and ROS, by inducing the activity of SOD, CAT, GPx, GR and glutathione S-transferase (GST) in the lung of aged rats (Ramesh et al. 2012).

Ageing is the main risk factor for major chronic diseases. There is cumulative evidence that oxidative stress is related to the pathology of ageing and many age-related chronic diseases, including type 2 diabetes, rheumatoid arthritis, atherosclerosis and neurodegenerative diseases (Liguori et al. 2018). Several lung diseases

such as COPD, IPF and lung cancer appear generally in the elderly. They are also characterized by increased pulmonary oxidative stress and have the prominent ageing hallmarks such as cellular senescence, telomere attrition, epigenetic alterations, loss of proteostasis, altered intercellular communication and ECM dysregulation as at differing extents depending on each disease (Meiners et al. 2015; Brandsma et al. 2017). The contribution of the increased pulmonary oxidative stress to the pathogenesis of diseases and the antioxidant therapies in fibrotic and diabetic lungs are discussed below.

17.3.2 Pulmonary Fibrosis

Pulmonary fibrosis is characterized by fibroblast activation, increase in myofibroblast number and overexpression and interstitial accumulation of several ECM components such as collagen, fibronectin, syndecan, decorin, hyaluronic acid, heparan sulphate and chondroitin sulphate (Venkatesan et al. 2011). The risk factors associated with pulmonary fibrosis include ionized radiation, asbestos, silica, metal powders, bacterial and viral exposures, some drugs used in the treatment of various diseases (such as bleomycin, amiodarone), gastro-oesophageal reflux, traumatic injuries, severe inflammation in the tissue, allergic responses and autoimmune reactions. Moreover, pulmonary fibrosis can be idiopathic. IPF affects approximately five million people worldwide (Meltzer and Noble 2008). It can be considered the most fatal of the interstitial lung diseases. The median survival is 3–5 years for the patients with IPF. Pro-fibrotic cytokines, chemokines and growth factors, including connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), endothelin-1 (ET-1), transforming growth factor-beta (TGF-β), tumour necrosis factor-alpha (TNF-α), IL-1 and IL-8, released from pulmonary epithelial, mesenchymal and inflammatory cells are involved in the regulation of the fibrotic response such as fibroblast/myofibroblast proliferation, myofibroblast differentiation, overproduction and their consequent accumulation in ECM.

Exposure of the lungs to various endogenous and exogenous oxidants may cause the induction of inflammation and fibrosis, leading to the production of ROS, and impaired antioxidant capacity and pulmonary redox balance. The increased oxidative and nitrosative stress can be seen in the patients with IPF. Nitrosative stress is mainly sourced from inducible NOS (iNOS) activation. Lung specimens collected from IPF patients show also signs of chronic oxidative damage (Global Initiative for Chronic Obstructive Pulmonary Disease 2016; Hancock et al. 2010; Rharass et al. 2014). Bronchoalveolar lavage fluid isolated from IPF patients demonstrates a high oxidative damage of proteins (Redza-Dutordoir and Averill-Bates 2016; Kutuk et al. 2017). Higher levels of H_2O_2 and products of free radical-mediated LPO, such as 8-isoprostane, have been found in the exhaled breath condensate of patients affected by IPF. In contrast, lower levels of antioxidants, such as the ROS scavenger GSH, have been found in epithelial lining fluid obtained from patients with IPF when compared to healthy control subjects (Meyer et al. 1995). Similar results were

obtained from rodent models of bleomycin-induced pulmonary fibrosis. Bleomycin is used for chemotherapy treatment. Pulmonary fibrosis can develop in patients undergoing bleomycin treatment. Bleomycin contains two regions which link DNA and iron. Bleomycin complexes with iron in the presence of molecular oxygen produce O_2^{-}• and • OH by Fenton's and Haber–Weiss' reactions, resulting in LPO of cell membranes, single- and double-strand DNA breaks and oxidation of DNA guanine residues in the alveolar epithelial cells. Additionally, TNF-α, IL-1, PDGF and TGF-β are released from alveolar macrophages and pulmonary epithelial and mesenchymal cells in animal models of bleomycin toxicity, causing pulmonary fibrosis and apoptosis of alveolar epithelial cells in an ROS-dependent manner (Liu et al. 2014; Ovet and Oztay 2014; Yilmaz et al. 2015). The suppressed expression of antioxidant enzymes by TGF-β1 contributes to the generation of pulmonary fibrosis. Bleomycin-induced pulmonary fibrosis murine models demonstrate increases in the bleomycin-induced oxidative stress species of lung lavage fluids, LPO products and TGF-β signalling and decreases in the levels of antioxidant enzymes (e.g. SOD, CAT, GPx). Antioxidant treatments with *N*-acetylcysteine (Yildirim et al. 2005), erdosteine (Sogut et al. 2004), aminoguanidine (Yildirim et al. 2004), caffeic acid phenethyl ester (Ozyurt et al. 2004), melatonin (Yildirim et al. 2006), ginkgo biloba (Iraz et al. 2006) and resveratrol (Sener et al. 2007) attenuate bleomycin-induced fibrosis and pulmonary oxidative stress in rodents and restore pulmonary antioxidant defence capacity.

Oxidative changes within the lung may pave the way for the generation of profibrotic tissue microenvironment. They influence the tissue homeostasis and the repair or regeneration capacity of the lung, by mediating various cellular behaviours (Ding et al. 2011). For example, the exogenous H_2O_2 can trigger senescence or apoptosis (Chen et al. 1998). And also exposure to chronic sublethal ROS results in stress-induced premature senescence of fibroblasts (Toussaint et al. 2000). Treatments with *N*-acetylcysteine (the small molecule antioxidant) reverse p21-induced growth arrest (Macip et al. 2002). While senescent epithelial cells are more susceptible to apoptosis, fibroblasts/myofibroblasts resist for apoptosis as a consequence of senescence (Hampel et al. 2004, 2005). H_2O_2 produced by myofibroblasts can induce alveolar epithelial cell apoptosis through paracrine signalling and cross-linking reactions of ECM components. Thus, it mediates the generation of pulmonary fibrosis (Waghray et al. 2005; Larios et al. 2001). Additionally, ROS production from mitochondria and membrane-bound NADPH oxidase-4 (NOX4) enzyme in alveolar epithelial cells leads to the damage of epithelial cells. This oxidant-mediated epithelial damage can reduce the regenerative capacity of injured epithelial cells and initiate pulmonary fibrotic response through alveolar epithelial cells in IPF patients and bleomycin-induced fibrosis (Waisberg et al. 2010; Camelo et al. 2014). The mitochondrial dysfunction and mtDNA damage may activate the apoptotic events in the alveolar epithelial cells (Kim et al. 2015; Liu and Chen 2017). Myofibroblasts, neutrophils and macrophages can induce epithelial cell apoptosis by paracrine H_2O_2 in areas adjacent to fibroblastic foci in the lungs of IPF patients (Uhal et al. 1998). Several growth factors, cytokines and some peptides [PDGF, CTGF, TGF-β, ET-1, angiotensin II, etc.] are released from the injured alveolar

epithelial cells. They can directly and indirectly mediate pro-fibrotic activation of mesenchymal and epithelial cells. The growth factors modulate cell proliferation, while TGF-β and ET-1 induce the differentiation of type 2 pneumocytes into myofibroblasts (Yilmaz et al. 2015; Jain et al. 2007). The differentiation processes of type 2 pneumocytes into myofibroblasts called epithelial mesenchymal transition (EMT) play a critical role in the generation of fibrotic foci in the lungs of IPF patients and bleomycin-induced experimental pulmonary fibrosis. In addition to type 2 pneumocytes, fibroblasts, endothelial cells, fibrocytes and pericytes are the sources of (myo)fibroblast (McAnulty 2007). ROS and TGF-β are interlinked by feed-forward and feedback mechanisms. In more detail, ROS are involved in both the production and activation of TGF-β1 and the generation of active TGF-β form (Barnes and Gorin 2011; Jobling et al. 2006; Radwan et al. 2012). ROS produced from mitochondria, NOX enzymes and peroxisomes can induce fibrotic responses such as myofibroblast differentiation, ECM production and contractility by activation of TGF-β1 signalling in the lung fibroblast (Richter and Kietzmann 2016). Additionally, increased ROS/RNS levels mediate pulmonary inflammatory responses by activating the NF-κB and AP-1 transcription factors, epigenetic alterations and expression of pro-inflammatory genes (Fig. 17.4) (Rahman et al. 2006). Moreover, AP-1 is necessary for the expression of collagen and fibronectin in response to TGF-β (Fig. 17.4) (Chung et al. 1996; Ignotz et al. 1987).

Membrane-bound NADPH oxidase enzymes (NOXs) are a primary source of $O_2^{-}\bullet$ production, which rapidly dismutates to H_2O_2 in pulmonary fibrosis. These protein complexes consist of a catalytic transmembrane-spanning subunit, cytosolic NADPH substrate and FAD cofactor-binding domains. Their functional activity is

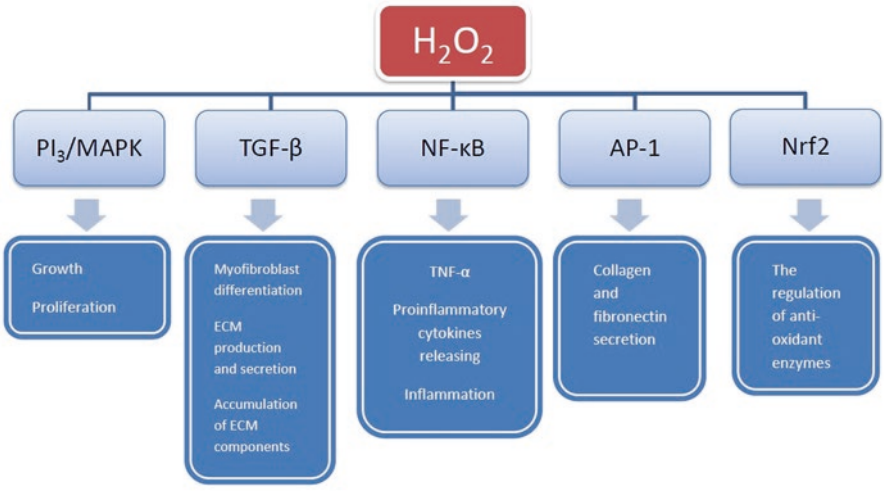

Fig. 17.4 The possible role of H_2O_2 via several signal pathways in the formation of pulmonary fibrosis. *PI3/MAPK* phosphatidylinositide-3-OH-kinase/mitogen-activated protein kinase, *TGF-β* transforming growth factor-beta, *NF-κB* nuclear factor-kappa B, *AP-1* activator protein 1, *Nrf2* nuclear factor-erythroid-related factor 2

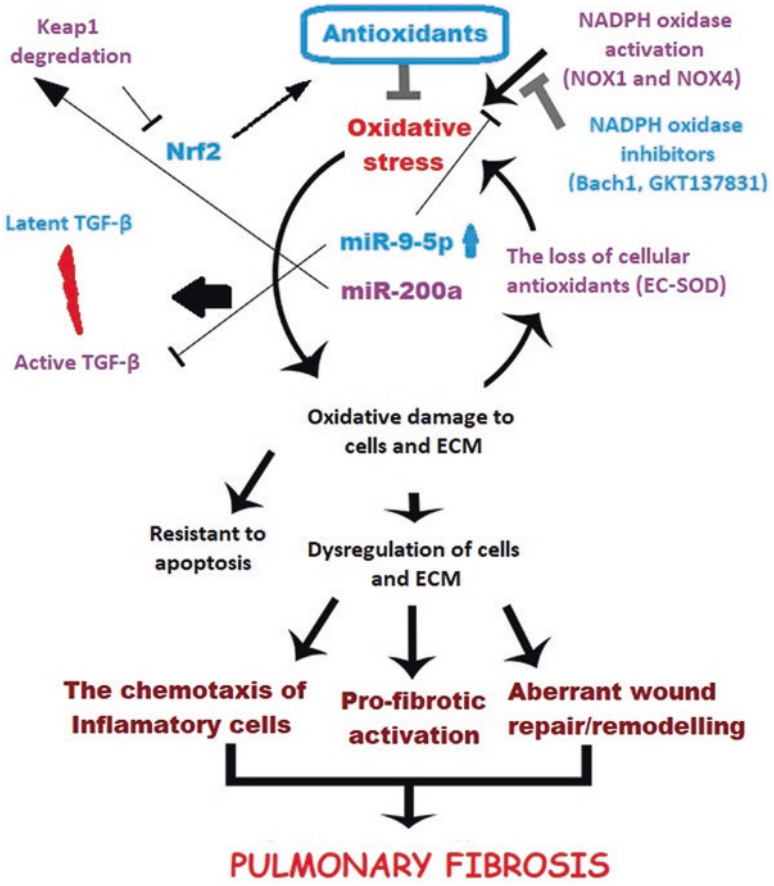

Fig. 17.5 The molecular mechanisms of oxidative stress on pulmonary fibrosis and its regulation via several antioxidants, transcription factors and miRNAs. *Keap1* Kelch-like ECH-associated protein 1, *Nrf2* nuclear factor-erythroid-related factor 2, *Bach1* BTB domain and CNC homolog 1, *EC-SOD* extracellular superoxide dismutase, *NOX* NADPH oxidase

determined in the cell types and subcellular compartments in which they are expressed (Bedard and Krause 2007). There are seven isoforms of NADPH oxidase. Among them, NOX4 provides a unique contribution to the production of ROS due to its high expression levels and functional activity (Martyn et al. 2006). H_2O_2 is produced within intracellular compartments as a result of NOX4 activation and acts as an intracellular signal molecule causing activation of tyrosine kinases and inactivation of protein tyrosine phosphatases, thereby influencing gene transcription downstream of TGF-β signalling (Figs. 17.4 and 17.5) (Liu et al. 2010a; Martin-Garrido et al. 2011). Additionally, TGF-β, a central mediator in the pathogenesis of pulmonary fibrosis, is known to cause elevated levels of NOX4 expression and activity, leading to increased oxidative stress. The expression of NOX4 enhanced by TGF-β can amplify the powerful fibrogenic response induced by TGF-β

(Martin-Garrido et al. 2011; Amara et al. 2010; Cucoranu et al. 2005; Hecker et al. 2009). NOX4 is selectively upregulated in the lungs of patients with IPF and expressed by myofibroblasts within fibrotic foci, vascular smooth muscle cells surrounding remodelled pulmonary arteries and hyperplastic alveolar epithelial cells (Amara et al. 2010; Hecker et al. 2009; Pache et al. 2011). Studies performed on fibrotic lung-derived fibroblasts demonstrated TGF-β-induced pro-fibrotic responses, which is regulated by NOX4. The RNAi-mediated knockdown of NOX4 inhibits TGF-β-dependent pro-fibrotic responses and subsequently cell proliferation, upregulation of alpha-smooth muscle actin (α-SMA) mRNA and the secretion of the ECM components such as fibronectin and collagen which are reduced in lung fibroblasts. Another study showed that NOX4-deficient mice were protected from bleomycin-induced acute lung injury and alveolar epithelial cell apoptosis preceding the onset of fibrosis. Type 2 pneumocytes isolated from the lungs of NOX4-deficient mice did not exhibit TGF-β1-induced intracellular ROS generation and endoplasmic reticulum stress-induced apoptosis (Carnesecchi et al. 2011). Another research group reports the reduced mRNA levels of lysocardiolipin acyltransferase in peripheral blood mononuclear cells in IPF patients. The overexpression of lysocardiolipin acyltransferase results in the regression of mitochondrial and intracellular oxidative stress induced by TGF-β, the reduction of NOX4 expression and differentiation of human lung fibroblasts (Huang et al. 2017).

Fibrosis is modulated by ROS through post-transcriptional and epigenetic mechanisms. Post-transcriptional regulation is largely achieved by miRNAs. Since the discovery of non-coding RNAs in humans, the cumulative knowledge about them and their functions such as cellular proliferation and tissue development, differentiation and repair show that miRNAs are promising molecules for IPF treatment (Pasquinelli et al. 2000; Wu et al. 2009; Zorio et al. 2009; Bartel 2004; Hwang and Mendell 2006). MiRNAs repressed translation by the mRNA degradation (Bartel 2004). Several miRNAs can respond to the redox state and to fibrotic stimuli (Fig. 17.5) (Pottier et al. 2014; Cheng et al. 2013). ROS can alter the biogenesis and processing of miRNAs. And also miRNAs can regulate the cellular redox homeostasis by modulating the nuclear factor-erythroid-related factor 2 (Nrf2)-driven antioxidant gene expression and some key enzymes that generate ROS (Cheng et al. 2013). MiRNAs play a role in the induction of fibrosis in different organs (Pottier et al. 2014). For example, the expression of miR-200 family is induced by oxidative stress conditions. The miR-200a targets the Keap1 3′-untranslated region (3′-UTR), leading to Keap1 mRNA degradation, and modulates the stability of the master antioxidant regulator Nrf2; thus, it regulates Nrf2 activation in breast cancer cells (Fig. 17.5) (Eades et al. 2011). Moreover, miR-200a has been described to promote EMT in cancer. The mirR-200 family together with miR-199 family generates the progression of liver fibrosis under oxidative stress (Xiao et al. 2015). However, it is known very little information about miRNAs regulated by both oxidative stress and pro-fibrotic symptoms involved in pulmonary fibrosis. The miR-21, whose expression is positively regulated by NF-κB, a redox-sensitive transcription factor, promotes pulmonary fibrosis in IPF patients and the bleomycin mouse model by inducing Smad-dependent TGF-β1 signalling (Liu et al. 2010b; Fierro-Fernández

et al. 2016). In another study, Fiero-Fernandez et al. identified the miR-9-5p-mediated TGF-β receptor type 2 and NOX4 expressions (Fierro-Fernández et al. 2015). They demonstrate the upregulation of miR-9-5p in oxidative stress conditions in human lung fibroblast treated with H_2O_2. MiR-9-5p inhibited the TGF-β1-induced fibrogenic pathway and myofibroblast differentiation in human lung fibroblasts and experimental pulmonary fibrosis. Researchers suggest that this may represent a potential mechanism contributing to the anti-fibrotic role of miR-9-5p in pulmonary fibrosis. Moreover, they detect miR-9-5p upregulation in the lungs of patients with IPF.

Yan et al. suggest that the upregulation of endogenous multiple antioxidants may be an efficient approach to prevent or treat pulmonary fibrosis, because the increased activities of the pulmonary antioxidant enzymes through various exogenous therapeutics have been linked with decreased lung fibrosis in a mouse model (Yuan et al. 2017). Nrf2, a redox-sensitive transcription factor, drives the endogen antioxidant mechanisms. It positively regulates the downstream antioxidant genes including GPx1, glutamate cysteine ligase, GST, GSH reductase, heme oxygenase-1 (Ho-1) and NADPH quinone oxidoreductase-1 (Swamy et al. 2016; Giudice et al. 2010; Jyrkkanen et al. 2011). Myofibroblasts isolated from IPF patients express low levels of Nrf2 when compared to those of controls. And also sulforaphane treatments attenuate the pulmonary fibrosis and oxidative stress, by increasing the expression of antioxidant enzymes, such as NOX, SOD, CAT and Ho-1, via upregulation of Nrf2 gene expression in the mice lungs. On the other hand, the inhibition of Nrf2 via a selective inhibitor or siRNA affects negatively antioxidant mechanisms, and this state leads to the increase of oxidants, the activation of TGF-β and the generation of pulmonary fibrosis. Additionally, the knockout of Nrf2 gene results in increased hydroxyproline content, inflammation and fibrosis in mice lung (Cho et al. 2004). The expression level of antioxidant molecules is determined by the balanced relationship between Nrf2 and Bach1 (BTB domain and CNC homolog 1). Bach1, as a competitive inhibitor, disturbs the interaction of Nrf2 and antioxidant response element. Pirfenidone is one of two currently drugs approved for IPF therapy. It has been reported both its regulatory effect on intracellular antioxidants and its inhibitory effects on the secretion of inflammatory cytokines and collagen synthesis. Pirfenidone also inhibits Bach1 mRNA and protein expressions in mouse lung fibroblasts treated with TGF-β1 and attenuates fibrosis in the lung of mice treated with bleomycin. Furthermore, it improves the mRNA and protein expressions of Nrf2, GPx1 and Ho-1. In the same study, after pirfenidone treatment to mice with pulmonary fibrosis, it has been reported the decreased levels of collagen 1a and IL-6 in supernatant of lung fibroblast, the decreased levels of MDA in serum and bronchoalveolar lavage fluids and the reduced production of ROS, infiltration of inflammatory cells and fibrosis degree in the lung (Yuan et al. 2017).

Clinical trials in patients with IPF have shown that the administration of antioxidant is not considered very effective on the reduced progression of disease and mortality, although the use of various antioxidants regressed pulmonary fibrosis in animal model of bleomycin-induced pulmonary fibrosis (Kandhare et al. 2016). In the IFIGENIA study, high doses of the antioxidant *N*-acetylcysteine combined with

prednisone and azathioprine are administered to patients with IPF. This treatment demonstrates a reduction in the decline in forced vital capacity and diffusion capacity in the patients (Demedts et al. 2005). Nevertheless, the use of antioxidant therapies has had limited success, and the lack of a placebo group in the original study raises questions as to the potential impact of drug–drug interactions on the outcome. The PANTHER trial sets out to address some of these questions, including the efficacy of treatment with *N*-acetylcysteine alone (Raghu et al. 2012). Since 2013 and 2014, pirfenidone, an anti-inflammatory and anti-fibrotic agent, and nintedanib, an inhibitory of receptor and cytoplasmic tyrosine kinase, have begun to be used in IPF therapy (Noble et al. 2011; Costabel et al. 2017; Crestani et al. 2018; Vancheri et al. 2018). However, although these drugs have positive effects on respiratory functions of patients with IPF, both drugs are used on patients with severe side effects such as diarrhoea, nausea and sensitivity to sunlight. Pirfenidone exhibits anti-fibrotic, antioxidant and anti-inflammatory effects in patients with IPF. It improves Nrf2 activation in addition to the upregulated expressions of Ho-1 and GPx1 mRNA and protein, by the inhibition of Bach1 in mouse lung fibroblasts induced by TGF-β1 and fibrotic lung tissues of mice (Yuan et al. 2017). In the future, the pharmacological inhibition or siRNA-mediated silencing of NOX4 enzyme (the primary enzymatic source of ROS in pulmonary fibrosis) and the use of resveratrol (a natural antioxidant) are recommended in the treatment of IPF, by depending on positive results obtained from in vitro and in vivo experimental studies. However, further clinical trials will be necessary in order to demonstrate their real beneficial effects.

17.3.3 Diabetic Lung

Diabetes is a metabolic disorder characterized by high blood glucose level. Clinical studies show that diabetic patients are under a high risk of pulmonary impairment when compared to nondiabetic subjects. Therefore, incidence of death due to pulmonary diseases among diabetic patients is greater than 50% in Japan (Asanuma et al. 1985). Diabetes can lead to lung damage, thickness of the basement membrane in the alveolar areas, impaired pulmonary surfactant system and reduction in the lung elastic recoil and lung-diffusing capacity (Oztay et al. 2008; Popov and Simionescu 1997; Ramirez et al. 1991). Moreover, the disruption of insulin signalling in hyperglycaemia and obesity is referred to as a strong risk factor for asthma and even COPD (Singh et al. 2013). Diabetes-induced damages can be considered as tissue-oxidative-damaging effects of chronic hyperglycaemia. Hyperglycaemia is the common feature of type 1 and type 2 diabetes. It induces the production of free radicals and secondary ends of LPO such as MDA and thiobarbituric acid reactive substances, generates the oxidative and nitrosative stresses in the lungs and subsequently contributes to lung injury and progression of pulmonary complications (Oztay et al. 2008; Ozansoy et al. 2005). Streptozotocin (STZ) is a toxic compound for the insulin-producing β-cells in mammalian pancreas. In experimental diabetes studies, diabetic rats and mice treated with STZ exhibit increased levels of ROS and MDA (the latter a LPO marker), oxidation of proteins and activities of nitric oxide

synthase enzymes in the lungs. In these animals, pulmonary oxidative stress-induced lung injury has been reported (Oztay et al. 2008, 2015, 2018; Eren et al. 2010). Additionally, it has been found the significant elevation of oxidative degradation of lipids and total nitrites and nitrotyrosine levels in the lungs of hyperglycemic rabbits (Gumieniczek et al. 2009). A study shows that diabetic rats characterized by high blood glucose and nitrate/nitrite levels have the intense immunoreactivities of endothelial and inducible nitric oxide synthases (eNOS and iNOS, respectively) in the epithelium of bronchi/bronchioles, alveolar macrophages and polymorph nuclear leucocytes in the lung (Oztay et al. 2008). Studies conducted in diabetic rats have revealed significant generation of mitochondrial superoxide at the site of NADH/ubiquinone oxidoreductase (complex I) (Coughlan et al. 2009). $O_2^{-}\bullet$ can react quickly with NO• to produce $ONOO^-$ that is very damaging. Proteins are the main targets of $ONOO^-$ and ROS. And also $ONOO^-$ can be able to change protein structure through cysteine amino acid oxidation in the peptide chain. Thus, proteins lose their function, and dysfunction of proteins affects lung cellular physiology adversely. The NO•/$ONOO^-$ pathway would be accepted as one of the mechanisms which leads to lung injury. It has been detected that overproduction of NO via eNOS and, specially, iNOS contributes to the pathological structural alterations in the diabetic rat lungs. In these diabetic rats, it has been observed that regression of NO• production through the inhibition of iNOS activity via a specific iNOS inhibitor named dexamethasone is accompanied by the improvement of structural alterations in the lung of diabetic rats (Oztay et al. 2008).

There are several mechanisms involved in the induction of pulmonary oxidative stress under hyperglycaemia, (I) increased flux of glucose through the polyol pathway, (II) intracellular formation of advanced glycation end products (AGEs), (III) mitochondrial dysfunction and (IV) the induction of inflammation, which consequently result in the generation of ROS (Fig. 17.6). The high glucose concentration inside the cell is tried to be reduced via polyol pathway. The polyol pathway is used

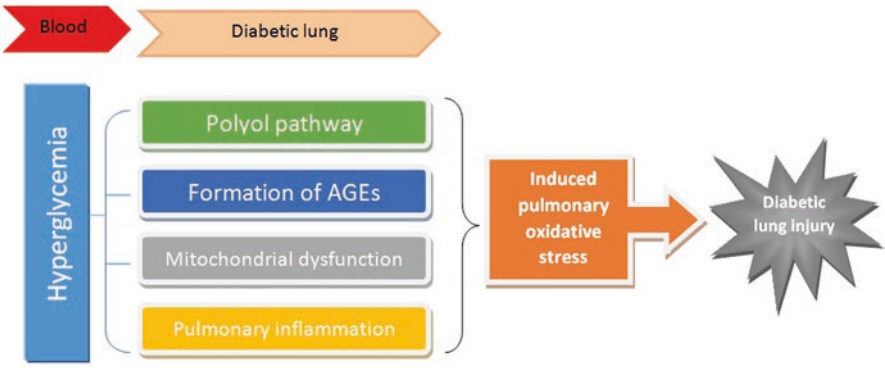

Fig. 17.6 Hyperglycaemia induces mainly the oxidative stress by several mechanisms, such as increased flux of glucose through the polyol pathway, intracellular formation of advanced glycation end products (AGEs), mitochondrial dysfunction and induction of inflammation. Increased pulmonary oxidative stress leads to lung injury

as a two-step pathway in the glucose metabolism. In the first step, glucose is reduced to sorbitol by aldose reductase. Sorbitol can induce cellular osmotic pressure, leading to cell death (Luo et al. 2016). Then, it is converted to fructose in the second step. An enzyme of polyol pathway competes with GRx for their cofactor NADPH, and NADPH is consumed by polyol pathway in diabetes. Increased nicotinamide adenine dinucleotide (NADH) causes more mitochondrial ROS production via NADH oxidase in the electron transport chain (Chung et al. 2003). The NADH/NAD^+, redox imbalance, mitochondrial abnormality and oxidative stress contribute to lung injury in diabetic rats (Wu et al. 2017). The excess generation of O_2^{-}• by mitochondria is related to tissue injury in diabetic rats (Wu et al. 2016). AGEs are overproduced as the irreversible products of non-enzymatic glycation of organic molecules such as proteins, nucleic acids and lipids in hyperglycemic environment (Nedić et al. 2013). The interaction of AGEs with their receptors so-called RAGE results in the production of O_2^{-}• by activating NADPH-oxidase and pulmonary inflammatory responses (Oczypok et al. 2017; Wautier et al. 2001). However, there is no significant alteration at the protein levels of RAGE despite increased ROS and MDA levels in the lungs of diabetic mice. It has been suggested that RAGE does not contribute to oxidative stress generation in mice lung under STZ-induced diabetes (Oztay et al. 2018). Inflammation, one of the several mechanisms contributing to pulmonary oxidative stress, can be potentially exacerbated by diabetes through the production of proinflammatory cytokines and the parenchymal infiltration of neutrophils and macrophages in rat lungs (Choi et al. 2008; Moral-Sanz et al. 2012). Neutrophils produce HOCl via abundantly MPO. The HOCl may interact with other ROS to yield $ONOO^-$, • OH, $^1O_2$ and O_3 (Hurst et al. 1991). MPO activities in the lung of the hyperglycemic rats are higher than in controls. Treatments with antidiabetic substances such as chard, insulin and chard plus insulin significantly result in the reduction of MPO activity, in the decreased MPO-induced ROS generation and consequently in the amelioration or reduction lung damage in hyperglycemic rats (Oztay et al. 2015). In addition to the above-mentioned mechanisms occur under hyperglycaemia-induced oxidative stress, XO is referred to as a new source of oxidative stress in the diabetic lung. It catalyses the oxidation of hypoxanthine to xanthine and can further catalyse the oxidation of xanthine to uric acid. Activated XO is involved in the O_2^{-}• production, which is converted to H_2O_2 by SOD or produces $ONOO^-$ by its reaction with NO•. XO is produced by pulmonary epithelial and endothelial cells in the lungs. Patients with COPD have a decrease in the oxidative stress, including GSH oxidation and LPO, when they receive allopurinol, an XO inhibitor (Dawson and Walters 2006). Several experimental studies on diabetes show an increase of the XO activity in the liver, heart, kidney and brain (Aliciguzel et al. 2003; Romagnoli et al. 2010). Additionally, another study reveals that the high XO activity contributes to the generation of ROS in the lung of hyperglycemic rat. The administration of chard extracts containing potent XO inhibitors such as polyphenols, flavonoids and saponins to diabetic rats significantly protects the lung against XO-derived ROS formation (Oztay et al. 2015; Mroczek et al. 2012).

The increased production of oxidants and the decreased in the capacity of pulmonary antioxidant defence system are the most effective reasons of the lung

cellular stress and structural damage in the lungs of diabetic mice, rats and rabbits (Ozansoy et al. 2005; Oztay et al. 2018; Gumieniczek et al. 2002; Kinalski et al. 2000). For this reason, antioxidant therapy combined with hypoglycemic agents is accepted as an effective strategy for the reduction of hyperglycaemia-induced complications in the lung. Strengthening the pulmonary antioxidant system after treatments of antidiabetic and antioxidant agents protects the lung from hyperglycaemia-induced oxidative damage, by the regulation of GSH redox cycle in the lungs of hyperglycemic rats (Oztay et al. 2015). Diabetes leads to a decrease in GSH level because of consumption of NADPH by polyol pathway. Thus, increased NADH causes ROS production by using NADH oxidases (Chung et al. 2003). Decreased levels of GSH and reduced activities of SOD, CAT and GP_x have been reported in plasma and tissues of diabetic patients and animals (Gumieniczek et al. 2009; El Boghdady and Badr 2012; Karthik and Ravikumar 2011). Because GSH is a substrate and cofactor of GST, the GST activity could be low due to the low GSH content. Diabetes depressed the GSH level and activity of several pulmonary antioxidant enzymes including SOD, CAT, GPx and GST in the lungs of STZ-treated rats and rabbits (Ozansoy et al. 2005; Oztay et al. 2015; Gumieniczek et al. 2009; Gumieniczek et al. 2002). In these animals, antidiabetic treatments including pioglitazone, repaglinide and chard improve the GSH level and antioxidant enzyme activities and then ameliorate structural lung damage. Additionally, insulin therapy reverts hyperglycaemia and removes the pulmonary oxidative/nitrosative stress by activating antioxidant enzymes and inhibiting the production of free radicals and reducing diabetic complications in the lung (Oztay et al. 2008, 2015; Sindhu et al. 2004). Exendin-4 is a peptide which regulates insulin production/secretion and reduces blood glucose in diabetes. The regression of hyperglycaemia and the reduced levels of the lung MDA and ROS via exendin-4 administration to diabetic mice attenuate hyperglycaemia-mediated abnormal structural alterations in the lungs (Oztay et al. 2018). Chard has the antioxidant properties in addition to its reducing effect of hyperglycaemia and protects against hyperglycaemia-induced tissue damage (kidney, liver and lung) (Oztay et al. 2015; Gezginci-Oktayoglu et al. 2014; Yanardag et al. 2002). Its antioxidant behaviour depends on the rich content with flavonoids and saponins which are known to have strong antioxidant and free radical scavenging properties. Chard treatments with and without insulin result in the reduction of hyperglycaemia-induced pulmonary oxidative stress, in the induction of pulmonary GSH level and in the antioxidant enzyme activities, thus contributing to the health of the lung in diabetic rats. Alternatively, exogenous SOD administration to diabetic rats could reverse structural changes in the lungs of these animals (Forgiarini Jr et al. 2010).

Diabetic lungs are characterized by the overproduction of ROS/RNS, the induced LPO, a decrease in the expression and/or activity of the antioxidant system members and oxidative stress-depending lung injury (Forgiarini et al. 2009; Liang et al. 2007). Diabetes contributes to the oxidative stress initiated by non-enzymatic sources. For example, auto-oxidation of glucose generates • OH. Chronically enhanced uptake of glucose can induce cell death by generating glucotoxicity. Oxidation of oxygen and the subsequent production of ROS increase after high

glucose consumption at mitochondrial electron transport system. The overproduction of mitochondrial ROS can damage the mitochondrial membrane structure and alter the pore permeability of mitochondrial membrane. Cytochrome c and other proteins released from open pores of mitochondrial membrane into the cytosol activate caspase-3 through the released proteins which promote cell apoptosis (Smart and Li 2007). In diabetic rat lung, high glucose results in redox imbalance, mitochondrial abnormalities and caspase-3-induced apoptosis (Wu et al. 2016). The pulmonary epithelium covering the inner surface of alveoli is highly sensitive to oxidative stress-induced cell death. The pulmonary epithelium consists of two types of epithelial cell: type 1 pneumocytes and type 2 pneumocytes. Type 1 pneumocytes are assigned for respiratory gas exchange. Because they have a poor antioxidant enzyme system, they are affected from oxidative stress and can die. The blood gas analysis showed some abnormalities in gas exchange of diabetic animals. They are characterized by a reduction in PaO_2 and an increase in $PaCO_2$, thus indicating a reduction in diffusion capacity (Forgiarini et al. 2009). Type 2 pneumocytes, having a more powerful antioxidant defence system, produce surfactant proteins which reduce surface tension in the alveoli. STZ disrupts the ultrastructure of type 2 pneumocytes, reduces the surfactant protein biosynthesis and impairs the secretion of surfactant proteins in the rat lungs (Foster et al. 2010). Diabetes reduces pro-surfactant protein-C and surfactant protein-D expressions in hyperglycemic rats (Oztay et al. 2015). Additionally, type 2 pneumocytes play a role in the regeneration of the pulmonary epithelium and the survival of pulmonary endothelium following lung injury. They proliferate and afterwards differentiate into the cells of the pulmonary epithelium as a response to tissue damage (Kayalar and Oztay 2014). Oztay et al. detect pulmonary epithelial cell hyperplasia and a lot of cleaved caspase-3^+ and proliferative cells in pulmonary epithelium of the diabetic mice (Oztay et al. 2018). These findings can be accepted as the signs of diabetes-mediated pulmonary epithelial death. On the other hand, hyperglycaemia-mediated ROS induces protein carbonylation leading to protein dysfunction. The protein carbonyl content is accepted as an early marker of oxidative damage to proteins. Increased protein carbonyl content occurs in human with type 1 and type 2 diabetes (Brownlee 2001) and diabetic animals (Cakatay et al. 2003; Dursun et al. 2005). Furthermore, it was found increased protein carbonyl content in diabetic rat lung (Eren et al. 2010). Increased protein carbonyl content is accompanied by the lung damage in hyperglycemic rats. Thus, the reduction of oxidative stress and protein carbonyl content by antidiabetic agents is effective on prevention of protein oxidation, which is thought to be involved in the lung damage in hyperglycemic rats (Oztay et al. 2015). Additionally, nuclear and mitochondrial DNA are exposed to oxidant attacks under the increased oxidative stress. The increased 8-OHdG level which is one of the predominant forms of free radical-induced oxidative lesions in patients with type 2 diabetes has been related to the occurrence of DNA damage in the diabetic lungs (Nishikawa et al. 2003).

17.4 Conclusion

It is well known that increased pulmonary oxidative stress is an important risk factor for the lung ageing, pulmonary fibrosis and diabetic lung injury. Although antioxidant treatments alone were effective on the decreased progression of the lung diseases, they were not completely successful in the improvement of the lung diseases or in the regression of ROS-/RNS-mediated structural and functional alterations in the lung. It appears that antioxidant therapies combined with various molecules effective on the regression of the disease pathogenesis, instead of antioxidant therapies alone, can be more useful in the treatment of lung diseases related to pulmonary oxidative stress. And also, large clinical trials are needed to perform their real beneficial effects.

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Mitochondrial Alterations and Oxidative Stress in Cystic Fibrosis

18

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Abstract

Cystic fibrosis (CF) is the most frequent autosomal recessive disease and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Since the discovery of the deletion in the phenylalanine 508 site (ΔF508) of the *CFTR* gene, the study of its function as chloride channel occupied most investigations. Now, we know that CFTR is also involved in the GSH and HCO_3^- transport, and its function could regulate the mitochondrial function and ROS production. In this way, the notion of the CFTR as a simple chloride channel has begun to change toward a more complex function as molecular hub that integrates different cellular signals. There is a growing body of evidence that shows mitochondrial dysfunctions and increased oxidative stress in CF. Here, we review the mitochondrial defects induced by the altered function of the CFTR in CF, focusing on oxidative stress and inflammation as targets for therapy.

