Oxidative Stress in Applied Basic Research and Clinical Practice

Nirmal K. Ganguly
Surinder K. Jindal
Shyam Biswal
Peter J. Barnes
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Studies on Respiratory Disorders

Humana Press

Oxidative Stress in Applied Basic Research and Clinical Practice

Editor-in-Chief
Donald Armstrong

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Note from the Editor-in-Chief

All books in this series illustrate point-of-care testing and critically evaluate the potential of antioxidant supplementation in various medical disorders associated with oxidative stress. Future volumes will be updated as warranted by emerging new technology, or from studies reporting clinical trials.

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Preface

Oxygen species constitute an important vehicle of damage in disease pathogenesis including several respiratory diseases. Although the information has been available for more than four decades, it had been difficult to attribute a specific role to oxidative stress in a cause-and-effect relationship. In respiratory medicine, some of the earlier studies had focused on pulmonary infections, including tuberculosis. Advances in the study of volatile organic components in the expired air have made it possible to examine some of the hitherto not understood mechanisms in different pulmonary diseases, particularly the airway disorders. We now recognize the wide spread involvement of oxygen species as well as of nitrogen-free radicals in airway diseases, such as asthma and chronic obstructive pulmonary disease. Numerous reports have appeared in the last two decades which demonstrate an imbalance of oxidant–antioxidant mechanisms in many other respiratory disorders such as the interstitial lung diseases, granulomatous disorders (e.g. sarcoidosis), asbestosis, muscle dysfunction, pulmonary hypertension, and thoracic cancers.

It is the therapeutic potential of antioxidant drugs in the management of diseases which has made the subject as particularly interesting to the clinicians. Unfortunately, we do not yet have a drug known for its proven therapeutic efficacy for almost any disorder. Numerous drugs are under investigation for possible supplemental roles in therapy of different disorders. One hopes for rapid development of drugs which, in addition to the primary therapy, will be able to act on specific target species for disease arrest and/or reversal.

We have written this monograph with a dual purpose—first to review the existing and up-to-date knowledge on oxidative stress in different respiratory diseases, and secondly to sensitize the clinicians to continue to look to a broader scene of pathogenetic spectrum of diseases for expansion of the therapeutic armamentarium. We do hope that this monograph shall help not only the specialist pulmonologists but all others who are interested and engaged in the subject of oxidative damage.

Chandigarh, India — Surinder K. Jindal
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Chapter 1
Introduction to Oxidative Stress and Antioxidant Therapy in Respiratory Disorder

Francesco Galli, Massimo Conese, Luigi Maiuri, Roberto Gambari, Desirée Bartolini, Marta Piroddi, Silvia Ciffolilli, and Giulio Cabrini

1.1 Reactive Oxygen Species and Oxidative Stress: A Definition

Reactive oxygen species (ROS) is the collective term for all highly reactive forms derived from the chemistry of molecular oxygen encompassing also N, S, and Cl containing forms and many others that include derivatives of biomolecule oxidation such as lipid hydroperoxides, reactive carbonyls, and radical intermediates of amino acid species. (reviewed in [119]). ROS have long been the subject of toxicology studies aimed at defining their role as dangerous molecules causing oxidative harms to various components of cells and body fluids. In fact, the chemistry of free radicals originating from radiation chemistry at the early beginning of the last century, developed into biology and medicine as the chemistry of oxidative stress. The term was first used in the title of a publication by Beutler and his coworkers in 1970 [139]

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who were studying oxidative pathways associated with glutathione metabolism of the red blood cell, but for a first attempt to provide a definition to such a toxicology condition, we have to wait since 1985 when Helmut Sies in his book "Oxidative stress" [174] clearly depicted the nature of harmful toxicants for ROS involved in biological processes, highlighting their potential to produce cellular damage if not properly counteracted by the homeostatic intervention of antioxidant and detoxification defense systems.

In the later years, a more comprehensive interpretation of the biochemistry and toxicology of ROS has been provided also fostering a revision of the concept of oxidative stress. Actually the chemistry of ROS is not always negative, being for instance involved in the host defense response and cell killing activity of phagocytes; under physiological conditions, ROS are steadily formed during a number of biochemical reactions as redox active intermediates of the cellular metabolism playing a key role as signaling molecules (reviewed in [154]) and possibly as pacesetters of metabolic rate and lifespan of living organisms [119].

1.1.1 Cellular ROS

It is generally accepted that H_2O_2 is the predominant intracellular ROS with physiological role in redox signaling [17, 158], but challenging cells in culture with a high level of H_2O_2 (i.e., 1 mM) can easily lead to extensive damage and even cell death. In contrast, it has been known that moderate levels of H_2O_2 can increase cell proliferation and that the flux of intracellular H_2O_2 is consistently elevated in various cancer cell lines [178, 185, 188]. The O–O linkage of H_2O_2, although weak compared to that of dioxygen (O_2), renders the molecule relatively stable compared to radical species, allowing H_2O_2 time to encounter and react with specific targets that it oxidizes at discrete sites [46]. By contrast, the propensity of the more reactive radical species, $O_2^{\bullet-}$, to become quickly dismuted, spontaneously and enzymatically, to H_2O_2, as well as its lack of diffusibility, limits its range of targets to those within the immediate vicinity of the $O_2^{\bullet-}$ source [65].

$O_2^{\bullet-}$ is slow to react with negatively-charged molecules, which does confer some specificity [80]. From a signaling perspective, $^{\bullet}OH$ is unsuitable as a result of the high oxidation rate constant that it has for most biomolecules, which is approximately equal to its rate of diffusion, resulting in highly nonspecific oxidation [80]. The lack of enzymatic-removal of the peroxyl and alkoxyl radicals, as well as their aggressive reactivity, means that their reactions with proteins are more likely to occur as irreversible oxidation events, leading to degradation of the damaged protein (reviewed in [17, 61, 62] and references therein). $^1O_2$ seldom occurs intracellularly and so is unlikely to contribute to physiological signal transduction, whereas HOCl, which is produced by myeloperoxidase (MPO) enzymes within neutrophils, is an established bactericidal agent that has also been proposed to function as signaling mediator in immune cells [132, 171].

1.1.2 *ROS as Signaling Molecules: The "Redox Hypothesis" of Oxidative Stress*

Cellular ROS such as the superoxide anion ($O_2^{\bullet -}$), H_2O_2, and NOx as peroxynitrite ($ONOO^-$) are all redox players of metabolic pathways which are capable of initiating the signaling of a broad variety of cellular processes that are regulated by redox-sensitive components, such as proliferation and survival (MAP kinases, PI3 kinase, PTEN, and protein tyrosine phosphatases), ROS homeostasis, and antioxidant gene regulation (thioredoxin, peroxiredoxin, Ref-1, and Nrf-2), mitochondrial oxidative stress, apoptosis (Bcl2/Bax, cardiolipin/cyt c), and aging (p66Shc), iron homeostasis through iron–sulfur cluster proteins (IRE-IRP), ATM-regulated DNA damage response, and receptor activation (e.g., αIIbβ3 integrin in platelet activation) (reviewed in [17, 22, 84, 136, 140, 143]).

The signaling function of ROS has been described in diverse physiological conditions such as those activated in hypoxic microenvironments [20]. The molecular response to hypoxia requires fast-acting mechanisms acting within a wide range of partial pressures of O_2 [147]. Intracellular O_2 sensing is an evolutionary conserved feature, and the best characterized molecular responses to hypoxia are umpired through transcriptional activation [12]. The transcription factor, hypoxia-inducible factor 1 (HIF-1), is an important mediator of these adaptive responses, and its activation by hypoxia involves O_2-dependent posttranslational modifications and nuclear translocation [50, 87, 157, 175]. Through the induction of the expression of its target genes, HIF-1 coordinately regulates tissue O_2 delivery and energy metabolism [12]. Other transcription factors such as nuclear factor κB (NF-κB) are also redox sensitive and are activated in pro-oxidant and hypoxic conditions [157, 181].

The redox state of thiol systems forms basis of the signaling effect of ROS (extensively reviewed in [9, 193]) and is controlled through thioredoxin (Trx) and glutathione (GSH)-dependent reactions. Trx and GSH systems are maintained under stable, but non-equilibrium conditions, due to continuous oxidation of cell thiols at a rate of about 0.5 % of the total thiol pool per minute. Both radical and non-radical oxidants, the latter includes peroxides, aldehydes, quinones, and epoxides, are generated enzymatically from both endogenous and exogenous precursors and can modify these thiols. As a mean to avoid this, cells are equipped with a complex machinery of H_2O_2 and thiol-regulating enzymes such as that of the peroxiredoxin (Prx)/sulfiredoxin system and that of thioredoxin–thioredoxin reductase/nicotinamide adenine dinucleotide phosphate (NADPH) system and glutaredoxins (reviewed in [9, 154, 158, 193]).

In redox signaling pathways, ROS effector proteins generally have a highly reactive Cys residue, of which oxidation changes the protein function, so as to activate signal transmission to downstream targets [192]. Among the ROS effectors, protein phosphatases, Trx and Prx family proteins own special domains/motifs to preserve the reactivity of Cys (redox-active Cys) and use them to react to ROS [71, 78, 96, 158].

Starting from such an exquisite signaling role of ROS, a complementary hypothesis for oxidative stress in disease has been proposed, which is termed the

"redox hypothesis" [152]. In this respect, oxidative stress can occur as a consequence of disruption of H_2O_2 regulating systems and thiol redox circuits, which normally function in cell signaling and physiological regulation. Because of the non-equilibrium conditions in the thiol pathways, aberrant generation of such a burden of oxidants at rates comparable to normal oxidation may be sufficient to disrupt function.

1.2 ROS: The Damage and the Response

1.2.1 ROS and Oxidative Damage

ROS are generated as xenobiotics or endobiotics in a number of processes of relevance to human toxicology. Exogenous ROS can be inhaled for instance during smoking or by the exposure to air pollution, ozone, and other toxicants. As far as endogenous processes are concerned, the exposure to physiological or noxious stimuli can activate different ROS-generating enzymes of specialized cells such as some leukocyte subsets and epithelial cells. These include NOX, Dual oxidase (Duox), MPO, inducible NO synthase (iNOS), and others, which ultimately can produce ROS at different extents [17].

One of the strongest ROS-generating process is that occurring as part of the cell-mediated immunity in the host response to pathogens. Activated neutrophils and in general phagocytes, give origin to the so called "respiratory burst" [199], that is a sudden and potent generation of ROS addressed to operate the bacterial killing. In the airways, the level of this response can assume abnormal proportions in the case of extended lesions that are observed, for instance, in the recurrent pulmonary infections of cystic fibrosis (CF) patients [62]. In these subjects, such an inflammatory environment may further exacerbate by the concomitance of the genetic defect that impairs the local feedback of the inflammatory response also weakening immune-homeostatic events at the systemic level. Uncontrolled inflammation is a well-recognized cause of oxidative stress and degeneration in the surroundings of a lesion. Here, the exposure to high levels of ROS produces cellular damage and even death by apoptosis or necrosis.

Depending on the type (molecular nature and intensity) and spatial distribution of the injurious event, such a ROS-generating machinery can lead to either acute or chronic and diffused events of toxicity. One of the most severe example of oxidative stress associated with acute inflammation is that of sepsis associated with multi-organ failure [62], while a typical condition of oxidative stress associated with chronic of micro-inflammation and molecular degeneration of tissues and eventually of the entire organism, is that which is observed in diabetic and kidney disease patients [98, 101], as well as in autoimmune diseases such as rheumatoid arthritis and LES [172], and neurodegenerative diseases [28].

According to the free radical theory of aging, a sustained exposure to high levels of ROS by chronic inflammation is believed to produce the cumulative damage of

cells and tissues, which is thought to be responsible for accelerated aging and age-related disorders [119]. The rate of production of ROS and inflammatory mediators in the setting of a chronic lesion can be even slightly higher than that observed during normal cellular metabolism, but its consistency over time and the presence of an altered distribution through redox pathways, can be at the origin of damages to subcellular components in the cytosolic and plasmalemma as well as in critical organelles such as mitochondria, endoplasmic reticulum, and nucleus. This may lead to a vicious cycle of ROS leakage essentially from mitochondria and peroxisomes, which ultimately can impair the physiological signaling of cellular ROS through redox-sensitive pathways described in the previous section.

1.2.2 ROS as Mediators of Tissue Reprogramming, Adaptation, and Repair

The exposure to damaging levels of ROS can result in a series of compensatory and adaptive responses that include the transcriptional activation of detoxification, antioxidant, and repair genes. For instance, cellular stresses due to ischemia/reperfusion injury or chronic exposure to fibrotic "initiators" (toxins, elevated glucose levels, etc.) increases expression of enzymes that generate ROS (NADPH oxidases (NOXs), NOX proteins, etc.) with concomitant reductions in ROS scavengers, such as glutathione peroxidase (GPx), catalase, and manganese/zinc superoxide dismutases (SODs) [205]. Increased oxidative state and a downstream redox-dependent genomic re-programming then affects cellular growth and starts processes of repair [31]. In various organ systems such as pulmonary, renal, and cardiovascular, NOX isoforms and their constituent subunit complexes play a key role in tissue reprogramming and adaptation. NOX proteins are multi-subunit enzymes that catalyze the reduction of oxygen using NADPH. ROS, generated by NOX, impact different signaling pathways that contribute to the pathophysiology of chronic lesions [18, 183]. The mechanisms of these responses and that of ROS generation vary depending on the specific collection of NOX isoenzymes expressed in different cell types or organs. These enzymes control, for instance stromal myofibroblast differentiation and fate, and are effectors of normal and pathologic tissue repair [15, 74, 75] impacting on the expression of critical fibrogenic genes. NOX expression is up-regulated in several models of induced fibrosis [43, 48, 98]. The potent pro-fibrogenic factor TGF-β1activates NOX4 and mediates myofibroblast recruitment in the kidney and bleomycin-injured lung and in idiopathic pulmonary fibrosis [5, 25, 27].