Keywords

Cystic fibrosis · CFTR · Mitochondrial complex I · Reactive oxygen species · Innate immunity

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18.1 Introduction

Cystic fibrosis (CF) is an autosomal recessive multisystemic disorder characterized by chronic lung damage and exocrine pancreatic insufficiency, among other symptoms (Quinton 2007; O'Sullivan and Freedman 2009). However, the leading cause of death is lung failure in the first three decades of life (Zielenski 2000). Before 1989, the gene affected in this hereditary disease was unknown, and different theories were postulated on its etiology; but an unexpected breakthrough happened in that year when the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene was cloned from a CF patient, and a deletion of three base pairs was found in both copies of the gene (Riordan et al. 1989; Rommens et al. 1989; Tsui et al. 1988). The mutation caused the loss of a phenylalanine at position 508 of the CFTR protein (ΔF508) that impaired the correct folding of the channel. More than 2000 mutations have been described for the *CFTR* gene (Liu et al. 2017), although with an occurrence of 66 %, ΔF508 is the most abundant in CF patients (Kerem et al. 1997; Mateu et al. 2002). However, considering the same *CFTR* gene mutation type, it has been reported a different degree of severity of the disease, probably because of genetic and environmental influences, infectious events, and differences in treatments (Noone and Knowles 2001).

The discovery of the *CFTR* caused a profound impact in the future research of the CF disease that was focused in the study of its codified product. In this way, the CFTR was characterized as a membrane glycoprotein commonly located in the apical membrane of epithelial cells and has also been reported in non-epithelial cells from several tissues (Horowitz et al. 1993; Lange et al. 2006; Levesque et al. 1992; Mulberg et al. 1994; Tizzano and Buchwald 1993). Initially, the CFTR was reported as a cAMP-regulated chloride channel (Anderson et al. 1991; Bear and Ling 1993; Gregory et al. 1990; Jefferson et al. 1990; Rich et al. 1990; Rommens et al. 1991; Sheppard and Welsh 1999), although recently it has been also involved in the transport of bicarbonate (Chan et al. 2006) and glutathione (Kogan et al. 2003). This channel has an intracytoplasmic regulatory domain (R domain) that is activated by protein kinase A (PKA) and protein kinase C (PKC) (Schwiebert et al. 1999). Also, the analysis of the secondary structure of CFTR showed that it has two nucleotide-binding domains (NBD1 and NBD2) that hydrolyze ATP to regulate the channel activity (superfamily of ATP-binding cassette transporter proteins) (Schwiebert 1999). More recently, several reports suggest that CFTR could be acting as a calcium-activated chloride channel (reviewed in (Billet and Hanrahan 2013; Kunzelmann and Mehta 2013)). In fact, it has been reported the existence of CFTR binding to isolated calmodulin domains/lobes and a PKA-independent CFTR activation by calmodulin (Bozoky et al. 2017), supporting a possible role of CFTR as a molecular hub (Kunzelmann and Mehta 2013; Bozoky et al. 2017).

In the last few years, new evidence suggests that CFTR function is close related to some mitochondrial functions, particularly in the mitochondrial electron transport chain (mETC) and redox pathways, with possible consequences in the inflammatory response regulation in CF. Mitochondria are responsible to generate a large amount of energy for the most of cellular functions by producing ATP through the

oxidative phosphorylation process (Vakifahmetoglu-Norberg et al. 2017). This process supposes the consumption of 90–95% of the total cellular oxygen and leads to the production of anion superoxide (O_2^-) as a result of electron leakage from the mETC, principally from the mitochondrial complex I (mCx-I) and complex III (mCx-III) (Mailloux et al. 2013; Quinlan et al. 2013). The O_2^- production is a common consequence of the oxidative phosphorylation and is controlled by the antioxidant system of the cells. However, in pathological conditions, the mETC impairment leads to an increased mitochondrial ROS (mtROS) production that could not be scavenged with deleterious effects to the cell, such as lipid peroxidation and mitochondrial DNA (mtDNA) damage (Cadenas and Davies 2000). Also, the redox imbalance could cause an elevated oxidative stress state that leads to inflammation with increased production and secretion of pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and IL-8 (Nakahira et al. 2011; Oh et al. 2014; Yu et al. 2014; Rimessi et al. 2016; Valdivieso et al. 2018).

Here, we review the most significant findings on CFTR-induced mitochondrial defects and oxidative stress and its consequences on the pro-inflammatory phenotype observed in CF in absence of infection.

18.2 The Old Theory of Mitochondrial Defects

Before the discovery of the *CFTR* mutation, numerous reports of Shapiro and Feigal suggested the existence of mitochondrial abnormalities in CF (Feigal and Shapiro 1979; Feigal et al. 1982; Friedman et al. 2011; Shapiro 1988; Shapiro 1989; Shapiro et al. 1979; Shapiro et al. 1982). In 1979, they found alterations on mitochondrial calcium uptake and oxygen consumption (Feigal and Shapiro 1979) and alterations in the optimal pH of mtCx-I in fibroblasts isolated from CF patients (Shapiro et al. 1979). Afterward they reported that the increased calcium uptake in mitochondria was associated with an altered respiratory system activity (Feigal et al. 1982) and a reduced *Km* of the mCx-I in whole cell homogenates of CF cultured skin fibroblasts (Shapiro et al. 1982). Following these initial works, several studies reported mitochondrial changes in CF (Shapiro 1988; Shapiro 1989; Dechecchi et al. 1988; Feigal and Shapiro 1986; von Ruecker et al. 1984; Waller et al. 1984). However, when CFTR was cloned and found to be a chloride channel (Riordan et al. 1989; Rommens et al. 1989; Tsui et al. 1988), the hypotheses of metabolic alterations in CF were disregarded, and most of the work was focused on CFTR function.

18.3 The Mitochondrial Theory Revival

18.3.1 Mitochondrial Complex I Impairment

In the last years, several findings revived the earlier work of Shapiro and colleagues, suggesting the existence of mitochondrial defects in CF. In 2004, Day et al. (2004) found decreased glutathione levels in epithelial lining fluid of CFTR-knockout

mice, the last finding also in agreement with the early work of Shapiro et al. regarding glutathione (Shapiro 1973; Shapiro et al. 1974, 1975, 1976).

In 1994, Santa Coloma's laboratory began to study the CFTR-dependent gene expression by differential display analysis. Among them, it was reported the upregulation of c-Src (Gonzalez-Guerrico et al. 2002) and the down-modulation of *MT-ND4* (a mitochondrial DNA-encoded gene) (Valdivieso et al. 2007) and *CISD1* (a nuclear DNA-encoded gene) (Taminelli et al. 2008) in CF cells. Interestingly, *CISD1* encodes for a mitochondrial protein, also named as mitoNEET, that was found associated with proteins that belong to the mtCx-I (Colca et al. 2004). The crystal structure of this protein showed a [2Fe2S] cluster (Hou et al. 2007; Lin et al. 2007; Paddock et al. 2007; Wiley et al. 2007a) and several mitochondrial functions it have been proposed for this protein, like modulator of the oxidative capacity of cells (Wiley et al. 2007b), sensor of the intracellular redox state (Paddock et al. 2007; Zhou et al. 2010; Zuris et al. 2012), and mediator for the transference of a [2Fe-2S] cluster to apoproteins (Zuris et al. 2011, 2012), and it has recently been associated to the formation of intermitochondrial junctions (Vernay et al. 2017). However, the implication of this protein in CF has not been determined yet. On the other hand, *MT-ND4* is a mitochondrial gene that encodes for ND4 (MT-ND4) subunit of the mitochondrial complex I (mtCx-I) (Chomyn 2001) that is the entry point of electrons to the oxidative phosphorylation system (OXPHOS) (Carroll et al. 2006). ND4 subunit constitutes a fundamental component for the assembly and correct activity of mtCx-I, and its absence has been associated to some mitochondrial diseases (Chomyn 2001; Majander et al. 1991, 1996). Taking into account the downregulation of *MT-ND4* observed in CF and the importance of this subunit, we found a decreased mtCx-I activity in airway cell lines from CF patients (IB3-1 and CFDE cells) compared to the same cells ectopically expressing wt-CFTR (S9 and CFDE/6Rep cells) (Fig. 18.1) (Valdivieso et al. 2012; Valdivieso and Santa-Coloma 2013). Also, we demonstrated in colon carcinoma cells (T84 and Caco-2) that the inhibition of the CFTR activity, by pharmacological inhibitors or shRNAi, caused a decreased mCx-I activity (Valdivieso et al. 2012).

In agreement with our results, Kelly-Aubert et al. reported a decrease in mtCx-I activity in CF cells compared with cells expressing wt-CFTR and also by using a *CFTR*-knockout mouse (Kelly-Aubert et al. 2011). These authors proposed that the reduction in the activity of mtCx-I was caused by oxidation due to a decrease in the glutathione levels (Fig. 18.1) (Kelly-Aubert et al. 2011).

Recently, Atlante et al. performed a characterization of the mitochondrial function in airway cells either homozygous for the Δ508-CFTR allele or stably expressing wt-CFTR (Atlante et al. 2016). In agreement with the previous results, they found an impairment in oxygen consumption, mitochondrial membrane potential ($\Delta\Psi$) generation, adenine nucleotide translocator-dependent ADP/ATP exchange and both mitochondrial complex I and IV activities in CF cells (Atlante et al. 2016). Interestingly, it was observed an improvement in the mitochondrial parameters affected in CF cells by the treatment with the small molecules VX-809 and 4,6,4′ trimethylangelicin (correctors of Δ508-CFTR). These correctors restored the chloride secretion induced by CFTR to values found in the airway cells expressing wt-CFTR.

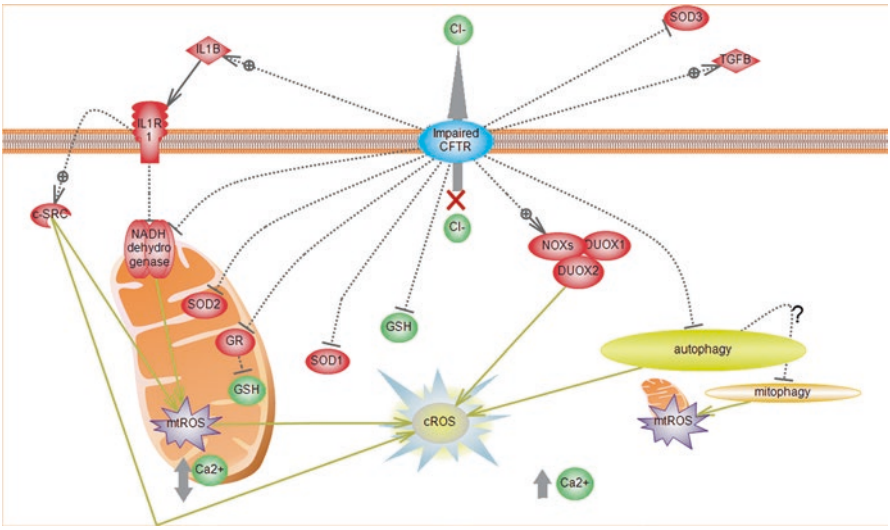

Fig. 18.1 Representative scheme of mitochondrial alterations and oxidative stress in CF. The graph illustrates the interactions among different proteins, kinases or small molecules involved in the mitochondrial alterations and ROS production caused by the impairment of the CFTR function. Arrows with the + symbol represent stimulations and those with ⊣ symbol represent inhibition. The (?) symbol indicates an unknown path. Green arrows indicate ROS generation. The figure was build using the Pathway Studio (v10, Elsevier).

Altogether, these observations suggest that the mtCx-I activity and the mitochondrial bioenergetics are altered in CF, thus proposing the mitochondrial function as a potential target for CF therapy.

18.3.2 Mitochondrial Calcium Homeostasis Implication in CF

Alterations in Ca^{2+} homeostasis have been largely studied in CF and show a complex relationship between CFTR and Ca^{2+} signaling (Feigal and Shapiro 1979; Feigal et al. 1982; Antigny et al. 2011a, b; Philippe et al. 2015, 2017; Martins et al. 2011; Balghi et al. 2011) (reviewed in (Antigny et al. 2011a)). This section will focus only in the mitochondrial Ca^{2+} homeostasis findings.

Diverse abnormalities in calcium homeostasis were observed in CF patients, previously to the discovery of the CFTR mutation (Blomfield et al. 1973, 1976; Botelho et al. 1973; Donnell and Cleland 1961; Wotman et al. 1971). For example, Feigal and Shapiro found increased intracellular calcium concentration ($[Ca^{2+}]_i$) in fibroblasts derived from CF patients (Feigal and Shapiro 1979) and an increased mitochondrial Ca^{2+} uptake attributed to alterations in mitochondrial oxidative phosphorylation (Feigal et al. 1982; Feigal and Shapiro 1986). In contrast, Antigny et al. found a decreased mitochondrial Ca^{2+} uptake in CF airway epithelial cells (ΔF508 homozygous) (Antigny et al. 2009). Through histamine stimulation, these

authors observed a reduced Ca^{2+} uptake in mitochondria from CF cells compared to non-CF cells (from human trachea serous gland cells) (Antigny et al. 2009). Also, the changes in mitochondrial Ca^{2+} homeostasis were linked to a decreased mitochondrial membrane potential (Ψm) caused by changes in the oxidative phosphorylation (Antigny et al. 2009).

Recently, Philippe et al. studied the impact of ΔF508-CFTR mutation on organelles Ca^{2+} homeostasis using targeted Ca^{2+} cameleon probes in CF (CFBE) and non-CF (16HBE) bronchial epithelial cell lines (Philippe et al. 2015). The authors found an increased endoplasmic reticulum (ER) Ca^{2+} content accompanied by deregulated SERCA and PMCA pump activities and mitochondrial Ca^{2+} alterations in CF cells. Considering that mitochondria play an important role in Ca^{2+} global homeostasis, in ER Ca^{2+} release and in Ca^{2+} influx, the impaired function of this organelle in CF could be critical for the cellular Ca^{2+} signaling. In contrast with previous observations of Antigny et al. (2009), these authors demonstrated that mitochondria Ca^{2+} uptake was increased in CF epithelial cells during store-operated Ca^{2+} entry (SOCE) stimulated by thapsigargin (TG) (Philippe et al. 2015). Interestingly, restoring the normal traffic of ΔF508-CFTR using the VX-809 corrector normalized SOCE in CF cells, although the ΔF508-CFTR correction was not enough to normalize the increased mitochondrial Ca^{2+} uptake observed. These results suggest that this alteration was independent of the ΔF508-CFTR accumulation in the ER (Philippe et al. 2015). Further research should be directed to study the mechanisms involved in the regulation of the mitochondrial Ca^{2+} uptake by the CFTR impairment.

18.4 Oxidative Stress and Inflammation in CF

On the last few years, there has been increase in the number of reports that demonstrate a close relationship between an oxidative stress and inflammation in CF (Day et al. 2004; Valdivieso and Santa-Coloma 2013; Kelly-Aubert et al. 2011; Atlante et al. 2016; Clauzure et al. 2014, 2017; Velsor et al. 2006; Massip-Copiz et al. 2017). The mechanisms proposed for the increased levels of ROS production are diverse and nonexclusive. Here, we will review the main findings about oxidative stress and the link with inflammation in CF.

18.4.1 Antioxidant Defense Defects

The expression and activity of the superoxide dismutases plays a fundamental role in the cellular antioxidant defense through the dismutation of O_2^- in oxygen (O_2) and H_2O_2. In this regard, it has been reported alterations in the protein expression of the superoxide dismutase Mn-SOD (SOD2, mitochondrial localization) and Cu/Zn-SOD (SOD1, cytosol localization) in pancreatic and tracheal cells derived from CF patients (Rottner et al. 2011). Also, the activity of extracellular SOD (EC-SOD, SOD3) was decreased in CF cells, without changes in its protein expression level (Rottner et al. 2011).

On the other hand, the ratio between reduced and oxidized glutathione (GSH/GSSG) is an important factor that affects the cellular redox balance and its susceptibility to ROS. In addition to the chloride transport function, the CFTR channel has been linked to the transport of extracellular glutathione (eGSH). Thus, it has been proposed that the deficiency in eGSH transport through the CFTR in CF might cause an imbalance between GSH/GSSG outside cells (Kogan et al. 2003; Gao et al. 1999; Linsdell and Hanrahan 1998; Velsor et al. 2001), producing a decreased antioxidant protection of the extracellular space against oxidative stress (Velsor et al. 2001). In agreement with these observations, Velsor et al. reported a decrease in the mitochondrial GSH (mGSH) in *CFTR*-knockout mice (*CFTR* −/−) and in tracheal cells from CF patients, accompanied by an increased ROS production (Velsor et al. 2006). Also, Kelly-Aubert et al. have reported low mGSH levels, associated to a decrease in the mtCx-I activity, in CF cells and *CFTR*-knockout mice. These authors also showed that the mCx-I defect was reverted to control by treating cells with GSH monoethyl ester, a membrane-permeable analog of GSH (Kelly-Aubert et al. 2011).

Another defect in the antioxidant defense in CF has been reported by Trudel et al. that observed a decreased expression and activity of the peroxiredoxin 6 (Prdx6) enzyme in *CFTR*-knockout mice (Trudel et al. 2009). Prdx6 through GSH as an electron donor reduces H_2O_2, fatty acid hydroperoxides, and phospholipid hydroperoxides (Trudel et al. 2009; Fisher et al. 1999). This protein plays an important role in the defense against oxidative damage in lung (Wang et al. 2006).

In agreement with the existence of an alteration in the antioxidant defense in CF, de Bari et al. have recently reported a decreased glutathione reductase (GR) activity. This reduction was not dependent on the protein level in CFBE41o- cells (CF cells expressing the ΔF508 mutation) (de Bari et al. 2018). Also, these authors found increased levels of the activity and expression of NADPH oxidase (NOX), suggesting that an impaired CFTR causes an imbalance between the NOX and GR that contribute to the GSH decrease and the ROS overproduction (de Bari et al. 2018).

18.4.2 Mitochondrial and Cellular ROS Overproduction

The alteration in the antioxidant defense components could be produced or accompanied by a deregulated increase in ROS production, generating an oxidative vicious circle and enhancing the oxidative stress in CF.

In this regard, the decrease of the mCx-I induced by the malfunction of the CFTR channel could be responsible for an increase in both mitochondrial (mtROS) and cellular ROS (cROS) production (Valdivieso and Santa-Coloma 2013; Kelly-Aubert et al. 2011; Atlante et al. 2016; Clauzure et al. 2014), contributing to an increased oxidative stress. In this way, we have reported, both in IB3-1 CF cells and in Caco-2/ pRS26 cells (colon cancer cells transfected with CFTR shRNAi), increased levels of the pro-inflammatory cytokine IL-1β. Interestingly, the increase in IL-1β levels induced, through an autocrine effect, the inhibition of the mCx-I activity and partially the ROS production (Fig. 18.1) (Clauzure et al. 2014). The IL-1β effect on

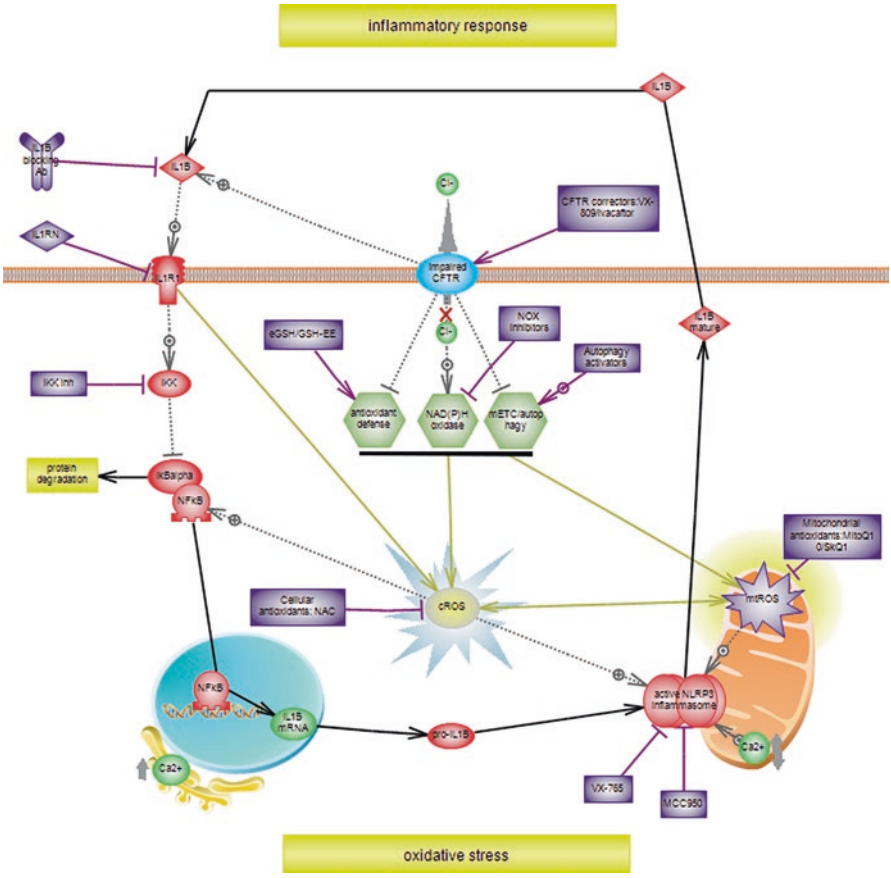

Fig. 18.2 Graphical abstract of oxidative stress and inflammation in CF. The graphic summarizes the alterations that were reported to include oxidative stress and inflammation in CF. In purple are indicated the possible targets of treatment in CF. Arrows with the + symbol represent stimulations and those with ⊣ symbol represent inhibition. Green arrows indicate ROS generation. The figure was build using the Pathway Studio (v10, Elsevier).

mCx-I and ROS levels, both mtROS and cROS, was reversed by using an anti-IL-1β monoclonal antibody that blocks IL-1β signaling, an interleukin-1 receptor antagonist (IL1RN), or using pharmacological inhibitors of NF-kB activation (IKK inhibitor III/BMS-345541) (Fig. 18.2) (Clauzure et al. 2014). Since the IL-1β autocrine loop was observed in the absence of bacterial infections, this effect appears to be the primary characteristic of CF cells. Thus, the autocrine effect of IL-1β could act as a bridge connecting the activity of the CFTR with the activity of the mCx-I and the ROS production.

The *CFTR* mutation has been recently associated to an impaired defense of neonatal alveolar macrophages (AM), via the TGF-β signaling and GSH alteration

(Fig. 18.1), in a mouse KO-CFTR model and macrophage-like cell line (THP-1) transfected with siRNA-CFTR (Gauthier et al. 2017). The treatment of the KO-CFTR AM with exogenous GSH was able to reduce mtROS generation and TGF-β expression (Gauthier et al. 2017). Alterations in neonatal AM phenotype support the idea that a redox imbalance might cause defects in the immune defense system before infection in CF. Thus, a precondition to the initiation of a lung inflammation/infection process could be determined for the CFTR deficiency.

In addition to the increase in mtROS generation, some NADPH oxidases (NOXs family) (Bedard and Krause 2007) have been also implicated in the increased oxidative stress observed in CF. In particular, the NADPH oxidases DUOX1/2 and NOX2 activities were increased in the epithelial line CFTR deficient IB3-1 cells, when these cells were incubated in an hypertonic environment, compared to control S9 cells (IB3-1 cells expressing wt-CFTR) (Pongnimitprasert et al. 2012). In these conditions, the pro-inflammatory cytokines IL-6 and IL-8 were increased in IB3-1 cells, and this increment was inhibited by treatment with diphenyleneiodonium (DPI), a NOX inhibitor. These data suggest a role of NOXs in the pro-inflammatory cytokine production mediated by oxidative stress in airway CFTR-deficient cells (Pongnimitprasert et al. 2012).

In agreement with these results, we have found in IB3-1 CF cells and Caco-2 cells with downregulated CFTR (shRNAi-CFTR) an increased c-Src activity that was regulated by the IL-1β autocrine loop. The inhibition of the c-Src activity with PP2 inhibitor decreased the cROS and mROS levels observed in CFTR-deficient cells to those observed in wt-CFTR control cells (Massip-Copiz et al. 2017). Interestingly, the inhibition of NOX 1–4 with GKT137831 inhibitor restored the cROS levels in CFTR-deficient cells compared with control cells. These results suggest that the oxidative stress observed in CF could be mediated, at least in part, through an increased activity of NOXs induced by the CFTR impairment (Fig. 18.1) (Massip-Copiz et al. 2017). However, further research is needed to determine a relation between c-Src and NOXs activities.

Altogether, the pathways involved in both mtROS and cROS overproduction could be considered as potential targets for the treatment of the inflammatory phenotype observed in CF.

18.4.3 Oxidative Stress and Autophagy in CF Inflammation

Oxidative stress leads to damage of cellular macromolecules such as DNA, lipids, and proteins (Halliwell 2007). The upregulation of autophagy by oxidative stress has been proposed for several pathologies and cellular models (Azad et al. 2009; Kiffin et al. 2006; Ryter and Choi 2015; Chen et al. 2007, 2009; Scherz-Shouval et al. 2007). In this context, the autophagy degrades oxidized substrates such as protein and phospholipids (Zhao et al. 2013), cleaning malfunctioning mitochondria that function as sources of ROS by a process known as mitophagy (Kubli and Gustafsson 2012).

In the last years, several studies suggest an impaired autophagy in CF with diverse consequences in this pathology, linking this alteration with inflammation in sterile condition (Fig. 18.1). Luciani et al. reported a ROS-mediated defective autophagy and lung inflammation that act through a vicious cycle of oxidation and inflammation (Luciani et al. 2010). Interestingly, these authors showed that the autophagy activation improves CFTR trafficking to the plasma membrane and reduces inflammation (Luciani et al. 2010, 2011, 2012). In agreement with these results, other authors have reported that the inactivation of the autophagy in macrophages induces mtROS production with a consequent activation of the NLRP3 (NODlike receptor pyrin domain containing 3) inflammasome in response to inflammatory stimuli (Nakahira et al. 2011; Ryter and Choi 2015; Schroder and Tschopp 2010; Zhou et al. 2011). Thus, considering that NLRP3 inflammasome activation is involved in the maturation and secretion of the pro-inflammatory cytokines IL-1β and IL-18, defective autophagy and oxidative stress would be intimately related to the pro-inflammatory process observed in CF.

On the other hand, the effect of the autophagy function has been studied in cells infected with bacteria. Abdulrahman et al. reported that treatment with rapamycin, an agent used to stimulate autophagy, rescued the defective autophagy in ΔF508-CFTR mouse macrophages, decreasing the infection with *Burkholderia cepacia* by enhancing the bacterial clearance (Abdulrahman et al. 2011). In this way, Junkins et al. have also reported that enhancement of autophagy in vivo was effective to promote bacterial clearance during lung *Pseudomonas aeruginosa* infection in mast cells and bronchial epithelial cells (Junkins et al. 2013).

Interestingly, it has recently been reported that activation of the autophagy, by different strategies, could improve ΔF508-CFTR stability, trafficking, expression, and function in the cell membrane, in some cases in combination with CFTR correctors such as VX-809 (Hutt et al. 2018; Reilly et al. 2017; Tazi et al. 2016).

These results highlight autophagy as a potential therapeutic target for CF in combination with the use of potentiators on ΔF508-CFTR function and antioxidants (Fig. 18.2). Thus, elucidate the underlying mechanisms of the autophagy alteration could be an interesting approach to improve CF treatments (Luciani et al. 2012; Hutt et al. 2018; Reilly et al. 2017; Tazi et al. 2016; Junkins et al. 2014; Zhang et al. 2018; Vu et al. 2017; De Stefano et al. 2014).

18.4.4 NLRP3 Inflammasome in CF

When talking about mitochondrial alterations, oxidative stress, and inflammation, it is inevitable to mention the activation of the NLRP3 inflammasome. This complex is formed by three proteins: NLR family pyrin domain containing 3 (NRLP3), caspase-1 (CASP1), and apoptosis-associated speck-like protein containing a CARD (ASC/PYCARD). The NLRP3 inflammasome activation is involved in IL-1β and IL-18 maturation and secretion (Gross et al. 2011) and requires two signals: (1) the priming signal that induces the upregulation and accumulation of IL-1β and inflammasome components and (2) signal of activation of the NLRP3 inflammasome

components in mitochondria to form the inflammasome complex, with the help of cardiolipin in the external mitochondrial membrane. Then a self-cleavage and activation of pro-CASP1 leads to the cleavage of pro-IL-1β to produce mature IL-1β. The activation of the NLRP3 inflammasome occurs by different danger- and pathogen-associated molecular patterns (DAMPs, PAMPs) (Dela Cruz and Kang 2018; Hennig et al. 2018) or by noncanonical activation (Kayagaki et al. 2015); all DAMPs and PAMPs signals appear to be integrated through mtROS signaling (Dela Cruz and Kang 2018).

The mitochondrial defects and oxidative stress reported in CF could be involved in the NLRP3 inflammasome activation, producing the inflammatory phenotype reported in this disease. As mentioned in the previous section, the inactivation of the autophagy and/or the mtROS induction caused by the CFTR impaired function could lead to the activation of the NLRP3 inflammasome and the maturation and secretion of the pro-inflammatory cytokines IL-1β and IL-18 (Nakahira et al. 2011; Ryter and Choi 2015; Schroder and Tschopp 2010; Zhou et al. 2011).

Recently, Rimessi et al. (Rimessi et al. 2015) reported mitochondrial perturbations dependent of *P. aeruginosa* infection involved in the inflammatory response in CF and that was mediated by Ca^{2+} signaling with participation of the mitochondrial Ca^{2+} uniporter (MCU) (Rimessi et al. 2015). Thus, this work suggests that Ca^{2+} homeostasis alterations in CF, in addition to oxidative stress, could have an important role in the NLRP3 activation and IL-1β maturation.

However, some caution should be taken regarding the mechanisms involved in the inflammatory phenotype observed in CF. Recently, Iannitti et al. (Iannitti et al. 2016) reported that the sustained production of the IL-1 receptor antagonist (IL-1Ra) could counteract the NLRP3 activity by contribution of the NLRC4 inflammasome (Iannitti et al. 2016). NLRC4 activation, like NLRP3 activation, is also sensitive to calcium signaling; although contrary to the NLRP3 activation, Ca^{2+} restrains NLRC4 activation (Wen et al. 2013). Defective NLRC4 activity has been observed in murine and human CF, for instance, in Nlrc4 −/− mice it has been suggested that NLRC4 may act as a negative regulator of NLRP3 (Cohen and Prince 2013). In this regard, Iannitti et al. reported that defective NLRC4 activation in murine and human CF bronchial epithelial cells resulted in an impaired production of IL-1Ra, which has the capacity to inhibit NLRP3 inflammasome activation (Iannitti et al. 2016).

18.5 Concluding Remarks

The elucidation of the molecular mechanisms by which CFTR regulates the mitochondrial abnormalities reported in CF (Fig. 18.1) requires further investigation. Altogether, differential expression of genes and alterations on mitochondrial calcium homeostasis, complex I activity, increased reactive oxygen species, diminished cellular antioxidant defenses, autophagy defects, and cytokines production, all appear to occur because of CFTR failure (Fig. 18.1). These alterations might produce changes on inflammatory responses and predispose cells with impaired CFTR to be more sensible to posterior bacterial infection than normal cells.

Figure 18.2 is a resume of the complexity of the relationships among CFTR activity, mitochondria, and oxidative stress described in this chapter. The net result supposes the production of pro-inflammatory cytokines from epithelial cells, previous to bacterial infection. Even though the source of the increased pro-inflammatory cytokines is still to be defined, their levels in sputum of CF patients reflect high lung levels that could lead to lung damage. The increased cytokines levels and inflammasome activation might explain the chronic lung inflammation and damage observed in CF patients. How these alterations might influence the innate immune response to infections in CF requires further research.

A complete understanding of these interactions, their mechanisms of action, and the relative importance of each pathway will help to better define the CF phenotype and clinical manifestations and to find possible new targets for CF therapy (Fig. 18.2).

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Part IV

Degenerative Lung Diseases

Oxidative Stress in Chronic Obstructive Pulmonary Disease

19

Mariana A. Antunes, Fernanda F. Cruz, and Patricia R. M. Rocco

Abstract

Chronic obstructive pulmonary disease (COPD), which comprises chronic bronchitis and emphysema phenotypes, is characterized by airflow obstruction and pulmonary inflammation and tissue disruption. It is also associated with several extrapulmonary manifestations and frequently occurs simultaneously with other disorders that have an additive influence on patients' quality of life. COPD is usually a disease of aging, and the main risk factor for its development is smoking. A particularly remarkable etiologic factor that drives COPD pathogenesis is oxidative and carbonyl stress which originates in the pulmonary milieu after prolonged exposure to cigarette smoke or to the by-products of combustion of biomass fuels. The fact that COPD progresses even after smoking cessation is probably attributable to numerous exogenous and, especially, endogenous sources of reactive oxygen species (ROS), although this involvement has yet to be proven. The function of key cells, as well as the levels of significant oxidant and antioxidant molecules, may be dysregulated in COPD. Oxidative stress has been related to increased expression of proinflammatory genes, inability to resolve inflammation, insensitivity to corticosteroids, impairment of endogenous antioxidant defenses, accelerated lung senescence, and elevated risk of developing emphysema.

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19.1 Introduction

Chronic obstructive pulmonary disease (COPD) is an important global health issue and is expected to become the third foremost cause of death worldwide by 2020. COPD is an avoidable, treatable disease, which is characterized by progressive airflow limitation that may be fully or partly irreversible (Decramer et al. 2012). This limitation is frequently linked to an augmented chronic inflammatory response in the airways and lungs, especially to noxious particles or gases. Exacerbations and concomitant disorders contribute to disease severity in individual patients (Decramer et al. 2012; Mirza et al. 2018). COPD results from a combination of genetic susceptibility and environmental exposures (Barnes 2000), and oxidative stress is considered one of the main predisposing factors for the development of this disorder.

Oxidative stress is characterized by a disequilibrium between oxidants generated by reactive oxygen species (ROS) and production of endogenous antioxidants. There are two main sources of oxidants in COPD: endogenous and exogenous (Fig. 19.1) (Marotta et al. 2011). The lungs are directly exposed to approximately 8000 L of air daily, containing oxygen, numerous pathogens, pollutants, and allergens (Canbaz et al. 2016; Meo and Suraya 2015). Several of these are environmental sources of airborne oxidative stress, including oxidant gases and ultrafine particulate material and nanoparticles from industrial pollution and car exhaust fumes (Kodgule and Salvi 2012). Each puff of cigarette smoke carries 10^{17} oxidant molecules (Yao and Rahman 2011). This exposure causes direct damage to airway epithelial cells, resulting in airway inflammation, involving several immune cells (i.e., neutrophils, macrophages, lymphocytes). Endogenous oxidants are generated by mitochondrial respiration (Liu et al. 2002) or by the inflammatory process. COPD patients exhibit greater activation of alveolar macrophages and peripheral blood neutrophils, with consequent increases in the release of ROS (such as the superoxide anion, O^{2-}; the hydroxyl radical, OH^{-}; and hydrogen peroxide, H_2O_2); if insufficiently counterbalanced by antioxidant factors, these will lead to further damage, including to lipids, proteins, and DNA (Fischer et al. 2015; Kirkham and Barnes 2013). Furthermore, COPD patients who experience frequent exacerbations express lower levels of glutathione (the main human endogenous antioxidant) in the bronchoalveolar lavage fluid (BALF) than stable COPD patients. Cigarette smoke may lead to COPD onset; however, once the disease is established, stopping smoking does not interrupt the oxidative stress process, and, consequently, disease

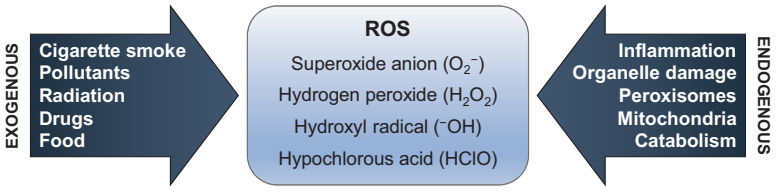

Fig. 19.1 Exogenous and endogenous sources of reactive oxidative species, such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and hypochlorous acid

progression occurs. The perpetuation of this oxidative stress is due to the persistence of endogenous sources, such as mitochondrial respiration.

Several oxidative stress biomarkers are known, including ROS. As ROS are in general too reactive and present a very short half-life, which hinders their direct detection in tissues or body fluids, it is more feasible to "measure" oxidative stress by detecting target products of oxidation, including lipid peroxidation end products and oxidized proteins. The exhaled breath of COPD patients may reveal enhanced markers of oxidative stress (e.g., hydrogen peroxide, carbon monoxide, and myeloperoxidase [MPO]), oxidative tissue damage (e.g., 8-isoprostane), and carbonyl stress (e.g., malondialdehyde [MDA]) (Kluchova et al. 2007). Additionally, increased levels of carbonyl adducts, such as 4-hydroxynonenal (Rahman et al. 2002), may also be suggestive of oxidative stress in COPD patients (Barreiro et al. 2010).

19.2 Cellular Sources of ROS in COPD

19.2.1 Neutrophils

Neutrophils are the major effector cells in COPD. Aberrant neutrophil phenotypes have been observed in patients with COPD, including such features as high speed and low migration accuracy (Sapey et al. 2011), high levels of activation and degranulation surface markers (CD63 expression), and activation of phosphoinositide 3 kinases (PI3K) (Sapey et al. 2014), a family of intracellular signal transducer enzymes that is linked to increased inflammation and proteinase activity (Sapey et al. 2011). Additionally, the neutrophils of COPD patients are reported to secrete elevated levels of ROS (Noguera et al. 2001; Rahman et al. 1996; Renkema et al. 1993). This inaccurate neutrophilic migration may result in unnecessary movement across a larger surface area and increased degranulation. Degranulation products, including inflammatory cytokines (i.e., interleukin 8), ROS, and neutrophil elastase, increase inflammatory signaling and protease charge in the lungs. Intracellular ROS excess alters the migratory accuracy of neutrophils by interacting with and reducing activity of the enzyme phosphatase and tensin homolog (PTEN) (Kuiper et al. 2011), which, in turn, allows buildup of phosphatidylinositol (3,4,5)-triphosphate, an important phosphoinositide related to the neutrophil migration.

Neutrophil proteinases and ROS are importantly correlated. Neutrophil elastase, a proteinase released during neutrophil activation, digests many extracellular matrix proteins (Korkmaz et al. 2008), contributing to apoptosis of epithelial cells (Ginzberg et al. 2001) and mucus hypersecretion by goblet cells (Takeyama et al. 1998). Neutrophil elastase is inhibited by α1-antitrypsin (α1AT) (Gadek et al. 1981). However, ROS may oxidize the methionine 358 residue in the active site of α1AT (Carp et al. 1982; Matheson et al. 1979), leading to the inactivation of this enzyme and propagating lung inflammation. Neutrophil myeloperoxidase catalyzes the oxidation of chloride ions (Cl^-) by H_2O_2 to generate the anionic ROS hypochlorite (OCl^-) or its conjugate acid, hypochlorous acid (HClO). HClO reacts with low-molecular-weight amines (i.e., nicotine) to produce chloramines that cross cellular membranes and induce intracellular protein damage (Fig. 19.2) (Salama and Snapka 2012).

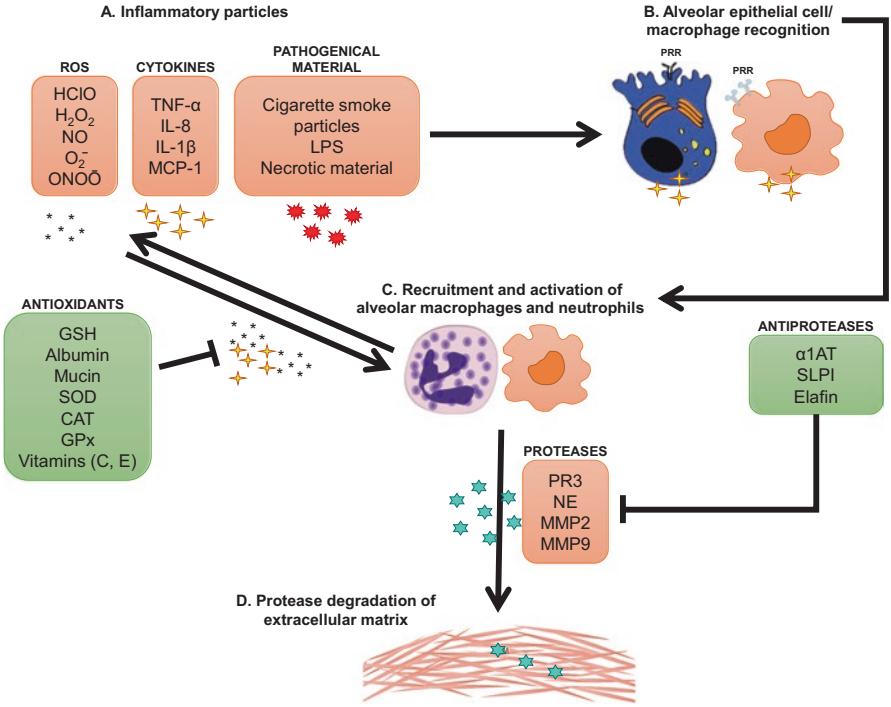

Fig. 19.2 Process of tissue damage in COPD, adapted from McGuinness and Sapey (McGuinness and Sapey 2017). (**a**). Influx of inflammatory particles to the lung. (**b**). Particles bind PRRs on the surface of lung epithelial cells or alveolar macrophages, initiating a signaling cascade that drives release of inflammatory cytokines. (**c**). Cytokines lead the recruitment, migration, and activation of peripheral neutrophils and monocytes, which mature into alveolar macrophages and locate in the alveolar surface. Activation of these cells culminates in further release of cytokines, proteases, and ROS, perpetuating inflammatory cell recruitment and activation. Antiproteases and antioxidants are physiologically present at very high levels, preventing excess damage to surrounding tissues and limiting a positive feedback loop. During COPD, these levels may be low, resulting in increased areas of tissue damage. (**d**). If poorly inhibited, proteases degrade extracellular matrix, leading to tissue destruction and features seen in COPD. O_2^- superoxide anion, *NO* nitric oxide, *SOD* superoxide dismutase, H_2O_2 hydrogen peroxide, *CAT* catalase, *GPx* glutathione peroxidase, *MPO* myeloperoxidase, *HClO* hypochlorous acid, $ONOO^-$ peroxynitrite, *TNF-α* tumor necrosis factor alpha, *IL-1β* interleukin 1β, *IL-8* interleukin 8, *MCP-1* monocyte chemoattractant protein 1, *LPS* lipopolysaccharide, *PRR* pattern recognition receptor, *GSH* glutathione, *PR3* proteinase 3, *NE* neutrophil elastase, *MMP9* matrix metalloproteinase 9, *α1AT* alpha-1 antitrypsin, *SLPI* secretory leukocyte protease inhibitor

19.2.2 Monocytes/Macrophages

Alveolar macrophages exert a key role in the resolution of inflammation by removing apoptotic or necrotic neutrophils through efferocytosis. In COPD, macrophages appear to secrete large amounts of ROS, proinflammatory cytokines (Aldonyte et al. 2003), and enzymes (Russell et al. 2002), as well as exhibit reduced intracellular

concentrations of thiol, an oxidative stress marker (Tager et al. 2000). In COPD, after macrophage activation, the bronchiolar epithelium exhibits increased expression of monocyte chemoattractant protein 1 (MCP-1), while macrophages present increased expression of the receptor C-C chemokine receptor type 2 (CCR2) (Tomaki et al. 2007), resulting in increased monocyte recruitment, which correlates with disease severity (Di Stefano et al. 1998). Finally, neutrophil elastase may cleave the phosphatidylserine receptor (Vandivier et al. 2002), a necessary factor for the recognition of apoptotic cells by macrophages, thus contributing to reduced efferocytosis in macrophages of patients with COPD, reduced bacterial clearance, and perpetuation of an inflammatory milieu.

Iron metabolism is also dysregulated in COPD-affected lungs. The overexpression of iron regulatory protein 2 and hemosiderin causes cellular and mitochondrial deposition of iron in alveolar tissue and macrophages (Cloonan et al. 2017; Mohan et al. 2017). In COPD, alveolar macrophages exhibit augmented expression of transferrin and ferritin, which mediate iron uptake and storage (Philippot et al. 2014). In smokers, iron-laden macrophages secrete high amounts of ferritin (Plautz et al. 2000; Wesselius et al. 1994), which helps catalysis of oxidative stress reactions in the alveoli.

19.3 Mitochondrial-Derived ROS in COPD

Continued oxidative stress after the cessation of cigarette smoking in COPD arises from endogenous sources (Kirkham and Barnes 2013), among which is mitochondrial respiration. Ordinarily, the mitochondrial electron transport chain "leaks" up to 2% of all electrons as ROS (Liu et al. 2002; Boveris and Chance 1973). Cigarette smoke also contains lipophilic components, including polycyclic aromatic hydrocarbons, aldehydes, amines, heavy metals, and phenolic compounds, which can easily cross cell membranes and reach the circulation. The lipophilic fraction of cigarette smoke accounts for a reduction in mitochondrial membrane potential, ATP production, and concomitant generation of mitochondrial ROS (van der Toorn et al. 2009).

Inflammatory cytokines play an important role in increasing production of mitochondrial ROS. When the airway smooth muscle cells of COPD patients are exposed to interleukin (IL)-1, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, larger amounts of mitochondrial-derived ROS are released (Kirkham and Barnes 2013). Matrix metalloproteinase (MMP)-2 appears to be implicated in mitochondrial damage: mitochondrial-derived ROS drive MMP-2 activation, resulting in a negative feedback loop that degrades mitochondrial membrane potential and impairs mitochondrial function (Zhou et al. 2007). MMP-2 seems to activate β-adrenergic receptor-stimulated activation of JNKs as well as cytochrome *c* release, inducing the JNK-dependent mitochondrial death pathway (Menon et al. 2006).

Protease-antiprotease imbalances are crucial for the pathogenesis of COPD. Cathepsins are one of the proteases secreted by neutrophils and macrophages that are significantly involved in this disease. One in particular, cathepsin E (CE), has been closely related to mitochondrial dysfunction in COPD. Although CE

has no proteolytic activity, its overexpression in COPD lungs contributes to pulmonary emphysema development by augmenting mitochondrial fission via induction of dynamin-related protein 1, Parkin, and the ubiquitin-proteasome system, favoring the mitochondrial fusion-fission imbalance and increasing caspase 3-mediated cell death (Fig. 19.3) (Zhang et al. 2014).

Rtp801, also known as Redd1 ("regulated in development and DNA damage responses"), is protein related to adverse environmental conditions and may be triggered by hypoxia or DNA damage. Expression of this protein, which is responsible for the inhibition of cell growth and protein synthesis driven by the mammalian target of rapamycin (mTOR) via activation of a negative regulator of mTOR, TSC-2 (tuberin), is upregulated in lungs with advanced emphysema. mTOR is a master regulator of cell growth, nutrient metabolism, protein synthesis, mRNA translation, cell motility, survival, and autophagy (Huang and Fingar 2014; Tang et al. 2014). Pharmacological or genetic inhibition of mTOR alters mitochondrial dynamics, shifting them toward increased mitochondrial fusion (Hayat 2014).

Homeostasis of mitochondrial morphology is kept in a fine balance between two converse processes, fusion and fission (Scheffler 2007; Tzagoloff 1982). Generally, fission is necessary to preserve an adequate amount of mitochondria during cellular growth and cell division, while fusion allows the unification of mitochondrial compartments. Mitochondrial fusion has been reported as a strategy to protect cells against mitochondrial DNA (mtDNA) mutations by allowing functional complementation of mtDNA gene products (Chan 2006). Elongated mitochondria are preserved from autophagy, exhibit more cristae and augmented activity of ATP

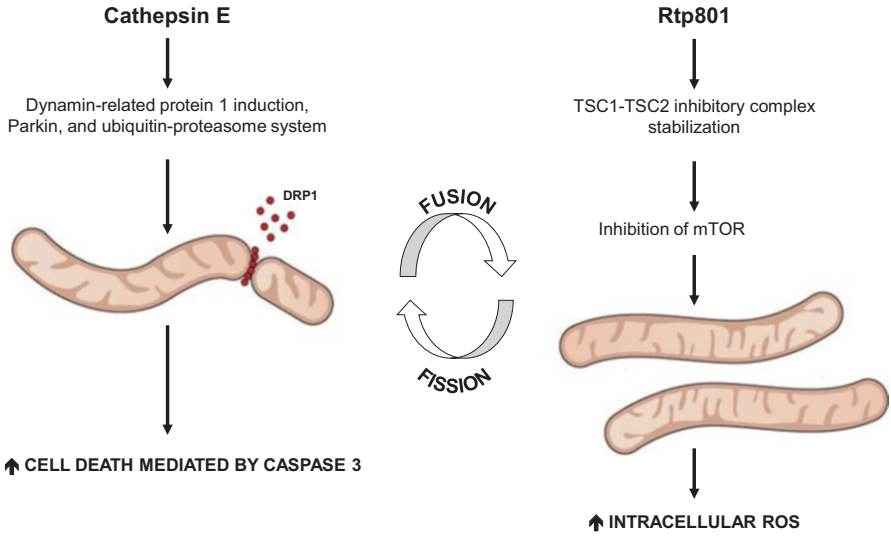

Fig. 19.3 Hypothesis for the impact of cathepsin E and Rtp801 on mitochondrial dynamics, adapted from Bialas et al. (2016). *DRP1* dynamin-related protein 1, *TSC1-TSC2* tumor suppressor genes mutated in the tuberous sclerosis complex syndrome, which constitute an inhibitory complex of mTOR, *mTOR* mammalian target of rapamycin, *ROS* reactive oxygen species

synthase, and are also able to maintain ATP production (Gomes et al. 2011). Nevertheless, prolonged permanence of mitochondria in the elongated state may lead to higher production of intracellular ROS and lower mitochondrial respiration, conducting to cellular senescence (Yoon et al. 2006), which is a major factor in the pathogenesis of COPD (Conti et al. 2015).

19.4 Oxidative Stress and Biological Molecules

ROS react with biological molecules, including lipids, proteins, DNA, RNA, and mitochondrial DNA, and lead to epithelial cell injury and death, which contributes to COPD progression. The major outcome of this process is the production of reactive carbonyls and their interaction with proteins, also known as protein carbonylation. Carbonylation is increasingly recognized as a major driver of the underlying pathology associated with several chronic diseases (Negre-Salvayre et al. 2008). In COPD, the process of reactive carbonyl accumulation and subsequent protein carbonylation, known as carbonyl stress, correlates with disease severity, which can be deduced by the decline in FEV_1 (forced expiratory volume in the first second) (Rahman et al. 2002).

ROS also induce lipid peroxidation, which is simultaneously a consequence of oxidative stress and a cause of oxidative damage. Lipids are susceptible to oxidation by both enzymatic and nonenzymatic oxidants. Protein cross-linkage and inactivation of several cellular proteins may be promoted by the lipid peroxidation products (Siu and Draper 1982). In COPD, they stimulate pulmonary inflammation (Rahman and Adcock 2006), leading to alveolar wall destruction and emphysema development. 4-Hydroxy-2,3-nonenal is a lipid peroxidation product associated with accumulation of cytoplasmic Ca^{2+}, expression of nuclear factor-kappa B (NF-κB) and proinflammatory cytokines, mitochondrial dysfunction, and apoptosis (Breitzig et al. 2016; Horvath et al. 2001). NF-κB expression and activation is increased in COPD and positively correlates with airflow limitation (Di Stefano et al. 2002). Inflammatory pulmonary diseases, including COPD, are characterized by accentuated expression of inflammatory genes that are regulated by inflammatory transcription factors, including NF-κB. Gene expressions are regulated by acetylation of central histones via coactivators that exhibit intrinsic histone acetyltransferase (HAT) activity. In contrast, gene repression is mainly mediated via histone deacetylases (HDACs) and other corepressors. Carbonylation reduces the activity and expression of a crucial factor for the suppressive effect of corticosteroids on activated inflammatory genes, the transcriptional corepressor histone deacetylase 2 (HDAC2) (Ito et al. 2004; Meja et al. 2008). Oxidative stress activates the enzyme PI3K-δ, which also leads to reduced activity and expression of HDAC2 in COPD patients. Downregulation of HDAC2 activity decreases the stability of the transcription factor nuclear erythroid-2-related factor 2 (Nrf2), which regulates many cellular antioxidant and detoxification enzymes, and controls the expression of many antioxidant and cytoprotective genes (Kobayashi and Yamamoto 2006). The oxidative stress-dependent reduction of another protein deacetylase, sirtuin (SIRT)-1, induces elevation of MMP9 expression in COPD. Sirtuins are

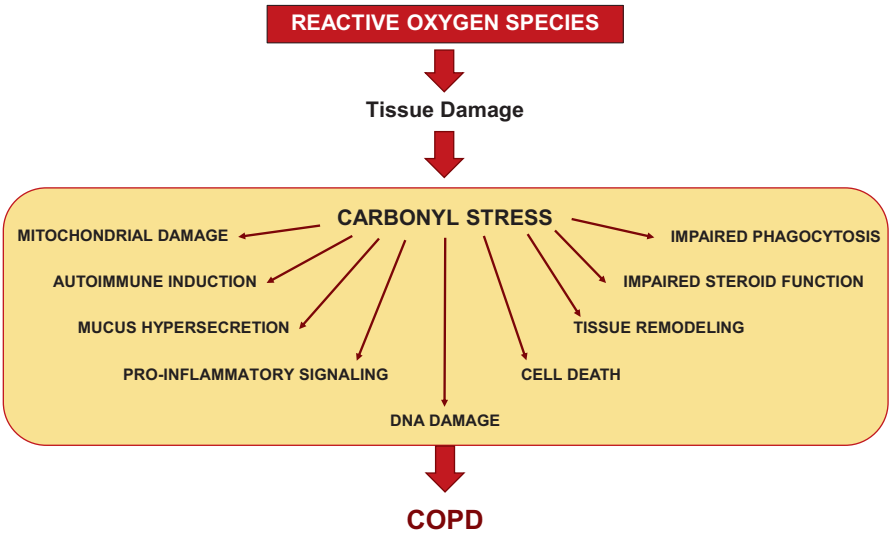

Fig. 19.4 Role of reactive oxygen species and carbonyl stress in the pathophysiology of COPD. Carbonyl adducts are associated with higher COPD mortality and morbidity, since they induce DNA and mitochondrial damage, impair phagocytosis and steroid function, induce autoimmune and proinflammatory signaling, and promote cell death, extracellular matrix remodeling, and mucus hypersecretion

enzymes that catalyze NAD^+-dependent deacetylation in target proteins and have been associated with aging, metabolism, and stress resistance. Their downregulation seems to accelerate the aging process and, consequently, contribute to pulmonary emphysema development (Nakamaru et al. 2009).

Phagocytosis is impaired in COPD (Donnelly and Barnes 2012), and impaired removal of apoptotic cells induces necrosis and helps to perpetuate the inflammatory component of COPD (Vandivier et al. 2006). Oxidative/carbonyl stress can affect phagocytosis by intracellular or extracellular mechanisms. Intracellularly, oxidative stress activates RhoA (Ras homolog gene family, member A), which impairs phagocytosis by inducing abnormalities in cytoskeletal reorganization (Richens et al. 2009). Outside the cell, oxidative/carbonyl stress results in the carbonylation of tissue proteins, which promotes competition for the same pattern recognition receptors on the surface of alveolar macrophages which are responsible for recognizing and phagocytizing proteins modified by carbonyl and apoptotic cells (Fig. 19.4) (Kirkham et al. 2004).

19.5 Reduced Antioxidant Defense in COPD

The lungs are continuously exposed to external and endogenous sources of oxidative stress. For this reason, the respiratory system relies on several strategies for achieving efficient antioxidant defenses, where reduced glutathione (GSH) is

particularly relevant. A significant portion of the total glutathione produced within the mitochondria is intended to balance endogenous production of ROS as a metabolism by-product (Rahman et al. 2005). The synthesis of GSH is regulated by the expression and activity of the glutamate-cysteine ligase enzyme (Shi et al. 1994). However, bronchial epithelial cells and alveolar macrophages of smokers and patients with COPD reveal reduced amounts of GSH (Harju et al. 2002), as well as other GSH-dependent antioxidant enzymes, including class-pi glutathione S-transferase (GST), glutathione S-transferase mu 1 (GSTM1), and glutathione peroxidase (Tomaki et al. 2007). As a protection mechanism against lung exposure to environmental oxidants, epithelial lining fluid is reported to possess several antioxidants, such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), and uric acid. There is a clear correlation between reduced levels of antioxidants (such as α-tocopherol and ascorbic acid) in the lung and pulmonary dysfunction in COPD (Fig. 19.5). Moreover, long-term supplementation with antioxidants has been shown

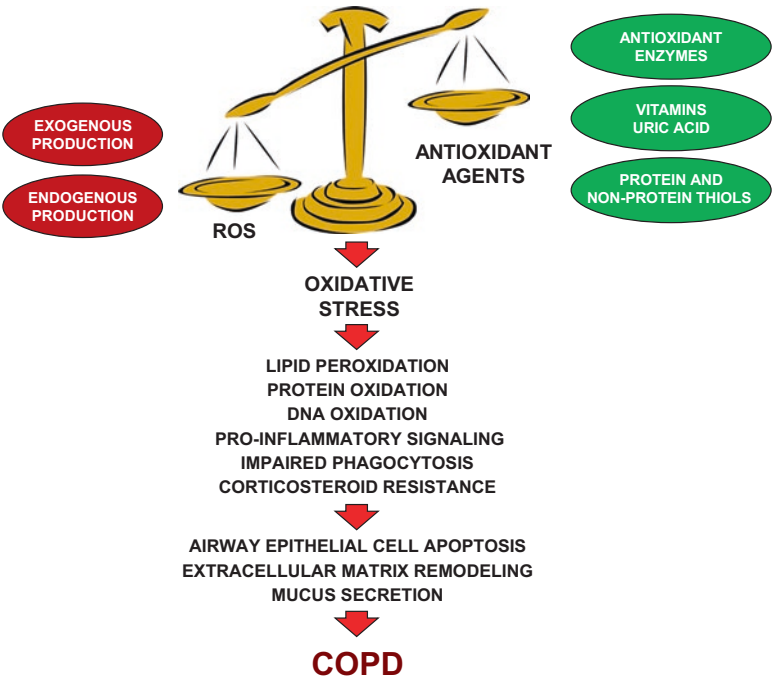

Fig. 19.5 Role of oxidative stress in the pathophysiology of COPD, adapted from Zinellu et al. (2016). The oxidant-antioxidant imbalance leads to harmful lung damage. Oxidative stress activates intracellular signaling pathways that lead to the release of inflammatory mediators, impair phagocytosis of apoptotic cells, and weaken the ability of corticosteroids to repress inflammatory gene expression. Inflammation, lipid peroxidation, protein oxidation, and DNA damage lead to tissue damage, alteration of protein functions and gene expression, extracellular matrix remodeling, and mucus secretion

to reduce the risk of developing chronic lung disease by 10%, as well as reduce carbonyl stress in the lungs (Agler et al. 2011; de Batlle et al. 2010).

Expression of transforming growth factor-β (TGF-β) is often increased in the lungs of patients with COPD. High levels of TGF-β inhibit expression of the antioxidant enzymes catalase and superoxide dismutase 2 (SOD2, also known as manganese-SOD) in airway smooth muscle cells (Michaeloudes et al. 2011).These enzymes play a critical role in counterbalancing mitochondrial ROS and are regulated by a transcription factor known as FOXO3. However, deficient FOXO3 activity has been described in COPD (Hwang et al. 2011).

19.6 Conclusion

High activity of ROS and metalloproteases has been implicated in all stages of COPD. As proteases are involved in physiological processes such as the immune response as well as in pathological conditions, further research into the molecular pathways of COPD is warranted to help define protease-based therapeutic targets.

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Oxidative Stress-Induced Autophagy Impairment and Pathogenesis of Chronic Obstructive Lung Diseases

20

Manish Bodas and Neeraj Vij

Abstract

Chronic lung diseases are one of the foremost ailments in the modern society, and a burgeoning increase in their occurrence is a significant challenge for the medical professionals as well as basic and clinical researchers. Based on the pathophysiology of the disease, chronic lung diseases can be classified into chronic "obstructive" or "restrictive" lung diseases. Irrespective of the lung disease condition, the two most common and interrelated mechanisms that initiate and/or worsen disease pathogenesis and progression are inflammation and oxidative stress. Hence, in this chapter, we first describe the central role of oxidative stress in the pathogenesis of chronic obstructive lung diseases and subsequently focus on the interrelationships of oxidative stress with aging, CFTR dysfunction, and the key homeostatic processes, proteostasis and/or autophagy. Moreover, we also present a perspective on targeting oxidative stress for augmentation of proteostasis and/or autophagy to control the pathogenesis of chronic obstructive lung diseases as well as to promote healthy lung aging.

20.1 Introduction

Chronic lung diseases are one of the foremost ailments in the modern society, and a burgeoning increase in their occurrence is a significant challenge for the medical professionals as well as basic and clinical researchers. Based on the

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pathophysiology of the disease, chronic lung diseases can be classified into chronic "obstructive" or "restrictive" lung diseases. Primarily, subjects with chronic obstructive lung diseases, such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), asthma, and bronchiectasis, have difficulty in exhaling air out of their lungs due to narrowing of the airways. Conversely, people with chronic restrictive lung diseases, such as idiopathic pulmonary fibrosis (IPF), sarcoidosis, amyotrophic lateral sclerosis (ALS), and interstitial lung disease (ILD), have trouble in inhalation, due to stiffness in the lung walls, resulting in an inability to fully expand their lungs (WebMD). Although chronic restrictive pulmonary diseases such as IPF are comparatively more severe and have a life expectancy of only 2–4 years once diagnosed (Fraser and Hoyles 2016), chronic obstructive lung diseases are much more widespread and thus present a significant socioeconomic burden on humans worldwide. Irrespective of the lung disease condition, the two most common and interrelated mechanisms that initiate and/or worsen disease pathogenesis and progression are inflammation and oxidative stress. The initiation of inflammatory-oxidative stress in the lungs stems from numerous factors such as exposure to all forms of tobacco smoke [first- and secondhand cigarette smoke (CS), e-cigarettes, waterpipe smoke] (Vij et al. 2018; Ni et al. 2015; Bodas et al. 2016a; Shivalingappa et al. 2015), biomass smoke (Capistrano et al. 2017), environmental air pollution (e.g., wildfires) (Roscioli et al. 2018), occupational agents (De Matteis et al. 2017), genetic mutations (e.g., CF) (Ziady and Hansen 2014), and lung infections (Nichols and Chmiel 2015; Rogers and Cismowski 2018). Exposure to these hazardous chemical and/or infectious agents results in the generation of reactive oxygen (or nitrogen) species (ROS/RNS), which functions as an integral component of the body's inherent immune defense mechanism(s) (Thannickal and Fanburg 2000; Deng et al. 2012). Although tightly regulated, low levels of ROS generation are protective during the initial stages of injury or infection (Zuo et al. 2015), but if ROS is not nullified efficiently by the body's integral antioxidant defense mechanisms, it promotes cellular- and tissue-specific deleterious phenomena termed "oxidative stress" (Zuo et al. 2015). Briefly, activation of specific inflammatory-immune cells upon exposure to external agents is the major source of ROS/RNS (Zuo et al. 2015; Bullone and Lavoie 2017; Domej et al. 2014; Lao et al. 2014), which could damage cellular proteins, lipids, and DNA (Phaniendra et al. 2015; Boukhenouna et al. 2018). This cellular damage, if left uncontrolled, results in hampering of key cellular homeostatic mechanisms such as cell proliferation (Matés et al. 2008), proteostasis and/or autophagy (Bodas et al. 2012), inflammatory signaling, etc. (Domej et al. 2014; Lao et al. 2014; Holguin 2013). The collective influence of these dysregulations leads to initiation and progression of chronic obstructive lung diseases (Holguin 2013; Comhair and Erzurum 2002). Therefore, it is obvious that controlling the elevated inflammatory-oxidative stress forms the rationale for numerous strategies utilized for preventive and therapeutic applications in numerous lung disorders (Rahman and Adcock 2006; Liguori et al. 2018; MacNee 2001), and several comprehensive articles have described the benefits and limitations of targeting ROS-mediated oxidative stress in chronic lung diseases (Bullone and Lavoie 2017; Boukhenouna et al. 2018; Rahman and Adcock 2006; Villegas et al. 2014; Ciofu and

Lykkesfeldt 2014; Galli et al. 2012). Hence, in this chapter, we first describe the central role of oxidative stress in the pathogenesis of chronic obstructive lung diseases and subsequently focus on the interrelationships of oxidative stress with aging, CFTR dysfunction, and the key homeostatic processes, proteostasis and/or autophagy. Moreover, we also present a perspective on targeting oxidative stress for augmentation of proteostasis and/or autophagy to control the pathogenesis of chronic obstructive lung diseases as well as to promote healthy lung aging.

20.2 Role of Oxidative Stress in Pathogenesis of Chronic Obstructive Lung Diseases

The scientific know-how about the crucial role of oxidative stress in lung diseases is well documented. Due to its direct contact with the external environment, our respiratory system is recurrently exposed to noxious agents that trigger the first line of inflammatory-immune defense mechanisms to protect self and eliminate the chemical or biological toxins. Generation of ROS is one such initial cellular protective response which plays a vital role in regulating numerous cellular functions in response to the external stimulus (Schieber and Chandel 2014). Since ROS is highly reactive and has the capacity to participate in chemical reactions to generate other more harmful toxic metabolites such as peroxynitrite (McGuinness and Sapey 2017), it is absolutely essential that an equilibrium is maintained (McGuinness and Sapey 2017). Thus, nature has bestowed upon us the inherent antioxidant defense mechanisms to overpower the uncontrolled activation of ROS (Comhair and Erzurum 2002; Rahman and Adcock 2006; McGuinness and Sapey 2017; Lü et al. 2010; He et al. 2017; Boutten et al. 2010). In certain circumstances, these protective mechanisms falter, leading to an imbalance of prooxidants and antioxidants, which forms the basis of initiation and progression of chronic oxidative stress-mediated obstructive lung diseases (Bullone and Lavoie 2017; Domej et al. 2014; Galli et al. 2012; MacNee 2005; Fischer et al. 2015; Ahmad et al. 2012). In the pulmonary environment, this imbalance may arise due to high ROS generation triggered by external stimuli, as well as endogenous sources causing oxidative stress (Boukhenouna et al. 2018). The subsequent deleterious effects of oxidative stress on lung cellular injury are mediated primarily via DNA damage (McGuinness and Sapey 2017; Neofytou et al. 2012; Zahiruddin et al. 2018; Chan et al. 2017; Bacsi et al. 2016), protein oxidative damage (Boukhenouna et al. 2018; Rahman and Adcock 2006; Ciencewicki et al. 2008), lipid peroxidation (Domej et al. 2014; Ciencewicki et al. 2008), and impaired phagocytosis (Ni et al. 2015; Boukhenouna et al. 2018; Pehote et al. 2017; Bodas et al. 2018a; Phipps et al. 2010; Tschernig et al. 2015; Berenson et al. 2013; Vandivier et al. 2009). These cellular changes manifest into complex disease-causing pathophysiological changes such as airway remodeling, mucus metaplasia, airway or cellular apoptosis, and senescence (Zinellu et al. 2016). Thus, oxidative stress-mediated changes to the lung cellular structure and function play a central role in the development of chronic lung diseases such as COPD, CF, and asthma (Holguin 2013; Park et al. 2009), even

though the source(s) of initiating oxidative stress might be different (Park et al. 2009).

As an example, oxidative DNA damage to lung epithelial as well as endothelial cells (Neofytou et al. 2012) and peripheral blood mononuclear cells (PBMCs) (Paschalaki et al. 2013) is related to COPD pathogenesis (Caramori et al. 2011). Moreover, both DNA and RNA damage in the alveolar wall cells of emphysema subjects correlate with emphysema disease severity and thus could be used as good indicators of COPD-emphysema pathogenesis (Deslee et al. 2009). Oxidative stress-mediated DNA damage leads to generation of 8-hydroxyguanine (8-OH-Gua) and its 2′-deoxynucleoside equivalent, 8-hydroxy-2′-deoxyguanosine (8-OHdG) (Igishi et al. 2003), which are both by-products of damage to the DNA base guanine. 8-OHdG is a common and useful marker of oxidative DNA damage in the peripheral lung of smokers and COPD subjects (Caramori et al. 2011), although few studies suggest no significant difference in 8-OHdG levels between COPD subjects and normal controls (Barreiro et al. 2013). Additionally, elevated levels of 8-OHdG are reported in bronchial and alveolar epithelial type II cells upon CS exposure in animal models (Aoshiba et al. 2003), indicating that smoking-induced oxidative DNA damage contributes to lung cellular injury. Moreover, oxidative stress-related damage to lipids, another crucial biomolecule, is well documented as an important mechanism of COPD-emphysema pathogenesis (Zinellu et al. 2016). Most commonly, when ROS is generated near the plasma membrane, it causes oxidation of membrane phospholipids (Rahman and Adcock 2006), which is termed as "lipid peroxidation." In COPD subjects, increased levels of malondialdehyde (MDA), a product of lipid peroxidation, have been extensively documented as a biomarker of oxidative stress (Zinellu et al. 2016; Paliogiannis et al. 2018). Some studies also claim that plasma MDA levels correlate with increasing severity of COPD lung disease (Zinellu et al. 2016; Arja et al. 2013; Ahmad et al. 2013; Kluchová et al. 2007; Bartoli et al. 2011). Once generated, MDA has the potential to damage proteins by forming protein cross-linkages (Siu and Draper 1982) that eventually ensues sustained inflammation resulting in alveolar wall damage and initiation of emphysema (Arunachalam et al. 2010). Other products of lipid peroxidation are also implicated in COPD pathogenesis, such as 4-hydroxy-2,3-nonenal (Takimoto et al. 2012), acrolein (Moretto et al. 2012), and F2-isoprostanes (Rahman and Adcock 2006; Zinellu et al. 2016), which have been shown to have numerous deleterious effects such as neutrophil chemoattraction, induction of NF-κB-mediated inflammation (Yadav and Ramana 2013), mitochondrial dysfunction (Anderson et al. 2012), smooth muscle constriction (Voynow and Kummarapurugu 2011), and cell death (Hauck and Bernlohr 2016). Apart from the above lipid peroxidation products, other bioactive lipids generated from sphingolipid metabolism, such as ceramide and lactosylceramide, play a crucial role in COPD-emphysema pathogenesis (Bodas et al. 2011a, 2015, 2018a). In fact, intratracheal instillation of ceramide leads to alveolar cell death and emphysema-like phenotype in mice (Petrache et al. 2005, 2006), and inhibitors of de novo ceramide synthesis protect mice from *Pa*-LPS-induced lung injury (Bodas et al. 2011a). Moreover, lung ceramide levels are elevated in human subjects with smoking-induced emphysema (Bodas et al. 2011a;

Elias and Lee 2005), which implies that ceramide accumulation is an important mediator of COPD-emphysema pathogenesis and a prominent therapeutic target (Tibboel et al. 2013). Apart from DNA, RNA, and lipids, oxidative damage to proteins is equally pathogenic in COPD-emphysema. Elevated ROS levels can lead to both reversible and irreversible changes in protein structure and thus modify their functions (Cai and Yan 2013). Protein carbonylation is the most common by-product of oxidative damage to proteins (Kirkham and Barnes 2013; Kirkham et al. 2011), and as expected, elevated levels of protein carbonyl groups are found in the plasma of COPD subjects (Kluchová et al. 2007; Kirkham et al. 2011), with some studies establishing a positive correlation between increasing protein carbonyls and disease severity (Rahman et al. 2002). Moreover, oxidized proteins can trigger NF-κB-mediated inflammatory gene activation and depletion in endogenous anti-proteases, which can facilitate COPD-emphysema pathogenesis (Kirkham and Rahman 2006). Another aspect of oxidative modification of proteins in COPD lungs is the increased amount of misfolded and/or damaged proteins (Chen et al. 2018; Kelsen 2016; Kenche et al. 2013) and inefficient protein turnover mechanisms such as the proteasome and autophagic machinery (Balch et al. 2014; Monick et al. 2010; Somborac-Bacura et al. 2013). These key homeostatic mechanisms are meant to clear off the misfolded or damaged nonfunctional proteins, which otherwise tend to form toxic cellular aggregates or "aggresomes" (Balch et al. 2014; Min et al. 2011). We and others have clearly demonstrated the crucial role of CS-induced ROS activation and resulting impairment of proteostasis and/or autophagy mechanism(s) promoting COPD-emphysema pathogenesis, using both in vitro and in vivo (murine) preclinical studies, and human COPD lung tissue samples with increasing severity of emphysema (GOLD 0 to GOLD IV) (Vij et al. 2018; Bodas et al. 2012; Tran et al. 2015; Bodas et al. 2016b, 2016c; van Rijt et al. 2012; Fujii et al. 2012).

Similar to COPD, CF subjects also possess elevated levels of oxidative stress biomarkers (Galli et al. 2012), thus indicating that oxidative stress-mediated cellular injury plays a crucial role in CF lung disease pathogenesis (Ziady and Hansen 2014; Hector et al. 2014). Elevated urine 8-OHdG levels are supportive of the notion that oxidative DNA damage is present in CF subjects, although these authors did not find a correlation between increased 8-OHdG levels and lung function decline (Brown et al. 1995). In another study, two widely accepted markers of oxidative stress, 8-OHdG and TBARS, were elevated in the lungs of CFTR KO mice (Galli et al. 2012; Day et al. 2004), thereby confirming the presence of inherent oxidative stress in the absence of functional CFTR, independent of other causal factors such as infection or exposure to environment noxious agents. In addition, increased levels of other lipid peroxidation and protein oxidation markers are also reported in CF patients (Olveira et al. 2017), providing further solid evidence of the pathogenic role of oxidative stress in CF. One of the most potent antioxidant peptides protecting the lung epithelial lining is "glutathione" (GSH), and CFTR is the predominant GSH transporter (Gould et al. 2012), although other transporters are also known. Diminished GSH levels are reported in epithelial lining fluid (ELF), bronchial lavage fluid (BALF), plasma, and blood neutrophils of CF subjects (Galli et al. 2012; Gould et al. 2012), indicating that lower than normal GSH level in the lung

and the peripheral immune system is an important biomarker of oxidative stress in CF (Roum et al. 1993).

Airway dysfunction in asthma, another chronic obstructive lung disease, is also directly associated with elevated oxidative stress (Bullone and Lavoie 2017; Erzurum 2016; Sahiner et al. 2011). Oxidative damage to DNA is reported in bronchial epithelial cells (Beas2B cells) exposed to house dust mite (HDM), a potent aeroallergen known to induce allergic asthma symptoms (Chan et al. 2017). The direct effect of HDM exposure on Beas2b cell cytotoxicity was attributed to ROS-/RNS-mediated DNA damage as quantified by CometChip assay and staining for DNA double-strand break marker, γH2AX (Chan et al. 2017). In another study, the ovalbumin (OVA)-induced murine model of asthma was used to demonstrate that oxidative stress-mediated DNA damage existed in the airways of asthmatic mice (Wang et al. 2018a). Moreover, in the OVA-induced mice, elevated inflammatory cell influx, especially eosinophils, and markers of lipid peroxidation, as well as antioxidant defense, correlate with lung function impairment (Wang et al. 2018a). In human subjects with asthma, several reports indicate increased levels of oxidative stress markers in the sputum (Antus 2016). Specifically, markers such as 8-OHdG, MDA, 8-isoprostane (only in severe asthma subjects), and 3-nitrotyrosine are elevated in asthmatic subjects (Antus 2016), further asserting the crucial role of redox imbalance in asthma pathogenesis.