In respiratory diseases, there is an increased expression of multiple inflammatory proteins in the respiratory tract, including cytokines, chemokines, and adhesion molecules. Chemokines have been shown to regulate inflammation and immune cell differentiation. Moreover, many of the known inflammatory target proteins, such as matrix metalloproteinase-9 (MMP-9), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), cyclooxygenase-2 (COX-2), and

cytosolic phospholipase A2 (cPLA2), are associated with airway and lung inflammation in response to various stimuli [110, 180]. Injurious environmental stimuli can access the lung through either the airways or the pulmonary and systemic circulations. The time course and intensity of responses by resident and circulating cells may be regulated by various inflammatory components of cell signaling, including Src family kinases (SFKs), protein kinase C (PKC) [1], growth factor tyrosine kinase receptors, NADPH/ROS [180], PI3K/Akt, MAPKs, NF-κB, activator protein-1 (AP-1), and other signaling molecules. These regulate both key inflammatory signaling transduction pathways and target proteins involved in airway and lung inflammation.

1.2.3 Antioxidants and Antioxidant Therapy

Constitutive and inducible antioxidant and detoxification genes are available as a line of defense against oxidative stress of tissues and body fluids, and this is at least in part implemented by exogenous antioxidants introduced with the diet [61]. Actually, malnourished cystic fibrosis subjects are believed to have a higher risk of exposure to oxidative stress by the chronic inflammation of the airways [62, 81].

The exposure of tissues to increasing fluxes of ROS, is associated with a compensatory response of cells thus training inducible components of the defense systems [205]. This hormetic effect of ROS is produced for instance in muscular workout by the exposure to sub-maximal conditions of oxidative stress [118].

The largest clinical trials carried out in the last decade have clearly demonstrated that acting with exogenous antioxidants to counteract the pathogenic effects of oxidative stress in chronic diseases remains a chimera [62]. Antioxidant therapy and specific nutritional intervention (e.g., use of antioxidant supplements of functional foods) can be recommended only in the case of proven malnutrition or severe inflammation and exposure to oxidative stress as a generic measure of prevention. This could be the case of most severe respiratory syndromes, particularly of cystic fibrosis that show a combination of severe nutritional and immune-inflammatory symptoms in the presence of increased biomarkers of oxidative stress.

1.2.4 Noncoding RNAs: Emerging Mediators and Possible Therapeutic Agents in Oxidative Stress

Besides the direct effects of ROS on redox-sensitive transcription factors and regulatory proteins described in the previous sections, other levels of control by these species on cell functions are emerging that include for instance translational regulation by noncoding RNAs. MicroRNAs (miRNAs, miRs) are a family of small (19–25 nucleotides in length) noncoding RNAs that regulate gene expression by sequence-selective targeting of mRNAs, leading to a translational repression or mRNA

degradation, depending on the degree of complementarity between miRNAs and the target mRNA sequences [73, 184]. Since their discovery and first characterization, the number of miRNA sequences deposited in the miRBase databases is growing [94, 179] and tools to screen them as individual or pathway-associated entities and to interpret their functions, are now available and in continuous implementation [2]. Considering that a single miRNA can target several mRNAs and a single mRNA might contain in the 3′ UTR sequence several signals for miRNA recognition, it is calculated that at least 10–40 % of human mRNAs are a target for miRNAs [4, 176]. Hence, great interest is concentrated on the identification of validated targets of miRNAs. This specific field of miRNA research has confirmed that the complex networks constituted by miRNAs and RNA targets coding for structural and regulatory proteins lead to the control of highly regulated biological functions, such as differentiation, cell cycle, and apoptosis [73, 184]. Low expression of a given miRNA is expected to be linked with a potential expression of target mRNAs. Conversely, high expression of miRNAs is expected to induce low expression of biological functions of the target mRNAs.

With respect to oxidative stress, recently available publications (Table 1.1) strongly suggest that several miRNAs are induced by oxidative stress [1, 11, 39, 40, 62, 70, 113, 117, 131, 170, 180, 185, 189]. These oxidative stress-responsive miRNAs may play a role linking the imbalanced redox state with deregulated expression of critical genes. Although in its infancy, research on oxidative stress-responsive miRNAs and their regulation of target genes may provide new insights in understanding disorders also disclosing innovative therapeutic strategies (miRNA therapeutics).

In order to identify putative miRNAs involved in oxidative stress, different authors have induced an oxidative stress to cellular systems and followed changes of expression of miRNAs and associated target mRNAs. Analysis of miRNA profiles revealed down-regulation of miR-150, miR-142-5p, miR-122, and up-regulation of miR-34c, miR-34b-5p, and miR-29b. Moreover, several papers, in addition to the identification of the oxidative-stress-modulated miRNAs, also reported the target mRNA(s), allowing a more complete dissection of the loops linking oxidative-stress—miRNA—target gene alterations—biological functions. For instance, miR743a, miR-335, miR-34a, miR-200c, miR-145, miR-205, miR-320, Let-7, miR-23, miR144, and miR-451 have been identified as miRNAs involved in oxidative stress. In addition to the implications concerning basic science, these results are of great interest with respect to possible future therapeutic strategies based on mimicking miRNA activity or targeting miRNAs, depending on the role of the considered miRNA. In fact, the so called “miRNA replacement therapy” or “miRNA targeting therapeutic” approaches have been recently the object of several reviews and, in the case of oximiRNAs, might lead to a control of oxidative stress. For instance, if a miRNA is down-regulated in conditions of oxidative stress, the miRNA replacement approach leads to antioxidant effects; conversely, if a miRNA is up-regulated following oxidative stress, its targeting by specific antagomiR might reverse its induced oxidative damage.

Table 1.1 Selected examples of microRNAs involved in oxidative stress (oximiRNAs)

Experimental system	microRNA	Target mRNA/pathway	Biological effects	References
Mouse hippocampal HT22 line	miR-743a	Malate dehydrogenase (mdh)	Negative regulation of *mdh2* at posttranscriptional level by directly targeting the *mdh2* 3′ UTR	[189]
Primary mesangial cells of renal glomeruli from male Wistar rats	miR-335 and miR-34a	Superoxide dismutase 2 (SOD2) and thioredoxin reductase 2 (Txnrd2)	Contribution to renal aging by inhibition of intracellular pathways involving the mitochondrial antioxidative enzymes SOD2 and Txnrd2	[11]
Human umbilical vein endothelial cells (HUVEC) exposed to H_2O_2	miR-200c	ZEB1 (zinc finger E-box binding homeobox 1)	Induction of endothelial cell apoptosis and senescence	[113]
Cardiomyocytes exposed to H_2O_2	miR-145	Bnip3 (Bcl2/adenovirus E1B 19 kDa-interacting protein 3)	Protection of cardiomyocytes from hydrogen peroxide H_2O_2-induced apoptosis through targeting the mitochondrial pathway	[62]
Renal tubular cells	miR-205	3′ UTR of the prolyl hydroxylase 1 (PHD1/EGLN2) mRNA	Modulation of both intracellular ROS levels and ER stress state	[131]
Human lung adenocarcincoma	miR-320	Conserved sites of the PFKm (phosphofructokinase, muscle) 3′ UTR	Control of mitochondrial oxidative stress, a central mechanism in the up-regulation of glycolysis of cancer	[180]
Human hepatocytes	Let-7	Bach1, a heme-dependent transcription factor	Enhancement of heme oxygenase activity	[40]
Human retinal pigment epithelial (RPE) cells	miR-23	3′ UTR of Fas mRNA	Protection of RPE cells against oxidative damage caused by H_2O_2 induced Fas up-regulation	[1]
Erythrocytes from sickle cell disease patients; K562 cells	miR-144	NRF2 (nuclear factor-erythroid 2-related factor 2)	Increased sensitivity to H_2O_2-induced oxidative stress through NRF2 inhibition	[170]
G1E and G1E-ER4 erythroid cells	miR-451	14-3-3zeta, a phospho-serine/threonine-binding protein	Facilitation of FoxO3-regulated antioxidant enzymes, protecting against erythroid oxidant stress	[117]

1.3 Chronic Pulmonary Diseases and Biomarkers of Oxidative Stress

Oxidative stress has been implicated in the pathogenesis of various lung disorders such as asthma, chronic obstructive pulmonary disease (COPD), acute lung injury, pulmonary fibrosis, pulmonary hypertension, and lung cancer [53, 142]. Here we introduce the role of oxidative stress in the lung disease of three paradigmatic respiratory diseases of both acquired (COPD and asthma) and genetic (cystic fibrosis) nature.

Bronchial asthma and COPD are currently global health problems with a major economic and social impact. Presently, their diagnosis, staging, and monitoring are based on spirometric measures, such as forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) [30]. However, since spirometric measures do require long follow-up periods to determine whether interventions tested in clinical trials obtain clinical relevant changes in patient status, surrogate outcome measures capable of predicting long-term responses have been intensely sought for, such as those based on the evaluation of oxidative stress.

1.3.1 Chronic Obstructive Pulmonary Disease

Current diagnosis of COPD includes an assessment of smoking and/or occupational exposures, a history of cough, sputum and dyspnea, and a measure of airflow obstruction by means of spirometry [30]. The interaction of host factors with the environment generates the pathologic triad of COPD: persistent inflammation, protease–antiprotease imbalance, and oxidative stress [59]. The inflammatory response of lungs affected by COPD is characterized by a massive infiltration of polymorphonuclear neutrophils. Chronic cigarette smoking and wood smoke inhalation expose the respiratory tree and lungs to ROS, resulting in oxidative stress and injury. This triggers the production of other ROS and lipid peroxidation and subsequent pulmonary inflammation [148]. The oxidant burden in the lungs is further enhanced by the release of ROS from alveolar macrophages and sequestered neutrophils in the lung. Moreover, the oxidative burden to the lungs of individuals with COPD is compounded by alterations in the antioxidant defenses [59]. Smoking also exposes various components of the blood in the pulmonary microvasculature, i.e., red cells, plasma, and leukocytes, to an increased oxidant burden of ROS, either directly by diffusion into the blood or indirectly from the ROS generated from activated inflammatory cells in the lung and/or peripheral leukocytes [134, 150]. Consequently, oxidative stress is increased in the lungs of patients with COPD compared to healthy subjects, and also compared to smokers without COPD [112].

Lipid peroxidation products are elevated in sputum [42, 88], exhaled breath condensate (EBC) [42, 91, 129], and plasma [189] of patients with stable COPD. Markers of oxidative stress are increased even further during exacerbation of

COPD [49] and in patients with very severe form of this disease [93]. Patients with COPD exacerbation had the highest levels of 8-isoprostane (8-iso-$PGF_{2\alpha}$), a widely used marker of peroxidation of arachidonic acid, in the induced sputum and EBC as compared to nonsmokers, healthy smokers, and symptomatic smokers [120]. These results are consistent with a study showing that the levels of 8-isoprostane were higher in the EBC of patients with COPD exacerbations compared to healthy nonsmoking subjects [23].

At the same time, the antioxidant mechanisms are attenuated in these patients, as indicated by reduced levels of glutathione (GSH) in the lungs [54], lowered GPx activity in erythrocytes, [55] and lower antioxidant capacity in plasma during exacerbations of COPD [149]. However, Rahman et al. failed to document any relationship between plasma antioxidant capacity and spirometric variables [151]. The antioxidant capacity in plasma would be less valuable in relation to the measurement of airway obstruction, due to high intraindividual variability in oxidative stress in plasma caused by smoking. There seems to be less variability in antioxidative enzymes measured in erythrocytes, and indeed a significant relationship between GPx activity in erythrocytes and pulmonary function in patients with COPD has been found [83, 90]. Nadeem et al. observed significant differences between the severity of COPD, as assessed by GOLD criteria and the oxidant/antioxidant status [134, 144]. Thus, stage III COPD patients had lower plasma antioxidant capacity and higher levels of total blood GSH as compared to stage II of COPD. Furthermore, plasma ferric reducing antioxidant power (FRAP) had positive whereas total blood GSH had a significant negative correlation with the severity of airway obstruction, suggesting that the extracellular antioxidant decrease as the severity increases whereas major intracellular redox buffer increases to compensate this deficit in the extracellular milieu. In the study by Gumral et al., the levels of erythrocyte malondialdehyde (MDA), a measure of lipid peroxidation, were significantly higher in the exacerbation period of COPD patients than in the stable period, and this was paralleled by an increase in GPx and glutathione reductase (GRd) activities, as well as by a depression in serum melatonin levels, in the exacerbation period [68]. Overall, these findings confirm that exacerbation is associated with elevated levels of oxidative stress, which may contribute to its pathogenesis. Finally, it has been suggested that there is an association between systemic inflammation and systemic oxidative stress reflected by erythrocytic GPx in patients with acute exacerbations of COPD [187].