The correlation of lung disease development with aging is buttressed by numerous studies indicating that aged individuals are more prone to oxidative insults (Hanania et al. 2011). This results from an age-related gradual decrease in the inherent antioxidant and DNA repair mechanisms (Hanania et al. 2011). The "oxidative stress theory of aging" describes that the progressive accumulation of oxidized cellular molecules such as DNA, proteins, and lipids, due to accumulation of ROS/RNS with age, leads to organ dysfunction (Salmon et al. 2010), including the lungs (Meiners et al. 2015). Some newer studies have challenged the oxidative stress theory (Gladyshev 2014), based on the findings where, in some models, overexpression of antioxidant proteins does not increase the life span (Gladyshev 2014; Mockett et al. 2010). Moreover, the finding that anaerobic organisms also age, and their life span is shorter than cells grown in aerobic conditions, further provides proof that ROS accumulation may not be the primary cause of aging (Moskovitz et al. 2001). Nonetheless, several recent studies still support the notion that increasing oxidative/nitrative stress with aging plays a crucial role in the advent of age-related chronic diseases, such as cardiovascular disease (CVD), COPD, asthma, neurodegenerative diseases, chronic kidney diseases, and diabetes (Liguori et al. 2018; Tan et al. 2018). Thus, several biomarkers of oxidative damage have been identified in the aging individual, and a recent review describes these markers in the specific context of the different age-related diseases (Liguori et al. 2018). Additionally, it is widely agreed upon that exposure to noxious agents such as CS, etc., leads to premature or accelerated lung aging, which contributes significantly to pathogenesis of COPD-emphysema (Mercado et al. 2015).

20.3 Mechanisms of Oxidative Stress Generation in the Airways

The generation of oxidative stress in the airways is regulated by several factors that may be endogenous or exogenous (Valavanidis et al. 2009). The most predominant reactive oxygen molecule is "ROS" that can be either generated endogenously within the cell or can enter our airways upon exposure to external noxious agents such as cigarette smoke (CS), allergens, infections, heavy metals, certain drugs, air pollutants including the indoor air pollution from burning biomass fuels (Chan et al. 2017; Postma et al. 2015; Paulin and Hansel 2016; Assad et al. 2016; Rushton 2007; Jomova and Valko 2011), and poor diet (Agrawal and Prakash 2014; Baffi et al. 2016; Li et al. 2016). In the airway, the activation of myriad types of inflammatory and/or other cells such as macrophages, neutrophils, eosinophils, lymphocytes, and epithelial/endothelial cells leads to the generation of superoxide ($O_2^{\bullet -}$), which is converted to H_2O_2 by the enzyme superoxide dismutase (SOD) (Park et al. 2009). Although H_2O_2 is not a free radical, it can form the highly reactive hydroxyl radical ($^{\bullet}OH$) as a secondary reaction with ferrous ion (Fe^{2+}) via the Fenton or Haber-Weiss reaction (Park et al. 2009). The action of the neutrophil-specific enzyme, myeloperoxidase (MPO), can catalyze H_2O_2 to generate hypochlorous acid (HOCl), which is a potent oxidizing species (Salama et al. 2014). Additionally, another reactive species, nitric oxide (NO), is formed from L-arginine by the action of the enzyme nitric oxide synthase (NOS), and this NO can further react with $O_2^{\bullet -}$ to form the highly reactive peroxynitrite (ONOO-) (Beckman and Koppenol 1996), which is capable of extensive cellular damage in the lung through its potent oxidizing property (Virág et al. 2003; Saleh et al. 1998). The predominant sources of endogenous ROS generation include the NADPH oxidase enzyme complex, mitochondrial oxidative phosphorylation (OXPHOS), and cytochrome P-450 (Park et al. 2009), and all these contribute to the pathogenesis of oxidative stress-related lung diseases.

20.4 Mechanisms of Oxidative Stress-Induced Lung Aging and Chronic Obstructive Lung Disease Pathogenesis

The continuous process of aging leads to a progressive buildup of ROS, which gradually inflicts its damaging effects on all cellular biomolecules such as DNA, proteins, and lipids (Liguori et al. 2018; Finkel and Holbrook 2000). The obvious impact of these changes is a time-dependent decline in organ function and finally organ failure (Meiners et al. 2015; Kirkwood 2005). Like other organs in the body, our lungs also age, and this begins not in old age, but right after we reach the age of 25 years. Our lung function as measured by forced expiratory volume slowly declines after age 20–25, by about 1% per year without any disease (Hanania et al. 2011; Sharma and Goodwin 2006). Moreover, structural and molecular/cellular changes in the lungs are evident as the lung ages, which include increased inflammation, enlarged airspaces, decreased overall surface area, and diminished elastic recoil (Mercado et al. 2015; Fukuchi 2009). Lung aging is now considered an

important risk factor for the incidence of COPD (Kukrety et al. 2018), and several reports support the hypothesis that CS (or other environmental factors) induces oxidative stress in the airways which is the primary mechanism that triggers classical features of aging, albeit much earlier in the life (Mercado et al. 2015; Ito and Barnes 2009). This forms the basis of the concept of "accelerated lung aging," where several hallmark features of normal aging, such as telomere shortening (Cordoba-Lanus et al. 2017), epigenetic changes, impaired proteostasis, dysregulated cellular repair potential due to stem cell exhaustion, cellular senescence, and autophagy impairment, become evident in the lungs of middle-aged COPD subjects (Meiners et al. 2015). It should be noted that normal levels of ROS are required to maintain healthy aging and thus aging is not the cause of COPD incidence, rather a risk factor. This means that exposure to CS or other noxious agents generates higher than normal levels of ROS, which is an add-on to the gradual protective increase in ROS levels in the aging individual. This results in excessive ROS accumulation that mediates chronic inflammation, alveolar or airway cell senescence, endothelial cell dysfunction, and loss of alveolar epithelial cells, resulting in COPD-emphysema pathogenesis (Mercado et al. 2015). Mechanistically, ROS mediates induction of PI3K/mTOR pathway and resulting autophagy decline is observed in aging and COPD subjects (Mercado et al. 2015), and one can envision that exposure to CS and environmental factors accelerates the mTOR activation, thus contributing to COPD pathogenesis.

Aging and COPD are also associated with decrease in mitochondrial biogenesis and function which when coupled with impaired mitophagy (Ahmad et al. 2015) results in accumulation of damaged mitochondria that are an important source of endogenous ROS. Recent studies have refuted the concept of free radical theory of mitochondrial damage with aging (Meiners et al. 2015; Sanz and Stefanatos 2008), similar to the emerging ideas that the free radical theory of aging is dead (Gladyshev 2014). Nonetheless, the key role of mitochondrial ROS in COPD is supported by the findings that antioxidants such as superoxide dismutase, catalase, heme oxygenase, and glutathione are related to mitochondrial function in COPD (Slebos et al. 2007). Moreover, changes in mitochondrial shape (fragmentation) are observed in bronchial epithelial cells from COPD subjects, which is also evident in cells exposed to CS (Mercado et al. 2015; Mizumura et al. 2014; Hara et al. 2013). Treatment with CSE disturbs the critical balance between mitochondrial fusion and fission, thus disturbing mitochondrial functions (Mizumura et al. 2014; Hara et al. 2013). Moreover, mechanistic studies performed in human airway smooth muscle cells (ASM cells) demonstrate that CS exposure increased the expression of dynamin-related protein 1 (Drp1), the mitochondrial fission protein, with a concurrent decrease in the mitochondrial fusion protein, mitofusin 2 (Mfn2), and this was attributed to CS-induced ROS activation (Aravamudan et al. 2014). Further, these changes in the mitochondrial structure correlate with diminished mitochondrial function in ASM cells (Aravamudan et al. 2017); and the ASM of CS-exposed mice exhibit pronounced modulation of proteins involved in mitochondrial function (Aravamudan et al. 2017), thus confirming the role of CS/ROS in altering mitochondrial homeostasis in the airways. Since the distally located alveolar epithelial cells

are crucial in emphysema pathogenesis, the response of these cells to CS in terms of mitochondrial function is important to understand. In a study using mouse alveolar epithelial cells (MLE12) and primary mouse alveolar epithelial cells, it was reported that mild and nontoxic doses of CSE caused mitochondrial hyperfusion, with a simultaneous increase in Mfn2 and elevated mitochondrial metabolism (Ballweg et al. 2014). The authors explain this as an adaptive pro-survival response of mitochondria in alveolar epithelial cells, as a mechanism to manage the mild stress generated by nontoxic CSE concentrations (Ballweg et al. 2014). Further, a sustained mitochondrial hyperfusion might render the mitochondria more susceptible to stresses such as chronic CS exposure, via attenuation of mitochondrial quality control (Figge et al. 2013). Additionally, hyperfused or elongated mitochondria are resistant to autophagic removal (mitophagy) (Gomes and Scorrano 2013) and make the mitochondrial network more susceptible to continuous stress, as observed in aging muscle cells. Thus, the authors conclude that CSE-induced mitochondrial hyperfusion might be a protective response initially but may play a crucial role in age-related COPD pathogenesis through increased cellular senescence and dysregulated mitochondrial quality control (Ballweg et al. 2014).

As described above, mitochondrial quality control is regulated by mitophagy, which is the specific autophagy process that eliminates damaged mitochondria (Ito et al. 2015). Again, low or moderate levels of ROS might induce mitophagy (Frank et al. 2012) but other reports suggest mitophagy impairment in COPD lungs (Ahmad et al. 2015). In fact, crucial proteins that regulate mitophagy such as SIRT1, SIRT6, PGC1-α, and prohibitin-1 (PBH1) are reduced in COPD (Mercado et al. 2015), leading to accumulation of damaged mitochondria. This could further negatively impact mitochondrial biogenesis through excessive ROS activation (Ito et al. 2015). Eventually, hampered mitochondrial biogenesis and clearance leads to apoptosis, cellular senescence (Mercado et al. 2015; Ahmad et al. 2015), and reduction in lung-resident stem cells necessary to replenish the different lung cell populations that are lost due to chronic CS-mediated oxidative insult (Mercado et al. 2015). A similar decrease in lung repair capacity either due to a reduction in the number of stem cells or impaired regenerative capacity in response to stress is also observed in the aging lung and thus contributes to the COPD-emphysema pathogenesis in the elderly (Meiners et al. 2015; Shaykhiev and Crystal 2014; Ryan et al. 2014). In the upper airways, four types of lung-resident cells maintain the integrity of the airway epithelium, namely, ciliated, secretory, intermediate, and basal cells (Crystal 2014). The secretory and ciliated cells are fully differentiated cell types that function to protect the lungs in the events of exposure to environmental agents and attack by pathogenic microorganisms. While these are crucial functions, the most important role of replenishing these differentiated cells is attributed to the cytokeratin-5 (KRT5)-positive basal cells, which are the stem/progenitor cells for the ciliated and secretory cells (Crystal 2014). Moreover, cigarette smoking is known to cause significant alterations in these basal cells, resulting in distorted transcriptome (Ryan et al. 2014), stem cell fatigue, basal cell hyperplasia, and altered differentiation (Crystal 2014). The characteristic features of distorted basal cell differentiation in response to CS exposure are loss of ciliated cells, decreased cilia length, mucus cell

hyperplasia, and squamous cell metaplasia. The basal cells derived from healthy smokers also show altered transcriptome and epigenetic changes, while those derived from smokers with clinically evident COPD demonstrate stem cell fatigue and inability to construct a normal healthy airway epithelium (Crystal 2014). These studies highlight the crucial role of CS-/oxidative stress-induced loss of basal progenitor cell functions involved in COPD pathogenesis. In the distal lung, the alveolar epithelial type II (ATII) cells are the stem/progenitor cells, which regenerate into the more functionally relevant alveolar type I cells (ATI) (Mercado et al. 2015). Exposure to alveolar epithelial type I-like cells (ATI-like cells), which are in the process of differentiating into ATII cells, leads to disruption of mitochondrial membrane potential, apoptosis and necrosis, along with a concurrent increase in the protective Nrf2 response (Kosmider et al. 2011). Moreover, Nrf2 knockdown sensitized the cells to CSE-induced cell death, while its overexpression conferred protection, highlighting the key role of Nrf2-dependent antioxidant mechanism in protecting the alveolar epithelial stem cells against CSE-mediated toxicity (Kosmider et al. 2011). A similar protective effect was observed by treatment with antioxidants such as N-acetylcysteine (NAC) and trolox, implicating that CSE-induced oxidative stress is mediating the damage to ATI-like cells (Kosmider et al. 2011), which may contribute to failed regeneration of the ATI cells in COPD subjects.

Another important aspect of CS-/aging-associated lung function decline and COPD-emphysema pathogenesis is the dysregulated autophagy process (Vij et al. 2018; Monick et al. 2010; Tran et al. 2015), which functions to degrade several cellular components via the autophagosome-lysosome pathway (Mizushima et al. 2008). There are several reports that autophagy declines with aging, which results in gradual accumulation of toxic cellular debris, resulting in inflammatory-oxidative stress responses (Martinez-Lopez et al. 2015). In smoking individuals, an excessive activation of ROS worsens and accelerates the autophagy impairment, thereby leading to formation of perinuclear p62+ aggresome bodies which we have shown to correlate with increasing severity of COPD-emphysema (Tran et al. 2015).

Although, early findings have pre-maturely reported that autophagy induction is deleterious in COPD leading to autophagic cell death (Chen et al. 2010), we and others have, since clearly shown that CS- and aging-mediated increase in inflammatory-oxidative stress/ROS leads to proteasome (van Rijt et al. 2012; Yamada et al. 2015) and autophagy impairment resulting in formation of aggresome bodies (Vij et al. 2018; Bodas et al. 2016b, c, 2018a), which play an important role in the pathogenesis of COPD-emphysema. Moreover, a relatively recent study from our group described that CS-mediated aggresome sequestration of transcription factor EB (TFEB) renders it unavailable to function as the master autophagy regulator and thus impairs autophagy (Bodas et al. 2016b). Further mechanistic investigation showed that TFEB knockdown elevates oxidative stress, autophagy impairment, and senescence, while TFEB-inducing drug, gemfibrozil, ameliorates these deleterious effects, thus confirming the crucial role of TFEB/autophagy impairment in COPD-emphysema pathogenesis (Bodas et al. 2016b). Moreover, it is appreciated that the aging process leads to a progressive decline in lung function. Our recent data also corroborates this, as lungs of aged mice demonstrate autophagy

impairment and formation of aggresome bodies (Vij et al. 2018). The critical role of autophagy impairment in COPD-emphysema pathogenesis and lung aging is supported by findings from various studies demonstrating the accumulation of ubiquitinated proteins and p62 that accelerates cellular senescence, thus contributing to COPD-emphysema pathogenesis (Fujii et al. 2012; Kuwano et al. 2016).

20.5 Oxidative Stress and CF Pathogenesis

Cystic fibrosis (CF) is a predominantly Caucasian disease caused by mutations in the CFTR gene (Galli et al. 2012; Cantin et al. 2015). The genesis of oxidative stress in the CF airways is believed to be congenital (Kettle et al. 2014), as the lungs of newly developed CF animal models and infants diagnosed with CF demonstrate an inherent deficiency of the crucial antioxidant glutathione, thus predisposing CF subjects to oxidative insults (Kettle et al. 2014). Moreover, the classical features of CF, such as airway obstruction and infection, are present in very early life, which is followed by inflammation in the first few weeks after birth (Nichols and Chmiel 2015; Wine 2010). Several studies have shown that the absence of a functional plasma membrane CFTR can itself cause oxidative stress by elevating ROS levels (Galli et al. 2012; Luciani et al. 2010). Moreover, pharmacological rescue of mutant F508del-CFTR controls inflammatory-oxidative stress in CF airways (Romani et al. 2017; Stincardini et al. 2018), thereby validating the role of CFTR in regulating oxidative stress and resulting CF pathogenesis. Additionally, with progressing age of CF subjects, the increasing infections, especially by the bacteria *Pseudomonas aeruginosa* (*Pa*), lead to recruitment of inflammatory cells such as neutrophils, macrophages, and lymphocytes (Nichols and Chmiel 2015). Activation of neutrophils generates reactive species such as superoxide, hydrogen peroxide, hydroxyl radical, and the myeloperoxidase enzyme, all of which contribute to the clearance of pathogens in the CF airways (Nichols and Chmiel 2015). If these reactive intermediates are not dampened by the antioxidants, it results in the development of inflammatory-oxidative stress in the CF airways (Ziady and Hansen 2014), eventually leading to chronic lung tissue damage. Another important cellular factor which is involved in regulating oxidative stress in the CF airways is the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Ziady and Hansen 2014). Nrf2 is a transcription factor which governs the antioxidant response gene expression and is one of the primary cellular defense mechanisms that maintains redox balance. Under steady-state conditions, Nrf2 is restricted in the cytoplasm by its inhibitor, Kelch-like ECH-associated protein 1 (Keap1), which promotes the ubiquitination and subsequent degradation of Nrf2. On encountering stress, Keap1 is oxidized leading to its dissociation from Nrf2, thus permitting Nrf2 to enter the nucleus and initiate transcription of numerous protective antioxidant proteins (Ziady and Hansen 2014). In CF airway epithelial cells, the elevated oxidative stress may be attributed to dysfunctional Nrf2 antioxidant response. Indeed, it is reported that CF airway epithelial cells display decreased levels of Nrf2-regulated antioxidant proteins potentially resulting in a redox imbalance. Additionally, a dramatic decrease in Nrf2 activity is

observed in CF cells as compared to non-CF cells, and this was also evident in conditions of CFTR inhibition, thus pointing toward the possible pathogenic mechanism of dysregulated redox homeostasis in the absence of a functional CFTR (Ziady and Hansen 2014). A recent study from our group demonstrated that *Pa*-LPS-mediated decrease in Nrf2 levels in the *Cftr*$^{+/+}$ mice was restored by co-treatment with the HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA) (Bodas et al. 2018b), although, in our study, no significant decrease in Nrf2 levels was observed in *Pa*-LPS-treated *Cftr*$^{-/-}$ mice, as compared to saline-treated controls. This suggests that Nrf2 may be involved in regulating infection-mediated oxidative stress, while further elaborate studies are necessary to delineate its role in the absence of CFTR.

Comprehensive studies in CF models and human subjects demonstrate low levels of nitric oxide (NO), a reactive intermediate responsible in maintaining airway tone (Zaman et al. 2016). This reduced NO concentration correlates with diminished lung function and elevated inflammation in CF patients (Zaman et al. 2006), thus suggesting its crucial role in regulating CF pathogenesis. An elevated production of the highly toxic peroxynitrite by the reaction of NO with O_2 and an increased production of asymmetric dimethylarginine (ADMA) are thought to be responsible for low NO bioavailability in the CF airways (Jones et al. 2000). Further mechanistic studies indicate that CF lungs have a low abundance of S-nitrosoglutathione (GSNO), an endogenous NO donor, which was attributed to increased levels of the GSNO catabolizing enzyme GSNO reductase (GSNOR) (Barnett and Buxton 2017). Thus, recent studies have tested the efficacy of specific GSNOR inhibitors, such as N91115 (Donaldson et al. 2017) or N6022 (Green et al. 2012), to restore GSNO levels and thus control inflammatory-oxidative stress in the CF lungs. Additionally, GSNO augmentation also facilitates the rescue of the mutant F508del-CFTR to the plasma membrane (PM), thereby correcting the root cause of CF lung disease (Bodas et al. 2016c; Zaman et al. 2006, 2014). Apart from the above potential therapeutic strategy, several antioxidants both chemical and natural compounds have been clinically evaluated in CF subjects, with promising outcomes (Galli et al. 2012). Some of the natural antioxidant compounds used in CF are curcumin, apigenin, genistein, quercetin, and hesperidin (Galli et al. 2012), although improvement in bioavailability, through novel drug delivery systems, precludes their clinical use. These drugs have been used as CFTR potentiators as well as antioxidants in CF, but further clinical studies are warranted before clinical use. The most widely evaluated antioxidant therapy for CF is GSH and its prodrug N-acetylcysteine (NAC) (Galli et al. 2012). Mechanistically, GSH augmentation in CF can benefit in several ways such as restoring antioxidant defense, control the activity of reactive chlorinated compounds generated by the MPO from activated neutrophils, and also protect the airway epithelium from the toxic effects of pyocyanin (PCN), a deleterious endotoxin released by *Pa (Schwarzer* et al. 2008*)*. Treatment with NAC has been scarcely evaluated in CF animal models, where it is known to facilitate controlling of mucus buildup and limit inflammation in the intestine of the CF murine model (De Lisle et al. 2007). Moreover, NAC might provide additional benefit in CF lung disease by restoring accumulation of misfolded proteins in aggregates that are

known to cause inflammation in CF airways (Galli et al. 2012; Luciani et al. 2010). This effect of NAC was attributed to its Beclin-1 restoring capacity, which helps correct the autophagy defect, along with improving the forward trafficking of F508del-CFTR to the PM (Luciani et al. 2010). The large collection of preclinical data on GSH supplementation and some studies on NAC provided the rationale for its clinical testing in CF subjects, albeit with not much success.

We and others have demonstrated the crucial pathogenic role of ceramide accumulation in COPD and CF lung disease (Bodas et al. 2011a, b, 2018a; Becker et al. 2010a, b; Grassme et al. 2017). In CF, it is believed that absence of PM-resident functional CFTR mediates high ROS levels, which in turn leads to increase in membrane ceramide accumulation (Teichgraber et al. 2008; Dumitru et al. 2007). This accumulation of ceramide results in elevated inflammatory-apoptotic stress through activation of NF-κB signaling pathway and thus plays an important role in causing lung injury in CF (Bodas and Vij 2010; Grassme et al. 2013). In fact, we recently demonstrated that cigarette smoke (CS)-induced ROS can induce ceramide accumulation in the PM as well as in perinuclear aggresome bodies, which could possibly justify the chronic activation of inflammatory-apoptotic signaling in the lungs of COPD subjects (Bodas et al. 2018a). Moreover, we described that ceramide accumulation correlates with decreasing lung function and increasing GOLD stages of COPD-emphysema (Bodas et al. 2018a). Unexpectedly, a similar trend is not observed for sphingosine, the downstream metabolite of ceramide, thus implying that a sphingolipid imbalance exists in lungs of COPD subjects with emphysema (Bodas et al. 2018a). Further, we showed that this CS-/ROS-induced sphingolipid imbalance can be corrected by the antioxidant drug, cysteamine, which also has potent autophagy-inducing properties (Bodas et al. 2018a). Moreover, we also confirmed that both cigarette smoke extract (CSE) and H_2O_2 induced elevated ROS levels triggering rapid PM translocation of acid sphingomyelinase (ASM), the enzyme which breaks down sphingomyelin to ceramide, while this translocation was prevented by treatment with antioxidant, cysteamine (Bodas et al. 2018a). Thus, these recent findings suggest the precise mechanism for CS-/ROS-mediated membrane ceramide accumulation. Although ceramide accumulation in CF is well documented (Becker et al. 2010a, b; Brodlie et al. 2010), and cysteamine has been tested in preclinical and clinical studies to correct several CF-related abnormalities (Charrier et al. 2014; De Stefano et al. 2014), it remains to be tested if the antioxidant property of cysteamine can control ceramide accumulation in CF cells and/or murine models.

20.6 Oxidative Stress and Asthma Progression

Asthma is a chronic inflammatory lung condition which is primarily triggered through exposure to allergens, which induces endogenous generation of free radicals such as ROS, hydrogen peroxide, and peroxynitrite (Erzurum 2016). An increase in ROS species induces chemical signatures that attract immune cells such as neutrophils and eosinophils into the airways (Cho and Moon 2010). The

subsequent activation of these inflammatory cells leads to further ROS generation, which contributes to sustained higher than normal levels of ROS in the asthmatic lungs (Sahiner et al. 2011; Cho and Moon 2010). This elevated ROS influences several characteristics of asthma pathophysiology by inducing airway hyperresponsiveness (AHR), epithelial apoptosis, mucus overproduction, airway remodeling, and decreased mucociliary clearance of pathogenic microorganisms (Bullone and Lavoie 2017). Additionally, other factors such as exposure to environmental pollutants, CS, and infections exacerbate asthma symptoms, as all these agents contribute to uncontrollable levels of oxidants in the airways. A recent study provides direct evidence for the effect of common allergens on the increase in ROS/RNS levels and its possible contribution to asthma. Exposure of Beas2b cells to house dust mites (HDM) results in DNA damage, through induction of ROS levels, increased mitochondrial oxidative stress, and elevated nitrosative stress (Chan et al. 2017). Moreover, HDM-induced DNA damage could be reversed by common antioxidants GSH and catalase, thus proving that allergens can mediate lung epithelial injury via production of ROS/RNS (Chan et al. 2017). Additionally, elevated oxidative stress is known to cause several deleterious changes to airway epithelial cells including increase in mucus secretion, cilia dysfunction, and shedding of the epithelial layer, which play crucial roles in asthma pathogenesis (Sahiner et al. 2011). Another hallmark feature of asthma is airway hyperresponsiveness caused by hyper-contractile ASM and increased ASM mass. Oxidative stress is associated with both these phenomena in the asthmatic airways, possibly through overexpression of NADPH oxidase-4 (NOX4) (Sutcliffe et al. 2012). Several recent and interesting studies correlate asthma and obesity, and the term "obese asthma" has emerged to explain the pathophysiological features found in such patients (Singh et al. 2015; Diaz and Farzan 2014). Notably, both obesity and asthma have elevated oxidative stress and share the common phenotypes of increased airway neutrophilia and mitochondrial dysfunction (Bhatraju and Agrawal 2017). The asthmatic airways demonstrate an imbalance in oxidant and antioxidants, and this correlates with decreased lung function (Bullone and Lavoie 2017). Specifically, decreased levels of catalase, superoxide dismutase (SOD), and glutathione peroxidase are found in lungs of asthmatic patients (Comhair and Erzurum 2002). Therefore, all above studies indicate the pathogenic role of oxidative stress in asthma pathogenesis and progression.

Recently the theory of oxi-inflamm-aging was described which states that the capacity of the endogenous antioxidants to counteract the generation of ROS decreases with aging, which contributes to the development of age-associated inflammatory environment (Bullone and Lavoie 2017). It is argued that some diseases have a different clinical profile in the elderly as compared to the young population, and adult- or late-onset asthma is one such disease. A cross-sectional study was performed in adults with severe asthma, and it was observed that the severity of asthma increased with each year of life until 45 years of age but increased at a slower rate after this age (Zein et al. 2015). The interesting finding of this study was that age alone, independent of asthma duration, was more strongly associated with risk of severe asthma. This might explain the clinical findings that even though asthma is a disease of the young, the asthma-related mortality and overall hospital

visits and associated costs are much greater in the elderly population (Hanania et al. 2011; Zein et al. 2015). Further studies are essential to delineate the asthma phenotype in the elderly, although it is clear that age-associated oxi-inflamm-aging might significantly contribute to severity of asthma in the aged population (Bullone and Lavoie 2017).

20.7 Central Role of Oxidative Stress-Mediated Autophagy Impairment in Pathogenesis of COPD

Our airways are constantly exposed to numerous harmful agents, where all forms of tobacco smoke, biomass smoke, and nicotine-containing e-cigarette vapor are the causal agents of COPD-emphysema initiation and progression (Bodas and Vij 2017). We and others have clearly shown that generation of oxidative-nitrosative stress and inflammation upon exposure to the above noxious agents plays a central role in mediating autophagy flux impairment in lung cells and murine lungs (Vij et al. 2018; Bodas et al. 2016a, b, 2018a; Fujii et al. 2012; Bodas and Vij 2017). Further, a series of comprehensive studies using a variety of preclinical models and human cells and/or tissues have demonstrated that tobacco smoke or e-cig vapor (eCV) impairs autophagy which mediates formation and accumulation of aggresome bodies that contributes to initiation and progression of chronic inflammatory-apoptotic responses, senescence, and COPD-emphysema (Vij et al. 2018; Shivalingappa et al. 2015; Tran et al. 2015). Moreover, we also reported that lungs of aged mice also show accumulation of aggresome bodies (similar to the lungs of COPD-emphysema subjects and CS-exposed mice), thus suggesting this as a common pathogenic mechanism to initiate CS- or aging-related cellular senescence and emphysema (Vij et al. 2018). Studies from other groups have shown the importance of CS-induced mitochondrial dysfunction and mitophagy as a pathogenic mechanism in COPD-emphysema (Ahmad et al. 2015; Ballweg et al. 2014), although conflicting reports exist (Mizumura et al. 2014). The "parkin RBR E3 ubiquitin protein ligase" (Parkin, Prkn) knockout murine model of impaired mitophagy was recently used to clarify the role of mitophagy in CS-induced emphysema (Ito et al. 2015). It was found that CS exposure of Prkn KO mice induced more severe emphysematous changes, accumulation of damaged mitochondria, increased oxidative stress, and accelerated cellular senescence, as compared to CS-exposed WT mice (Ito et al. 2015). Moreover, PRKN overexpression-mediated mitophagy induction was sufficient to attenuate CSE-induced mitochondrial ROS and cellular senescence (Ito et al. 2015). These studies verify the critical role of functional mitophagy in protecting against CS-induced emphysema and other pathological features associated with COPD. In a recent interesting study, exposure of small airway epithelial cells (SAEC) and primary bronchial epithelial cells to wildfire smoke extract (WFSE) was found to inhibit autophagy flux, along with increased apoptosis and barrier dysfunction (Roscioli et al. 2018). In a scenario where incidences of wildfire smoke (WFS) exposure are increasing in many parts of the world, and exposure to WFS has been statistically correlated with increased COPD exacerbations (Roscioli

et al. 2018), these findings provide a potential therapeutic strategy to decrease COPD severity using autophagy augmentation. Several mechanisms of CS-/ROS-mediated autophagy impairment have been described which provides potential druggable targets to control COPD-emphysema lung disease. The mammalian target of rapamycin (mTOR) is a crucial regulator of autophagy, and activation of mTOR is associated with autophagy inhibition (Wang et al. 2018b). The lungs of COPD subjects show an activation of both mTORC1 and mTORC2, and lung cell senescence strongly correlated with increased mTOR activity (Houssaini et al. 2018). Moreover, transgenic mice with deletion of tuberous sclerosis complex heterodimer TSC1 (a negative mTORC1 regulator) were generated to create a model of mTORC1 overactivation (Houssaini et al. 2018). These TSC1 KO mice demonstrated increased inflammation and cellular senescence, but more importantly these mice developed emphysematous changes in their lungs even in the absence of any other stimuli (Houssaini et al. 2018). The treatment of these mice with rapamycin, a well-known mTOR inhibitor and autophagy inducer, nullified the inflammatory and senescence phenotype, and also reduced emphysema, thus identifying mTOR overactivity as a crucial mediator of lung senescence and emphysema in COPD (Houssaini et al. 2018). The authors conclude that since mTOR is an essential negative regulator of autophagy, activation of mTOR in COPD lungs might be a mechanism of diminished autophagy, as lungs of TSC1 KO mice showed decreased levels of autophagy proteins (Houssaini et al. 2018). In a separate study, mTOR activation has been linked to oxidative stress-induced cellular senescence and is also found to be associated with CS-induced oxidative stress (Yoshida et al. 2010). In a stark contrast to the above findings, a similarly timed study found that mTOR levels were significantly decreased, while the levels of tuberous sclerosis complex heterodimer TSC2 (TSC2) were elevated in the airway epithelium of COPD subjects and lungs isolated from mice exposed to chronic CS (Wang et al. 2018b). Moreover, CSE treatment of bronchial epithelial cells led to TSC2 activation, mTOR inhibition, and concurrent induction of autophagy (Wang et al. 2018b). They eventually conclude that mTOR activation may be a potential therapeutic strategy to control CS-induced COPD-emphysema. Thus, it is clear that further detailed analysis of the mTOR-autophagy signaling axis will be necessary before it can be used as a therapeutic target.

Recent studies from our group have described the role of CS/ROS-induced TFEB sequestration into perinuclear aggresome bodies as a mechanism of autophagy impairment in COPD-emphysema (Bodas et al. 2016b). TFEB is a master regulator of important genes that regulate lysosomal biogenesis and autophagy (Bodas et al. 2016b). Interestingly, the active mTORC1 complex regulates the function of TFEB by mediating its phosphorylation (Napolitano et al. 2018; Roczniak-Ferguson et al. 2012), which leads to its cytoplasmic retention. Under conditions of stress or alterations in lysosomal function, TFEB translocates to the nucleus, possibly due to inactivation of mTORC1 function, to trigger transcription of genes involved in autophagy and lysosomal biogenesis. Furthermore, we went on to demonstrate that knockdown of TFEB using shRNA, in Beas2b cells, leads to a significant increase in accumulation of ubiquitinated proteins in the insoluble protein fraction, along with increase

in ROS levels and cellular senescence (Bodas et al. 2016b). These studies identified a novel mechanism for CS-/ROS-mediated autophagy impairment in COPD-emphysema, and indeed pharmacological induction of TFEB provides therapeutic benefit in lung cells and mice treated with CS, via autophagy restoration, which is discussed in a separate section below. In a detailed study to identify the role of CFTR in regulating autophagy, Luciani et al. demonstrated that a defective CFTR leads to autophagy impairment and formation of aggresome bodies through ROS activation (Luciani et al. 2010). They propose that elevated ROS due to loss of functional CFTR mediates activation of transglutaminase 2 (TG2), leading to cross-linking and subsequent aggresome trapping of Beclin-1 (BECN1), a key protein required for formation of autophagosome, thus blocking autophagy, as observed by increased accumulation of p62 (Luciani et al. 2010). In the context of COPD, a recent study shows that circulating BECN1 levels are decreased in smokers and COPD subjects as compared to healthy controls (Schlemmer et al. 2018). Moreover, the authors also demonstrate a correlation between circulating BECN1 levels and airflow obstruction, and markers of accelerated aging, thus providing preliminary data showing that defective autophagy is indeed present in smokers with COPD, and BECN1 could be a potential mechanism and quantitative marker of autophagy impairment in COPD subjects (Schlemmer et al. 2018). Since this study only investigates serum BECN1 levels, further studies are warranted to verify if a similar mechanism operates to cause an autophagy defect in the lungs of COPD subjects.

20.8 Oxidative Stress-Induced Acquired CFTR Dysfunction Exacerbates Chronic Lung Disease Development

CFTR dysfunction in cystic fibrosis (CF) is caused by the mutation phenylalanine-508 (F508-del) which results in a misfolded CFTR protein that is incapable of reaching to the plasma membrane (PM), thus hampering chloride transport (Welsh et al. 1993). Moreover, there is considerable evidence supporting the critical role of membrane-resident CFTR in regulating the innate and adaptive immune responses in CF (Bodas and Vij 2010; Bruscia and Bonfield 2016). In COPD, several studies now support the concept of acquired CFTR dysfunction (Dransfield et al. 2013; Rab et al. 2013; Shi et al. 2018), where CS-/ROS-mediated decrease (but not complete absence) in the functional PM-resident CFTR protein results in NF-κB-mediated inflammatory-oxidative stress and emphysema. It is intriguing that CFTR was studied as a susceptibility gene for COPD as early as 1995 (Luisetti and Pignatti 1995), although the first study that directly correlates CFTR protein loss (in vitro) with CS was conducted by Cantin AM et al. in 2006 (Cantin et al. 2006a, b). The authors found that CS decreases expression of CFTR gene, protein, and function in Calu3 and T84 cell lines, while nasal respiratory epithelial cells of smokers demonstrate acquired CFTR deficiency (Cantin et al. 2006a). CS is composed of numerous noxious agents and cadmium is one of its major toxic components, as well as a by-product of smelters (thus causing environmental contamination) (Rennolds et al. 2010). Treatment of human airway epithelial cells and mice with cadmium leads to

decreased CFTR protein levels and hampered chloride transport, which was corrected by the antioxidant alpha-tocopherol, suggesting that cadmium-induced ROS negatively affects CFTR levels (Rennolds et al. 2010). COPD is also characterized by airway surface liquid (ASL) dehydration and diminished mucociliary clearance (Seys et al. 2015). The primary mechanism first demonstrated for this pathogenic feature is the CS-induced acquired CFTR dysfunction in smokers and COPD subjects (Bodas et al. 2011a, b). On similar lines, it was shown that CS induces rapid CFTR internalization into detergent insoluble perinuclear aggresome-like bodies, which led to ASL dehydration, thus rendering smokers with increased risk of bronchitis and pulmonary infections (Clunes et al. 2012). Although CS inflicts injury in the whole lung, the damage to the lower airways (alveoli) plays a crucial role in emphysema pathogenesis. Therefore, Dransfield et al. investigated nasal potential difference and lower airway potential difference (LAPD) as a measure of CFTR activity in healthy nonsmokers and smokers with and without COPD (Dransfield et al. 2013), confirming our initial reports (Bodas et al. 2011a, b). Indeed, they report that CFTR activity was decreased in smokers with and without COPD, as compared to nonsmokers, thus suggesting the role of smoking in causing acquired CFTR dysfunction in the lower airways of COPD subjects (Dransfield et al. 2013). Several reports support the notion that CS and its specific oxidant-generating components are capable of mediating acquired CFTR dysfunction (Shi et al. 2018; Rennolds et al. 2010), and a defective CFTR by itself can lead to further ROS generation (Luciani et al. 2010). This creates a vicious cycle that mediates ceramide accumulation (Brodlie et al. 2010), autophagy impairment (Luciani et al. 2010), lung cell apoptosis (Becker et al. 2010a), and impaired pathogen clearance (Ni et al. 2015), all of which contribute to COPD-emphysema progression and chronicity. We have previously shown that a decrease or loss of membrane CFTR leads to increased ceramide levels in the lipid rafts, and this correlates with increasing severity of COPD-emphysema (Bodas et al. 2011a). In a follow-up study, we verified that absence of membrane-localized CFTR worsens CS-induced ceramide accumulation, apoptosis, and defective autophagy responses, thus providing mechanistic insights of acquired CFTR dysfunction-mediated lung injury and emphysema (Bodas et al. 2011b). Recently, we described a novel pathogenic mechanism in COPD-emphysema, where CS/ROS leads to both membrane and aggresome accumulation of ceramide, which results in severe inflammatory-apoptotic responses (Bodas et al. 2018a). Mechanistically, we demonstrate that membrane and intracellular ceramide accumulation is mediated by two distinct mechanisms, albeit both being regulated by ROS. Specifically, ROS-induced autophagy and lipophagy impairment leads to aggresome accumulation of ceramide, while acquired CFTR dysfunction-induced ASM translocation to the PM caused membrane ceramide accumulation (Bodas et al. 2018a). Thus, all these studies highlight the key role of CS/oxidative stress in mediating acquired CFTR dysfunction and its implications in COPD-emphysema. Based on these studies, it seems obvious that compounds or drugs that enhance CFTR membrane stability and/or channel function could be potentially utilized to control CS-induced oxidative stress and resulting COPD-emphysema. Indeed, the CFTR potentiator, ivacaftor, was shown to provide benefit

from CS-induced reduced mucus transport through increasing ASL levels (Sloane et al. 2012). These encouraging results pave the way for several other CFTR potentiators and/or corrector drugs as future therapeutic agents for chronic bronchitis and COPD-emphysema.

20.9 Pathogenic Effects of CFTR Dysfunction-Mediated Autophagy Impairment in Progression of CF (Genetic) and COPD (Acquired)

We and others have clearly demonstrated that a complete loss (in CF) or partial decrease (in COPD) in PM-resident functional CFTR leads to autophagy impairment that regulates several hallmark pathophysiological features of both CF and COPD (Bodas et al. 2011a, b, 2016c; Luciani et al. 2010; Vij 2016; Valle and Vij 2012). The foremost pathogenic feature of both CF and COPD is the defective autophagy-mediated accumulation of aggresome bodies (Luciani et al. 2011), which are aggregates of misfolded or damaged proteins or organelles (Kopito 2000). In fact, the F508-del CFTR is by itself a misfolded aggresome-prone protein, and loss of PM-CFTR leads to ROS activation (Luciani et al. 2010). Further, ROS-mediated TG2 SUMOylation leads to aggresome sequestration of PPARγ, which is an anti-inflammatory protein (Luciani et al. 2010). This is proposed as one of the mechanisms of ROS-induced inflammation in CF airways. Additionally, TG2 also triggers cross-linking of key autophagy protein, Beclin-1 (BECN1), and promotes its aggresome sequestration (Luciani et al. 2010). This cross-linked BECN1 dissociates the PI3K complex III away from the endoplasmic reticulum (ER) resulting in sequestration of BECN1 interactome into aggresomes, which causes autophagy impairment due to unavailability of important autophagy proteins to form autophagosome. Since p62 is an autophagy substrate, defective autophagy leads to accumulation of p62 that further promotes aggresome formation. Moreover, accumulation of damaged mitochondria generates more ROS that creates a vicious cycle resulting in chronic inflammatory-oxidative stress in the CF lungs. Furthermore, evidence from our studies demonstrated that the absence of CFTR (CFBE41o- cells) leads to perinuclear accumulation of microtubule-associated protein 1 light chain-3 (LC3), both basally and post-CSE treatment (Bodas et al. 2011a, b). Moreover, co-localization of p62 and LC3 in CFTR-deficient cells and murine lungs confirmed that autophagy is indeed impaired in the absence of CFTR (Bodas et al. 2011b). Additional confirmatory evidence comes from our recent studies, where restoration of the PM-resident functional CFTR corrects the CS-induced autophagy impairment and inflammatory-oxidative stress in COPD-emphysema (Bodas et al. 2016c), while studies from other groups indicate that restoration of autophagy impairment rescues the mutant F508-del CFTR to the PM (Zhang et al. 2018; Junkins et al. 2014), thus providing therapeutic benefit in chronic CF lung disease. Collectively, these findings indicate the strong rationale for autophagy induction as a therapeutic strategy for treatment of CF and COPD lung disease, as discussed below in detail.

20.10 Autophagy Augmentation Controls Underlying Pathogenic Mechanism(s) of Lung Aging and Chronic Obstructive Lung Diseases

As discussed above, autophagy impairment is one of the most important pathogenic mechanisms implicated in aging, CF, and COPD lung disease. Even though the mechanisms of autophagy impairment may be different in CF and COPD, activation of ROS higher than what is required for normal cellular functions is the primary upstream mediator of autophagy impairment. Our comprehensive studies have clearly shown that first- and secondhand CS, e-cigarette vapor (eCV), waterpipe smoke extract (WPSE), nicotine (a major toxic component of CS), and aging all mediate autophagy impairment in lung cells or mice (Vij et al. 2018; Ni et al. 2015; Bodas et al. 2016a; Shivalingappa et al. 2015; Tran et al. 2015; Bodas and Vij 2017). Moreover, ROS activation was the primary driver of autophagy impairment, suggesting that antioxidants and/or autophagy inducers might regress the deleterious effects of all these noxious agents. The overactivation of ROS (oxidative stress) inflicts a damaging effect on a wide variety of key cellular processes related to lung disease development, such as cell survival, immune response, pathogen clearance, cellular senescence, and proteostasis/autophagy. It is intriguing but well demonstrated that CS-/ROS-mediated-acquired CFTR dysfunction can be cited as an important regulator and mediator of almost all the above cellular processes (Fig. 20.1). In fact, acquired CFTR dysfunction, or the decrease in PM-resident functional CFTR protein, has been shown to (1) elevate inflammatory-oxidative stress, (2) induce membrane- and intracellular- (aggresome) ceramide accumulation promoting severe lung cellular injury, (3) hamper bacterial clearance resulting in chronic infections and inflammation, and (4) cause autophagy impairment leading to aggresome formation, all of which are key mediators of COPD-emphysema pathogenesis (Tran et al. 2015). Therefore, it was tempting and logical to see whether drugs or compounds, those that induce CFTR trafficking to the PM and/or augment autophagy, can modulate the key pathogenic mechanisms described above and thus slow down or reverse COPD-emphysema symptoms. We have extensively evaluated several autophagy-inducing drugs in the in vitro and in vivo preclinical models of CS/eCV/WPSE and nicotine exposure and found that autophagy augmentation can control CS-/ROS-mediated inflammatory-oxidative stress and ceramide accumulation and improve bacterial clearance while reducing cellular senescence and preventing aggresome accumulation, mostly by restoring CFTR expression and function. Based on our multiple comprehensive studies, we present a strong case in favor of correcting the oxidative stress-mediated proteostasis and autophagy impairment in both CF and COPD-emphysema to control lung disease progression. The following section highlights the utility of drugs or compounds that augment proteostasis and autophagy to correct the underlying mechanisms of chronic lung disease progression.

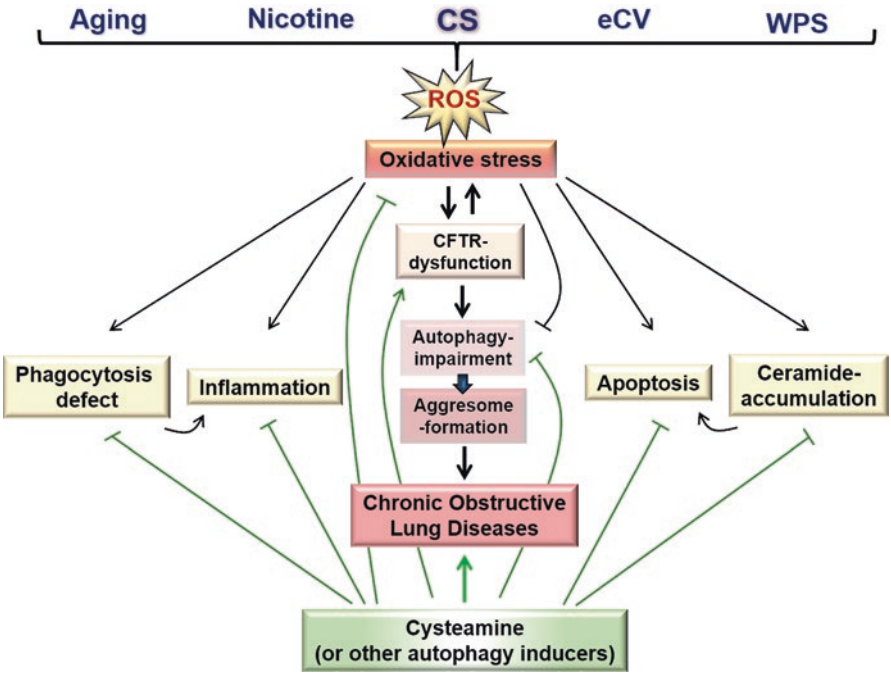

Fig. 20.1 *The multipronged mechanism of action of cysteamine in controlling oxidative stress-induced chronic lung disease pathogenesis*. The accumulation and activation of ROS due to aging or exposure to external noxious agents, such as cigarette smoke (CS), waterpipe smoke (WPS), and nicotine, leads to oxidative stress, which causes acquired CFTR dysfunction. A dysfunctional CFTR also leads to further increase in ROS levels, thus forming a vicious cycle, in which the pathogenic mechanisms initiated by ROS, such as phagocytosis impairment, inflammation, apoptosis, and ceramide accumulation, can all be modulated by a nonfunctional CFTR. The central mechanism governing all these features is oxidative stress-mediated autophagy impairment, which can be dependent or independent of CFTR dysfunction. The oxidative stress-mediated autophagy impairment and resulting aggresome formation are critical for chronic obstructive lung disease pathogenesis. In addition, autophagy augmentation by an antioxidant drug such as cysteamine has multiple benefits in alleviating the key pathogenic features of chronic lung disease progression. Briefly, treatment with cysteamine controls oxidative stress, improves pathogen clearance, dampens inflammation, and reduces ceramide levels as well as cellular apoptosis. The potential mechanisms of these beneficial effects are mediated by CFTR/autophagy augmentation that regulates almost all the pathogenic features involved in CF and COPD lung disease progression or lung aging. Thus, we support the ongoing clinical evaluation of this potent drug in CF, COPD, as well as lung aging and variety of inflammatory-oxidative stress-induced lung condition as a novel therapeutic or intervention strategy.

20.11 Proteostasis and Autophagy Augmentation as an Intervention Strategy to Regulate Lung Aging and Chronic Obstructive Lung Disease Progression

Based on several concrete studies from our and other groups, we can safely state that ROS-mediated autophagy impairment plays a central role in pathogenesis of CF and COPD-emphysema. Numerous drugs or compounds are available as autophagy inducers and we have tested a few of them in our pursuit to develop a potent therapeutic for controlling CF and COPD-emphysema lung disease progression. Cysteamine is one of the most widely studied and a potent antioxidant drug with autophagy-inducing properties (Bodas et al. 2018a; De Stefano et al. 2014; Zhang et al. 2018; Vu et al. 2017; Ferrari et al. 2017). It is an FDA-approved drug for nephropathic cystinosis and is commercially available as Cystagon® and Procysbi® for oral administration (Besouw et al. 2013). Numerous studies have tested the efficacy of cysteamine and its other modified forms or nano-based formulations in controlling CF lung disease (Charrier et al. 2014; Vij 2016; Vu et al. 2017; Brockman et al. 2017; Tosco et al. 2016). Cysteamine is a multipronged drug and displays several beneficial properties such as antioxidant, bactericidal, mucolytic, anti-inflammatory, anti-biofilm, and autophagy inducer (Fig. 20.1) (Bodas et al. 2018a). All these features are quite necessary to control pathogenesis of CF lung disease, and thus a clinical study was conducted using cysteamine and epigallocatechin gallate (EGCG) as a combinatorial drug strategy (Tosco et al. 2016). The therapeutic benefits of this strategy were accredited to cysteamine-mediated autophagy induction and resulting restoration of F508del-CFTR to the PM, followed by enhanced stability of the PM-resident CFTR protein via inhibition of protein kinase CK2, by EGCG (Tosco et al. 2016). The autophagy restoring mechanism of action of cysteamine is substantially delineated. Briefly, cysteamine-mediated inhibition of transglutamine-2 (TG2) leads to the disintegration of the aggresome assembly, which is sequestering key autophagy proteins and F508del-CFTR, thus allowing forward trafficking of CFTR, restoring autophagy, and decreasing p62 levels (Ferrari et al. 2017; Villella et al. 2013; Esposito et al. 2016). Based on cysteamine's strong candidature in CF, we had tested this drug in our in vitro and murine model of CS exposure. Data emerging from that study showed that CS-induced accumulation of polyubiquitinated proteins into p62+ aggresome bodies was significantly controlled by cysteamine (Vij et al. 2018). Moreover, cysteamine was also able to control CS-induced apoptosis and cellular senescence in Beas2b cells and murine lungs, while it also demonstrated its synergestic antibacterial potential in a model of CS exposure + *Pa* infection (Vij et al. 2018). These studies provided substantial evidence that cysteamine could be further tested as an antioxidant drug with autophagy-inducing properties to limit COPD-emphysema pathogenesis and allow healthy lung aging. In a recent report, we also demonstrated that cysteamine inhibited the translocation of acid sphingomyelinase (ASM) enzyme to the PM, thus blocking the enzymatic degradation of sphingomyelin to ceramide, a pathogenic bioactive lipid that mediates chronic inflammatory-apoptotic signaling in CF and COPD lung disease (Bodas et al. 2018a). In parallel we also report that CS-induced intracellular

ceramide accumulation (lipophagy dysfunction) into perinuclear aggresome bodies was controlled by cysteamine, by virtue of its autophagy-inducing properties (Bodas et al. 2018a). The further confirmation of cysteamine's beneficial actions comes from our findings that nicotine and eCV exposure-mediated autophagy impairment and resulting apoptosis, and senescence, could be nullified by the ROS quenching action of cysteamine (Bodas et al. 2016a; Shivalingappa et al. 2015). These studies warrant further clinical investigation of cysteamine in smokers with COPD-emphysema. The clinical utility of cysteamine is hampered by its poor bioavailability and the need of a high dose that might be difficult to achieve in vivo. To this end, attempts have been made to improve cysteamine's bioavailability, as well as to lower the effective dose, such as by conjugating it with a ω-3 fatty acid (docosahexaenoic acid, DHA), which can by itself induce autophagy by the AMPK pathway (Vu et al. 2017). This cysteamine conjugate was able to effectively rescue F508del-CFTR to the PM at a much lower concentration, thus warranting its further clinical evaluation (Vu et al. 2017). In another study, nine "prodrugs" of γ-glutamyl-cysteamine were tested in cultured kidney cells, and these were able to successfully deliver cysteamine into these cells with much lower toxicity and improved bioavailability (Frost et al. 2016). In another relatively recent report, we demonstrated that CS-induced aggresome sequestration of transcription factor EB (TFEB), the master autophagy regulator, into aggresome bodies was corrected by TFEB-inducing drugs gemfibrozil (GEM) or fisetin (Bodas et al. 2016b). Additionally, TFEB induction significantly decreased CSE-induced autophagy impairment and resulting aggresome formation by diminishing ROS activation (Bodas et al. 2016b). Moreover, in vivo studies showed that CS-induced autophagy impairment, inflammation, apoptosis, and the resulting emphysema were controlled by GEM-mediated TFEB induction (Bodas et al. 2016b). In the context of CS-induced phagocytosis defect, we have previously demonstrated that TFEB/autophagy induction by fisetin can control *Pa* infection in a murine macrophage cell line (Raw 264.7) (Pehote et al. 2017), although further preclinical studies are warranted to confirm the utility of this drug in mediating autophagy-mediated pathogen clearance from CF or COPD airways.

20.12 Conclusion and Perspective

To summarize, the critical role of elevated oxidative stress in the pathogenesis of chronic obstructive lung diseases is well established. Based on this knowledge, several antioxidant drugs and natural compounds with antioxidant properties are available that can be used to control chronic lung diseases. Although, these strategies are helpful, they may come with issues such as toxicity, low bioavailability, and/or drug delivery problems. Since we and others have shown the prominent central role of autophagy in dampening inflammatory-oxidative stress responses in the lung and solid evidence that autophagy impairment is the key player in mediating most of the pathogenic features of chronic lung disease progression, we propose the utility of cysteamine or other autophagy/proteostasis augmenting drugs for controlling CF or

COPD-emphysema pathogenesis as well allowing healthy lung aging. Recent studies have helped improve the bioavailability of cysteamine, and novel drug delivery approaches to deliver the drug past the thick mucus barrier in the CF airways are emerging. Therefore, we believe that using one drug that has multiple benefits is better than multiple drugs, as the multidrug strategy may cause various side effects and it may be difficult to zero-in on the doses of the drugs for in vivo or clinical application, as multiple drugs have different pharmacokinetic properties. Thus, we support the ongoing clinical translation studies aimed to standardize the efficacy of cysteamine, in controlling critical phenotypic features of chronic lung disease progression such as inflammatory-oxidative stress, cellular senescence, and apoptosis while improving bacterial clearance, *via* induction of functional CFTR and autophagy augmentation, in order to restrict chronic lung disease pathogenesis and progression.

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Part V

Prevention and Therapeutics

Therapeutic Targeting of Oxidative Stress and Inflammation in Asthma and COPD and Pharmacological Interventions with Phytochemicals

21

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Abstract

Asthma and chronic obstructive pulmonary disease (COPD) are the common respiratory diseases posing immense burden on human health. Incidence of asthma and COPD are increasing significantly in recent decade around the world. There is abundant evidence that these disorders are mediated by oxidative stress which plays a key role in the initiation and augmentation of inflammation. Currently available western drugs are associated with severe side effects and resistance, and hence, there is a need of new drugs which can halt the progression of disease. Use of herbal medicine to treat the ailment is known to mankind from ancient times. Phytoconstituents, apart from their antioxidant capacity, possess anti-inflammatory effect. This property can be utilized for the treatment of asthma and COPD, where oxidative stress and inflammation plays a major role in the progression of the disease.

The present chapter deals with the brief explanation of interplay between oxidative stress and inflammation in asthma and COPD. Phytochemicals that showed promising effect against these disorders in the animal models and their molecular mechanism involved for the protection are described briefly.

21.1 Introduction

Respiratory diseases pose an immense burden on human health throughout the world. According to the World Health Organization (WHO), chronic respiratory conditions affect more than 1 billion people with an estimated 235 million cases of

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asthma, more than 200 million cases of chronic obstructive pulmonary disease (COPD), 65 million suffering from moderate to severe COPD, over 100 million experiencing sleep-disordered breathing, 8.7 million people ensuing tuberculosis annually and millions living with pulmonary hypertension, and more than 50 million people struggling with occupational lung diseases. Asthma and COPD are the most common obstructive respiratory disorders (World Health Organization 2012, 2013a, b; http://www.who.int/gard/publications/chronic_respiratory_diseases.pdf; http://www.who.int/gard/news_events/1-3.GARD-06-07-K1.pdf). Asthma is a chronic inflammatory disorder of the airways associated with an increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or early in the morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible, either spontaneously or with treatment (Bateman et al. 2008). On the other hand COPD is partially reversible airflow obstruction characterized by the limitation of airflow which is usually progressive and associated with an abnormal response of the lungs to noxious particles or gases (Pauwels et al. 2001). There are some important similarities as well as differences between asthma and COPD (Barnes et al. 2009). Both are chronic inflammatory diseases that involve structural changes in the small airways and cause airflow limitation (Jeffery 1998; Wiggs et al. 1990), resulting from gene-environment interactions and characterized by mucus and bronchoconstriction. However, they both differ in the nature of inflammation and the anatomical sites involved in the disease (Jeffery 1998). Asthma affects only the airways, while COPD affects the airways as well as the parenchyma. Secondly, the nature of inflammation is primarily eosinophilic and CD4-driven in asthma and neutrophilic and CD8-driven in COPD (Keatings et al. 1996a). This later difference affects the response to pharmacological agents as evidenced by the fact that inhaled corticosteroids are effective against the eosinophilic inflammation in asthma but largely ineffective against the primarily neutrophilic inflammation seen in COPD (Keatings et al. 1997). WHO predicts that COPD will become the third leading cause of death worldwide by 2030 (http://www.who.int/gard/news_events/1-3.GARD-06-07-K1.pdf).

Oxidative stress and inflammation play a major role in the pathogenesis of asthma and COPD (Paul et al. 2001; Rahman and Adcock 2006a). Persistent chronic inflammation leads to generation of reactive oxygen species (ROS); prolonged exposure to ROS causes oxidative stress, which leads to overactivity of the immune system (Kleniewska and Pawliczak 2017). Oxidative stress plays a central role in upregulating inflammatory events by activating gene expression of pro-inflammatory cytokines (Zuo et al. 2013). The lung is an organ with large surface area and highly supplied with blood vessels making it susceptible to oxidative damage (Wei Sheg et al. 2014). Both exogenous and endogenous factors play a major role in the production of ROS. Cigarette smoke, vehicle exhaust, gases like ozone, and sulfur dioxide are some of the exogenous factors that play a major role in the production of ROS, whereas the endogenous production of ROS is linked with mitochondria, microsomes, enzymes, and phagocytes. Lungs have a well-defined antioxidant

system to protect against ROS. However, the imbalance between these systems leads to asthma and COPD (Zuo et al. 2013; Sahiner et al. 2011).

Corticosteroids are the effective anti-inflammatory drugs used for the treatment of asthma and COPD; however, they are associated with severe side effects and resistance (Barnes 2006, 2013; Adcock et al. 2008). In recent times much attention is focused on the usage of natural products for the treatment of various disorders because of their minimal side effects associated with them. Natural products show protective effect in various diseases by their antioxidant and anti-inflammatory properties. Several phytoconstituents and extracts have been evaluated for their effectiveness in treating asthma and COPD and were found to be beneficial. In this chapter, we briefly discuss about the role of oxidative stress and inflammation in asthma and COPD, protective effect of phytoconstituents in relevant animal models of asthma and COPD.

21.2 Role of Oxidative Stress in Asthma

Generation of ROS is a continuous process which takes place in a cell under normal physiological conditions. Excessive production of ROS shows deleterious effect on a wide range of biological molecules like carbohydrates, proteins, lipids, and mitochondria of cell affecting its function (Gutteridge and Halliwell 2000). There are numerous evidences suggesting that endogenous and exogenous ROS and RNS (reactive nitrogen species) play a major role in the pathogenesis of asthma and factors of asthma severity (Bowler 2004). Asthma is characterized by the presence of amplified levels of RNS and ROS in sputum and breath condensates, which further enhance epithelial permeability, increase mucus secretion, and induce smooth muscle contraction and airway hyperresponsiveness (Rogers and Cismowski 2018). The major enzymatic antioxidants in lungs are superoxide dismutase (SOD), catalase, glutathione S-transferase, and thioredoxin, and nonenzymatic antioxidants include glutathione, cysteine, homocysteine, urate, and ascorbate (Wei Sheg et al. 2014). When ROS overcomes endogenous antioxidant protective response, it leads to deleterious effects and activation of various pathways that have a role in the pathogenesis of asthma (Nadeem et al. 2008).

21.2.1 Interplay Between Oxidative Stress and Inflammation in Asthma

There are numerous evidences to prove that overproduction of ROS can evoke inflammatory responses. The inflammatory cells of asthmatics have an increased capability to generate free radicals compared to controls, which further contribute to high concentrations of ROS (Kleniewska and Pawliczak 2017). This is confirmed by the studies that showed increased generation of superoxide anion radicals by inflammatory cells from peripheral blood and bronchoalveolar lavage (BAL) fluid of asthmatic subjects than those from normal controls (Nadeem et al. 2003).

Asthmatic patients demonstrated increased production of ROS by many cell types within the lung including macrophages, antigen-presenting cells (APCs), neutrophils, and eosinophils. On the other hand, ROS can directly stimulate histamine release from mast cells and mucus secretion from airway epithelial cells. ROS are also known to modify the properties of endothelial barrier dysfunction and increase permeability to fluid, macromolecules, and inflammatory cells resulting in bronchial hyperreactivity which is a characteristic of asthma (Park et al. 2009; Rahman and Adcock 2006b).

Oxidants in the lungs which are inhaled or produced by the inflammatory cells act as secondary messengers and activate signal transduction pathways (Lee and Yang 2012). ROS are known to activate the transcription factors like NF-κB and AP-1, which lead to increased inflammatory gene transcription. They play an important role in inflammatory and immune response in most of the cells and are an essential factor that contribute to asthma progression by activating gene coding for pro-inflammatory cytokines (Lee and Yang 2012; Barnes and Adcock 1997). The airways of asthmatic patients have predominant NF-κB activity especially in epithelial cells and macrophages (Imanifooladi et al. 2010) (Fig. 21.1).

21.2.1.1 NF-κB Signaling Pathway

ROS acts as a second messenger for the degradation of IκB, which holds NF-κB in the cytoplasm. Hyperoxic conditions enhance the activation of IKK which leads to enhanced phosphorylation and degradation of IκB promoting the nuclear translocation of NF-κB and its binding to DNA. Many of the inflammatory mediators produced in the airways are regulated by NF-κB pathway, which includes pro-inflammatory cytokines like IL-1β and TNF-α. This pathway also induces genes of many inflammatory cytokines like IL-4, IL-5, IL-9, and IL-15 (Park et al. 2009; Lee and Yang 2012). Further, p50-deficient mice lack the production of IL-4, IL-5, and IL-13 which are supposed to play divergent roles in asthma pathogenesis (Ziegelbauer et al. 2005). Adhesion molecules, such as ICAM-1 (intercellular adhesion molecule-l) and VCAM-1 (vascular cell adhesion molecule-l), are also upregulated by NF-κB pathway (Fig. 21.1). RANTES and eotaxin which attract eosinophils are increased by the activation of NF-κB. Further, the expression of INOS (inducible nitric oxide synthase) is also increased which causes augmented nitric oxide exhalation in asthma patients (Stütz and Woisetschläger 1999; Mori et al. 1999; Sugiura and Ichinose 2008).

21.2.1.2 Activator Protein-1

c-Fos and c-Jun are the other important transcription factors that play a major role in inflammatory process underlying asthma. They dimerize to form homodimeric (Jun/Jun) and heterodimeric (Fos-Jun) complexes of the activator protein (AP)-1 family (Janssen et al. 1997). AP-1 is known to be involved in oxidant signaling, pathogenesis of lung injury, apoptosis, and immune responses (Karin et al. 1997; Shaulian and Karin 2002). AP-1 is also an important contributor to the expression of Th2 cytokines, IL-4, IL-5, and IL-13 (Raju et al. 2014). There is an evidence of augmented c-Fos expression in the epithelial cells of asthmatic patients (Barnes and

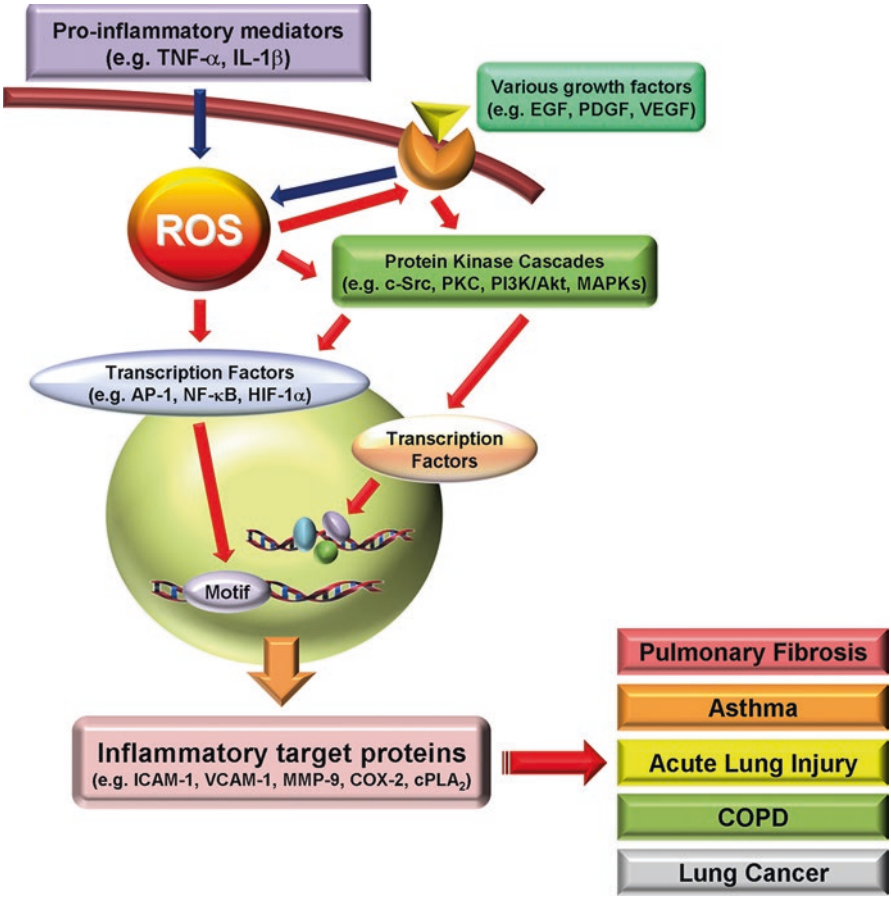

Fig. 21.1 General overview of the role of ROS in different respiratory diseases which activates transcriptional factors like NF-κB, AP-1, and HIF-1α which in turn produces inflammatory target proteins

Adcock 1998). Studies have indicated that oxidants like O_2^- and H_2O_2 can upregulate the transcriptional factors like Fos and Jun (Amstad et al. 1990; Kiyoshi et al. 1991). In a recent study, SIRT 1 (sirtuin 1) decreased c-Fos/c-Jun acetylation, thereby inhibiting the transcription of AP-1 which subsequently reduced the expression of COX-2 and PGE_2 (Zhang et al. 2010). In a study, a small molecule inhibitor of redox-regulated NF-κB and activator protein-1 transcription blocked allergic airway inflammation in a mouse model of asthma (Ziegelbauer et al. 2005). These results clearly show that ROS activates AP-1 which in turn produces inflammatory mediators (Fig. 21.2).

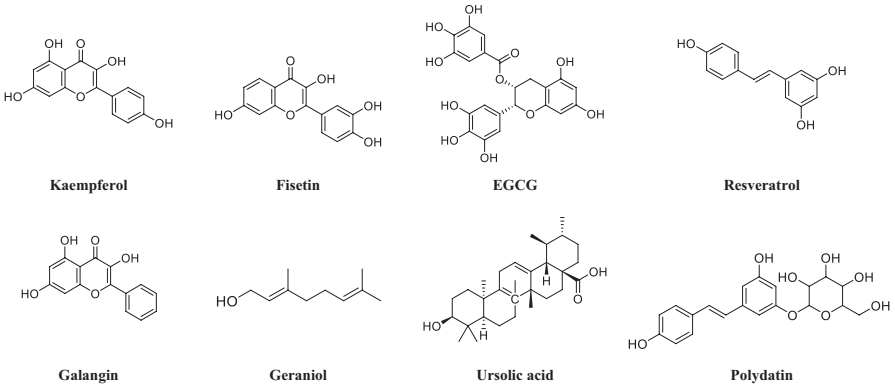

Fig. 21.2 Structures of phytochemicals effective against asthma

21.2.1.3 Hypoxia-Inducible Factor 1

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor, which is made up of two subunits HIF-1α and HIF-1β. HIF-1β is constitutively expressed, whereas HIF-1α is degraded by ubiquitin-protease system; under hypoxic conditions this subunit is stabilized, and it is translocated into the nucleus and induces hypoxia-responsive genes like inflammatory genes and VEGFA (vascular endothelial growth factor A) (Lee et al. 2007). ROS increase vascular permeability by inducing VEGFA expression through upregulation of HIF-1α (Lee and Yang 2012). ROS have also been known to stabilize HIF-1α under hypoxic or non-hypoxic conditions and promote its translocation into the nucleus (Lee et al. 2006). Haddad et al. reported that TNF-α increased the accumulation of ROS and activation of HIF-1α (Haddad and Land 2001). HIF-1α levels are increased when they are exposed to ROS and the levels are decreased when they are subjected to antioxidants (Park et al. 2009).

21.3 Oxidative Stress Markers in Asthma

Direct measurement of free radicals is difficult because of their short-lived nature. It is well known that overproduction of ROS has detrimental effects on biological molecules like lipids and protein. Therefore, oxidative stress is measured indirectly by studying its effects on the biomolecules.

The most studied markers are isoprostanes, malondialdehyde (MDA), myeloperoxidase (MPO), and thiobarbituric acid. The markers of protein damage studied are nitrotyrosine and bromotyrosine (Nadeem et al. 2008). Isoprostanes are prostaglandin-like molecules which are produced during oxidative damage on polyunsaturated fatty acids (PUFA) present in the cell. Some of the isoprostanes increase airway hyperresponsiveness (AHR) and smooth muscle constriction; apart from these effects, isoprostanes (8-iso-$PGF_{2\alpha}$) can also enhance the binding of macrophages to endothelial cells and stimulate the secretion of IL-18 by regulating

mitogen-activated protein kinase (MAPK) pathway. They are present in detectable amounts in the biological fluids and unaffected by fat in the diet. Asthma is characterized by increased lipid peroxidation as evident from increased level of isoprostane 8-iso-$PGF_{2\alpha}$, in asthmatics compared to normal group. Further evidences include the urinary excretion of isoprostane in the urine of mild atopic asthmatics (Voynow and Kummarapurugu 2011). It is reported that increased levels of isoprostane 15-F_{2t}-isoP were observed in serum and urine samples of asthmatics compared to normal people (Wedes et al. 2009).

MDA is a product of lipid peroxidation which is a consequence of oxidative stress (Wood et al. 2003). The levels of MDA are measured in blood, sputum, bronchoalveolar lavage fluid, and exhaled breath condensate (EBC). MDA levels in EBC are high in asthmatics compared to normal (Nadeem et al. 2008). Ethane, another product of lipid peroxidation, is also upregulated in asthmatics (Paul et al. 2001). Further more, the damaged lipids alter the structure of structural proteins in the cell membrane, which leads to proteolytic degradation (Yao and Rahman 2011a).

21.4 Animal Models of Asthma

Animal models of asthma are required to understand the pathophysiology of the disease and to study the efficacy of compounds against it. Several species like mice, rats, guinea pigs, and primates are used in the studies, and different methods are used to induce the asthma in these animals (Zosky and Sly 2007; Stevenson and Belvisi 2008). Some of the commonly used methods are described below.

21.4.1 Ovalbumin-Induced Model of Asthma

Ovalbumin (OVA)-induced allergic inflammation is the most commonly used model to study asthma. OVA is an antigen present in egg whites, which is administered along with an adjuvant to enhance immunogenicity. The most frequently used adjuvant are aluminum hydroxide or alum and ricin. A usual protocol would be sensitization of animals to OVA along with adjuvant through i.p route. The second dose can be given 1–2 weeks later; this is followed by sensitization of OVA to the airways generally by aerosols. The animals develop AHR in 24–48 h. In this model, there is an increased IgE level, Th2-subtype cytokine secretion, bronchoconstriction, edema, and increased mucus secretion which are characteristic features of asthma. The main limitation of the model is the development of tolerance on chronic exposure of OVA [53 54].

21.4.2 House Dust Mite Model of Asthma

House dust mite (HDM, *Dermatophagoides* sp.) is the common allergen worldwide; 50–85% of asthmatics are HDM allergic (Gregory and Lloyd 2011). HDM

extracts have different allergens and contribute to its allergic lung inflammation. The model is advantageous over OVA induced asthma, in that, the model can replicate the features of chronic asthma and airway remodeling upon longer exposure to HDM. Asthma-like features, i.e., increased IgE and increased APC, lymphocytes and eosinophils are reported to develop by administering HDM intranasally for 10 days (Mullane and Williams 2014; Stevenson and Birrell 2011).

21.5 Phytochemicals Effective Against Asthma

21.5.1 Kaempferol

Kaempferol is a flavonoid, a polyphenolic compound commonly present in medicinal plants like *Euphorbia pekinensis* Rupr., *Ginkgo biloba* L., *Hypericum perforatum* L., *Phyllanthus emblica* L., *Ribes nigrum* L., and *Rosmarinus officinalis* L. It is also present in apples, tomatoes, broccoli, grapes, and other edible foods. The protective role of kaempferol is well studied. It is an antioxidant, anti-apoptotic, and anti-inflammatory compound (Calderon-Montano et al. 2011). Gong et al. reported abrogation of eosinophil deposition and degranulation in lung tissue of ovalbumin (OVA)-induced asthma in mice by kaempferol treatment through downregulation of NF-κB pathway (Gong et al. 2011). Chung et al. evaluated the effect of kaempferol and kaempferol-3-O-rhamnoside, water-soluble form of kaempferol on OVA-challenged mice; results demonstrated that pretreatment of kaempferol inhibited Th2-related cytokine level (IL-4, IL-5, and IL-13) by antioxidant effect. In contrast, kaempferol-3-O-rhamnoside had lower antioxidant effect compared to parent kaempferol but showed higher inhibitory effect on Th2 cytokines, TNF-α level, and IgE production (Chung et al. 2015).

21.5.2 Fisetin

Fisetin is a flavonoid present in foods including fruits like strawberry and apple and vegetables like onions and cucumber (Sung et al. 2007). It has demonstrated diverse beneficial effects like antioxidant, anti-inflammatory, neuroprotective, anticancer, antidiabetic, antiviral, as well as anti-angiogenesis effects in both in vitro and in vivo models. In a study, prophylactic treatment with fisetin in OVA-challenged mice decreased airway hyperresponsiveness, mucus hypersecretion, and Th2 cytokine level (IL-4, IL-5, and IL-13) in bronchoalveolar lavage fluid which was increased during OVA administration. Fisetin markedly inhibited p65 nuclear translocation, thereby inhibiting NF-κB pathway. These results corroborated with in vitro study on human lung cell lines where fisetin suppressed NF-κB reporter gene expression (Goh et al. 2012).

21.5.3 Epigallocatechin Gallate

Epigallocatechin gallate (EGCG) is a catechin present in green tea leaves, oolong tea, and black tea leaves. It has antioxidant and anti-inflammatory properties and is proven effective in disorders of the cardiovascular system, ulcerative colitis, kidney disorders, and cancer (Eng et al. 2017). Kim et al. reported the protective effect of EGCG on toluene diisocyanate-induced airway inflammation in a murine model of asthma; treatment with EGCG reduced the generation of ROS and MMP-9 expression; decreased the number of inflammatory cells like eosinophils, macrophages, and neutrophils; and reduced the level of TNF-α in BAL fluids (Kim et al. 2006). In another study on OVA-induced asthma in mice, EGCG reduced the count of neutrophils and eosinophils in BAL; decreased airway resistance; decreased cytokine levels of IL-4, IL-6, and TNF-α; and improved proportion of Th17/Treg cells and exerted its effect through TGF-β1 signaling pathway (Shan et al. 2018).