According to the analysis made by Comandini et al., 8-isoprostane is the only biomarker of response to tobacco smoke exposure associated with COPD activity, which is expressed at higher levels in healthy smokers than in nonsmokers and at higher levels in COPD than in healthy smokers [35]. On the other hand, SOD is a biomarker negatively associated with COPD and/or tobacco smoke exposure, while MPO and eosinophil peroxidase (EPO) are variably associated with COPD and/or tobacco smoke (Table 1.2). Moreover, erythrocyte SOD activity is elevated in COPD exacerbation compared with stable COPD [68]. Also, in patients with COPD associated with wood smoke exposure and tobacco smoking in the previous 10 years, an inverse correlation between plasma MDA and SOD with FEV_1 was found,

Table 1.2 Most relevant biomarkers of oxidative stress in respiratory diseases

Lung disease	Sample	Biomarker	Outcome
COPD	EBC	8-Isoprostane	Increased levels
			Increased in exacerbation
	Serum	SOD	Lower levels
			Increased in exacerbations
	Eo, Neu	MPO, EPO	Variably associated
Asthma	EBC	8-Isoprostane	Increased levels
			Increased in exacerbation
		MDA	Increased in exacerbation
	Serum	SOD	Lower levels and activity
	Eo, Neu	MPO, EPO	Increased levels
	Urine	3-Bromotyrosine	Increased levels
Cystic fibrosis	Plasma/urine	8-Isoprostane	Increased levels
			Increased in exacerbations
	BAL, plasma	GSH	Lowered levels
	Plasma	Fat-soluble vitamins	Lowered levels
		Vitamin C	Normal to low levels
			Decline with age

Abbreviations: *BAL* bronchoalveolar lavage, *EBC* exhaled breath condensate, *Eo* eosinophils, *EPO* eosinophil peroxidase, *GSH* reduced form of glutathione, *MDA* malondialdehyde, *MPO* myeloperoxidase, *Neu* neutrophils, *SOD* superoxide dismutase

indicating that the disease progressed and oxidative stress continued even after smoke cessation [126]. Similarly, the decline in symptoms, despite persistent neutrophilic airway inflammation and oxidative stress (8-isoprostane in induced sputum), was observed in COPD patients 3 months after the cessation of smoking [103]. This finding suggests that clinical improvement does not necessarily correlate with objective assessment of disease or that these biomarkers may not be the best ones in regard to clinical relevance in COPD and/or that the mechanisms of COPD are still poorly known. Furthermore, these results raise the questions whether some of these markers may be predictive of which patient goes on to develop further lung damage and in which patient the disease processes may be arrested.

None of the biomarkers of oxidative stress has been studied in response to therapy (corticosteroids) or in longitudinal studies in order to assess their robustness and predictivity of acute exacerbations. As recently reviewed by Fischer et al., an association between genetic polymorphisms and surrogate biomarkers of oxidative stress and inflammation appear to exist in relationship with the susceptibility of COPD, but not of disease severity and progression [59].

1.3.2 Allergic Asthma

Allergic asthma is a chronic inflammatory airway disease determined by repeated exposure to allergens. Eosinophils represent the major inflammatory cell type

infiltrating the airways, although neutrophils massively invade the lungs in corticosteroid-resistant form of severe asthma [15]. The ROS produced by these leukocytes likely play an important role in the pathophysiology of asthma because several of the characteristic changes in the airways can be produced by the action of ROS [14]. ROS cause tissue damage, constriction of smooth muscles, increase in vascular permeability, mediator release, and bronchoconstriction [36, 167].

Increased oxidative stress in asthma has been studied in plasma [76, 133, 150, 203], BAL [204], EBC [16, 41, 60, 141, 196], and urine [56, 197]. In addition, EPO and MPO are increased in peripheral blood, induced sputum, and BAL fluid of patients with asthma [3, 115, 127]. On the other hand, changes in antioxidant defenses have been reported, mainly in plasma [133, 150], lung cells [182], BAL [85, 203], and induced sputum [19].

EBC has proven to be a useful biological sample for assessing oxidative stress in asthma and linking oxidative stress and asthma pathophysiology [105]. An inverse correlation between H_2O_2, FEV_1, peak expiratory flow (PEF), and metacholine hyperresponsiveness has been reported [6, 79, 104]. These studies also showed that H_2O_2 levels in stable asthmatic patients treated with inhaled corticosteroids (ICS) were lower compared with steroid-naïve patients and similar to normal subjects [6, 79, 104]. Finally, in two randomized double-blind placebo-controlled clinical trials, ICS and the oral administration of the lipid extract of New Zealand green-lipped mussel significantly decreased H_2O_2 levels [7, 57], whereas montelukast had no effect on H_2O_2 [169]. The EBC levels of 8-isoprostane increase in asthma in association with its severity [128, 168], and exhaled 8-isoprostane were found to be increased in relation with asthma exacerbation frequency [13, 86] (Table 1.2). ICS seem to have no effect on 8-isoprostane levels [125, 128, 172, 206], with two studies reporting a positive effect in specific conditions (exacerbation and aspirin sensitivity) but with 8-isoprostane levels still remaining higher than normal after treatment [8, 13]. Finally, a leukotriene receptor antagonist showed no effect on 8-isoprostane concentration in EBC of children with asthma [130].

MDA levels in EBC increased during asthma exacerbations whereas GSH levels decreased. After steroid treatment, MDA levels decreased whereas GSH levels increased [41]. In a study evaluating aldehydes [(MDA), acrolein, *n*-hexanal (C6), *n*-heptanal (C7), *n*-nonanal (C9), 4-hydroxynonenal (HNE), and 4-hydroxyhexenal (HHE)] in EBC and induced sputum in asthmatics, no significant correlation between each other was observed, indicating that the two samples must be evaluated independently [42].

Children with asthma have increased plasma levels of MDA and lower than normal levels of GSH. Furthermore, the higher MDA and lower GSH levels correlated with the severity of asthma [69]. SOD activity, but not Mn-SOD or Cu/Zn-SOD protein, was lower in asthmatic serum as compared with control, and activity loss was significantly related to airflow limitation. Further, serum SOD activity demonstrated an inverse correlation with circulating levels of 3-bromotyrosine, a posttranslational modification of proteins produced by the EPO system of eosinophils [39, 40]. Levels of plasma GPx and SOD and of reduced glutathione, ascorbic acid, α-tocopherol, lycopene, and β-carotene were significantly lower in children

with asthma as compared with healthy controls [164]. Serum SOD activity is related to asthma lung function, and its relationship appears to be unique to asthma since serum antioxidant capacity in COPDs is unrelated to airflow limitation [37, 39, 40, 145, 149].

Lipid peroxidation as well as antioxidant enzyme activities in erythrocytes was studied in patients with asthmatic exacerbation and in stable period [68]. MDA levels were significantly higher, whereas GPx and GRd activities, and catalase activity were lower and higher, respectively, in exacerbation periods than in the stable period. Levels of melatonin, a potent-free radical scavenger, were depressed during the exacerbation periods. Accordingly, serum ROS levels were significantly higher in patients with acute exacerbation of asthma than in patients with stable asthma or healthy subjects [177].

SOD activity is significantly lower in epithelial lining fluid and airway epithelial cells in asthmatic patients compared with those in the healthy controls, and the airway reactivity is inversely related to SOD activity [38–40]. Lower SOD activity may be partly due to the increased oxidative and nitrative stress in the asthmatic airway and serves as a sensitive marker of airway redox and asthma severity [36]. In addition to lower SOD activity, Cu/Zn-SOD protein is decreased in cells recovered by BAL and by bronchial brushing in asthmatic patients compared to healthy subjects [182]. Oxidation and nitration of Mn-SOD are also present in the asthmatic airway, correlating with the severity of asthma [39, 40]. Catalase activity in BAL fluid is lower in patients with asthma as compared with those in healthy controls, due to nitration and oxidation of the enzyme [64]. Thus, as in COPD, the loss of antioxidant activity reflects the oxidant stress in the airway.

There are little data on the correlation of biomarkers of inflammation and oxidative stress with the clinical picture of asthma, disease progression, and therapeutic response, thus their diagnostic value should be evaluated further [51]. However, recent data point out to the usefulness of bromotyrosine, a noninvasive marker of eosinophil-catalyzed protein oxidation. Asthmatic children with high baseline levels of urinary bromotyrosine were 18.1-fold more likely to have inadequately controlled asthma and 4.0-fold more likely to have an asthma exacerbation over the ensuing 6 weeks [198].

In summary, the stable end-products of the ROS-mediated reactive pathways may be used as reliable markers of oxidative stress in patients with asthma (Table 1.2). Identification of noninvasive biomarkers of oxidative stress in patients with asthma will be critical for enabling assessment of treatment outcomes [36].

1.3.3 Cystic Fibrosis

Cystic fibrosis (CF) is a lethal autosomal recessive disorder caused by mutations in the CF Transmembrane Conductance Regulator (*CFTR*) gene located on the chromosome 7. The CFTR protein is mainly expressed in the apical membrane of epithelial cells lining the airway mucosa and submucosal glands, acting not only as a

chloride channel, but also as a regulator of transport of other molecules, including GSH. The redox unbalance in the CF lungs has been attributed to different causes [29, 62]. An abnormal generation of ROS by airway epithelial cells, determined by CFTR-related intrinsic defects, is compounded by a sustained neutrophil activation by recurrent infections. A constitutive defect of glutathione metabolism together with a lowered intake and absorption of fat-soluble antioxidant vitamins contribute to a defective antioxidant protection, which is believed to exacerbate stress indices along with the progression of clinical status [10, 81, 203]. Besides targeting different biomolecules to damage epithelial cells and extracellular fluid components of the airways, oxidants can contribute to the pathophysiology of CF by exacerbating inflammation [26, 32], and being synergic in the induction of mucins with neutrophil elastase [58].

Many indices of oxidative stress, including the levels of protein oxidation and lipid peroxidation products, have been studied in CF plasma [10, 34, 100, 202], buccal mucosal cells [10], EBC [10, 109, 129, 159, 160], and BAL [33, 72].

Several studies have tested whether markers of oxidative stress may reflect the onset, severity, and response to therapy for an acute exacerbation (Table 1.2). For example, the levels of 8-isoprostane in the EBC negatively correlated with the respiratory function [129]. Robroeks et al. found that the presence of CF was best indicated by 8-isoprostane and nitrite in EBC, similarly as during an acute exacerbation [159]. In a following study aimed at investigating the relationship between lung function, structural lung changes, and noninvasive biomarkers, FVC was significantly predicted by H_2O_2, while total lung capacity was significantly predicted by 8-isoprostane, nitrate, and H_2O_2 in EBC [160]. Overall, these studies indicated that noninvasive biomarkers of oxidative stress may help in the follow-up of CF patients.

Biomarkers of oxidative stress are increased in patients with an acute exacerbation, but not in stable condition, as compared with those in healthy controls [121, 122, 155, 202]. However, not all the biomarkers are useful in this context. Breath isoprene, a volatile product of lipid peroxidation, was significantly lower in patients during exacerbation than in controls and increased to normal values following treatment [122]. The treatment of an acute exacerbation with antibiotic therapy brings to a diminished oxidative stress at the systemic level [121, 122, 153], but not at the pulmonary level [155, 202], indicating the potential for more targeted antioxidant supplementation in CF (see below). Breath hydrogen peroxide levels are not elevated in stable CF patients as compared with healthy controls [77]. However, it has been shown that CF patients with an acute pulmonary exacerbation have abnormally high concentration of H_2O_2 in exhaled air, which decrease during intravenous antibiotic treatment [82], suggesting that appropriate biomarkers should be investigated accordingly to the lung compartment under study. Interestingly, in CF patients with infective exacerbations, treatment with intravenous antibiotics resulted in increased plasma levels of antioxidants, with a parallel decrease in lipid oxidation [153].

As regards the antioxidants (Table 1.2), significantly reduced GSH levels are present in the BAL fluid of adult CF patients [161], and low levels of GSH have been observed in plasma [161] and blood neutrophils [186], suggesting altered

systemic GSH homeostasis in CF. Interestingly, the GSH content in sputum samples is higher in CF patients than in healthy subjects [44], indicating a disparity in GSH levels between the lower and the upper respiratory tracts.

Exocrine pancreatic insufficiency and a diminished bile acid pool cause malabsorption of fat-soluble antioxidants such as tocopherols, carotenoids, and coenzyme Q10 (Co-Q10), which are believed to contribute to the oxidative stress of CF (Table 1.2). Levels of plasma carotenoids such as β-carotene, β-cryptoxanthin, and total lycopene were significantly lower in CF patients as compared to those in healthy controls, and this was accompanied by higher susceptibility to lipid peroxidation [10, 99, 156, 163, 200]. The levels of α-tocopherol and vitamin C in plasma, buccal mucosal cells, and EBC decreased significantly with age in association with a decreased respiratory function as well as with an increased oxidative stress markers, such as protein carbonyls, thiobarbituric acid-reactive substances (TBA), and 8-isoprostane [10]. In a longitudinal study, persistently low levels of Co-Q10 were found more prevalent in patients with pancreatic insufficiency [97].

Levels of plasma vitamin C have been found decreased or normal as compared to healthy controls, nevertheless age- and disease-related decline of this hydrosoluble antioxidant was reported in CF patients [10]. CF children are reported to have lowered blood selenium and erythrocyte selenium-dependent GPx activity [137], normal plasma selenium, and lowered erythrocyte GPx activity [201], and even normal levels of these two parameters [102]. Plasma oligoelements, in particular, zinc, appear to be in the normal range at baseline [137, 190, 191, 203]. Neve et al. found that plasma zinc concentrations were significantly lower in patients with moderate-to-severe growth retardation and with severe pulmonary disease as compared to patients without growth failure and with moderate pulmonary disease, whereas erythrocyte zinc and copper levels were higher than normal [137]. These results suggest a compensatory up-regulation of the erythrocyte Cu/Zn-SOD by the exposure of erythroid precursor cells to ROS and/or other CF-derived stressors. These findings have to be confirmed by further studies.

A lower level of erythrocyte SOD activity was found by Best et al., whereas Wood et al. found that the activity of erythrocyte SOD and plasma 8-isoprostane were in the normal range at baseline [21, 203].

Some pilot studies investigating the effect of GSH inhalation or that of oral GSH prodrug *N*-acetylcysteine (NAC) were able to demonstrate increased GSH levels in the epithelial lining fluid in association with improved lung function [24, 45, 66, 162, 183, 186, 195]. However, indices of oxidative damage were found to be unaffected by aerosolized GSH treatment [66, 67]. Both aerosol and oral formulations are still under investigation as for safety, tolerability, and efficacy [62, 135].

Supplementation with single or combined antioxidants produces poor responsiveness in CF as concerning oxidative stress biomarkers. For example, supplementation of vitamin C together with other antioxidants as vitamin E did not significantly affect the levels of plasma 8-isoprostane and erythrocyte SOD activity [203]. This failure may depend on the dose of the supplement, since for vitamin E, unlike vitamin C, successful high-dose treatment appears to lower oxidative stress markers, such as TBA-MDA complexes, and to correct the total antioxidant capacity

of plasma [163]. In another study, β-carotene supplementation was observed to decrease MDA concentrations in plasma and to enhance the resistance of low-density lipoproteins to oxidation [200]. More recently, the use of a CF-tailored multivitamin formulation (commercial name AquaDEKs®) resulted in the normalization of β-carotene levels but with minor improvements on respiratory and growth parameters and with no increase of urinary 8-isoprostane levels [166]. In another study, this multivitamin preparation normalized MDA levels in plasma and increased SOD activity and sulfhydryl groups in erythrocytes [165], indicating that larger randomized controlled trials are deserved.

1.3.3.1 The Emerging Role of Oxidative Stress and Autophagy in Cystic Fibrosis Lung Disease

Cellular homeostasis is deranged in CF airways as a result of increased intracellular levels of ROS, induced by defective CFTR function. Increased ROS levels induce posttranslational changes of tissue transglutaminase (TG2), a multifunctional protein [138] that can function as a rheostat of posttranslational network in CF epithelial cells [106]. In the presence of high Ca^{2+} levels, TG2 works as a cross-linking enzyme, catalyzing several posttranslational modifications of target proteins. TG2 is up-regulated in CF epithelial cells at the transcriptional and even more at the post-transcriptional levels [114]. Indeed, TG2 undergoes a posttranslational modification, the small ubiquitin like-modifier SUMOylation, as the result of ROS-induced increase of the SUMO E3 ligase protein inhibitor of activated STAT (PIAS)y [106] which can orchestrate SUMO modifications in response to either oxidative or genotoxic stress [111]. SUMOylation is a key player of the posttranslational network as it regulates transcription, nuclear translocation, stress responses, and chromatin structure and influences intracellular localization, stability, and function of modified proteins [63, 123, 146]. SUMOylation of lysines in TG2 (SUMO consensus sequence: ψ_kxE) is incompatible with the ubiquitination of these residues, leading to the inhibition of TG2 ubiquitination, thereby preventing its proteasomal degradation. High TG2 levels can in turn sustain ROS, as TG2 may stimulate the activity of the mitochondrial respiratory chains [116].

These ROS-mediated posttranslational changes of TG2 protein, induced by defective CFTR function, can switch off the posttranslational regulatory mechanisms and may have functional implications in epithelial homeostasis. Sustained TG2 activation leads to cross-linking, increased ubiquitination, and functional sequestration of TG2 substrates, among which are the gamma forms of peroxisome proliferator-activated receptor (PPARγ) and IkBa [47, 114, 142], thus favoring inflammation in CF airways [106]. TG2-mediated protein cross-linking may lead to proteasome overload [52] favoring protein aggregation; in fact, misfolded or post-translationally modified proteins that cannot be degraded by the proteasome machinery, are stocked in the cytoplasm in the form of aggresomes [89, 173]. Therefore, the proteostasis of CF epithelia is affected by a combination of genetic defects (resulting from the misfolded CFTR protein) and posttranslational alterations (mediated by ROS/TG2 axis).

Such an impaired redox balance in CF airways compromises the ability of CF cells to re-establish homeostasis in response to stress, either constitutive or induced by bacterial challenges. Indeed, it inhibits the activation of autophagy, a mechanism that cells adopt in response to stress. Autophagy is pivotal in promoting cellular clearance of protein aggregates and removal of ROS sources, such as damaged mitochondria [89, 92, 95, 124]. Thus, autophagy machinery should have been highly activated in CF environment. By contrast, human and mouse CF airways exhibit a pronounced defect in autophagy, as indicated by reduced autophagosome formation together with accumulation of sequestosome 1 (p62/SQSTM1), a major autophagic substrate. In CF airways, autophagy is impaired in spite of the normal expression of major autophagy genes [107], as sustained TG2 activation results in cross-linking of Beclin 1 (BECN1), a major player of autophagosome formation. This dislodges type III phosphatidylinositol 3-kinase (PtdIns3K, also known as hVps34, a protein that belongs to the BECN1 interactome) away from the endoplasmic reticulum (ER), thus impairing the generation of phosphatidylinositol 3-phosphate (PtdIns3P) [194], that is pivotal in both autophagosome formation and endosomal trafficking. Therefore, ROS-mediated TG2 activation generates a vicious feed-forward loop that impairs the regulation of proteostasis and sustains inflammation in human and mouse CF airways.

A defective autophagic response to bacterial infection has also been reported in murine CF macrophages. Reduced autophagosome formation in CF macrophages promotes Burkholderia cenocepacia survival and hypersecretion of IL-1b [1].

Targeting ROS by means of a catalase-SOD mimetic (EUK-134) or the enforced Mn-SOD expression, or inhibiting TG2 by cystamine (or by its reduced form of cysteamine), can rescue autophagy, restore proteostasis, and control airway inflammation in CF [107].

Both EUK-134 and cystamine also favor F508del-CFTR trafficking to the epithelial surface in CF cell lines, in primary brushed nasal epithelial cells from F508del-CFTR homozygous patients and in lungs from F508del-CFTR homozygous mice [107, 108]. These treatments also stabilize a functional rescued F508del-CFTR at the plasma membrane of airway epithelial cells and their effects extend well beyond drug washout. Indeed, the ROS/TG2-mediated inhibition of autophagy, consequent to functional depletion of CFTR, favors CHIP-mediated CFTR ubiquitination at the plasma membrane, thus diverting CFTR recycling to lysosomal degradation [194]. This indicates that targeting oxidative stress in CF epithelia can either favor F508del-CFTR trafficking or prevent CFTR plasma membrane disposal [108, 194]. These evidences may have relevant implication in CFTR-repairing therapies [5], to restore autophagy, by means of ROS-modulators or TG2 inhibitors, can favor the beneficial action of CFTR potentiators in F508del-CFTR homozygous patients [108, 194].

The “anti-inflammatory” effects of both EUK-134 and cystamine also extend well beyond drug withdrawal unless CFTR is inhibited or depleted during the washout period [108, 194]. This indicates their anti-inflammatory properties rely on their ability to rescue and stabilize a functional CFTR at the epithelial surface. Altogether, these findings might open a new scenario in the design of new anti-inflammatory strategies for CF patients.

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Chapter 2
Reactive Oxygen and Nitrogen Species: General Considerations

Veena Dhawan

2.1 Introduction

Oxidative stress is defined as a natural physiological process in the biological systems where the presence of free oxygen radicals overpowers the radical scavenging mechanisms, thus creating an imbalance between the oxidants and the antioxidants. Historically, recognition of the presence of free radicals in the living cells was first demonstrated in 1954 [1]. Soon thereafter, a therapy based on free radicals and radiation chemistry was proposed for ageing [2]. Numerous studies in literature have shown that free radicals are involved in the etiology of several human diseases, as well as in ageing [3]. Harman described free radicals as "Pandora's box of evils which account for cellular damage, mutagenesis, cancer and degenerative diseases" [2]. Based on the volume of research on this subject, it is believed that the cross talk between various risk factors converges on a final common pathway of oxidative stress through which they exert their deleterious effects in causing various diseases.

Oxidative stress represents a state of increased levels of reactive oxygen species (ROS), also termed as "oxygen-derived species" or "oxidants." The function is controlled physiologically by concentration of oxygen, signal transduction, and maintenance of redox homeostasis. The science of redox regulation is a rapidly growing field of research that has impact on almost every discipline involving biological systems which have not only adapted to the coexistence of damaging free radicals but also developed mechanisms of using free radicals to their advantage. Numerous data exist in the literature that both ROS and reactive nitrogen species (RNS) are produced in a well-regulated manner to help maintain homeostasis at the cellular level in the normal healthy tissues, play an important role as second messengers, and regulate cellular function by modulating signaling pathways [3].

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Overproduction of ROS, as well as the deficiency of enzymatic and nonenzymatic antioxidant defense mechanism creates an imbalance in the equilibration of prooxidant/antioxidant status which governs a wide array of diverse disorders. ROS elicit and regulate divergent effects on cellular functions, e.g., cell growth and differentiation, growth factor signaling, mitogenic responses, modulation of extracellular matrix production and breakdown apoptosis, inactivation of nitric oxide (NO), oxygen sensing, and stimulation of proinflammatory genes and many kinases [4].

2.2 Free Radicals

A free radical is defined as a molecule that contains one or more unpaired electrons in a single orbit. Molecular oxygen has two and nitric oxide ($NO^{\bullet}$) has one unpaired electron which can exist independently and thus justify their free radical characters. A chemical reaction shall involve the transfer of one single electron. Any related reactive species that leads to free radical generation or other species that result from free radical reactions can also be included in this category. Cells use oxygen to generate energy and form free radicals as a result of ATP production by the mitochondria [5]. Free radicals become a part of the propagative chain reaction whereby they combine with other radicals to form other more damaging species, unless the chain is terminated by chain breaking antioxidants to form a species which is nontoxic [5]. All organisms possess inherent cellular defenses to overcome oxidative stress that are collectively termed as antioxidants. Free radicals have very short life, e.g., in milli-, micro-, or nanoseconds, and readily react with lipids, DNA, and proteins causing damage and form harmful products such as lipid peroxides and other lipid adducts. The consequent protein damage results in loss of enzyme activity, while DNA damage can result in mutagenesis and carcinogenesis [6].

2.3 Generation of ROS

Oxygen is essential to aerobic life but, paradoxically, it can be toxic even at atmospheric concentrations. ROS/RNS are formed as byproducts of normal metabolism in aerobic organisms. ROS is a broader term; it includes many reactive species, e.g., superoxide ($O_2^{\bullet}$), hydroxyl ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkyl radical, alkoxyl ($RO^{\bullet}$) radicals, singlet oxygen (O) and semiquinone radical ($HQ^{\bullet}$), and ozone (O_3) (Table 2.1). Hydroxyl radicals are formed in the presence of metals and hydrogen peroxide (Fenton reaction); peroxynitrite might play a small role in hydroxyl radical formation. In this process, certain non-radicals are also produced that are either oxidizing agents or easily converted into radicals, such as HOCl, ozone, H_2O_2, and lipid peroxides with no unpaired electrons. H_2O_2 and lipid peroxides also serve as a source of highly reactive $^{\bullet}OH$, $ROO^{\bullet}$, and $RO^{\bullet}$ radicals. $O_2^{\bullet-}$ reacts quickly with very few molecules, whereas hydroxyl radical $OH^{\bullet}$ has an extremely high rate of reactivity [7].

Table 2.1 Different types of ROS and RNS produced in the cell

Reactive Oxygen Species (ROS)

Radicals:	
$O_2^{\cdot -}$	Superoxide
$OH^{\cdot}$	Hydroxyl
$RO_2^{\cdot}$	Peroxyl
$RO^{\cdot}$	Alkoxyl
$HO_2^{\cdot}$	Hydroperoxyl

Non-Radicals:	
H_2O_2	Hydrogen peroxide
$HOCl^-$	Hypochlorous acid
O_3	Ozone
$^1O_2$	Singlet oxygen
$ONOO^-$	Peroxynitrite

Reactive Nitrogen Species (RNS)

Radicals:	
$NO^{\cdot}$	Nitric Oxide
$NO_2^{\cdot}$	Nitrogen dioxide

Non-Radicals:	
$ONOO^-$	Peroxynitrite
ROONO	Alkyl peroxynitrites
N_2O_3	Dinitrogen trioxide
N_2O_4	Dinitrogen tetroxide
HNO_2	Nitrous acid
NO_2^+	Nitronium anion
NO^-	Nitroxyl anion
NO^+	Nitrosyl cation
NO_2Cl	Nitryl chloride

2.4 Sources of Reactive Oxygen Species

Normal metabolic processes in all the aerobic conditions constitute a major source of ROS. The cellular sources include the electron transport chain of mitochondria and endoplasmic reticulum [8]. ROS are produced by all cell types, e.g., the neutrophils, monocytes, macrophages, and the cytotoxic lymphocytes, and can be formed by the action of many enzymes. The important enzymatic sources responsible for ROS production include NAD(P)H oxidase, xanthine oxidase (XO), and uncoupled form of nitric oxide synthase (NOS). The other enzyme sources are myeloperoxidase (MPO), aldehyde oxidase, cyclooxygenase, lipoxygenase, dehydrogenase, tryptophan dioxygenase, and flavoprotein dehydrogenase [9].

In nonphagocytic cells, a variety of cytokines such as TNF-α, IL-1, and interferon (IFN)-γ are shown to generate ROS essential for their signaling by binding to cytokine receptors. Several growth factors are capable of generating ROS by binding to different receptors in nonphagocytic cells and initiate mitogenic signaling. Depending on their isoforms, they either inhibit or activate NADPH oxidase activity for H_2O_2 production [10, 11]. All receptor serine/threonine kinases in mammalian cells belong to the TGF-β superfamily. TGF-β1 is shown to stimulate ROS production in a variety of cell types [12].