21.5.4 Resveratrol

Resveratrol is a polyphenolic compound present in families like Vitaceae, Dipterocarpaceae, Gnetaceae, Cyperaceae, and Leguminosae. It is present in various food and food products such as grapes, wine, grape juice, mulberries, and cranberries (Pangeni et al. 2014). The effect of resveratrol against house dust mite (HDM)-induced asthma was studied in a mouse model. Treatment with resveratrol decreased the levels of IL-6, IL-17, TNF-α, and TGF-β in BALF which were upregulated in HDM-treated mice per se. It also suppressed the IgE-induced expression of Syk (spleen associated tyrosine kinase) in RBL-2H3 cells (Chen et al. 2015). In another study on OVA-induced asthma, administration of resveratrol decreased 8-isoprostane level by its antioxidant effect and decreased activation of PI3K-Akt signaling by restoring the expression of INPP4A (inositol polyphosphate 4-phosphatase) (Aich et al. 2012).

21.5.5 Galangin

Galangin belongs to chemical class of flavanol, mainly present in medicinal plants *Alpinia officinarum* and *Helichrysum aureonitens* and foods like honey. It has various pharmacological properties like anti-inflammatory, antioxidant, and anti-fibrotic (Mak et al. 2018). Zha et al. studied the effect of galangin on TNF-α-stimulated human ASMC (airway smooth muscle cells); they also studied the effect of galangin on OVA-induced asthma. The results of their study demonstrated that galangin inhibited the NF-κB pathway in TNF-α-stimulated human ASMC, whereas it decreased Th2 cytokine (IL-4, IL-5, and IL-13) levels in BALF, decreased IgE, and suppressed NF-κB activity in OVA-induced asthma (Zha et al. 2013). Another study reported that treatment with galangin attenuated TGF-β-induced ROS production in human ASMC and decreased OVA-specific IgE level in serum as well as reduced

α-SMA and MMP-9 expression and VEGF and TGF-β1 expression in OVA-induced asthma model (Liu et al. 2015).

21.5.6 Geraniol

Geraniol is a monoterpene alcohol present in geranium, lemon, and other essential oils in medicinal plants. Previous studies reported the antioxidative, antimicrobial, antitumor, and anti-inflammatory activities of geraniol (Lei et al. 2018). Xue et al. reported the protective effect of geraniol in OVA-induced asthma. Geraniol increased Nrf-2 expression and increased GST and SOD activities in OVA-challenged mice showing its antioxidant effect by decreasing oxidative stress through Nrf2/ARE pathway as well as improving Th1/Th2 balance in lungs (Xue et al. 2016).

21.5.7 Polydatin

Polydatin is a glucoside mainly extracted from the plant *Polygonum cuspidatum*, a natural antioxidant known to have many medicinal properties. Polydatin is well known for its anti-inflammatory, analgesic, cardioprotective, and antitumor activities (Du et al. 2013). Polydatin was evaluated against OVA-induced asthma in a mouse model; OVA-treated mice showed increased ROS and TGF-β and decreased Nrf-2 activity. However, polydatin treatment enhanced the antioxidant NQO1 enzyme activity and increased Nrf2 and HO-1 expression. These results conclude that polydatin effectively decreased the ROS production and fibrosis by increasing Nrf2 activation (Zeng et al. 2018).

21.5.8 Ursolic Acid

Ursolic acid (UA) is a pentacyclic triterpenoid carboxylic compound present in many medicinal plants, belonging to Lamiaceae family. UA is known for its hepatoprotective, cardioprotective, antitumor, antidiabetic, and inflammatory properties (López-Hortas et al. 2017). Kim et al. reported the protective effect of ursolic acid against ovalbumin-induced asthma by decreasing the influx of inflammatory cells in BAL and the level of Th2 cytokines and IgE (Kim et al. 2013).

21.6 Oxidative Stress in COPD

COPD is characterized by progressive airflow obstruction that is generally not reversible. Smoking is the major risk factor for COPD; each puff of cigarette smoke includes 10^{17} oxidants/free radicals (Church and Pryor 1985). Once the disease is established, the production of ROS doesn't halt even after the cessation of smoking due to generation of ROS from mitochondrial respiration. Other factors like air

pollution, occupational dust, and infections exacerbate COPD (Białas et al. 2016). One of the major factors for the pathogenesis of COPD is the imbalance between oxidants and antioxidants (Domej et al. 2014). There are numerous markers like H_2O_2 and 4-hydroxy-2-nonenal to identify the ROS-induced damage in COPD. Many studies have confirmed that markers of oxidative stress are increased in the lungs and systemically (blood) in COPD patients. Hydrogen peroxide concentration is increased in the exhaled breath of smokers with COPD compared to non-smokers (Rahman 2005). A product of lipid peroxidation, 4-hydroxy-2-nonenal, is seen higher in the bronchial secretions of COPD patients as compared to the normal control, and its level is elevated in the smokers without COPD compared to non-smokers. Sputum of COPD patients had increased concentration of nitrotyrosine compared to healthy controls and asthmatics (Petruzzelli et al. 1997). The TBARS concentration is increased in lungs and breath condensate in COPD patients. Moreover ROS has deleterious effects on nucleic acids; 8-hydroxyguanosine (8-OHG) is the oxidized product of RNA and is prevalent in lung tissue of emphysema (Fischer et al. 2011).

The human body has an antioxidant defense mechanism to prevent the damage caused by ROS. Numerous enzymes for detoxification of aldehydes are increased in mice subjected to cigarette smoke. The total antioxidant capacity in COPD patients is decreased compared to normal control. In a study there was a decrease in the mRNA of GSTP1 (glutathione S-transferase P), GSTM1 (glutathione S-transferase mu 1), EPHX (epoxide hydrolase 1), and TIMP2 (tissue inhibitor of metalloproteinases) in lung tissues of COPD subjects (Yao and Rahman 2011b). Several studies have shown a clear association between reduced levels of the antioxidants in the lungs, such as tocopherol and ascorbic acid, and deteriorating pulmonary function in COPD (Rahman 2005). Nrf-2 is a transcription factor which regulates antioxidant proteins. A study indicated that exposure of cigarette smoke to Nrf-2-deficient mice had amplified inflammation, apoptosis, and exacerbated emphysema. Nrf2 and Nrf2 activators have great prospective for shielding against RNS in tobacco smoke, particularly in COPD patients (Tuder et al. 2006).

The consequence of ROS is imbalance of protease/anti-protease in the lungs, which is observed in the emphysema. There is an amplified burden of elastase on the lungs due to deficiency of α1-antitrypsin. A study indicated that cigarette smoke can inactivate anti-proteases. As a result there is significant accumulation of macrophages and neutrophils which can release proteases like matrix metalloproteinases (MMP) and cathepsins which guide the degradation of α1-antitrypsin. Cigarette smoke is implicated to have a role in the apoptosis of pulmonary endothelial cells and apoptosis is an early event occurring in alveolar tissue devastation in the emphysema (Rahman 2005; Fischer et al. 2011).

21.6.1 Interplay of Oxidative Stress and Inflammation in COPD

Inflammation in COPD is not a separate thing by itself but is integrally related to oxidative stress. Inflammation is characterized by release of pro-inflammatory

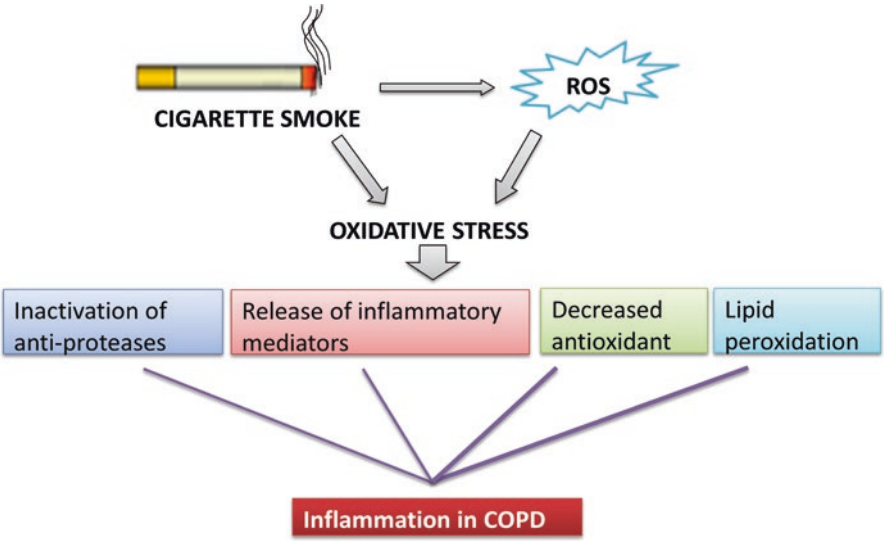

Fig. 21.3 Mechanism of ROS-mediated lung inflammation in COPD. Oxidative stress generated causes inactivation of anti-proteases and, release of inflammatory mediators and lipid peroxidation products leads to inflammation in COPD

mediators in the lung by neutrophils, B-cells, macrophages, T-cells, eosinophils, and mast cells (Rennard and Barnes 2002). Oxidants in cigarette smoke can trigger macrophages to produce ROS, which can magnetize neutrophils in the lungs (Fig. 21.3).

It is reported that circulating neutrophils release more O_2 in smokers and COPD patients (Rennard and Barnes 2002). It has been studied that immune cells of COPD patients release more proteases in sputum and BAL than normal control. Neutrophils secrete several proteinases, including neutrophil elastase (NE) and cathepsin G which add to parenchymal devastation. Neutrophil deformability was increased by oxidants and enhanced its sequestration to the endothelial cells of blood vessels in the lungs (Fischer et al. 2011). Chronic inflammation evidenced by increased levels of IL-1β, IL-6, CXCL8/IL-8, GM-CSF, and TNF-α secreted by macrophages exposed to cigarette smoke is the characteristic feature of COPD (Yao and Rahman 2011b).

21.6.2 Inflammation and Gene Expression

There is an increase in concentration of TNF-α and IL-8 in the sputum of COPD patients. The genes for these inflammatory mediators are regulated by NF-κB pathway. There is an augmented expression of p65 protein of NF-κB in the epithelial cells of lungs in smokers and COPD patients (Keatings et al. 1996b). Many stimuli can trigger the activation of NF-κB; one of the stimuli is cigarette smoke. Cigarette smoke

and extracts of cigarette smoke can activate NF-κB in immune cells. Smokers with COPD and currently healthy smokers both increase DNA binding activity of NF-κB causing an increased release of inflammatory mediators such as nitric oxide, IL-8, IL-1, and prostaglandins thereby upregulating COX-2 enzyme (Rom et al. 2013).

21.6.3 Histone Modifications

Acetylation of histone proteins results in uncoiling of the DNA, thereby allowing transcription factor binding leading to gene transcription. Histone acetylation can be reversed by deactivating histones through removal of acetyl group. Histone deacetylases (HDAC) suppress gene expression by switching off gene transcription through recruiting co-suppressor proteins (Barnes 2009). In COPD, in peripheral lung airway biopsies, and in alveolar macrophages, there is a raise in the acetylation of histones coupled with the promoter region of inflammatory genes, such as IL-8, that are regulated by NF-κB, and the scale of acetylation increases with disease severeness. HDACs also have the capability to deacetylate non-histone proteins, such as NF-κB, thus modifying NF-κB-dependent pro-inflammatory gene transcription (Szulakowski et al. 2006). HDAC-2, one of the isoforms of HDAC, is necessary for the immunosuppressive actions of glucocorticoids. Reduced level of HDAC-2 is associated with increased pro-inflammatory response and reduced responsiveness to glucocorticoids in alveolar macrophages obtained from smokers (Ito et al. 2001). Thus cigarette smoke/oxidants not only decrease the activity of HDAC-2 in the macrophages and epithelial cells but also decrease the functions of glucocorticoids in COPD patients. Oxidative stress plays a major role in decreasing the activity of HDAC-2 by posttranslational modification which leads to proteolytic degradation of HDAC-2 (Adenuga et al. 2009).

21.6.4 Sirtuin 1

SIRT-1 (sirtuin 1) is the most studied human sirtuins reported to possess many physiological actions like anti-apoptotic, anti-inflammatory, and antiaging properties. SIRT-1 is a HDAC that removes acetyl group on the histones and silences the gene transcription (Rahman et al. 2012). The level of HDAC is affected by posttranslational modifications, oxidants, and aldehydes derived from lipid peroxidation which causes SIRT-1 phosphorylation in macrophages and mouse lungs (Caito et al. 2010). Increased activation of NF-κB is seen when SIRT-1 has been knocked down using siRNA; SIRT-1 also suppressed the activation of activator protein-1 which leads to downregulation of COX-2 enzyme suggesting that modulation of SIRT-1 can be a therapeutic target for COPD (Rajendrasozhan et al. 2008). Nrf-2 is the other transcription factor present in cell which imparts protection against ROS produced from cigarette smoke. Nrf2 activation leads to upregulation of many antioxidant genes and there is a decreased activation of Nrf-2 in the patients suffering from COPD (Tuder et al. 2006). Many phytochemicals can activate Nrf-2 and can protect against

deleterious effects of ROS. Combining Nrf-2 activators with other therapeutic drugs to treat COPD will have positive effects.

21.7 Phytochemicals in Protection Against COPD

21.7.1 Curcumin

Curcumin is a yellow-colored compound present in *C. longa* and is a perennial member of the Zingiberaceae family. Curcumin is a well-explored phytochemical for various pharmacological activities (Gupta et al. 2013). Curcumin inhibited PPE (porcine pancreatic elastase)-induced inflammation and emphysema by increasing antioxidants and inhibiting chemokine secretion. In another model with cigarette smoke, curcumin decreased the number of inflammatory cells in BAL and decreased protein carbonyl levels, indicator of oxidative stress in BALF (Suzuki et al. 2009). Jin et al. reported the protective effect of curcumin against LC (LPS and cigarette smoke)-induced COPD in a mouse model and LPS-stimulated BEAS-2B cells in vitro (Yuan et al. 2018).

21.7.2 *Trans*-Anethole

Trans-anethole, the major constituent obtained from anise, star anise, and fennel, has been reported to have anti-inflammatory, antioxidant, anticarcinogenic, neuroprotective, and vasoactive effects. In PPE/LPS (porcine pancreatic elastase/lipopolysaccharide)-induced COPD mouse model, pretreatment with anethole decreased the level of LDH (lactate dehydrogenase) in the BAL of animals, which was increased in PPE/LPS alone treated group of animals. The number of inflammatory cells like lymphocytes, neutrophils, and macrophages are increased in the PPE-/LPS *per se* treated group than in control group. Pretreatment with anethole significantly decreased the count of inflammatory cells. Pro-inflammatory cytokine levels are measured by ELISA which indicated the increased level of these cytokines in PPE/LPS group compared to control group and pretreatment with anethole decreased the level of these cytokines (Kim et al. 2017).

21.7.3 Andrographolide

Andrographolide, one of the diterpenoids, is purified from the aerial parts of plants of the genus *Andrographis*. Andrographolide is known to possess hepatoprotective, antiviral, anticancer, anti-inflammatory, and antithrombotic effects. Andrographolide has been studied against cigarette smoke-induced lung injury in mice. Andrographolide significantly decreased the total inflammatory cells and neutrophils. It suppressed the gene expression of GM-CSF (granulocyte-macrophage colony-stimulating factor), TNF-α, and MIP (macrophage inflammatory protein)-2α

and also decreased the levels of IL-1β, IP-10 (interferon gamma-induced protein), MCP-1 (monocyte chemoattractant protein), and KC (keratinocyte chemoattractant) in BALF, which were upregulated by exposure to cigarette smoke. Andrographolide markedly suppressed the levels of 3-NT (nitrotyrosine), 8-OHdG, and 8-isoprostane ameliorating oxidative damage to proteins, DNA, and lipids induced by cigarette smoking. Andrographolide by its antioxidant property promoted the GSH-related enzyme activity as well as increased the nuclear Nrf-2 levels (Guan et al. 2013).

21.7.4 Quercetin

Quercetin is a 3,3′,4′,5,7-pentahydroxyflavone found in many plants. Due to its polyphenol structure, quercetin has potent antioxidant effects. A broad spectrum of beneficial properties have been described for quercetin, including anti-inflammatory effects, atherosclerosis, thrombosis, hypertension, and arrhythmia. Quercetin was tested against elastase-/LPS-induced lung injury which showed features of COPD in mice (Fig. 21.4).

Elastase-/LPS-exposed mice treated with vehicle showed significantly increased levels of TBARS and iNOS, decreased levels of HMOX-1 (heme oxygenase-1) mRNA, and decreased ratio of *iNOS/HMOX-1*. In contrast, elastase-/LPS-exposed mice treated with quercetin showed significantly reduced TBARS, increased HMOX-1 mRNA, and decreased *iNOS/HMOX-1* compared to vehicle-treated controls. These results demonstrated the antioxidant effect of quercetin. Quercetin treatment of elastase-/LPS-exposed mice inhibited the MMP-9 and MMP-12 activities and increased both Sirt1 mRNA and protein levels. Quercetin treatment also decreased the levels of all chemokines and pro-inflammatory cytokines (Ganesan et al. 2010).

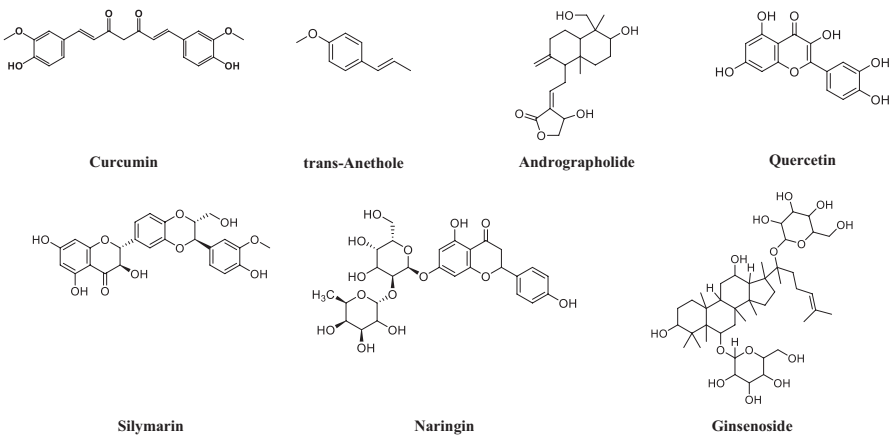

Fig. 21.4 Structures of phytochemicals effective against COPD

21.7.5 Silymarin

Silymarin is a flavonoid extracted from milk thistle (*Silybum marianum*) and has been extensively investigated for antioxidant, anti-inflammatory, anti-apoptotic, and anti-fibrotic properties. Silymarin is also studied against CS-induced airway inflammation. BALF of CS-treated mice per se had increased number of macrophages and neutrophils, whereas silymarin treated along with CS exposure decreased these inflammatory cells in BALF. Further, the pro-inflammatory cytokine levels (TNF-α, IL-1β, and IL-8) increased in CS-treated mice, and silymarin pretreatment decreased the levels of these cytokines. In conclusion silymarin had protective effects by inhibiting ERK/p38 MAPK pathway (Li et al. 2015). In another study silibinin, a major active component of silymarin, was studied and inhibited the pulmonary fibrosis in CS- and LPS-exposed mice by suppressing TGF-βb1/Smad 2/3 pathway (Ko et al. 2017).

21.7.6 Ginsenoside Rg1

Ginsenoside Rg1 is a major ginsenoside present in *Panax ginseng* and is known for many pharmacological properties like antiaging, immunoregulation, neuroregulation, lipid regulation, anti-thrombosis, and wound healing (Kim 2017). Ginsenoside Rg1 was studied against CS-induced COPD in rats. The results indicated that ginsenoside Rg1 decreased the pulmonary fibrosis by decreasing the expression of α-SMA (smooth muscle actin) and E-CAD (cadherin) partly by inhibiting TGF-β1/Smad pathway. Similar findings were observed in HBE cells exposed to CSE (Guan et al. 2017).

21.7.7 Naringin

Naringin is a flavonoid abundantly present in grapes and citrus family and is known to possess protective actions against hepatotoxicity, radiation-induced damage, ischemia reperfusion injury, neuroprotection, and nephrotoxicity. Naringin was studied for its effect on airway inflammation in a guinea pig model of chronic bronchitis induced by cigarette smoke. With repeated exposure to CS-induced cough, however, oral administration of naringin suppressed cough and reduced inflammatory cells in the lung tissue. Further, naringin markedly reduced the levels of IL-8, TNF- α, LTB4, and the MPO activity in BALF. Naringin dose dependently increased the levels of SOD, which was significantly decreased in the rats exposed to CS (Luo et al. 2012).

21.8 Conclusion

Despite the significant therapeutic effect of the synthetic drugs in the respiratory diseases like asthma and COPD, they are associated with resistance and severe adverse effects in many patients. Since ancient times, natural products are well known for their medicinal properties, which led to a paradigm shift toward phytochemicals for the development of new drugs. Phytochemicals of several classes like alkaloids, terpenoids, and polyphenols were evaluated against asthma and COPD, beyond their antioxidant property, and these compounds have shown profound anti-inflammatory property in the preclinical animal studies. These phytochemicals can be used in combination with other anti-inflammatory drugs or can be used alone in the treatment of asthma and COPD, allowing a decline in adverse drug reactions and cost. Further studies have to be conducted to judge their efficacy and safety for human use.

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Diallyl Trisulfide Prospectively Retrieves Arsenic Induced Lung Oxidative Stress, Inflammation Through the Activation of Nrf2/HO-1 Signaling

22

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Abstract

Background: Arsenic (As), an entrenched natural cancer-causing agent, has been found to cause oxidative pressure initiated fiery lung maladies in people upon unending introduction. The aim of our examination is to explore the ameliorative capability of garlic polysulfide, i.e., diallyl trisulfide (DATS), against As-initiated oxidative lung damage in rodents. **Methods:** Rats were directed with As (5 mg/kg BW) for about a month and treated as lethal control. The treatment convention was made by the pre-administration of DATS (40 mg/kg BW, PO) for about a month in As-treated rodents. Prooxidant and antioxidant status, oxidative markers, and Nrf2 and its related ARE and tissue morphometric investigations were performed in the lungs of rodents. **Results:** As inebriation essentially expanded the dimensions of free radicals, inflammatory cytokines, and lung edema (wet to dry weight proportion) when contrasted with control rodents. Moreover, lung oxidative pressure markers, for example, malonaldehyde (MDA) and myeloperoxidase (MPO), were likewise altogether expanded in As-treated rats. Histological and immunohistochemical (iNOS) analyses additionally uncovered that the As initiated provocative changes in the lung tissue of rodents. Pre-administration of DATS essentially improved the cell reinforcement status and restrained the oxidative pressure, cytokines, and Keap1 protein by means of the actuation of Nrf2 translocation into the nucleus. **Conclusion:** Morphometric,

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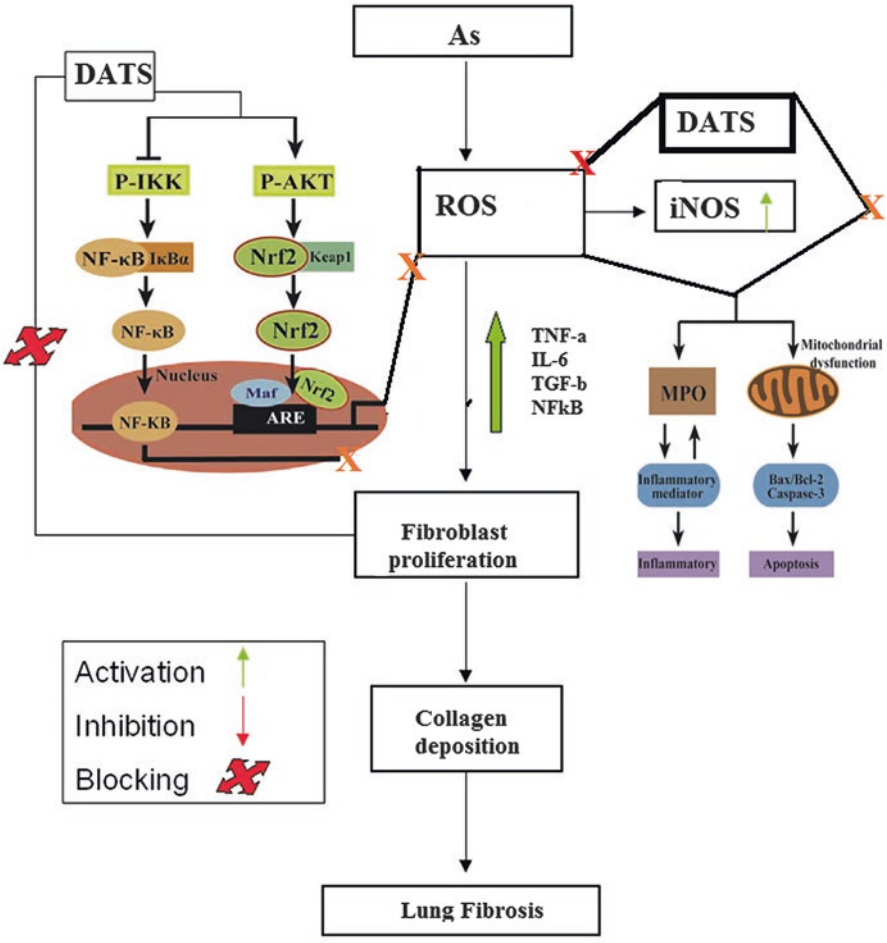
biochemical, histological, and immunohistochemical examination remarkably exhibited that DATS was proficient in limiting the fiery harm in lung tissue and improved the oxidative pressure. By and large, our outcomes prescribe that DATS may be a potential remedial contender for treating oxidative and inflammatory lung damage and hence warrants additionally examination.

Graphical Abstract



Graphical abstract

22.1 Introduction

Arsenic's (As) position as a biologically inescapable and epidemiologically basic metalloid is now starting to hurt an immense number of people far and wide, especially in the developing countries, since endless arsenic presentation has been experienced in light of radiations from mining activities, industrial or pesticide use, or sullied well water (Hughes et al. 2011; Mazumder and Dasgupta 2011). It is found in both inorganic and common structures and in different valence or oxidation states on the earth. Exposure to arsenic is connected with a broad assortment of dangers (Hughes et al. 2011). Harm of the skin, lung, kidney, liver, and urinary bladder are the basic developments related with these perilous effects (Smeester et al. 2011). Arsenic exposure is connected with an expanded danger of lung ailments, which may make it an irregular toxicant, including the abbreviation of inborn resistance and aspiratory injury (Kozul et al. 2012). Despite this broad collection of information about the hazardous effects of arsenic introduction, the precise mechanism of its lung lethality following intense and unending presentation remains perplexing. Curiously, the effect of arsenic presentation on lung function disability has additionally only been inconsistently reported. In light of the couple of epidemiological investigations detailed up until this point, decrements of lung function parameters have been found after ceaseless utilization of arsenic-debased drinking water, especially in individuals with arsenic related skin injuries (arsenicosis) (Nafees et al. 2011).

Introduction to arsenic is connected with an extended risk of lung ailments, which may make it a surprise toxicant, including the camouflage of characteristic opposition and pneumonic damage (Kozul et al. 2012). Besides these malignant effects, arsenic has also seemed to have critical sufficiency in treating individuals with broke confident or unmanageable extreme promyelocytic leukemia (Zhang et al. 2010). Arsenic-incited lung harm may be most associated with the reactive oxygen species (ROS). Arsenic -initiated unnecessary ROS have a direct hurtful effect on lung endothelial cells and may add to subsequent necrotic cell death (Brigham 1986). Arsenic drains glutathione and protein-bound sulfhydryl gatherings, which decrease reactive oxygen species (for instance, superoxide molecule, hydroxyl radicals, and hydrogen peroxide). The reactions of these ROS with cell biomolecules prompts lipid peroxidation, layer protein damage, cancer prevention agent irregularity, DNA damage, changed gene articulation, and apoptosis. The net consequence of arsenic's poisonous quality on the influenced cells is apoptosis or necrosis if not balanced by the cellular antioxidant process. Recently, common phytoantioxidants that go about as viable ROS foragers could give possible approaches to reduce the risk started by consistent arsenic introduction (Bhattacharya and Haldar 2012). Therefore, novel restorative techniques are required to decrease the troublesome health impacts related with the harmful effects of arsenic in the lung.

Garlic contains numerous organically and pharmacologically dynamic mixes like allicin, diallyldisulfide (DADS), and diallylsulfide (DATS), which are helpful to human wellbeing against various ailments (Chung 2006; Das and Chaudhuri 2014; Cao et al. 2018). DATS is an effective cancer prevention agent that shields cells from different oxidative pressure wounds. As of late, we have revealed the

antigenotoxic, hepato, cardio, and nephro defensive nature of DATS against the poisonous quality of arsenic in rodents (Sumedha and Miltonprabu 2013a, b, 2014a, Miltonprabu et al. 2017). The hypolipidemic and hypocholesteromic impacts of DATS in rodents have been recorded (Miltonprabu and Sumedha 2015). Likewise, investigations of DATS weakening the ROS generation and restraining mitochondrial lethality (Sumedha and Miltonprabu 2014b) has just been recently detailed from our lab. Thinking about all the above forerunners, the present examination was intended to assess the remedial impact of DATS against the lung oxidative danger actuated by arsenic in rats. This investigation sheds light on the inhibitory job of DATS against oxidative fibrosis instigated by As and the vital job of ROS and incendiary cytokines amid the improvement of fibrotic changes in the lungs. Our speculation proposes that the defensive impact of DATS may be ascribed to its cell reinforcement and calming properties that could restrain the activity of proinflammatory cytokines amid As-prompted lung damage.

22.2 Materials and Methods

22.2.1 Chemicals

Sodium arsenate (Na3AsO4), diallyl trisulfide (DATS), and BSA were bought from Sigma Chemical Co., St. Louis, MO, USA. Bax, Bcl-2, Bcl-xl, Nrf2, HO1, γGCS,GAPDH, β-actin, lamin, and NQO1 antibodies were obtained from Santa-Cruz Biotechnology, Inc., USA. Every single other synthetic and solvents were of guaranteed logical evaluation and bought from S.D. Fine Chemicals, Mumbai or Himedia Laboratories Pvt. Ltd., Mumbai, India. Reagent packs were obtained from Span Diagnostics, Mumbai, India.

22.2.2 Animals and Diet

Sound grown-up male albino rats of Wistar strain, reproduced and raised in Central Animal House, Bureau of Experimental Medicine, Rajah Muthiah Medical College, and Annamalai University, were utilized for the test. Rats were liked to maintain a strategic distance from complexities of the estrous cycle. Rats of equivalent weight (160–180 g) were chosen and housed in polypropylene confines fixed with husk and kept in a semi common light/dull condition (12 h light/12 h dim). The rats had free access to water and were provided with standard pellet diet (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd., Bangalore, India), constituting protein (22.21%), fat (3.32%), fiber (3.11%), offset with sugars (> 67%), nutrients, and minerals. Rats dealing with and test methodology were endorsed by the Institutional Animal Ethics Committee, Annamalai University (Registration number: 885/2012/CPCSEA) and they were minded as per the "Guide for the consideration and utilization of lab creatures" and "Council with the end goal of control and supervision on exploratory animals."

22.2.3 Experimental Plan

In the present investigation, arsenic (As) was given by means of the caudal vein as sodium arsenate in ordinary saline (0.9%) at a portion of 5 mg/kg body weight/day for about a month, which was 1/10 of the oral LD_{50} values in rodents. Control (group 1) only got the vehicles, test rodents were subdivided into two groups (3 and 4). Drug control group (group 2) was administered with DATS (40 mg/kg. bw/day) (dissolved in 10% of Tween 80) only. In the examination, a total of 24 rodents were utilized. The rodents were arbitrarily partitioned into 4 groups of 6 rats in each.

Group 1: (n = 6) Control rodents got the ordinary saline (0.9%) and the vehicle for about a month
Group 2: (n = 6) Rats directed with DATS broke down in Tween 80 (40 mg/kg. bw) for about a month
Group 3: (n = 6) Rats were given arsenic by means of caudal vein (5 mg/kg. bw) broke up in normal saline for about a month.
Group4: (n = 6) Rats pre-managed with DATS (40 mg/kg. bw) 90 min before the administration of As (5 mg/kg. bw/day) for about a month.

After the last treatment, rodents were fasted medium-term and anesthetized with pentobarbital sodium (35 mg/kg, IP) and euthanized by cervical beheading. Blood was collected in unheparinized tubes. The privilege and left lung tissues were fixed in 10% formalin for histological examination. A little segment of lung tissue was homogenized in 5.0 ml of 0.1 M Tris-HCl buffer (pH 7.4). The homogenate was centrifuged and the supernatant was utilized for the estimation of different biochemical parameters.

22.2.4 Determination of Arsenic Concentration

The arsenic concentration in the lung tissue was calculated using a hydride vapor generation system (PerkinElmer MHS-10) fitted with an atomic absorption spectrophotometer (AAS, PerkinElmer AAnalyst 100). Results are expressed as μg As/g wet weight for lungs.

22.2.5 Wet-to-Dry Lung Weight Proportion (W/D Proportion)

After willful extermination of rodents, the lungs were promptly weighed to get the wet weight, and afterward put in an oven at 60 °C for 48 h and weighed to acquire the dry weight. The proportion of the wet lung to the dry lung was determined to evaluate the lung edema.

22.2.6 Determination of Oxidative Stress Markers

Investigation of tissue thiobarbituric acid reactive substances (TBARS) level as a marker of lipid peroxidation was performed by the spectrophotometry technique (Ohkawa et al. 1979). This strategy was utilized to acquire a spectrophotometric estimation of the color created amid the response to thiobarbituric acid (TBA) with MDA at 535 nm. The MDA level is expressed as nmol/g-tissue protein. Myeloperoxidase (MPO) action, an index of the level of neutrophil accumulation, was estimated in tissues with ELISA unit (Bioxytech MPO-EIA, USA). The absorbance was perused at 405 nm utilizing Multi-Detection Micro Plate Reader. Quantification was accomplished by the development of a standard curve utilizing the known concentration of MPO. Results were communicated as ng/mg tissue protein (Schneider and Issekutz 1996).

22.2.7 Assurance of Responsive Oxygen Species (ROS)

The level of superoxide radicals ($O_2^{\bullet-}$) in lung tissue was determined by the spectrophotometric method of Pick and Keisari (1981). The outcomes were communicated as responsive radiance units (RLU) per 1 min for every milligram dry weight (RLU/min/mg dry weight). The hydrogen peroxide (H_2O_2) level was evaluated by the spectrophotometric technique of Pick and Keisari (1981). The H_2O_2 content of the example was expressed as μmole/min/mg protein. Hydroxyl radical (OH^-) generation was evaluated by the method of Puntarulo and Cederbaum (1988). The hydroxyl radical content of the sample was expressed as μmoles/min/mg protein.

22.2.8 Estimation of Pro-inflammatory Cytokines in Broncho Alveolar Lavage Fluid (BALF)

Toward the finish of the experimental period, the right lungs were ligated at the right main bronchus. At that point, the left lungs were lavaged with 1 ml of autoclaved PBS multiple times. The recuperation proportion of the liquid was around 80% (4 ml), the BALF was promptly centrifuged at 1500 rpm for 3 min at 4 °C, and the supernatants were put away at –70 °C until required for consequent tests. The levels of TNF-α, IL-1β, and TGF-β in BALF were determined by enzyme-linked immunasorbent (ELISA) kits.

22.2.9 Estimation of Non-enzymatic Antioxidants

Glutathione (GSH) level was estimated utilizing the technique of Moron et al. (1979) and was expressed as μg/mg protein. Vitamin E (a-tocopherol) level was evaluated by the strategy of Desai (1984) and was expressed as μg/mg protein.

22.2.10 Estimation of Enzymatic Antioxidants

Tissue superoxide dismutase activity was resolved from its capacity to restrain the auto-oxidation of pyrogallol as per the technique of Marklund and Marklund (1974). The enzymic action was expressed as U/mg protein. Catalase action was measured utilizing the strategy of Sinha (1972) and its action was expressed as U/mg protein (1 U is the measure of compound that uses l μmol of hydrogen peroxide/min). Glutathione peroxidase activity was determined by the technique of Rotruck et al. (1973). The unit was expressed as U/mg protein (1 U is the measure of catalyst that changes over l μmol of GSH to GSSG within the sight of H_2O_2/min). Glutathione reductase action was measured by the technique of Stall et al. (1969). The movement of GR was expressed as moles of NADPH oxidized/min/mg protein of cell separately. Glutathione-S-transferase was measured by the strategy of Habig et al. (1973). GST action was expressed as U/mg protein (1 U is the measure of chemical that conjugates l μmole of CDNB with GSH/min).

22.2.11 Immunohistochemistry of iNOS and NF-kB

Immunohistochemical localization of iNOS and NF-kB was performed by utilizing iNOS and NF-kB monoclonal antibodies from Lab vision Inc., Fremont, CA, USA. The avidin– biotin complex procedure was utilized. Quantitative estimations were completed utilizing an image analysis system (Leica Qwin 500 C Imaging System Ltd., Cambridge, England) to quantify the mean of optical thickness of iNOS response at an amplification of 400× in 10 non-covering fields from every creature in all gatherings.

22.2.12 Collagen Specific Assays

Hydroxyproline content is considered to be an essential biochemical marker for parenchymal collagen content and was evaluated in the lung tissue homogenate and BALF by the technique of Neuman and Logan (1950). Tissue segments were stained with Sirius red to distinguish collagen filaments under captivated magnifying instrument (Olympus B×50) as portrayed prior (Chen et al. 2003). Sirius red (Direct Red 80, Sigma, St. Louis, USA) was dissolved in a saturated picric acid solution at a concentration of 1 g/l to prepare the staining solution. Collagen appeared in yellow/orange shading when pictured under polarized light.