A number of stimuli, e.g., angiotensin II (Ang II), serotonin, 5-hydroxytryptamine (5-HT), bradykinin, thrombin, and endothelin (ET), are shown to generate ROS in different cells by binding to G protein-coupled receptors. Neurotransmitters, by binding to ion channel-linked receptors, mediate rapid synaptic signaling. Relatively little is known about ROS signaling by ion channel-linked receptors [10, 13] (Fig. 2.1).

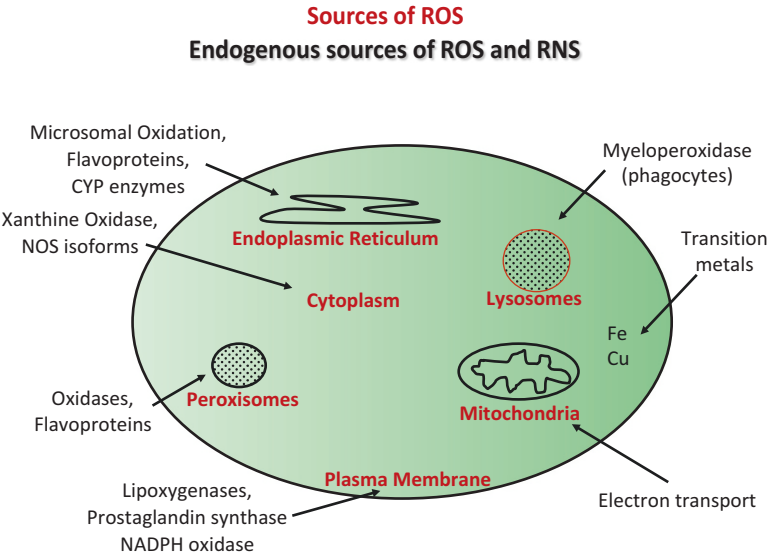

Fig. 2.1 Various endogenous sources of ROS and RNS in the cell

2.5 NAD(P)H Oxidase

NAD(P)H oxidase is a membrane-bound enzyme complex which represents a major source of $O_2^{\bullet-}$ in the body. It is present in various cells, e.g., the endothelial cells, smooth muscle cells, fibroblasts, monocytes, and macrophages [14]. Although NAD(P)H oxidases were originally considered as enzymes expressed only in the phagocytic cells, the recent evidence indicates that there is an entire family of NAD(P)H oxidases. The new homologs are now designated the Nox family of NAD(P)H oxidases. The family includes seven members such as Nox1, Nox2 (gp91phox), Nox3, Nox4, Nox5, Duox1, and Duox2 [6, 14]. They are expressed in many tissues and mediate diverse biological functions. The NAD(P)H oxidase found in neutrophils has five subunits: p22phox, p47phox (or NOXO1), p67phox (or NOXA1), and p40phox (phox stands for *ph*agocyte *ox*idase), and the catalytic subunit gp91phox (or its homologs, Nox1 and Nox4) also termed Nox2. In quiescent cells, NAD(P)H oxidase exists in an unassembled state, i.e., p22phox and gp91phox are present in the membrane whereas p47phox, p67phox, and p40phox exist in the cytosol.

A number of stimuli activate NAD(P)H oxidase whereby p47phox becomes phosphorylated and the cytosolic subunits form a complex that translocates to the membrane and convert the oxidase into an assembled and active form which transfers electrons from the substrate to O_2, forming $O_2^{\bullet-}$ [15]. Therapy based on free radicals and radiation chemistry was proposed for ageing [2]. In the first step one

electron is added to the molecular oxygen in a univalent reduction to generate superoxide anion ($O_2^{\bullet-}$) using NADPH or NADH as the electron donor:

$$2O_2 + NAD(P)H \rightarrow 2O_2^{\bullet-} + NAD(P)^+ + H^+.$$

Superoxide anion can be generated both enzymatically, e.g., during the NADPH phagocytic oxidase reaction in neutrophils, and nonenzymatically in the mitochondrial respiratory chain.

2.6 Regulation of NAD(P)H Oxidase Activity

The mechanism behind interaction of NAD(P)H oxidase subunits in cells and how they generate $O_2^{\bullet-}$ is not fully understood. Plentiful evidence exists that Nox enzymes are crucial for normal biological responses and contribute to the pathophysiology of several diseases, yet their regulation and function remain unclear. NAD(P)H oxidase responds to the stimuli of many growth factors, cytokines, mechanical forces, metabolic factors, and G protein-coupled receptor agonists. Ang II is the most potent regulator of NAD(P)H oxidase that activates NAD(P)H oxidase through stimulation of various signaling pathways and through transcriptional regulation of oxidase subunits [16].

2.7 Xanthine Oxidase

Xanthine oxidoreductase (XOR) is another important enzymatic source of ROS which belongs to metalloflavoprotein family [17]. XOR (EC 1.17.1.4) catalyzes the oxidation of hypoxanthine and xanthine to form uric acid. XOR is shown to exist in two forms: xanthine oxidase (XO) and xanthine dehydrogenase (XDH). The enzyme catalyzes the reduction of O_2, leading to the formation of superoxide ($O_2^{\bullet-}$) and H_2O_2; it is proposed as a central mechanism of oxidative injury.

Principle reaction catalyzed by xanthine oxidase (XO) is the oxidation of xanthine into uric acid:

$$XO + H_2O + O_2 \rightarrow \text{Uric acid} + H_2O_2.$$

This process is accompanied by production of superoxide:

$$XO + O_2 \rightarrow XO^{-1} + O_2^{\bullet-}.$$

The concentration of circulating XOR is low under physiological conditions, but it increases dramatically in certain diseases. Most of the circulating XOR form exists in the oxidase form. Once in circulation, XOR has the ability to initiate oxidative damage in remote organs with intrinsically low XOR content. XO can generate

nitric oxide ($NO^{\bullet}$) by catalyzing the reduction of nitrate to nitrite and nitrite to $NO^{\bullet}$ in the presence of NADH as an electron donor. $NO^{\bullet}$ or $ONOO^{-}$ has been proposed as feedback inhibitor of XO via disruption of the critical molybdenum (Mo) center of the enzyme. The Mo cofactor or sulfate moieties in the XOR protein are critical components which are responsible for transcriptional and posttranslational regulations of XOR activity [6, 18]. H_2O_2 has also been shown to inhibit XOR activity by deactivating the Mo center. Phosphorylation has also been cited as a mechanism of posttranslational modification of XOR.

Commercially available allopurinol and metabolite oxypurinol are the nonselective inhibitors of XOR that prevent oxidation of xanthine to uric acid. Febuxostat is also shown to inhibit the oxidized and reduced forms of XOR selectively without affecting other enzymes of purine and pyrimidine metabolism [19].

2.8 Generation of Reactive Nitrogen Species

RNS is a collective term that includes nitric oxide radical ($NO^{\bullet}$), peroxynitrite ($ONOO^{-}$), nitrogen dioxide radical ($NO_2^{\bullet}$), and other oxides of nitrogen and products arising when $NO^{\bullet}$ reacts with $O_2^{\bullet-}$, $RO^{\bullet}$, and $H^{\bullet}NO^{\bullet}$ [20]. $NO^{\bullet}$ was initially discovered in 1980 as a vasodilating substance secreted by the endothelium, termed as EDRF [21]. Subsequently, this factor was termed as $NO^{\bullet}$. In 1992, $NO^{\bullet}$ was chosen as "molecule of the year" [22]. In 1998 Furchgott, Ignarro, and Murad were awarded the Nobel Prize in Physiology and Medicine for their discovery of $NO^{\bullet}$ as a signaling molecule in the cardiovascular system [23–25].

$NO^{\bullet}$ plays significant role in cellular signaling, vasodilation, and immune response. It is a highly reactive small uncharged molecule containing one unpaired electron, therefore considered a free radical. It has a half-life of 15 s and can readily diffuse across the membrane due to its uncharged state. Endogenous $NO^{\bullet}$ is formed in the biological tissues via the action of NOS where L-arginine and oxygen are converted into $NO^{\bullet}$ and citrulline via a five-electron oxidative process. The reaction requires the presence of many cofactors such as FAD, FMN, NADPH, tetrahydrobiopterin, and heme [26, 27].

2.9 Nitric Oxide Synthase

Conversion of L-arginine to L-citrulline and nitric oxide is carried out by NOS but under uncoupling conditions, these enzymes also produce superoxide:

$$\text{NOS} + \text{L-Arginine} + O_2^{\bullet-} + \text{NADPH} \rightarrow NO^{\bullet} + \text{Citrulline} + NADP^{+}.$$

$$\text{NOS (Fe (II) heme)} + O_2^{\bullet-} \rightarrow \text{NOS (Fe (III) heme)} + O^{\bullet-}.$$

There are three known isoforms of NOS with different activities; two of the NOS forms are constitutively expressed in neuronal cells (nNOS) or in the endothelial cells (eNOS) [28, 29]. These constitutively expressed NOS isoforms are regulated via calcium levels. As the intracellular calcium levels increase, calcium forms a complex with calmodulin (a calcium binding protein) which then binds to NOS and causes its activation. Activated NOS synthesizes small amounts of $NO^{\bullet}$ till calcium levels decrease. This intermittent production of $NO^{\bullet}$ is responsible for transmission of signals and is sufficient to maintain a basal vasodilator tone [30–32]. $NO^{\bullet}$ as a vasodilator has been shown to inhibit leukocyte interaction with the endothelium, inhibit platelet aggregation and cell adhesion, and control cell proliferation [33]. In oxidative stress conditions, $NO^{\bullet}$ is consumed, thereby causing various problems.

Another isoform of NOS which is subject to regulation by inflammatory mediators is expressed in macrophages, and is termed as iNOS [34]. iNOS is independent of calcium and calmodulin ions. Once activated, it generates large amounts of $NO^{\bullet}$ for as long as the inflammatory stimulus is present and kills or inhibits pathogens. All the NOS are homologous and have different regulation controls and activities. iNOS is regulated by phosphorylation/dephosphorylation via protein kinases; in its phosphorylated form, the activity is decreased. eNOS can also be regulated via phosphorylation/dephosphorylation. iNOS can also bind calmodulin, though calcium has little effect on its activity. In contrast to other signaling molecules which act through receptors, $NO^{\bullet}$ diffuses out of the cell where it is produced and diffuses in target cells to transmit signals and interact with its molecular target, e.g., proteins, nucleic acids, and other free radicals like superoxide [35].

$NO^{\bullet}$ is shown to act through cyclic GMP (cGMP, a second messenger). By binding to iron in heme group of GC, it activates the enzyme whereby cGMP is produced which further activates other cellular processes [36]. $NO^{\bullet}$ causes auto-ADP ribosylation, i.e., ribosylation of a target without enzyme catalysis, e.g., by ADP ribosylation of glyceraldehyde 3-phosphate dehydrogenase, therefore inhibiting ATP production [37].

$NO^{\bullet}$ has also been shown to inhibit the activity of a number of enzymes including xanthine oxide, gluthathione peroxidase, cytochrome *c* oxidase, and NADPH oxidase. $NO^{\bullet}$ interacts with proteins by binding to iron, present as heme group or as an iron sulfur complex in enzymes, and either activates or deactivates the enzyme. $O_2^{\bullet-}$ plays a critical role in $NO^{\bullet}$-induced toxicity where $O_2^{\bullet-}$ and $NO^{\bullet}$ can combine in a radical–radical reaction which is extremely fast and form toxic product peroxynitrite. Peroxynitrite is a potent oxidant produced in various inflammatory and pathological conditions that can attack a wide variety of biological molecules. $ONOO^-$ directly attacks sulfhydryl groups in various target molecules [37] and also reacts by either one- or two-electron oxidation reactions [38]. Peroxynitrous acid (HOONO), which has $OH^{\bullet-}$-like properties, is formed by reacting with nitric acid, and has oxidant properties.

2.10 Antioxidant Defenses

"Antioxidants" can be defined as those substances that neutralize free radicals or their actions [39]. These are present in low concentrations and significantly prevent oxidation of that substrate. To counteract deleterious effects of oxidative stress, nature has endowed each cell with adequate protective antioxidant defenses which can be broadly categorized into enzymatic or nonenzymatic antioxidants based on their action in intracellular and extracellular compartments. Enzymatic antioxidants include superoxide dismutase (SOD) which catalyzes the dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2. SOD exists in three isoforms in mammalians, i.e., copper/zinc SOD (SOD1), mitochondrial SOD (Mn SOD, SOD2), and extracellular SOD (ecSOD, SOD3) [40, 41]. Glutathione peroxidase reduces H_2O_2 and lipid peroxides to water and lipid alcohols and in turn oxidizes glutathione to glutathione disulfide. Catalase catalyzes the conversion of H_2O_2 to water and molecular oxygen, and protects the cells from harmful effects of H_2O_2 produced within the cell. This enzyme is highly effective during augmented oxidative stress, as reduced levels of glutathione or glutathione peroxidase are available. Reduced glutathione plays a major role in the regulation of the intracellular redox state of the cells as it is a major source of reducing equivalents [42]. Thioredoxin reductase is responsible for thiol-dependent reductive processes in the cell [43]. Glutathione S-transferase and H_2O_2 can form spontaneously or can be formed by dismutation of $O_2^{\bullet-}$ catalyzed by SOD: $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$. Thioredoxins are low molecular weight proteins that contain a conserved dithiol motif which is responsible for a variety of biological functions. Sulfur switches are shown as sensors in redox signaling pathways which control and integrate metabolic pathways. Three major redox controls responsible for regulation of these switches are thioredoxins, GSH/GSsL, and Cys/Cyss [44].

The nonenzymatic category of antioxidant defenses includes low molecular weight molecules, e.g., glutathione, uric acid, vitamin A (retinoids), carotenoids particularly beta carotene with a high-antioxidant activity as it quenches free radicals, and α-tocopherol (vitamin E), a fat-soluble and free radical chain breaking antioxidant which, due to the presence of hydroxyl (–OH) group in its structure, is an effective hydrogen donor. Ascorbic acid (vitamin C) acts as a hydrogen donor and reverses oxidation, and can act both as an antioxidant and as a prooxidant. Fruits and vegetables in the diet are main source of vitamin C and other nonenzymatic antioxidants, e.g., flavonoids and related polyphenols. The concentration of these antioxidants is low and varies depending on their location. Bilirubin, lipoic acid, albumin, ferritin, ceruloplasmin, and transferrin also show antioxidant properties and can indirectly reduce or inhibit generation of reactive species (Table 2.2).

2.11 ROS/RNS Signaling

Data from different studies clearly demonstrate that reactive species act as second messengers and play a critical role in immune function and signal transduction thereby affecting cellular homeostasis [45]. ROS act as signaling molecules when

Table 2.2 Enzymatic and nonenzymatic antioxidants that protect against ROS/RNS generation

Enzymatic antioxidants	Nonenzymatic antioxidants
Thioredoxin (Trx)	Vitamins C, E, A
Peroxiredoxins (Prx)	Thiols
Glutaredoxin (Grx)	β-Carotene
Glutathione peroxidase (Gpx)	Polyphenols
Reduced glutathione (GSH)	NAC
Oxidized glutathione (GSSG)	Zinc, selenium
Glutathione reductase (GR)	Glutathione
Extracellular glutathione peroxidase (eGPx)	Uric acid
Catalase	Lycopene
Peroxidase	Allyl sulfide
Superoxide dismutase	Indoles
	Gallic acid
	Hesperitin
	Catechin
	Chrysin

present in low concentration, and promote cell proliferation and cell survival, whereas an increased concentration activates NF-κB and AP-1 [46, 47]. At extremely high levels or persistent cellular ROS, these are shown to promote cell death. Redox system regulates ROS-mediated signaling via direct oxidative modification of redox-sensitive signaling proteins. Multiple layers of regulation are reported at the level of signaling pathways [48]. Their actions are mediated through oxidative/nitrosative reactions. These molecules may attack cysteine residues on proteins via oxidative/nitrosative modifications and alter many proteins, e.g., transcription factors, kinases, and phosphatases, which in turn may affect downstream signaling cascades and alter cellular fate. In the presence of a transition metal, such as iron, hydrogen peroxide can be converted to the highly reactive hydroxyl ion which amplifies oxidative stress and its consequences [49].

Hypoxia-inducible factor (HIF) is a TF shown to regulate cellular metabolism and cell survival under hypoxic stress. By binding to hypoxia response element (HRE) in the promoter of many genes, HIF1α results in activation and suppression of several genes involved in metabolism, e.g., cell survival/death, angiogenesis, and invasion/metastasis [50]. HIF1α is regulated by oxygen requiring hydrolyzing enzymes and is also regulated via feedback regulation under hypoxia by increased expression of its own regulators. Increased ROS/RNS generation is shown to stabilize HIF1α via increased generation of $OH^{\bullet}$ radical from H_2O_2, by direct oxidative modification and by activating multiple signaling pathways which may render HIF1α inactive. Use of antioxidants has been shown to decrease HIFα activity [51].

One of the important signaling pathways involved in ROS regulation is that of serine/threonine AMP-activated protein kinase (AMPK) that contributes to the control of energy metabolism [52]. Silencing AMPKα1, a predominant catalytic subunit of enzyme in human umbilical vein endothelial cells (HUVEC), was shown to inhibit cell proliferation and ROS accumulation [53].

MAPK and SAPKs: MAPKs operate in a cascade fashion; the family includes ERK1/2, JNK, p38, ERK3/4, and BMK1/ERK5 pathways. The JNK and p38 kinase pathways are also known as SAPKs [54].

Several studies demonstrate that NF-kβ, a redox-sensitive TF, can be activated or inhibited in response to OS and is regulated via redox-mediated mechanism at multiple levels of activation pathways. AP-1 is a transcription factor involved in control of cell growth and apoptosis. MAPKs are shown to regulate AP-1, JNK, ERK, and p38 kinase pathways [55]. Redox-mediated regulation of AP-1 has been demonstrated at level of transcription and translation. Oxidative stress is shown to promote AP-1 activity by inhibition of histone deacetylases (HDAC), by activating MAPK pathways [56]. $NO^{\bullet}$ is also shown to modulate AP-1 through S-glutathionylation. Both experimental and human studies have provided sufficient evidence to show that OS can activate MAP kinase via Ras pathway. As far as SAPK pathways are concerned, they are differentially regulated depending on dose and duration of the stimuli and type of oxidative modification, and are regulated at multiple levels [57].

Phosphatidylinositol-3-kinase (*PI3K/Akt pathway*): Signal transduction via PI3 kinase plays an important role in the regulation of cell growth, proliferation, survival, and motility. Depending on the type and duration of ROS, PI3K signaling is activated or inhibited, thus modulating cell survival pathways. Activation of PI3K/Akt pathways is tightly kept in check by phosphatases. ROS are shown to activate or inhibit this pathway mainly through oxidative modification of cysteine-dependent phosphatases (CDPs) which results in sustained activation of PI3K/Akt signaling, whereas redox modification of kinases results in down-regulating PI3K/Akt signaling [58, 59]. Oxidative modifications of ubiquitin-proteosome or other proteases can also affect turnover of signaling proteins [60].

Nrf2–Keap 1 axis: An Nrf2–Keap 1 axis (NF-E2-related factor 2 protein) has been implicated in respiratory disorders and oxidative stress and ROS are shown to activate Nrf2 pathway. ROS disrupt Nrf2–Keap 1 association, whereby Keap 1 dissociates from Nrf2 and Nrf2 translocates to the nucleus from cytosol and binds antioxidant response element (ARE) in the regulatory region of many genes. Reports in Nrf2-deficient mice using microarray-based assays have suggested that Nrf2 modulates transcription of multiple genes whose protein products function as antioxidants, heat shock proteins, glutathione synthesis enzymes, proteasomes, and phase-2 detoxification enzymes [61, 62]. All these proteins are known to play a very crucial role in maintenance of cellular homeostasis against an onslaught of oxidative stress. Nrf2 has been implicated in protection against oxidative damage-induced injury, hyperoxia, nitrosative stress, ER stress, and exogenous prooxidants. The absence of Nrf2 is shown to promote apoptosis and modulate cell survival processes [63].

Recent reports also suggest a novel role of redox regulation in chromatin remodeling which affects death/survival signals at transcriptional levels. Posttranslational modifications of signaling proteins are also regulated through redox-mediated mechanism. There is a lot of cross talk at the level of redox regulation which, through modulation of signaling proteins, may affect cell survival mechanisms, transcription, and signal transduction (Fig. 2.2).

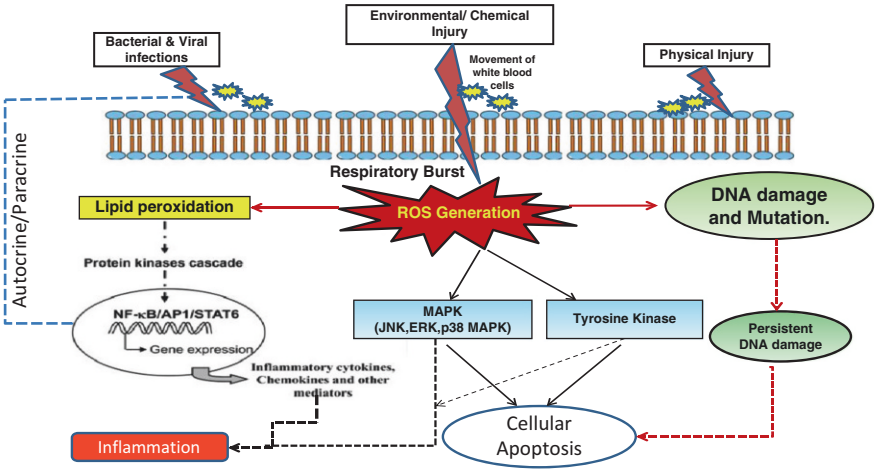

Fig. 2.2 Role of ROS and RNS in tissue damage. Inflammation begins with a reaction to an irritant or infection that is characterized by movement of fluid and white blood cells into extravascular tissue. This is followed by cell proliferation and involves tissue repair and regeneration. Generation of free radicals, e.g., ROS and RNS, follows leading to lipids, protein, and DNA damage via activation of transcription factors through signal transduction pathways such as MAPK and PKC leading to inflammation. Prolonged stimuli cause ROS:antioxidant imbalance and affect cell survival in terms of apoptosis and cell death

2.12 Respiratory System

Oxygen is essential to life, but at concentrations exceeding physiological limits, it may be hazardous to the cells. Lungs are directly exposed to very high oxygen concentration and thus are prone to high risk of developing oxidative stress. A variety of ROS/RNS are generated by inflammatory pulmonary cells. The ROS are produced in bulk from activated macrophages in a process known as "respiratory burst" which acts as a first line of defense against environmental triggers/pathogens. Apart from their role as a part of host defense in aerobic organisms, they play a different role independent of host defense. Neutrophils, eosinophils, alveolar macrophages, and epithelial cells and bronchial epithelial cells are the source of ROS/RNS in the lungs. There is also an array of antioxidant defenses present in the lung tissue and epithelial lining fluid to counteract onslaught of oxidative stress resulting in cellular adaptive and protective responses. ROS/RNS usually exert their action at the cellular level through signaling mechanisms which involves genetic regulation. Thus an oxidant:antioxidant imbalance can lead to a variety of respiratory diseases such as chronic obstructive pulmonary disease (COPD), asthma, and idiopathic pulmonary fibrosis (IPF).

ROS cause damage to the lipids, protein, and DNA resulting in lung injury and induce a variety of cellular responses, e.g., extracellular matrix remodeling in blood

vessels, increased mucus secretion, and alveolar repair responses. Atmospheric aerosols produced due to air pollution containing hazardous agents, e.g., diesel exhaust, soot, polycyclic aromatic compounds, mineral dusts, ozone, nitrogen dioxides, ultraviolet and ionizing radiation, and tobacco smoke, are the other factors which can damage biological molecules and initiate a cascade of events in the respiratory system. Allergenic proteins upon exposure to O_3 and NO_2 get sufficiently oxygenated and nitrated and thus form toxic products leading to inflammation and cellular damage.

The evidence is further strengthened by extensive amount of data available from both in vivo and in vitro studies as well as from studies using experimental animal models which support the view that ROS and RNS are important in maintaining respiratory homeostasis.

2.13 Pharmacological Inhibitors of ROS and RNS in Experimental/Clinical Trials

Redox system is involved in the maintenance of cellular homeostasis; alterations in redox homeostasis can promote cell death or cell survival depending on the type and duration of exposure to stimuli. Functional status of cellular antioxidant and redox-sensitive survival signaling pathways can significantly modulate the cell fate. Therefore, redox-based therapeutic/preventive strategies should be evolved which may maintain redox homeostasis to modulate redox-sensitive factors which govern cell fate.

Despite the extensively reported evidence, the pharmacological strategies to overcome the deleterious effects of the ROS and RNS have not been successful in clinical trials. There is a need to prove whether antioxidant therapy can prevent or overcome the damaging effects of ROS in life threatening situations. The compounds must be tested for their safety, toxicity, selectivity, bioavailability, and therapeutic efficacy. Combination therapy with these agents can also be tried to achieve synergistic clinical effects. A complete understanding of the molecular mechanisms of ROS/RNS, as well as epidemiological and randomized clinical trials in humans is needed before a drug can be routinely prescribed and used.

ROS and RNS induce DNA damage which activates PARP (poly (ADP-ribose) polymerase). Development of PARP inhibitors can be explored for therapy of the respiratory disorders. Neutralization of peroxynitrites and pharmacological inhibition of MMPs and PARP are promising new approaches in the experimental therapy [64]. Inhaled apocynin was shown to decrease ROS concentration in exhaled breath condensate (EBC) in mild asthmatics. In a completed clinical trial, effect of inhaled apocynin on ROS and NOS generation was demonstrated in 13 bronchial asthma and COPD patients. In comparison to placebo, H_2O_2 and NO_2 were shown to reduce in EBC of COPD subjects in response to nebulized apocynin, and showed no adverse side effects [65]. In an in vivo placebo-controlled crossover study in different age group of healthy subjects, fermented papaya preparation (FPP) supplementation

was shown to augment SOD, a potent enzymatic scavenger of O_2 [66]. In a mice model of ventilator-induced lung injury, amifostin preconditioning was reported to attenuate oxidative stress in the lung by scavenging ROS and RNS and by augmenting enzymatic antioxidants, and was proposed as a promising strategy for critically ill patients on extended mechanical ventilation [67, 68].

Erdosteine is a mucolytic agent for chronic pulmonary diseases and possesses antioxidant properties. Experimental data demonstrate beneficial effects of this drug, by reducing OFR generation and increasing enzymatic antioxidant cellular defenses [69]. Albumin and furosemide therapy has also been proposed to be beneficial in hypoproteinemic subjects with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), though data on outcome are lacking [70]. Therapies with GSH and its analogues have been used in clinical trials but did not demonstrate a positive outcome, and rather shown to result in generation of undesirable toxic products [71].