22.2.13 Western Blot Assay

Lung tissues were homogenized in RIPA lysis buffer (50 mM Tris– HCl, pH 7.4; 150 mM NaCl, 0.25% deoxycholicacid, 1%NP-40 and 1 mM EDTA) containing an EDTA-free protease inhibitor cocktail (Thermo logical, USA). After extraction of

protein, the protein samples (100 μg protein/path) were stacked and isolated on 10% sodium dodecylsulfate (SDS)- polyacrylamide (PAGE) followed by exchange into polyvinylidene difluoride (PVDF) membrane and blocking. At that point, the membranes were incubated overnight at 4 °C with a specific primary antibody against target genes followed by addition of the horse radish peroxidase-coupled secondary antibody (Abcam, Cambridge, UK). The immune-relative bands were visualized after treating with a chemiluminescence (ECL) reagent (Amersham International Plc., Buckinghamshire, UK) and were evaluated by densitometry and standardized with a housekeeping standard β-actin.

22.2.14 Histological Examination

The fixed lung tissue samples were dehydrated in graded ethanol series and implanted in paraffin. Every paraffin square was cut into 5-μm-thick cuts. At that point, these cuts were dewaxed in xylene, rehydrated in inclination alcohol series, and flushed with distilled water. For hematoxylin and eosin (H&E) staining, these slides were incubated with hematoxylin for 5 min and afterward with eosin for 3 min.

22.2.15 Statistical Evaluation

All the values were expressed as mean ± SD of various analyses. The statistical significance was evaluated by one-way analysis of variance using SPSS version 16.0 (SPSS, Cary, NC, USA) and the individual correlations were derived by Duncan's various range test (DMRT), followed by the post hoc test and least critical contrast (LSD). Values were considered statistically significant when $P < 0.05$.

22.3 Results

22.3.1 Impact of DATS on Arsenic Fixation and Wet-to-Dry Lung Weight Proportion (Edema (g/g))

Arsenic levels in the lung tissue of rats treated with As and As and DATS are given in Fig. 22.1a. A noteworthy increment in lung arsenic level was seen in As-inebriated rats when contrasted with the control gatherings. Pre-treatment with DATS fundamentally diminished the As fixation in the lung tissue when contrasted with that of As-only-treated rats.

Lung edema joined by a rise of wet-to-dry lung weight proportion was seen in control and treated rats. A huge increment in lung edema (wet-to-dry lung weight proportion) was seen in As-inebriated rats when contrasted with the control gatherings (Fig. 22.1b). Pre-treatment with DATS altogether diminished the As-initiated edema when contrasted with that of As-only-treated rodents. DATS-only-administrated rodents did not display any adjustments in the lungs.

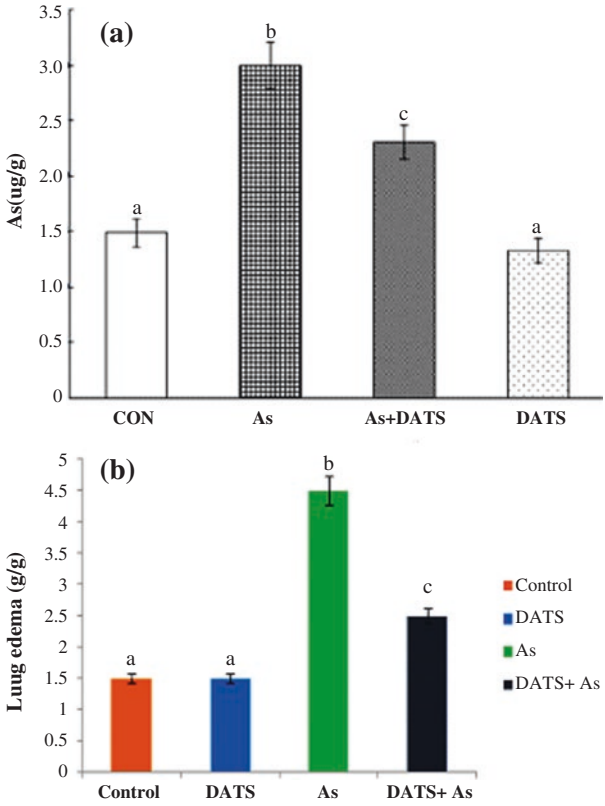


Fig. 22.1 Effect of DATS on As accumulation in the tissue (**a**) and lung edema (wet-to-dry lung weight ratio) (**b**) in control and experimental rats. Values are mean ± SD for 6 rats in each group; a, b, and c values are not sharing a common superscript letter a, b, and c differ significantly at $p < 0.05$ (DMRT)

22.3.2 Impact of DATS on Oxidative Stress Markers

The impact of DATS on the oxidative stress markers (TBARS and MPO) in the lungs of control and exploratory rodents are shown in Fig. 22.2. There was a critical increment in the dimensions of TBARS (Fig. 22.2a) and myeloperoxidase (MPO) (Fig. 22.2b) in the As-inebriated rodents when contrasted with the control rodents. Pre-oral administration of DATS to As-inebriated rodents altogether diminished the TBARS and MDA levels by its intense free radicals rummaging property. No critical changes were seen in the DATS-only-treated rodents.

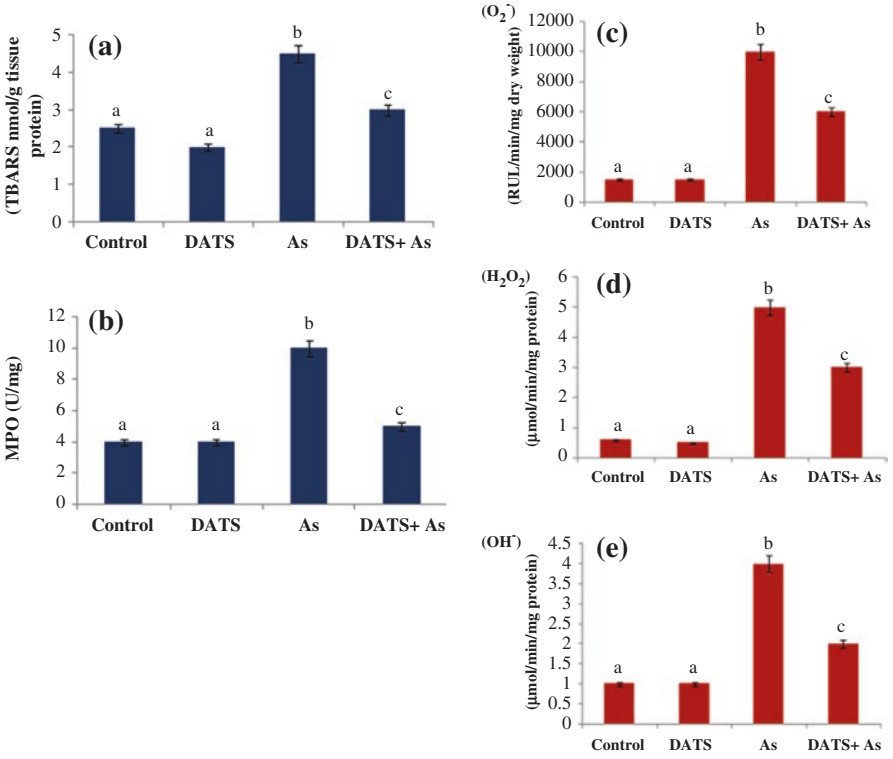


Fig. 22.2 Effect of DATS on As-induced lung oxidative stress marker TBARS (**a**), MPO (**b**), superoxide radicals (**c**), hydroxyl radical (**d**), and hydrogen peroxide (**e**) in control and experimental rats. Values are mean ± SD for 6 rats in each group; a, b, and c values are not sharing a common superscript letter (a, b and c) differ significantly at $p < 0.05$ (DMRT)

22.3.3 Impact of DATS on Free Radical Searching Capacity

Figure 22.2c–e demonstrates the free radical trenchant action of DATS on As instigated oxidative stress in control and treated rodents. The levels of superoxide radicals (2C), hydroxyl radical (2D), and hydrogen peroxide (2E), were altogether expanded in lung exposed to As. Preoral supplementation of DATS altogether diminished further production of these free radicals (ROS) when contrasted with the As-only-treated rodents.

22.3.4 Impact of DATS on As Prompted Pro-inflammatory Cytokines

The impact of DATS on As prompted pro-inflammatory cytokines in control and test rodents is shown in Fig. 22.3. As organization fundamentally expanded the dimensions of pro-inflammatory cytokines, for example, TNF-α (3A), TGF-β (3B),

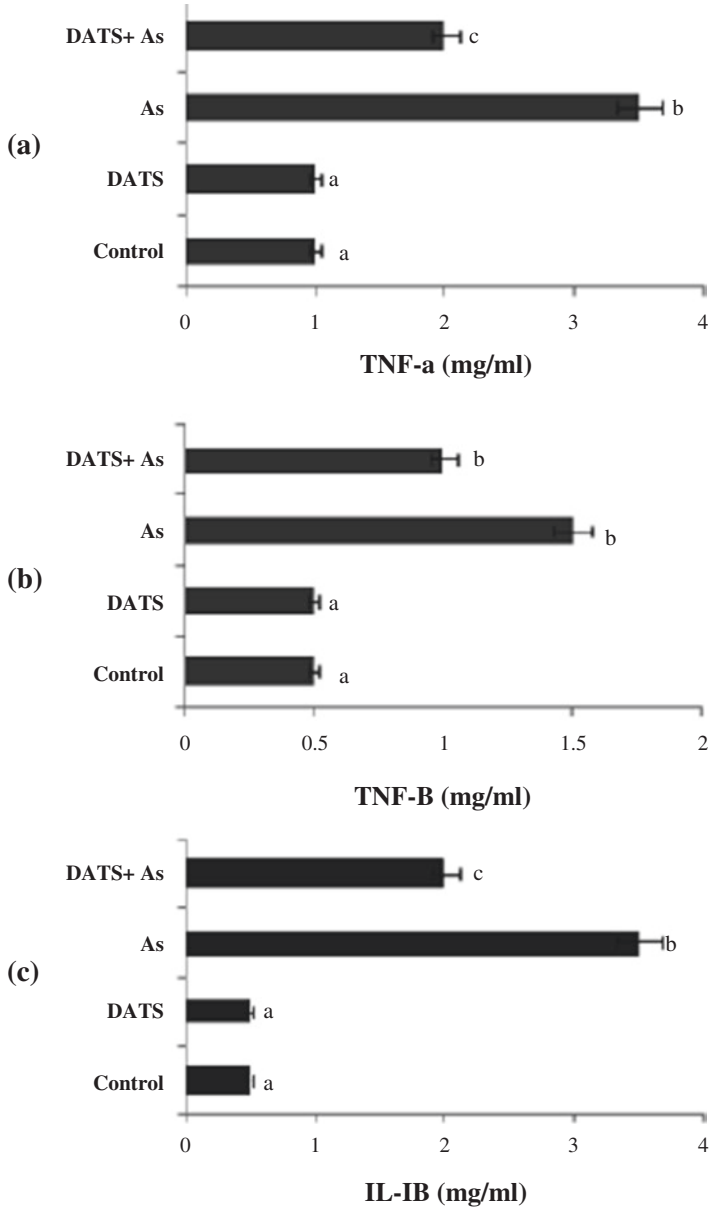


Fig. 22.3 Effect of DATS on As-induced lung inflammatory cytokines TNF-α (**a**), TGF-β (**b**), and IL-1β (**c**) in control and treated rats. Values are mean ± SD for 6 rats in each group; a, b and c values are not sharing a common superscript letter (a, b and c) differ significantly at $p < 0.05$ (DMRT)

and IL-1β (3C), in lungs treated with As. Pre-administration of DATS fundamentally diminished the pro-inflammatory cytokines strikingly when contrasted with that of control. DATS-only-administrated rodents likewise demonstrated some critical inhibitory impact on aggravation.

22.3.5 Impact of DATS on Enzymatic and Non-enzymatic Cell Reinforcement Levels

Table 22.1 demonstrates the impact of DATS on As-twisted enzymatic and non-enzymatic cell reinforcement levels of control and test rodents. There was a huge decline in the dimensions of enzymatic cell reinforcement, for example, SOD, CAT, GPx, GR, GST, and non-enzymatic antioxidants, for example, GSH and Vit. E in As-inebriated rodents when contrasted with control. This might be because improved dimensions of As prompted ROS that diminished these cellular antioxidants level. Pre-oral supplementation of DATS to As rodents altogether expanded the dimensions of both enzymatic and non-enzymatic antioxidants levels when contrasted with the As-only-inebriated rodents.

22.3.6 Expression of iNOS and NF-κB in Lung Tissue Segments

The lung tissue segment from control rodents demonstrated a low level of immunostaining for iNOS (Fig. 22.4a). As created an expanded articulation of iNOS in lung tissues, as shown in Fig. 22.4b. DATS treatment restrained the expression of this pro-inflammatory mediator of pneumonic fibrosis (Fig. 22.4c). The immunohistochemical staining was additionally measured and the consequence of the equivalent is portrayed in Fig. 22.4d. The control rodent lung tissue segment demonstrated a low level of immunostaining or NF-κB (Fig. 22.5a). As induction expanded the p65

Table 22.1 Effects of DATS and As on enzymatic and non-enzymatic antioxidant levels in control and experimental rats

Groups	Control	DATS	As	DATS + As
SOD (min/mg protein)	15. 52 ± 0.50[a]	16. 97 ± 0.37[a]	11. 77 ± 0.31[b]	13.35 ± 0.30[c]
CAT (min/mg protein)	81. 42 ± 10.20[a]	81.12 ± 12.49[a]	50. 59 ± 10.43[b]	66.37 ± 12.10[c]
GPx (min/mg protein)	12.31 ± 0.47[a]	13.97 ± 0.77[a]	10.35 ± 0.23[b]	12.87 ± 0.55[c]
GR (min/mg protein)	30.85 ± 9.96[a]	33.12 ± 9.72[a]	23.27 ± 7.65[b]	26.46 ± 0.49[c]
GST (min/mg protein)	12.37 ± 0.51[a]	12.72 ± 0.69[a]	10.07 ± 0.27[b]	13.88 ± 0.49[c]
GSH (μg/mg protein)	14.24 ± 0.52[a]	14.57 ± 0.57[a]	7.50 ± 0.41[b]	11.97 ± 0.35[c]
Vit. E (μg/mg protein)	0.83 ± 0.05[a]	0.85 ± 0.05[a]	0.58 ± 0.02[b]	0.98 ± 0.07[c]
Hydroxyproline (mg/g dried tissue)	9.51 ± 0.53[a]	9.39 ± 0.59[a]	14.71 ± 1.12[b]	11.34 ± 0.87[c]

Values are given as mean ± SD from six rats in each group

[a–c] values with different superscript letter (a–c) in the same row differ significantly at $P < 0.05$ (DMRT)

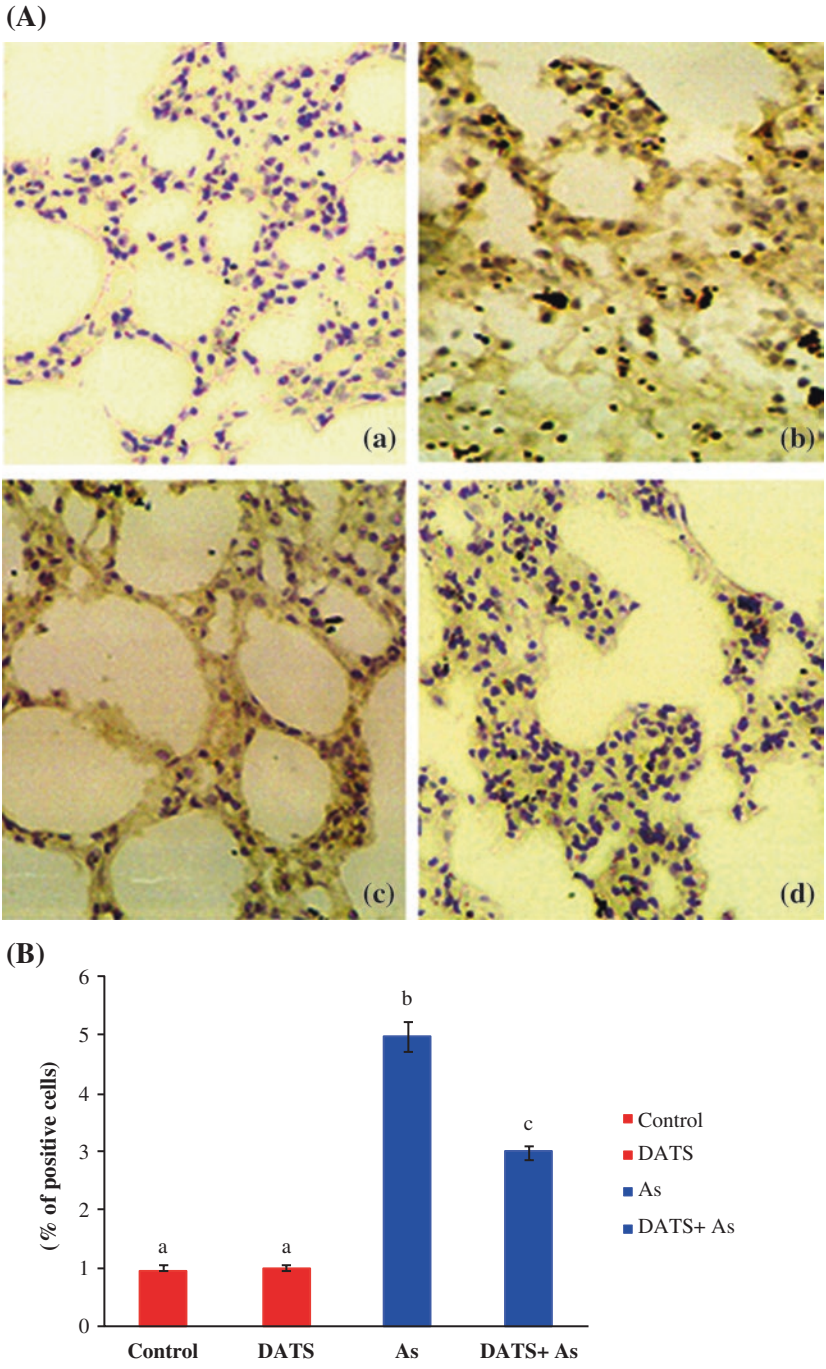


Fig. 22.4 Effect of DATS on As-induced iNOS expression in lung tissue by immunohistochemistry analysis in control and experimental rats. Control rats (**a**) showed no expression of iNOS in the lung tissue which was similar to that of the DATS alone treated rats (**b**). A significant increase of iNOS expression was observed in the lung tissue of As-treated rats (**c**) when compared to the control rats. Pre-administration of DATS showed a significant reduction in the over expression of iNOS in the lung tissue (**d**)

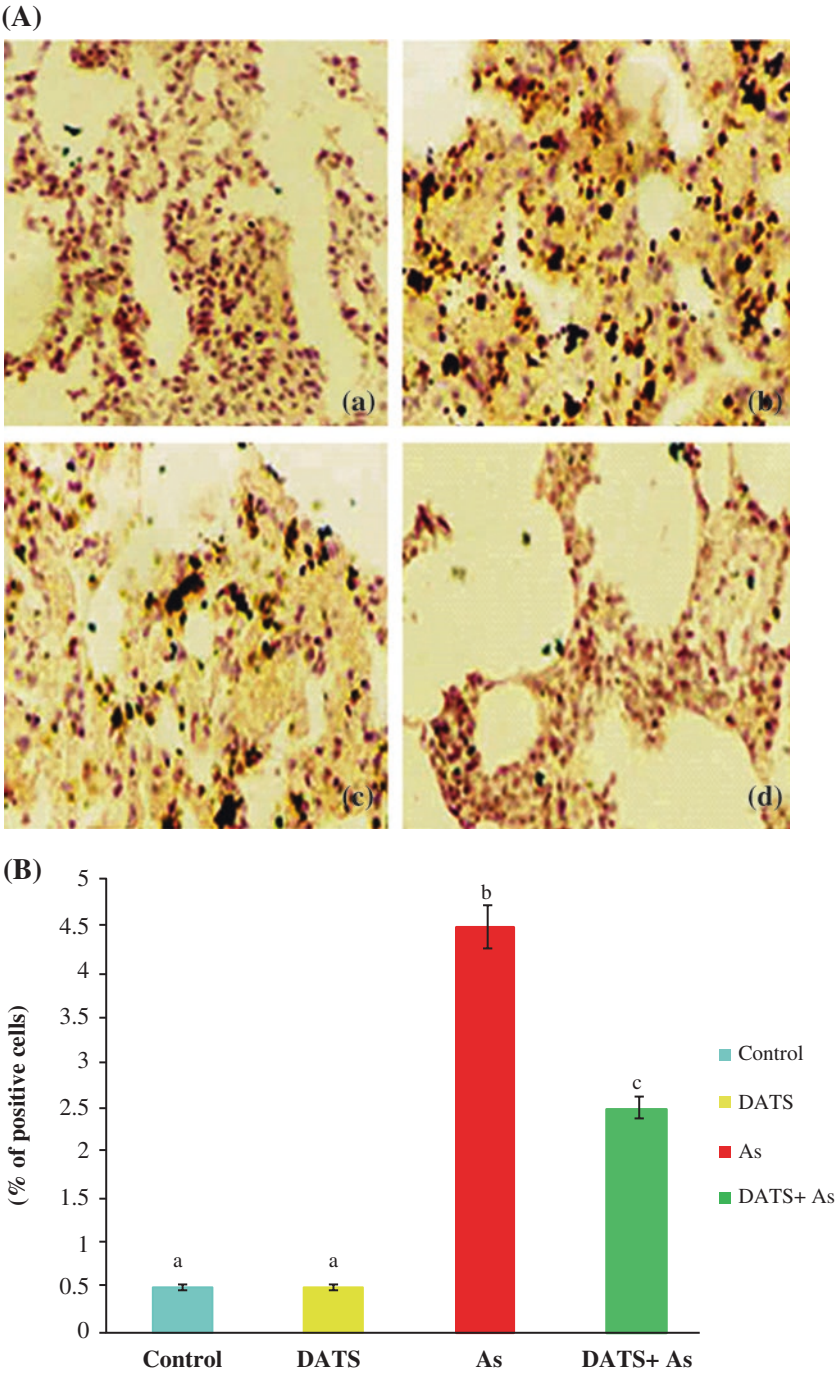


Fig. 22.5 Effect of DATS on As-induced NF-κB expression in lung tissue by immunohistochemistry analysis in control and experimental rats. Control rats (**a**) showed no expression of NF-κB in the lung tissue which was similar to that of the DATS alone treated rats (**b**). A significant increase of NF-κB expression was observed in the lung tissue of As-treated rats (**c**) when compared to the control rats. Pre-administration of DATS showed a significant reduction in the overexpression of NF-κB in the lung tissue (**d**)

dimensions of nuclear protein in the lung tissues, which was obvious from the exceptionally dark colored staining (Fig. 22.5b). Notwithstanding, treatment with DATS altogether restrained the expression of NF-κB (Fig. 22.5c) when contrasted with As-enlistment gathering. The immunohistochemical staining was measured and the consequence of the equivalent is shown in Fig. 22.5d.

22.3.7 DATS Diminishes Collagen Assemblage and Hydroxy Proline Level

Fig. 22.6 demonstrates the impacts of arsenic and DATS on Sirius red staining. Sirius red staining is exhibited as a technique for collagen accumulation and performed in locally defined tissue areas. When seen under a polarized light

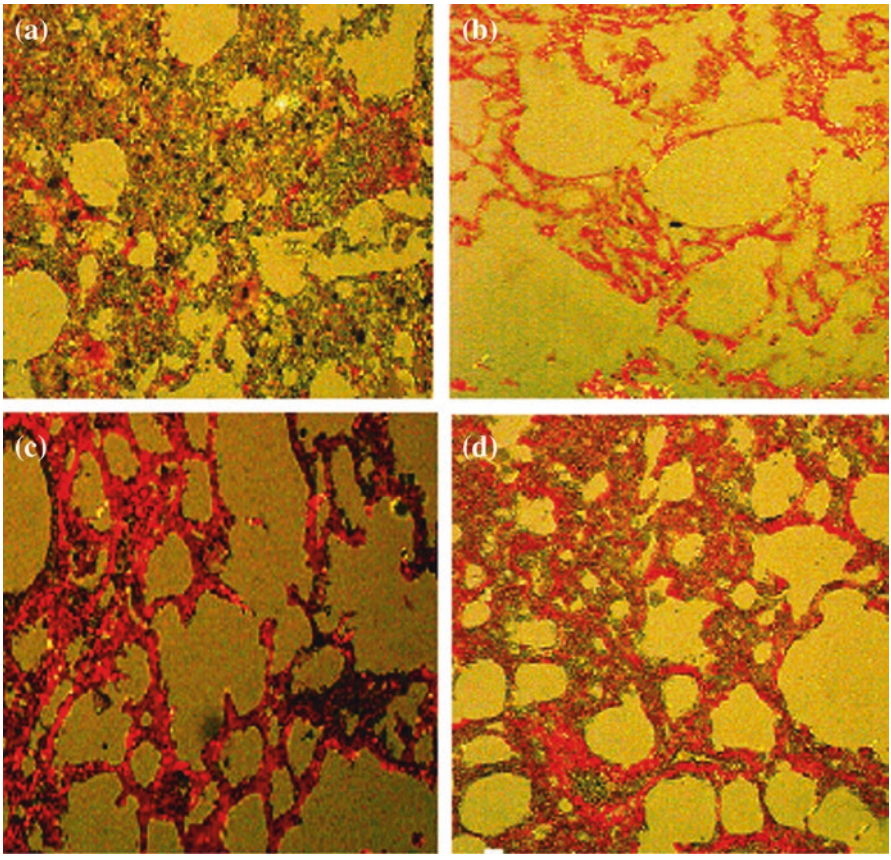


Fig. 22.6 Effect of DATS on As-induced collagen deposition in the lung tissue. Tissue sections were stained with picric acid and sirius red (Direct red 80). Collagen was in yellow/orange color when viewed under polarized microscope. (**a**) Control sections showing scarcely deposited collagen. (**b**) As-induced group shows increased collagen deposition. (**c**) As and DATS group shows comparatively less collagen deposition to As-induced group. (**d**) DATS alone treated group almost resembles control

microscope, arsenic-treated gathering (Fig. 22.6b) showed an expanded measure of collagen, which was kept in bewildered design in zones of fibrosis. Solid yellow/orange staining which was clear in arsenic-treated gathering was extensively decreased in DATS-treated gathering (Fig. 22.6c). Figure 22.6a and d compares control and DATS-only gatherings separately, which indicates insignificant collagen accumulation.

Considering hydroxyproline in lungs is solely gotten from collagen, the entire lung collagen content was evaluated by estimating the hydroxyproline levels. Table 22.1 delineates the dimensions of hydroxyproline in the lung tissues of control and test gatherings of rats. Arsenic-treated rats showed a critical increment in the dimension of hydroxyproline when contrasted with control rats. DATS treatment to arsenic-incited rats caused a noteworthy reduction in the dimensions of hydroxyproline when contrasted with arsenic-treated rats.

22.3.8 Impact of DATS on Apoptotic Markers in Lung

The Western blot investigation of Fig. 22.7 demonstrated the articulation of pro-apoptotic Bax, antiapoptotic Bcl2 and Bcl-xL, and TNF-α proteins in the lung tissues of control and test rodents. It was seen in the lung tissue of As-treated rodents that the dimension of pro-apoptotic Bax and TNF-α articulation fundamentally ($P < 0.05$) expanded with abatement in the dimension of Bcl2 and Bcl-xL proteins. Pre-treatment of DATS to As-inebriated rodents demonstrated a critical ($P < 0.05$) decline in the dimension of pro-apoptotic Bax and TNF-α articulation with noteworthy ($P < 0.05$) increment in the dimension of Bcl2 and Bcl-xL proteins in lung tissue when contrasted with the As-only-treated rodents.

22.3.9 Impact of DATS on Nrf2, γ-GCS, NQO1, and HO-1levels in Lung Tissue

Figure 22.8 demonstrates the Western blot investigation of Nrf2 enactment through HO-1, NQO1, and γ-GCS proteins in the lung tissues of control and treated rodents. The dimension of Nrf2, HO-1, NQO1, and γ-GCS protein expression was fundamentally ($P < 0.05$) diminished in the lung tissue of As-treated rodents. Pre-treatment of DATS to As-inebriated rodents demonstrated a critical ($P < 0.05$) increment in the expression of Nrf2, HO-1, NQO1, and γ-GCS protein in lung tissue when contrasted with the As-only-treated rodents.

22.3.10 DATS Improves Lung Histology

The impacts of arsenic and DATS on lung histology are given in Fig. 22.9. Measurably critical contrasts ($P < 0.05$) were seen between the control rats and the arsenic-treated rats in the histopathological examination of the lung tissue

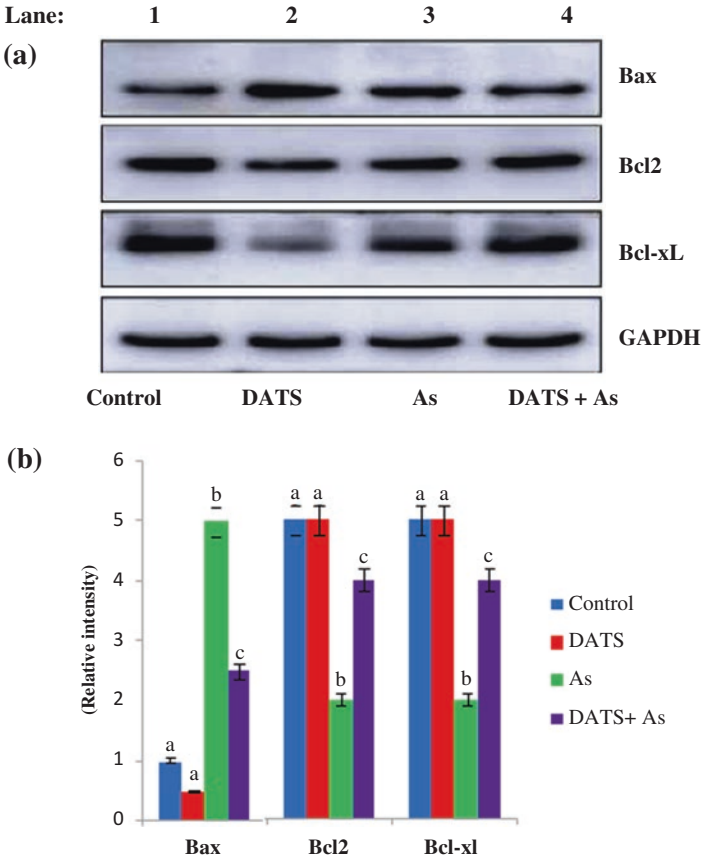


Fig. 22.7 (**a**) Effect of DATS on Bax, Bcl-2, and Bcl-xl protein expressions in the lung tissue of control and Fl-treated rats by western blot analysis. Lane1, Control; Lane 2, DATS; Lane3, As; Lane 4, DATS + As. (**b**) Effect of DATS on Bax, Bcl-2, and Bcl-xl protein band intensities scanned by densitometer in control and experimental rats. Values are mean ± SD for 6 rats in each group; a, b, and c Values are not sharing a common superscript letter (a, b and c) differ significantly at $p < 0.05$ (DMRT)

segments. In the arsenic-treated rats, contrasts in histopathogical parameters, for example, inflammation, alveolar damage, and vascular congestion, were found (Fig. 22.9c). Histopathological analysis revealed severe lung injury manifested with congestion and edema of interalveolar spaces, interalveolar inflammation, thickened alveolar wall, collapsed alveoli with bronchial erosion, and inconsistent fibrotic areas. Amid treatment with DATS, the parameters referenced above were essentially diminished (Fig. 22.9d). Control (Fig. 22.9a) and DATS-only-treated (Fig. 22.9b) rats did not exhibit any pathological derangement in their histomorphology.

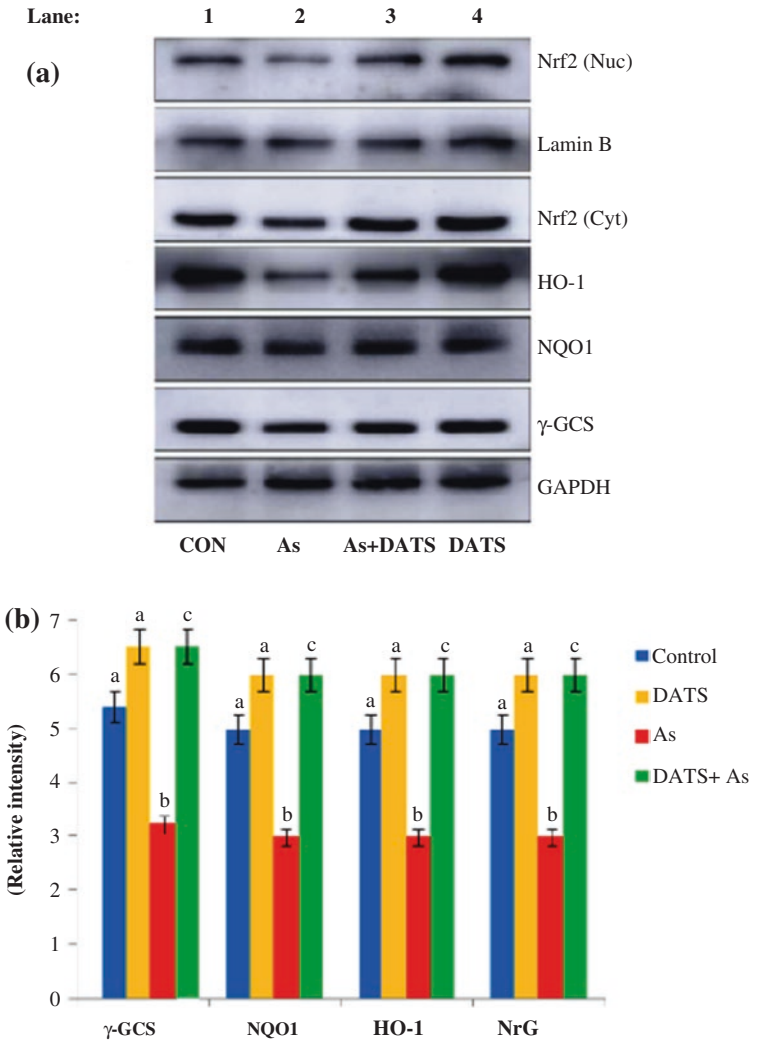


Fig. 22.8 (**a**). Effect of DATS on Nrf2, γ-GCS, NQO1, and HO-1levels protein expressions in the lung tissue of control and Fl treated rats by western blot analysis. Lane1.Control; Lane 2.DATS; Lane 3.As; Lane 4. DATS + As. DATS + As. (**b**). Effect of DATS on Nrf2, γ-GCS, NQO1, and HO-1levels protein band intensities scanned by densitometer in control and experimental rats. Values are mean ± SD for 6 rats in each group; a, b and c values are not sharing a common superscript letter (a, b and c) differ significantly at $p < 0.05$ (DMRT)

22.4 Discussion

Disregarding the way that an enormous number of people are still encountering various afflictions related with arsenic exposure, suitable approaches intended for ameliorating arsenic toxicity are quite inadequate. Arsenic from the environment

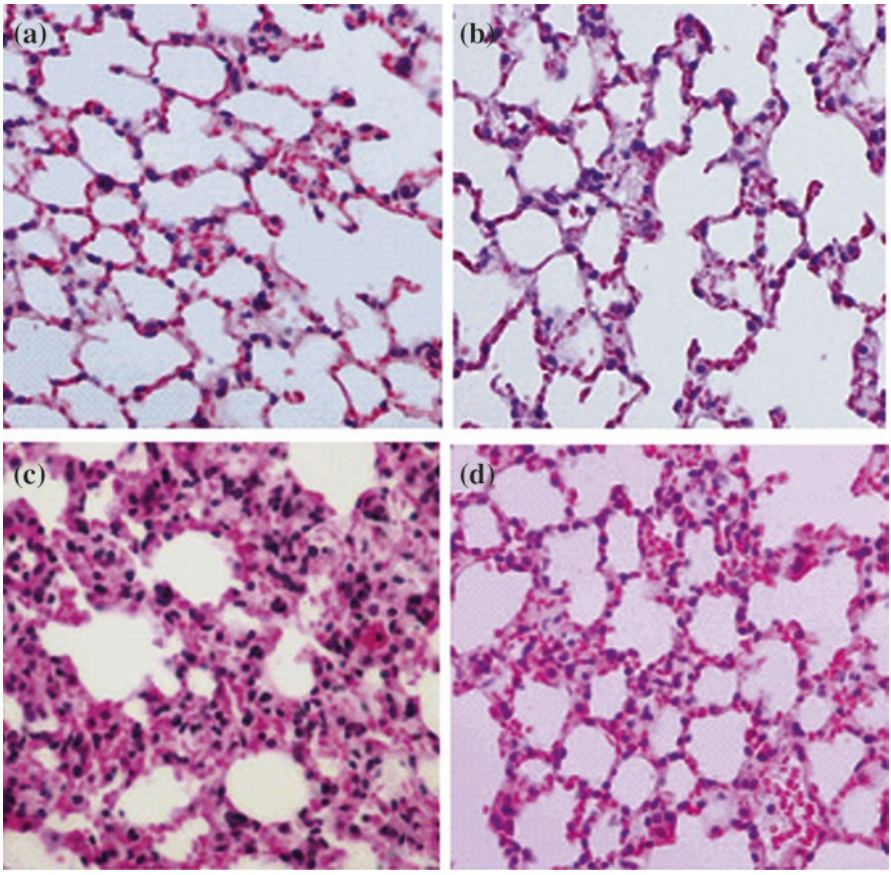


Fig. 22.9 Protective effect of DATS on As-induced alterations in the lung histoarchitecture of control and experimental rats. Control and DATS rats did not show any pathological alterations in the structure of lung tissue (**a, b**). As-treated rats showed extensive inflammation and fibrosis in the lung tissue (**c**). Preadministration of DATS significantly recuperated all the pathological alterations elicited by As (**d**)

instigates the ROS increment in the cells, which could results in apoptotic/necrotic cell death (Halder et al. 2013). Arsenic exposure is known to initiate the inflammatory response in the airway route, and constant introduction to arsenic can evoke or intensify bronchitis, pneumonia, asthma, and COPD (Mazumder et al. 2000; Smith et al. 2006). In our examination, we inspected the reaction to arsenic exposure and the strength of focusing on Nrf2 actuation by DATS. We showed the viability of Nrf2 enactment in forestalling sub-chronic and obsessive changes in the lung incited by momentary exposure to an eco-relevant dose of arsenic utilizing an in vivo model. It is astounding to see such considerable pathological changes in the lungs following a month of exposure. Strikingly, DATS corrected the arsenic-actuated biochemical/pathological changes and tissue damage in the lungs of rats, and it is

noteworthy that DATS-intervened protection is critically through the induction of Nrf2. Our outcomes outfit a solid premise that dietary mediation focusing on Nrf2 initiation is an extraordinary way to deal with correcting the oxidative lung maladies related with arsenic exposure, which may be unavoidable for people living in domains with an abnormal state of arsenic pollution in their drinking water or in their environment/ecosystem.