Many studies have been undertaken with inhibitors of major ROS generating enzymes which show promising results. Use of natural ROS scavengers and treatments with exogenous antioxidants are reported to attenuate deleterious effects of ROS. *N*-acetylcysteine (NAC), melatonin, resveratrol, vitamin C, mitochondria-targeted antioxidants such as mitoQ and mito vitamin E, lipoic acid, selenium (Se), and GSNO (a physiologic metabolite of GSH and $NO^{\bullet}$) have been developed and utilized for the prevention of oxidative stress in several diseases [72–76]. Thioredoxin has also been proposed as an attractive therapeutic approach for preventing and/or treating cardiopulmonary disorders [77]. $NO^{\bullet}$ prodrug JS-K has also been considered as a therapeutic option [78]. In cystic fibrosis, MPO has been shown to act as a phagocyte oxidase blocking $NO^{\bullet}$ bioavailability and is considered a potential therapeutic target [79].

In addition to pharmacological interventions, the data from several epidemiologic and observational studies suggest a positive association between antioxidant vitamin status and indicators of airway obstruction and pulmonary function [80, 81]. A meta-analysis of randomized controlled trials examining the role of iNO for treatment of ARDS or ALI in children and adults reported inconsistent results and prevented assessment of all outcomes [82]. Based on multiple in vitro and animal model studies, no specific pharmacologic approach for ARDS has been successfully validated in clinical trials [83]. GSH depletion in lung epithelial lining fluid has also been noted in COPD, IPF, and ARDS [84]. Two clinical trials with aerosolized buffered GSH in cystic fibrosis (CF) patients have shown promising results [85].

Published evidence from randomized clinical trials do not support the use of iNO in infants with hypoxemic respiratory failure despite its role in treatment of several diseases in neonates [86]. In very ill-ventilated preterm infants, iNO as a rescue therapy had failed, and increased the risk of severe IVH. Multiple pharmacological interventions such as with corticosteroids, prostaglandins, $NO^{\bullet}$, prostacyclin (PGI_2), surfactants, cisofylline, NAC, and fish oil have not shown any improvement in survival in ARDS [87, 88]. Low dose of iNO also did not demonstrate any substantial impact on duration of ventilator support or on death rate. iNO therapy was shown to improve oxygenation in patients with ALI or ARDS but was not

shown to reduce mortality [89]; rather it was proposed to be harmful [90]. The same effect was reported for iNO use in patients of acute chest syndrome with sickle cell disease [91].

Antioxidants are used as chemopreventive agents in models of cancer, but use of beta carotene and vitamin A in lung cancer prevention trials showed no chemopreventive effects, and rather increased the risk of lung cancer incidence and mortality in smokers. Targeting redox-sensitive signaling inhibitor molecules at signal transduction, transcription, or functional levels, inhibitors, mimetics, activators, and antisense nucleotides may be of potential therapeutic utility. In this regard, NF-κB and Nrf2 are particularly attractive targets as they are shown to regulate transcriptional expression of multiple antioxidant genes. Curcumin as NF-κB inhibitor, isothiocyanates as Nrf2 activator, and compounds activating Nrf2 via PI3K and PKC signaling have also been used [92].

NSAIDs have also been tried as cyclooxygenase inhibitors because of their free radical scavenging effect against an array of ROS and RNS. By inhibiting MPO, they are also shown to inhibit HOCl formation [93]. Hydroxytyrosol (HT), a phenolic compound present in olive oil, demonstrated strong antioxidant activity in porcine pulmonary artery endothelial cells (VECS). The mechanism of action of HT was shown via suppression of ROS and catalase expression through phosphorylation of AMPK pathway and by activating FOXO3a [94].

Studies using vitamin A and carotenoids have demonstrated beneficial effects in various diseases such as diarrhea, ischemic heart disease, immunological disorders, acute respiratory infections, and bronchial asthma. Reports on supplementation of exogenous antioxidants in several clinical trials have yielded controversial and mixed results due to lack of quality-controlled trials [95, 96].

Selenium supplementation was shown to increase GPx activity in a randomized placebo-controlled trial on oral Se supplementation on antioxidant levels in COPD patients [97]. Similarly, in a double-blind placebo-controlled trial using effect of 1-year supplementation with 200 IU/day vitamin E on the incidence and duration of respiratory infections in 617 elderly persons, a nonsignificant reduction in the duration of cold was observed [98]. Evidence from randomized and controlled studies suggested that the use of specialized nutritional formula containing eicosapentaenoic acid (EPA) + gamma linoleic acid and elevated antioxidants might offer physiologic and anti-inflammatory effects over standard formulas [99].

A randomized controlled clinical trial was conducted in 137 asthmatic adults to investigate the effects of a high-antioxidant diet (with lycopene), compared with that of a low-antioxidant diet (without lycopene) supplementation, for 14 weeks [100]. Increased fruit and vegetable intake resulted in improved clinical asthma outcomes. Antioxidant manipulation was shown to modify clinical outcomes of asthma; antioxidant withdrawal was associated with aggravation of inflammation, lung function, and symptoms of asthma [101].

eNOS derivatives play an important role in modulating pulmonary vascular tone and attenuating pulmonary hypertension. iNOS is also shown to contribute to the pathology of ALI and ARDS. Thus, L-arginine–NO•–cGMP pathway can serve as an important pharmacological target in the treatment of pulmonary vascular diseases [102].

Melatonin, a hormone with antioxidant properties, has been shown to provide significant protective effects with a remarkable safety profile in newborns which harbor increased oxidative stress. Also, long-term melatonin therapy in children and adults has not shown any significant complications. Similarly, none of the animal studies with maternal melatonin therapy or postnatal melatonin therapy have resulted in any side effects [103].

Acetylcysteine and carbocysteine have limited efficacy, and reported to be safe in children with upper and lower respiratory tract infections (ARTIs) without chronic bronchopulmonary diseases [104]. Nebulized or oral thiol derivatives administered to patient with cystic fibrosis were demonstrated to be ineffective [105]. In clinical trials on ARDS in ICU patients with impaired oxygenation, enteral administration of fish oil, antioxidants, and physiological amounts of arginine was found to improve oxygenation and clinical outcomes [106].

The various observations underscore the importance of controlled clinical trials for evaluation of benefits and risks of effective therapies. Several promising therapies are being currently investigated for the treatment of ARDS, and include use of exogenous surfactants, antioxidants, immunomodulating agents, HMG-CoA reductase inhibitors such as statins and β2-adrenergic receptor agonists and prostacyclin. Reports reveal that a single pharmacotherapy may not be effective [107].

Several explanations have been suggested by investigators for the failure of convincing evidence from antioxidant trials [108, 109]: (a) the cells employ homeostatic mechanisms to restrict the total allowable antioxidant activity; supplementation of antioxidants exogenously may decrease the rate of synthesis or uptake of antioxidants, so that total antioxidant potential remains unaltered; (b) the amount of antioxidant is insufficient and is not targeted to the site of excessive ROS production. In addition, it is plausible that complete removal of oxidants may lead to altered cellular signaling mechanisms, hence worse outcomes. Further, the potential of exogenous antioxidants in terms of relative specificity and efficiency to reduce each reactive species could be different. It was further emphasized that injury causing oxidants must be identified.

2.14 Methods of Detection of Markers

The presence of oxidative stress in the biological systems can be determined by markers/metabolites of oxidative stress, antioxidants (both enzymatic and nonenzymatic) in blood, urine, and tissue samples. In practice, the analytical measurement of oxidative stress markers is difficult due to the short half-life (in seconds) of such compounds. This can be determined biochemically. A variety of methods have been employed for the determination of free radicals and oxidative stress metabolites [110].

Electron spin resonance (ESR) spectroscopy, or electron paramagnetic resonance (EPR) spectroscopy, is the only analytical approach that enables direct detection of free radicals, such as $NO^{\bullet}$, superoxide, and hydroxyl radical. It is also able to detect

free radical-derived species, e.g., ascorbyl radical, tocopheroxyl radical, and heme-nitrosyl complexes with limited sensitivity [111].

Lipid peroxides, i.e., malondialdehyde (MDA), or other lipid adducts are determined as a measure of the cellular oxidant status; however, the method is nonspecific [112]. F2-isoprostanes, particularly, 8-iso-PGF2alpha, is shown to be a specific and reliable indicator of in vivo oxidative stress. This marker is also not affected by diet, and can be easily detected in the urine [113]. Recently, a d-ROM test has been developed to determine reactive oxygen metabolites (ROM) in the blood that determines mainly stable lipid hydroperoxides in the serum. Redox state of the GSH/GSSG pool in tissue and/or plasma as an indicator of oxidative stress in vivo can also be determined spectrophotometrically [114].

$NO^{\bullet}$ is extremely difficult to measure due to the short half-life and a very low concentration in biological fluids, and can be directly analyzed by NO analyzer. In routine practice, a simple spectrophotometric method is used to determine stable metabolites of $NO^{\bullet}$, e.g., nitrite (NO_2^-) and nitrate (NO_3^-) as indirect measures of NO production in vivo. These metabolites can also be determined by using mass spectrometry, gas and liquid chromatography, and electrophoretic methods. Asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, can also be determined by ELISA, HPLC, liquid chromatography–mass spectrometry (LC–MS), and GC–MS [115–117].

The antioxidants both enzymatic and nonenzymatic can easily be determined using spectrophotometric assays, commercially available enzymatic kits, and HPLC-based techniques. The total antioxidant capacity (TAC) in the plasma can also be determined by FRAP assay [118–120].

EBC is a novel noninvasive source of aerosol particles of exhaled breath which reflects consumption of airway lining fluid [121]. EBC has been used for determination of a large number of biomarkers or footprints of the presence of ROS/RNS activity in the lungs such as lipid peroxides, isoprostanes (8-iso PGF2α), H_2O_2, $NO^{\bullet}$ and $NO^{\bullet}$ metabolites, nitrated proteins such as nitrotyrosine and nitrosothiols, and DNA damage biomarker, e.g., 8-OH deoxyguanosine, cytokines, peptides, and cysteinyl leukotrienes. EBC can be used for an early assessment of airway inflammation and oxidative stress in respiratory disorders, thus is useful for making differential diagnosis of the airway disease and for monitoring the course of therapy. Increased levels of these biomarkers have been observed in smokers; patients of bronchitis, asthma, COPD, cystic fibrosis, and bronchiectasis; and in the presence of alteration of bronchomotor tone and pulmonary surfactant activity [87].

Future research should be directed in identifying potential biomarkers or genetic markers to facilitate diagnosis and to initiate use of novel cell-based therapies, e.g., mesenchymal stem cells which may reduce lung injury and facilitate repair.

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Chapter 3
Role of Exhaled Biomarkers, Volatiles, and Breath Condensate

Yan Liang and Lou Ann S. Brown

Abbreviations

AMP	Adenosine monophosphate
ALF	Airway lining fluid
CO	Carbon monoxide
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenases
Cys	Cysteine
eNose	Electronic nose
ELISA	Enzyme immunoassay
EBC	Exhaled breath condensate
FeNO	Fractional exhaled nitric oxide
GC/MS	Gas chromatography/mass spectrometry
GSH	Glutathione
H_2O_2	Hydrogen peroxide
5-LO	5-Lipoxygenases
LC/MS	Liquid chromatography/mass spectrometry
NO	Nitric oxide
PLA2	Phospholipase A2
$ONOO^-$	Peroxynitrite
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
VOCs	Volatile organic compounds

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3.1 Introduction

Since the 1960s, a simple breath test has been used as a tool to estimate ethanol concentrations in blood and the capacity to drive safely. Over the past 20 years, the further developments and miniaturization of gas chromatography with mass spectrometry analytical technologies have expanded breath analysis towards the development of fingerprints for specific diseases or respiratory pathogens. During exhalation, a chemically complex mixture is released that comprises volatile organic compounds (VOCs), condensed water vapor, and micro-droplets aerosolized from the fluid that covers the alveoli, bronchi, and mouth (Fig. 3.1a). In the VOCs, there are over 3,000 compounds inhaled or absorbed from the environment or produced during cellular metabolic processes [1]. When the exhaled breath is chilled, the exhaled breath condensate (EBC) can be collected, comprising variable-sized particles or droplets that are aerosolized from the airway lining fluid (ALF), distilled water that condenses from gas phase, and water-soluble volatiles that are exhaled and absorbed into the condensing breath (Fig. 3.1b) [2–5]. Since both the VOCs

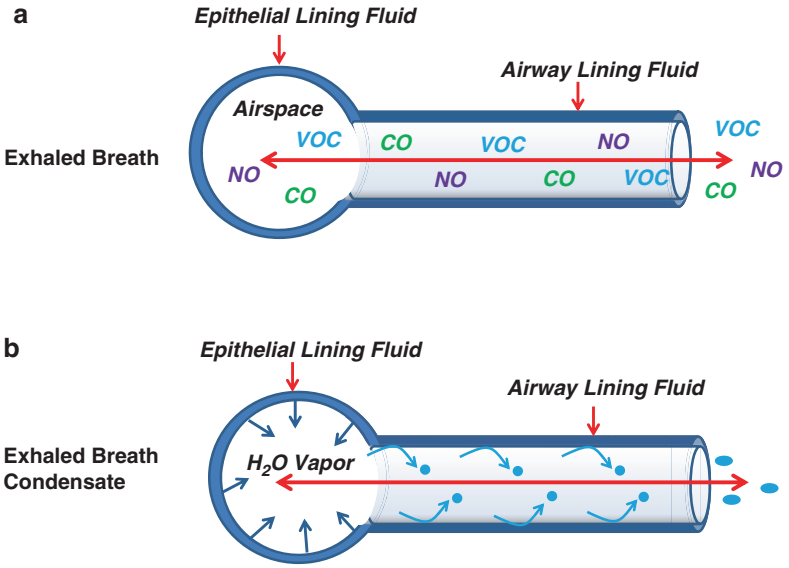

Fig. 3.1 Nonvolatile and volatile components in exhaled breath. In the exhaled breath, the gases nitric oxide (NO) and carbon monoxide (CO) as well as volatile organic compounds (VOCs) which are related to various metabolic processes within the body are readily detectable (**a**). When the stream of exhaled breath is chilled, the exhaled breath condensate (EBC) contains nonvolatile components mainly from the airway lining fluid. Water vapor is rapidly diffused from the lining fluid on the surface of the airway (bronchi) and airspace (alveolar) into the expiratory flow. The turbulence at the surface of the lining fluids generated during exhalation coupled with the raining out of the water vapor results in variable-sized droplets that are aerosolized from the lining fluid (**b**). These droplets reflect the alveolar and airway lining fluids which can be sampled in the condensate when the exhaled breath is chilled (**b**)

and EBC can be safely and easily collected from spontaneously breathing subjects or mechanically ventilated patients, it provides an opportunity for noninvasive assessment of different biomarkers. Once the signature profiles are identified and their predictive values validated, the profiles can be easily and noninvasively used to characterize phenotypes and improve accurate diagnoses and management decisions, monitor disease states, and determine therapeutic effectiveness.