Arsenic-initiated ROS caused the arrival of strong provocative substances and the development of reactive oxygen species in lungs. In our investigation, the pneumonic edema (wet-to-dry lung weight proportion) was fundamentally expanded in arsenic-treated rats when contrasted with the typical control rats. For the most part this is because the arsenic-instigated free radicals lead to lipid peroxidation inside the cell membrane responding specifically with polyunsaturated fatty oxidation, which is accepted to cause pneumonic edema in rodents. Inability to dispose of over-the-top ROS prompts consequent oxidative harm to the lung tissue (Zheng et al. 2012). Besides, pre-treatment with DATS altogether diminished the lung edema when contrasted with that of arsenic-treated rats. This is most likely because of the diminished ROS interceded lipid peroxidation in the lung tissue through the free radical scavenging/antioxidant nature of DATS (Miltonprabu et al. 2017).

Arsenic exposure stimulates the generation of free radicals, for example, superoxide and hydroxyl radical, and the amalgamation of collagen in the lungs increments with these radicals. After arsenic intoxication, cytokine dysregulation, and aggravation creation, fibroblasts are enacted and collagen synthesis is revived, while collagen decay is hindered. In our study, hydroxyproline content, which is a marker of collagen affidavit, expanded with arsenic treatment and with DATS treatment it essentially decreased. This impact of DATS can be clarified with conceivable systems, for example, hindering lung inflammation by subsequently diminishing free oxygen radicals creation just as detoxifying free radicals and accordingly restraining the fibroblast expansion (Kalayarasan et al. 2008). Arsenic causes demolition of the lung design, prompting pneumonic fibrosis that was portrayed by an expansion in hydroxyproline levels and collagen accumulation in the lungs. The defensive impact of DATS seen in this investigation may be because of its radical scavenging exercises that diminish the level of hydroxyproline content in As-prompted lung tissues. An expansion in lung hydroxyproline content due to As and the bettering impacts of different antioxidants have been accomplished (Arslan et al. 2002), which further help our discoveries.

Oxidative stress has long been recognized as a prime mechanism in the pathophysiology of arsenic-initiated lung disease. Lipid peroxidation, a marker of oxidative stress, is an autocatalytic, free radical interceded, ruinous procedure, wherein polyunsaturated fats in cell layers experience oxidation from outline lipid hydro peroxides (Brigham 1986). In our examination, the extended dimensions of lipid peroxides in arsenic-treated rodents suggested the indefensible generation of free radicals, which may reduce the cell enzymic and nonenzymic safeguards. DATS take-up particularly diminished the arsenic-actuated lipid peroxidation in lung. These outcomes demonstrated that DATS upgraded the membrane barrier, accordingly restraining arsenic passage into cells.

To quantify the hindrance of cell invasion, we dissected the impact of DATS on As-prompted cell collection in the lungs, utilizing tissue MPO level and histological investigation. The MPO action has been utilized as a potential biochemical marker for the tissue substance of polymorphonuclear leukocytes (Homma et al. 2004). In our investigation, As enlistment delivered a critical increment in the lung tissue MPO action when contrasted with the control rodents. We found that DATS organization delivered a stamped restraint of MPO level that may be because of its antioxidant action. The capacity of DATS to revive the aggregation of leukocytes in BALF and lung tissue has been recently detailed (Serrano-Mollar et al. 2002).

Arsenic-prompted ROS causes added substance harm through acceptance of provocative results by means of initiation of various translation factors, including the enactment of pro-inflammatory cytokines (Zheng et al. 2012). Our outcomes depicted that the level of pro-inflammatory cytokines, TNF-α, IL-1β, and TGF-β, are fundamentally expanded in the lung tissues in light of arsenic treatment. It has been accounted for that free radicals initiated alveolar and interstitial macrophages to express the early reaction of pro-inflammatory cytokines, IL-1β, TGF-β, and TNF-α (Zheng et al. 2012). The exorbitant arrival of early reaction pro-inflammatory cytokines triggers and strengthens the pneumonic inflammatory course. The inflammatory course can actuate the lung endothelial cells and epithelial cells to deliver chemokines which, thus, pull in inflammatory cells, for example, initiated neutrophils (a prima root of ROS), aggravating tissue damage (Chen et al. 2014). In spite of the fact that arsenic-initiated pro-inflammatory cytokines increase the provocative course and create tissue damage that decreases the accessibility of antioxidant agents in lung tissue to rummage the free radicals, the pre-organization of DATS altogether debilitated the dimensions of pro-inflammatory cytokines in lung tissue. This is in amicability with the consequences of past reports that DATS vanquishes the commencement of pro-inflammatory cytokines through its amazing anti-inflammatory and antioxidant activity (Kalayarasan et al. 2008).

The As-initiated pulmonary damage includes, as an underlying occasion, the generation of prooxidant species. Further harm is most likely inspired by expanded measures of ROS and RNS delivered by actuated inflammatory cells that are introduced into the lung amid arsenic exposure. In our present study, an expansion in the declaration of iNOS was recorded, which may escalate the generation of nitric oxide, further stimulating NF-κB. The utilization of DATS in the present examination brought about a huge decrease of nitric oxide synthase (iNOS) activity. NF-κB is a transcription factor comprising p65 and p50 subunits of the Rel protein family. It enacts the transcription of numerous cytokines, including TNF-α, IL-1, IL-6, and IL-8, which are accepted to be critical in accelerating the inflammatory reactions. In concurrence with past investigations, As actuated the NF-κB movement in lungs, proposing that pneumonic epithelial cells discharge NF-κB p65. Our discoveries are in concurrence with past reports, which demonstrate that cell reinforcements can repress oxidant-initiated NF-κB actuation (Gurujeylakshmi et al. 2000) along these lines applying the antifibrotic impact of DATS in the arsenic model of pneumonic fibrosis.

Endogenous cell reinforcement catalysts (SOD, CAT, and GPx) and non-enzymatic antioxidant GSH are critical for ensuring the lung tissue against oxidative harm. They are responsible for the detoxification of malicious oxygen radicals. Beforehand, it was exhibited that arsenic exposure lessened the antioxidant barrier and extended the oxidative stress in the lung tissue, which stimulated the pneumonic fibrosis (Zheng et al. 2012). In the present examination, the decrease in the proportion of SOD, CAT, GPx, and GSH in the lung tissue of arsenic-treated rodents might be a direct result of the extended release of free radicals, subsequent augmentation in lipid peroxidation, and disintegration of cell reinforcement guard. These impacts were fundamentally turned around by DATS, recommending its cell reinforcement restoration viability (Miltonprabu and Sumedha 2014) against arsenic-initiated oxidative stress in the lung tissue.

Being an imperative transcription factor for oxidative insult, Nrf2 initiates the cell reinforcement reaction component (ARE) encoded inside numerous cytoprotective qualities. Nrf2 stimulates the synthesis of different detoxification and antioxidant elements, including NQO1, HO-1, and γ-GCS, notwithstanding controlling cell guard exercises (Kleszczyński et al. 2016). γ-GCS is the rate-constraining protein of GSH amalgamation and its action demonstrates the oxidative stress prevention agent ability of tissues and cells (Jin et al. 2015). The communication of GSH with arsenic appeared to diminish the poisonous quality of free arsenic. In this investigation, DATS treatment adequately stimulates the nuclear translocation of Nrf2 and reversed arsenic-instigated decline in γ-GCS action, which advanced GSH articulation in the lung and improved the opposition of lung tissue to oxidative pressure. The downstream quality focuses of Nrf2, NQO1, and HO-1 levels were essentially expanded in the DATS-treated group. Conjointly, these outcomes proposed that DATS-enacted Nrf2 flagging advanced NQO1 and HO-1 articulation and expanded γ-GCS movement, in this manner upgrading antioxidant status of the cells and limiting and securing against arsenic-actuated lung damage.

Cooperation between Bcl-2 relatives causes the permeabilization of the external mitochondrial layer and advances cytochrome C transport over the mitochondrial membrane, in this manner turning on apoptosis. Apoptosis includes the actuation of pro-apoptotic part Bax and concealment of anti-apoptotic part Bcl-2 and Bcl-xl (Vucicevic et al. 2016; Thibaud et al. 2017). Arsenic actuates the reactive oxygen species, advances the declaration of target qualities, for example, Bax, and initiates apoptosis amid DNA damage. In light of our outcomes, arsenic incited the oxidative worry in lung cells by going about as an upstream flag to initiate p53 and cause lung apoptosis. Be that as it may, these impacts were reversed by DATS through promoting the levels of anti-apoptotic proteins in cells. Together, these outcomes recommended an anti-apoptotic nature of DATS in shielding lung cells from arsenic-initiated oxidative lung damage.

The immunohistochemistry and histological discoveries additionally firmly bolster our biochemical discoveries that DATS secures the arsenic-incited oxidative pressure interceded lung damage. Our light microscopic investigation of pulmonary histology shows that arsenic introduction brought about a recognizable thickening of the alveolar septa with expanded collagen synthesis, multiplication of fibroblasts,

hyperplasia of pneumocytes, and expanded iNOS and NF-kB articulation. Administration of DATS nearly reestablishes the typical pulmonary histoarchitecture with insignificant articulation of iNOS and NF-kB unmistakably depicted the antioxidant, anti-apoptotic, anti-inflammatory, and calming job of DATS in amending the arsenic-prompted oxidative lung injury.

22.5 Conclusion

Our data extrapolate that DATS might be a promising contender for the treatment of oxidative stress initiated lung fibrosis or, if nothing else, for maintaining a strategic distance from the headway of As-actuated lung fibrosis in the midst of antineoplastic treatment. DATS acts upon a multifaceted framework containing cytokines, chemokines, development components, and oxygen radicals obtained from inflammatory cells and almost controls the advancement of fibrosis by arsenic intoxication. Therefore, a broad work about the relationship of different signal molecules would shed all the more light on the effect of this garlic polysulfide against As-instigated oxidative pressure initiated aspiratory fibrosis.

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Molecular Therapeutic Targets in Tobacco-Induced Lung Pathology

23

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Abstract

Tobacco consumption globally is estimated to be around 20% and in India accounted for 12% of the world's smokers according to the World Health Organization (WHO) statistics. It is also estimated that around six million deaths occur every year and rises to eight million by the year 2030. The socioeconomic health burden is of great concern due to the rise in cigarette smoking and related deaths. Cigarette smoking is known to cause many disorders such as chronic obstructive pulmonary diseases (COPD), oral cancers, and pulmonary tumors. More than 6000 different chemicals contain approximately 100 known pulmonary carcinogens present in the cigarette smoke (CS). Inhaled polycyclic aromatic hydrocarbons (PAHs), constituents of CS, are deposited in the airway epithelium and the majority in the alveolar epithelium which detoxifies the cigarette constituents by way of drug-metabolizing enzymes such as cytochrome p450 (CYP450) enzymes. Reactive oxygen species (ROS) present in the CS and the CS-mediated generation of mitochondrial and cellular ROS have an impact on the cellular bio-constituents such as membrane lipids, proteins, and DNA. Studies suggest that oxidative stress causes lung inflammation by remodeling of membrane lipids and pulmonary surfactants via activation of phospholipase A_2 (PLA_2), cyclooxygenases (COX), and lipoxygenases which directly or indirectly activates proinflammatory cytokines. Considering the complexity of lung tissue and functions, understanding the cellular and tissue mechanisms of damage and strategies overcoming the deleterious effects mediated by the CS would be prime importance. This understanding helps target specific molecules that will help in developing therapeutic strategies to circumvent the CS-mediated disease burden, mortality, morbidity, and economic burden.

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23.1 Introduction

23.1.1 Tobacco and Lung Cancer

Globally tobacco smoke is considered to be the major risk factor for cancer and accounts for approximately 22% of cancer-related deaths (GBD 2015 Risk Factors Collaborators). WHO statistics show that globally about six million deaths annually and about 400,000 deaths alone in the USA are due to cigarette smoking (CS) (WHO report on the global tobacco epidemic 2011; Bhalla et al. 2009). It also estimates that by 2030 the death burden due to cigarette smoking rises by eight million annually. The major factors involved in the regulation of initiation and progression of pulmonary tumors depend on the type of tobacco (smoking and non-smoking forms/filter/non-filter) (Lee 2001), processing of tobacco (quality), smoking duration (Pesch et al. 2012), smoking onset age, smoking inhalation depth (Ramroth et al. 2011), interindividual genetic variability, nutritional habits, and lifestyle, to name a few (Subar et al. 1990; Koo et al. 1997). Reports suggest that CS also causes deaths due to cardiovascular diseases (10%), respiratory diseases (9%), and digestive diseases (7%) (Gvinianidze and Tsereteli 2012). CS causes chronic diseases, especially of the airways including chronic obstructive pulmonary disease (COPD) and cancer of the lungs, both of which pathophysiologically differ at the cellular levels. COPD arises due to tobacco-mediated extensive lung tissue injury brought about by apoptosis of epithelial cells. Lung cancer is characterized by uncontrolled clonal expansion of alveolar epithelial cells (Goldkorn et al. 2013). In smokers, the high risk of oxidative burden such as H_2O_2 in serum and breath patient samples suffering from COPD shows a direct link leading to a series of biological actions including the lung epithelial tissue damage through apoptosis (Goldkorn et al. 2013). The data from the COPD studies clearly show that (1) CS-produced particulates retain in the lung tissue causing injury, damage, and lung diseases and (2) the biological effects include overall molecular and cellular mechanisms of injury with all particles of CS. Understanding the clear mechanisms of tobacco-mediated lung disorders including cancer will help develop therapeutic strategies, thereby reducing the lung cancer risk.

23.2 Composition

The burning of tobacco is a very dynamic process and has two major events which result in the production of carcinogenic products. In the combustion region where temperatures range between 800 and 900 °C, tobacco undergoes complete pyrolysis,

and as the temperature drops in the range between 200 and 600 °C, tobacco undergoes partial combustion due to lack of oxygen (Johnson 1977). Thus tobacco smoke is a complex mixture (pyrolyzed and partially combustible product) of over 6000 chemicals including a high concentration of oxidants and free radicals. Cigarette smoke has two major components, the tar/particulate component and the gaseous component. The particulate/tar component, exclude part of alkaloids and water content, contains remarkably high concentrations of stable and long-lived radicals (10^{17} radicals/g). The gas phase of CS contains aromatic amines, N-nitrosamines, polycyclic aromatic hydrocarbons (PAHs), and radicals of carbon, nitrogen, and oxygen (10^{15} radicals/puff) such as nitric oxide, reactive olefins, dienes, etc., that typically have lifetimes less than 1 sec (Hoffmann et al. 2001; Diana 1993; Shishodia et al. 2003). Single puff from the cigarette has 10^{17} oxidant molecules with reactive oxygen species (ROS) and reactive nitrogen species (RNS) accounting for 10^{15} molecules. Cigarette smoke consists of more than 100 known strong carcinogens such as benzo(α)pyrene [polycyclic aromatic hydrocarbons (PAHs)], *N*-nitrosamines, and nitrosonornicotine; weak carcinogens such as acetaldehydes, formaldehydes, and tumor initiators such as urethane; and co-carcinogens such as catechol, phenol, and formaldehyde (Table 23.1) (Hecht 1999, 2003). The nicotine-derived N-nitrosamines such as nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and

Table 23.1 Pulmonary carcinogens in tobacco smoke

Carcinogen class	No. of compounds	Compound	Quantity in mainstream tobacco smoke (ng/cigarette)
Polycyclic aromatic hydrocarbons	10	Benzo[α]pyrene	20–40
		Benzo[*b*]fluoranthene	4–22
		Benzo[*j*]fluoranthene	6–21
		Benzo[*k*]fluoranthene	6–12
		Dibenzo[*a,i*]pyrene	1.7–3.2
		Indeno[*1,2,3-cd*]pyrene	4–20
		Dibenz[*a,h*]anthracene	4
		5-Methylchrysene	0.6
Aza-arenes	3	Dibenz[*a,h*]acridine	0.1
		7H-Dibenzo[*c,g*]carbazole	0.7
N-nitrosamines	7	N-Nitrosodiethylamine	ND–2.8
		4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	80–770
Miscellaneous organic compounds	15	1,3-butadiene	20–70 × 10^3
		Ethyl carbamate	20–38
Inorganic compounds	7	Nickel	0–510
		Chromium	0.2–500
		Cadmium	0–6670
		Polonium-210	0.03–1.0 pCi
		Arsenic	0–1400
		Hydrazine	24–43

Reprinted with permission Hoffmann and Hoffmann (1997)

N' -nitrosonornicotine are known to have tumorigenic potential (Amin et al. 1996). Cytochrome p450 enzymes metabolize about 500 different types of carcinogens including PAHs that have two or more aromatic and cyclic rings identified. Benzo(α) pyrene ($C_{20}H_{12}$), a key carcinogenic pollutant, belonging to the family of PAHs and found in cigarette smoke, has five aromatic rings in a fused, honeycomb-like structure (Agen et al. 2001). Benzo(α)pyrene has an equally distributed electronic density and metabolizes to carcinogen benzopyrene-7,8-dihydrodiol-9,10-epoxide in three enzymatic steps (Vahakangas and Pelkonen 1989). It is highly mutagenic as it can bind covalently to guanine residues of DNA at the N_2 position producing adducts (Weinstein et al. 1976). Oxidative stress damages mitochondrial DNA as it is also the organelle for oxygen-free radical source with poor DNA repair machinery (Backer and Weinstein 1980). The order of oxidative stress-mediated DNA component damage includes thymine > cytosine > adenine > guanine > deoxyribose sugar moiety (Saul et al. 1987). The formation of free radical intermediates, such as DNA adducts, is suspected of being involved in gene modifications. Thus, the ultimate targets for free radicals are usually DNA and RNA molecules in both nuclear and mitochondrial organelles. The irreversible nature of these alterations ultimately leads to malignant and mutagenic states (Trush et al. 1982). Therefore, it appears that cigarette smoking leaves a large number of particles/residues into the airway system which form adducts during the process of metabolism and clearance that ultimately bring about lung injury and disease.

23.3 Redox Reactions

ROS play a vital role in normal cellular homeostasis by regulating multiple biological processes and signal transduction cascades (Hoidal 2001; Sen and Packer 1996). The emerging role of ROS also dictates predominant role as redox regulator of GSH/GSSG ratio, glutathione cycle, and overall cellular redox homeostasis (Remacle et al. 1992; Aw 2003). Predominant production of free radicals in the lungs from CS that oxidizes many cellular biomolecules is due to an imbalance in the local and global ROS levels which counters the antioxidant defense system. The ROS level imbalance contributes directly toward the development of many lung diseases (Sen and Packer 1996; Artaud-Macari et al. 2013; Avti et al. 2006) including lung cancer (Filosto et al. 2011; Khan et al. 2008). However, it could also be that without causing many alterations in the ROS levels, low levels of CS could induce GSH, γ-glutamylcysteine synthetase (γ-GCS), and DNA synthesis and enhance the alveolar epithelial cell proliferation through activation of (mitogen-activated protein kinases) MAPKs (Kaushik et al. 2008). Glutathione peroxidase (GPx), which converts two GSH molecules per H_2O_2 utilized into oxidized glutathione (GSSG), also acts as a detoxifying enzyme which protects the lungs from the ROS-mediated DNA, proteins, and lipid damage. The nonenzymatic antioxidants in the lung-lining fluid include reduced glutathione (GSH), uric acid, albumin, vitamin C, and α-tocopherol, and enzymatic antioxidants include glutathione *S*-transferase, thioredoxin, superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase.

Toxic oxidants in the tobacco smoke, oxygen (H_2O_2, OH^-, O_2^-) and nitrogen radicals, have detrimental effects on the lung epithelial and endothelial cells leading to COPD with very little room for therapeutic strategies (Samara et al. 2011; Goldkorn and Filosto 2010). The epithelial cell lining fluid which covers the pulmonary epithelial cells and their plasma membranes is rich in antioxidants, acts as a primary line of defense, and reacts with CS-induced ROS and leads to the direct damage at this level without allowing the ROS to enter the cells through the plasma membrane and reach the circulation. On the other hand, the lipid-soluble components of the CS, such as aldehydes, phenolic compounds, and PAHs, can permeate the airway epithelial cells through the plasma membrane and create mitochondrial dysfunction leading to ROS-generated oxidative stress markers which enter the systemic circulation (Prokopczyk et al. 2002; van der Toorn et al. 2007) (Fig. 23.1).

The lipophilic constituents of CS alter the mitochondrial function balance through mitochondrial linked antioxidants such as GSH, thioredoxin, SOD, and heme oxygenase-1 levels (Nohl et al. 2003; Slebos et al. 2007). Due to the excess oxidative burden in tobacco consumers, the plasma levels of antioxidant vitamin C and its recycling are highly reduced (Kurata et al. 1998; Maranzana and Mehlhorn 1998; Kallner et al. 1981). Studies have shown that supplemental intake of approximately 35 mg/day of vitamin C might reduce tobacco-induced oxidative stress (Lykkesfeldt et al. 2000; Mays et al. 1999) considering the use of very high vitamin C doses that might further induce the oxidative burden (Hemila 1997; Podmore et al. 1998; Rehman et al. 1998). Studies also suggest that the vitamin C supplementation elevates the catalase activity which otherwise subverted during the smoking (Hilbert and Mohsenin 1996). It is also assumed that the strong oxidative stress conditions generated during the tobacco smoking and after cessation show evidence of protein oxidative and subsequent damage which was restored by the use of dietary intake of vitamin C (Banerjee et al. 2008; Panda et al. 1999, 2000). Use of flavonoids, such as liquiritin apioside (LA) isolated from the root of *Glycyrrhiza uralensis* (licorice), protected the CS-induced oxidative stress in A549 cells by restoring the TGF-β and TNF-α cytokines and reducing the GSH and apoptosis in these cells (Guan et al. 2012). In CS-exposed ICR mice, LA treatment inhibited the pulmonary inflammation reducing the infiltration of macrophages, neutrophils,

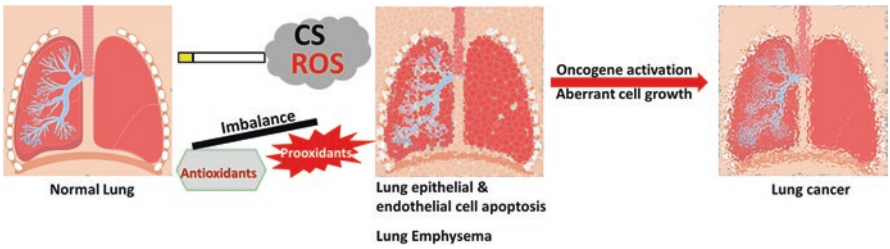

Fig. 23.1 Cigarette smoke-induced ROS generation causes aberrant cell death during lung emphysema and activation of oncogenes and lung epithelial cell proliferation toward the development of lung cancer

mucus-containing goblet cells, TNF-α, TGF-β, and myeloperoxidase activity while increasing the SOD activity (Guan et al. 2012). Other studies have shown that licochalcone A, a chalcone, and glycyrol, a benzofuran coumarin, also inhibit inducible nitric oxide synthase, cyclooxygenase-2, interleukin-1β, and interleukin-6 via regulation through GSH (Furusawa et al. 2009; Shin et al. 2008).

A GPx mimetic, ebselen, a selenium-based organic complex, acts as an inhibitor of cigarette smoke-enhanced reactive oxygen species-mediated BALF inflammation and IL-17A in the lungs of gpx-1 knockout mice as compared to wild-type mice (Duong et al. 2010). Treatment with ebselen also reduced the tobacco smoke-induced BALF neutrophils, macrophages, IL-17A, and proteolytic damage (Duong et al. 2010). This compound can inhibit the CS-mediated peroxynitrite from preventing the NF-kB/AP-1 activation and proinflammatory cytokine generation (Jozsef and Filep 2003). Therefore, GPx mimetics have the therapeutic potential in decreasing lung damage due to cigarette smoke. Similarly, SOD mimetics especially the metalloporphyrin-based compounds AEOL10150 and AEOL10113 decrease the CS-induced inflammation and restore the normal cellular antioxidant levels (Smith et al. 2002).

23.4 Cytochrome p450s

The lungs, composed of greater than 40 cell types with complex biological functions, form the primary organ for CS metabolism mediated especially through cytochrome p450-catalyzed redox reactions (Hukkanen et al. 2003; Ding and Kaminsky 2003). Generally, 10–20% of inhaled PAHs get deposited in the airway epithelium and the majority in the alveolar epithelium. The macrophages present in the airways clear the particulate constituents of the tobacco (Drath et al. 1979) and the airway epithelium detoxifies the tobacco by way of phase I and II xenobiotic-metabolizing enzymes (Crawford et al. 1998; Han et al. 2005). Phase I enzymes often catalyze most of the redox reaction including hydrolysis, and phase II enzymes mediate conjugation reactions. A large number of cytochrome p450 enzymes (phase I) are found in the different regions of the lungs. Activation of specific CYP450s to become metabolically active carcinogens or genotoxic carcinogens determines the overall risk in developing lung cancer (Smith et al. 2003; Rubin 2001; Yamazaki et al. 1992). The most common among the tobacco-induced CYPs include CYP1A1/2, CYP2D6, and CYP3A4. The three main factors hypothesized about the PAH-induced CYP isoforms include (1) an intracellular receptor that interacts with tobacco inducer, (2) the steric structures of each isoform and receptors that might be similar, and (3) distinct genomic response element for each isoform that selectively interacts with respective receptors. The CYPs oxidize PAHs and generate reactive metabolites that induce DNA mutations (Douben 2003; Sasaki 2013; Rodgman 2006). PAHs present in tobacco induce CYP1A1 and CYP1A2 (Avti et al. 2010; Hukkanen 2012) by binding to the transcription factor such as aryl hydrocarbon hydroxylase receptor to form a heterodimer which binds to the aryl hydrocarbon-responsive elements of the above genes (Miller 1998; Guengerich and Shimada

1998; Denison et al. 2002). The binding in turn methylates complete or partial *CYP1A1* gene promoters to about 33% of heavy smokers diagnosed with lung cancer (Anttila et al. 2003). On the other hand, bronchial biopsies of smokers have shown reduced activity of histone deacetylases as PAHs present in tobacco smoke epigenetically modify NHF4α and HNF1α transcription factors and acetylate lysine groups of histone proteins to upregulate CYP1A2 (Nebert and Dalton 2006; Pascussi et al. 2008; Marwick et al. 2004; Ito et al. 2001) (Fig. 23.2).

The CS components that induce xenobiotic-metabolizing enzymes result in the rapid clearance of therapeutic drugs that are used during smoking cessation therapy, thereby reducing the overall efficacy of drug therapy (Willey et al. 1997; Villard et al. 1994). The exact mechanisms underlying the rapid clearance are still nonconclusive. But it is seen in some cases even after the smoking cessation that CS and their components observed a modification of the CYP genes and result in CYP persistent elevated activity (Hirota et al. 2008). This persistently elevated activity of CYPs further impacts the overall pharmacokinetics and dynamics of the drug metabolism due to the direct effect either on the transcriptional factors or the epigenetic effects of the CYP isozymes (Launay et al. 2009; Faber and Fuhr 2004).

Studies have also shown that intake of excessive vitamin C, a dietary antioxidant, did not induce the tobacco-induced pulmonary CYP450 in guinea pigs (Koul et al. 1988). A later study has also shown that NDEA, another major constituent of tobacco, did not induce the pulmonary CYP450s pre-treated with vitamin C (Khanduja et al. 1990). Similarly, studies with vitamin C have shown that tobacco-induced CYP1A1, 1A2, and 2E1 expressions were reduced (Mori et al. 1997; Clarke et al. 1996; Ueta et al. 2001).

Some of the chemotherapeutic drugs, such as erlotinib, used in non-small cell lung cancer (NSCLC) treatment, have shown survival variability in patients with smoking and non-smoking backgrounds (Zugazagoitia et al. 2013). Erlotinib is a cell cycle inhibitor at a G1 phase which is usually EGFR-dependent proliferation in the lung tumor cells (Moyer et al. 1997; Petty et al. 2004). CYP3A4 majorly metabolizes

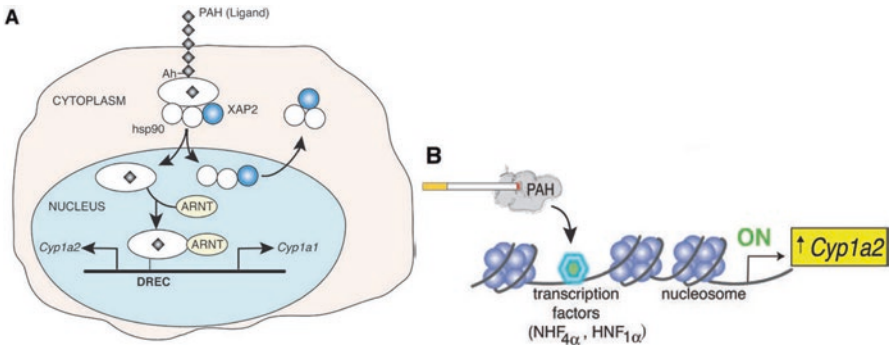

Fig. 23.2 PAH-induced direct (**a**) transcriptional and (**b**) epigenetic regulation of cytochrome P450 enzymes, CYP1A1, and CYP1A2. *PAH* polycyclic aromatic hydrocarbon, *Ah* aryl hydrocarbon, *ARNT* aryl hydrocarbon receptor nuclear translocator, *Hsp90* heat shock protein 90, *XAP2* X-associated protein (O'Malley et al. 2014)

erlotinib and minorly by CYP1A1 and 1A2 (Ling et al. 2006). The difference in the lung cancer survival variability during smoking conditions by the erlotinib variation accounts for the rapid catabolism and clearance which leaves lower plasma concentrations of the drug. Similarly, CYP3A and CYP2C8 class which also metabolize taxanes such as docetaxel and paclitaxel, which are anti-microtubule inhibitors and used as the first line of defense against NSCLC, have shown that smokers had a lower incidence of neutropenia and leukopenia without altering the pharmacokinetics of the drug, thereby showing a protective effect against the taxane therapy-mediated hematological toxicities (de Graan et al. 2012; Shou et al. 1998).

23.5 Phospholipase A_2 (PLA_2) Isoforms

PLA_2 isozymes are a major class of enzymes, with more than 30 isoforms (Murakami et al. 2011), that produce signaling molecules, such as lysophospholipids and arachidonic acid (AA), with a major role in lung cancer. Depending on their cellular localization, substrate specificity, and calcium dependence, PLA_2 are divided into at least four groups such as cytosolic PLA_2 ($cPLA_2$, 85 kDa), secreted PLA_2 ($sPLA_2$, 14–19 kDa), calcium-independent PLA_2 ($iPLA_2$, 85–89 kDa), platelet-activating factor-acetylhydrolase (PAF-AH)-activated PLA_2, and lipoprotein-associated PLA_2 (Lp-PLA_2) (Kudo and Murakami 2002). However, most of these isoforms act as housekeeping enzymes by hydrolyzing the fatty acids present in the *sn*-2 position of glycerophospholipids mainly involved in membrane phospholipid remodeling. Other evidence suggests that $cPLA_2$ acts on intracellular membranes such as perinuclear and endoplasmic reticulum whereas $sPLA_2$ acts on plasma membrane generating large quantities of arachidonic acid. Arachidonic acid acts as a substrate for COX-2. CS is also known to regulate the levels of COX-2 and release the prostaglandins (PGE2 and PGI2) and thromboxanes TXA2 from alveolar macrophages and lung dendritic cells (Huang and Chen 2011). The identified steps in the smoking-induced COPD and lung cancer include the (a) epithelial transformation, (b) ensuing angiogenesis, and (c) suppression of apoptosis (Martey et al. 2004). Nicotine is known to cause initiation and progression of solid tumors through COX-2 expression (Shin et al. 2004). $cPLA_2$ and $sPLA_2$ are emerging as pulmonary cancer targets (Lu and Dong 2017; Dong et al. 2014; Cummings 2007) due to the formation of arachidonic acid, lysophospholipids, and other active metabolites which play an important role in lung tumor cell growth, invasion, and metastasis (Kudo and Murakami 2002; Wang et al. 2014). $sPLA_2$ produces lysophosphatidylcholine and free fatty acids which induce protein kinase c δ and ε (PKC δ and ε), finally activating the MAPK pathway leading to $cPLA_2\alpha$ activation (Nishizuka 1992; Han et al. 2003; Rupprecht et al. 1999). $cPLA_2$ activates the $sPLA_2$ through a positive feedback loop which enhances the AA levels and promotes lung cancer (Sakai et al. 2012). Earlier it was shown that polycyclic aromatic hydrocarbons (PAHs) present in the smoke of the cigarettes also enhanced the apoptosis of endothelial cells by activating group IV and VI PLA_2s through the activation of arachidonic acid (Tithof et al. 2002). Alongside, AA also activates prostaglandin E_2 (PGE_2) which is involved

in suppression of immune response, angiogenesis, proliferation, and metastasis (Greenhough et al. 2009). The surfactant protein B (8 kDa), produced by alveolar type II cells, inhibits $sPLA_2$ which in turn suppresses lung cancer progression (Lee et al. 2017). Recently, studies from our lab have shown that cigarette- and tobacco-related products activate $cPLA_2$ isoforms IVA, IVB, and IVC which in turn alters both type I and II alveolar epithelial cell homeostases. Alteration in cellular homeostasis by cigarette smoke in both type I and II alveolar cells is brought about by decreased cell viability, increased membrane destabilization, activation of primary and secondary apoptotic signatures, and increased ROS generation through activation of ERK MAP kinases (Kumar et al. 2019). We have observed that targeting the specific $cPLA_2$ by ATK inhibitor reverses the tobacco-induced damage to the lung cells (Kumar et al. 2019; Yadav et al. 2016). Tobacco-induced changes are also observed in the colon cells, apart from alveolar cells, such as membrane destabilization, excess ROS generation, upregulation of $sPLA_2$ IID, and downregulation of $sPLA_2$ IB and $sPLA_2$ IVA. Gene silencing experiments on $sPLA_2$ IID during tobacco-induced changes in the colon pathologies have reversed the tobacco-induced damaging effects in colon cells (Sharma et al. 2018). Thus targeting specific PLA_2 isoform could provide therapeutic strategies during tobacco-induced pathology including lungs (Sharma et al. 2018) (Fig. 23.3).

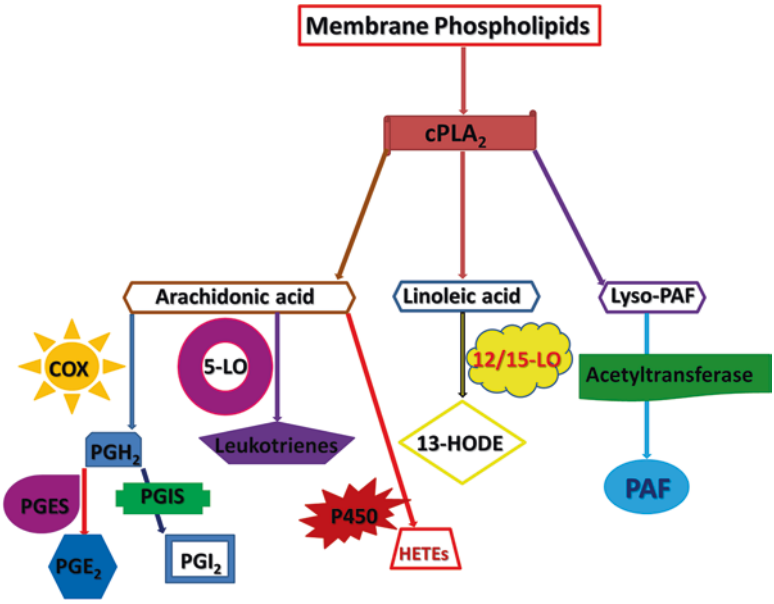

Fig. 23.3 cPLA2-metabolized lipid mediator's generation and signaling. *COX* cyclooxygenase, *PGH_2* prostaglandin H_2, PGIS prostaglandin I synthase, *PGES* prostaglandin E synthase, *PGE_2* prostaglandin E_2, *PGI_2* prostaglandin I_2, *LO* lipoxygenase, *P450* cytochrome p450, *HETEs* 5-hydroxyeicosatetraenoic acid, *13-HODE* 13-hydroxyoctadecadienoic acid, *Lyso-PAF* 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine-platelet-activating factor, *PAF* platelet-activating factor

23.6 Concluding Remarks

The xenobiotic-metabolizing pathways and pathways of cellular signaling that lead to an overall imbalance in the cellular apoptosis and proliferation of the lungs due to CS play a vital role in the inflammation and lung cancer. In the search for the lung molecular pathways and targets in pulmonary damage and carcinogenesis, ROS, CYP450, COX, and PLA_2 play as vital targets. All these targets suggested having critical roles in eliciting inflammation, cellular apoptosis, cell proliferation, and ultimately lung tumorigenesis. Inhibiting such targets by a plethora of agents either by natural antioxidants, vitamins, or synthetically designed drugs has a potential inhibitory effect on the overall CS-mediated damage on the lungs. Supplementation with natural antioxidants could improve the overall therapeutic strategies because synthetically designed drugs are being actively metabolized by CYP450s and making them least available at the target site.

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