3.2 Exhaled Volatile Biomarkers

Although the composition of the rich mixture depends on the health status of the individual, breath contains numerous gases and VOCs in trace amounts in the parts per billion to trillion range. The gases of current interest include nitric oxide (NO) and carbon monoxide (CO). The VOCs include hydrocarbons, alcohols, ketones, aldehydes, esters, and or heterocycles. Although relevant volatile biomarkers need to be identified before their possible clinical application can be determined, it is important to determine if the source of the compound of interest is endogenous and from metabolic processes. Alternatively, the potential biomarker may be due to an exogenous source such as the ambient air, a chemical modification of the immediate environment, or in response to a prior exposure. Exogenous VOCs penetrating the body as a result of environmental exposure can also be used to quantify the body burden. Although the lung constitutes its own microenvironment, the comparisons of blood, room air, and breath levels in parallel will help determine if the source of the volatile compound of interest is endogenous or exogenous. For collecting the breath, the Tedlar bags have been suggested to be superior over bags made with other polymers in terms of background emission, species stability, and reusability [6]. The important factors affecting sample integrity appear to be the degree to which the bag was filled and the molecular weight of the volatile where molecular masses greater than 90 exhibited losses of 20–40 %.

3.2.1 Exhaled Nitric Oxide

Nitric oxide (NO) is produced in our body from L-arginine by nitric oxide synthase to form L-citrulline. NO is an important cellular signalling molecule, which functions in modulating vascular tone, insulin secretion, airway tone, angiogenesis, and peristalsis [7–10]. Fractional exhaled nitric oxide (FeNO) is easily detectable by a chemiluminescence-based analysis in which the NO in a single breath exhalation reacts with ozone to form nitrogen dioxide in an excited state. When this excited state of nitric dioxide returns to its ground state, it emits light in quantities that are proportional to the amount of exhaled NO. There are three known isoforms of nitric oxide synthase, but the inducible isoform is the major contributor of increased FeNO in the exhaled breath because expression of inducible nitric oxide synthase,

upregulated by a wide range of inflammatory cytokines, is thought to be a surrogate marker of ongoing eosinophilic airway inflammation. However, factors such as age, atopy, medication use, therapy adherence, and airway infections also contribute to FeNO generation.

Elevation of FeNO has been used in the diagnosis of asthma in both adults and children. Despite initial enthusiasm, FeNO as a noninvasive marker of airway inflammation and the clinical usefulness of FeNO as a measure of asthma control are still debated. Tailoring asthma treatment based on FeNO measurements did not decrease asthma exacerbations or lead to better asthma control [11]. However, the heterogeneity of poorly controlled asthma between individuals limits the utility of FeNO alone as a biomarker of inflammation, but it may still be a valuable marker in asthma management when paired with multiple biomarkers for diagnosis and management. When asthma management was also guided by FeNO measurement, there was no improvement in the symptom-free days, but there were fewer asthma exacerbations associated with an increased leukotriene receptor antagonist use and an augmentation of the inhaled corticosteroid doses [12].

3.2.2 Exhaled Carbon Monoxide

Carbon monoxide (CO) is a low molecular weight gas that is a ubiquitous environmental product of organic combustion but is also produced endogenously during heme degradation catalyzed by heme oxygenase. Under stressful conditions, the intracellular CO levels increase in response to upregulation of heme oxygenase and have been proposed to be a key mediator of oxidative damage resulting from peroxynitrite ($ONOO^-$) production [13]. CO also has a very important biological activity as a signalling molecule with marked protective actions namely against apoptosis and endothelial oxidative damage [14]. In the absence of high background exposure, elevated CO levels in the breath can be detected with an electrochemical gas sensor. Similar to exhaled NO, exhaled CO has been evaluated as a candidate breath biomarker of pathophysiological states, including smoking status, and inflammatory diseases of the lung and other organs. Exhaled CO values have been evaluated as potential indicators of inflammation in asthma, stable chronic obstructive pulmonary disease (COPD) and exacerbations, cystic fibrosis, lung cancer, or during surgery or critical care. However, the utility of exhaled CO as a marker of inflammation and its potential value for diagnostic assessment remain unclear [15].

3.2.3 Exhaled Volatile Organic Compounds

Various metabolic processes within the body produce VOCs, which are released into the blood and will be passed on to the airway once the blood reaches the lungs. VOCs can also be taken up as pollutants from the environment by inhalation or ingestion.

VOCs include hydrocarbons, alcohols, ketones, aldehydes, and esters. The occurrence of inflammation and/or oxidative stress can result in unique VOC patterns, which are different from the healthy state. In a systematic literature search, patterns of exhaled VOCs have been applied to a broad range of patients, including subjects with severe disease and children [16]. This analysis concluded that profiles of VOCs are potentially able to accurately diagnose various pulmonary diseases but further standardization and validation of the diverse techniques need to be mastered before VOCs can be applied into clinical practice. Another potential use is based on the ingestion of isotopically labeled precursors and following exhalation of isotopically labeled metabolites.

In metabolomic analysis of breath of asthmatic and healthy children, a panel of eight candidate markers (1-(methylsulfanyl)propane, ethylbenzene, 1,4-dichlorobenzene, 4-isopropenyl-1-methylcyclohexene, 2-octenal, octadecyne, 1-isopropyl-3-methylbenzene, and 1,7-dimethylnaphthalene) were found to differentiate [17]. In other studies, asthma patients were differentiated from healthy controls based on their "breath-prints" but these studies were less successful in distinguishing mild asthmatics from severe asthmatics [18]. A set of 15 VOCs were also used to discriminate asthmatic patients from controls, classify patients according to inflammatory sputum phenotype, and asthma control [19]. Although FeNO and lung function were not predictive of asthma exacerbations, a panel of seven VOCs provided correct classification, sensitivity, and specificity [20]. In a study of recurrent wheezing in preschool children, analysis of VOCs by gas chromatography–time-of-flight mass spectrometry detected 913 different VOCs [21]. Using a panel of 28 VOCs, 73 % of the preschool children with recurrent wheezing were correctly classified. For COPD patients, their profile of exhaled VOCs as detected by an "electronic nose" (eNose; polymer-based gas sensor arrays) could be distinguished from that of asthmatic patients and correlated with the presence of eosinophils and neutrophils as well as myeloperoxidase in induced sputum [22].

More recently, VOC biomarkers and biomarker profiles associated with infections have evolved [23]. For *Mycobacterium tuberculosis*, four VOCs have been used to detect infection: methyl phenyl-acetate, methyl nicotinate, methyl p-anisate, and o-phenylanisole [24]. In other studies, a field-deployable, pulsed discharge helium ionization detector was used to detect VOCs due to mycobacterial volatiles in breath samples from tuberculosis patients [25]. In studies with infected mice, secondary electrospray ionization-mass spectrometry was used to characterize the breath profile of *Pseudomonas aeruginosa* and *Staphylococcus aureus* lung infections [26]. In patients with prolonged chemotherapy-induced neutropenia, eNose technology was used to detect a VOC profile characteristic of pulmonary invasive aspergillosis [27].

However, tongue biofilms also contain anaerobic bacteria that can contribute to the VOC profile breath requiring further research to identify the major source of different VOCs associated with systemic disease or metabolic disorders in the body [28].

For lung cancer, analysis of VOCs by gas chromatography/mass spectrometry (GC/MS) identified some alcohols, aldehydes, ketones, and hydrocarbons as potential biomarkers that differentiate between normal subjects and cancer patients [29–31].

Using solid-phase microextraction and GC/MS analysis of breath, 42 VOCs were recognized as potential lung cancer biomarkers to be used in the development of a diagnostic panel, particular when incorporated with patient data and smoking histories [32, 33]. When exhaled breath was collected over packed polymer Tenax tubes and analyzed by GC/MS, 18 VOCs were significantly altered in the breath profile of lung cancer subjects when compared to controls [34]. For patients with cystic fibrosis and primary ciliary dyskinesia, VOC analysis by eNose showed that there were significantly different breath profiles when compared to healthy controls or each other [35]. The VOC profiles were also different when patients with and without exacerbations were compared. Metabolomic analysis of VOCs in exhaled breath also discriminated between cystic fibrosis patients and controls but also between those patients with or without *Pseudomonas* colonization [36]. Patients living with transplanted lungs can also be discriminated from healthy subjects by exhaled VOC profile using the eNose [37]. However, plasma level of tacrolimus showed significant relationship with the VOC profile of lung-transplanted patients further highlighting that medications may have profound influence on the VOC profile.

Analysis of breath VOC profiles not associated with pulmonary manifestations has also been pursued. In addition to alcohol use, correlations between analyses of urine, serum, and breath profiles demonstrated that breath VOCs were also useful for identification of the use of other substances of abuse including methadone, amphetamine, methamphetamine, 6-acetylmorphine, morphine, benzoylecgonine, cocaine, diazepam, oxazepam, alprazolam, buprenorphine, and tetrahydrocannabinol [38]. Breath VOCs were evaluated in patients with end-stage renal disease for monitoring and therapy initiation under hemodialysis and increases were observed in exhaled concentrations of isoprene similar to that observed in serum at the end of hemodialysis [39]. In contrast, exhaled pentane increased at the onset of hemodialysis but returned to baseline levels afterwards. Exhaled acetone concentrations were significantly lower in diabetic patients when compared to non-diabetics. In patients after kidney transplantation, proton-transfer-reaction triple-quadrupole tandem mass spectrometry suggested that a C7-ketone and a branched C7-aldehyde were good biomarkers to monitor these patients [40]. Breath VOC analysis by proton-transfer-reaction time-of-flight mass spectrometry was also able to distinguish cirrhotic patients from healthy subjects and to discriminate those with well-compensated liver disease from those at more advanced severity stage [41].

3.3 Exhaled Breath Condensate

The EBC is collected from exhaled breath, usually through a cooling or freezing process [2, 4, 5, 42] and has been increasingly studied as a noninvasive research method for sampling the alveolar and airway space and recognized as another promising source of biomarkers [43, 44]. In order to generate a reproducible volume of EBC and sampling of the ALF, the recommended method is tidal breathing where the volume of air that is inhaled or exhaled is included in a single breath [45]. In EBC sample collection, pre-condensation conditions, such as ambient air and

environment temperature, should be recorded; the condenser's design, material, surface area, and cooling temperature should be well adjusted; and the subject's conditions, such as medications, tobacco smoking, food and drinks, and exercise, can have significant effects on EBC collection, and should be recoded and adjusted.

One principal contributor to EBC is the variable-sized particles or droplets that are aerosolized from the ALF which presumably reflect the ALF itself [5, 44, 46]. Droplet formation within the lungs during exhalation is largely in the airways where turbulence is encountered. The second principal contributor to EBC is distilled water that condenses from the gas phase but substantially dilutes the aerosolized ALF. The third component comprises water-soluble volatiles that are exhaled and absorbed into the condensing breath. These water-soluble volatile constituents are found in substantially higher concentrations and are therefore more readily assayed than the nonvolatile compounds. In the EBC, the condensing vapor phase water dilutes the components of the ALF by 20-fold to 30,000-fold. It needs to be pointed out that the dilution of these nonvolatile biomarkers by water vapor can vary dramatically and, to date, there is no gold standard for assessing the dilution of ALF biomarkers in the EBC [2, 44, 47, 48]. However, dilution factors that have been used include urea, cations, total protein concentration, or the conductivity of lyophilized EBC [42, 44, 49]. In addition to dilution, the ALF profile can be further altered by gross or microscopic salivary contamination. However, the ratios between various nonvolatile compounds in EBC are substantially different than the ratios in saliva suggesting that the ALF is a dominant source of EBC constituents. EBC can also be collected during mechanical ventilation but the condensate volumes are dependent on the ventilation volumes and the humidification of the inspiratory gas [50–52]. Although the concentrations of the different analytes will improve by decreasing the humidity of the ventilator gases, this may not be well tolerated by the patient. Substances measured in EBC include mediators of oxidative stress and inflammation such as arachidonic acid derivatives, reactive oxygen/nitrogen species, reduced and oxidized glutathione (GSH), adenosine, ammonia, hydrogen peroxide (H_2O_2), isoprostanes, leukotrienes, prostanoids, peptides, and inflammatory cytokines. Although the EBC has great potential as a source of biomarkers in many lung diseases, the low concentrations of compounds within the EBC present challenges in the standardization of sample collection and analysis. Validation is needed to confirm that the EBC analyte truly represents that present in the ALF, is reproducible, has normal values, and provides information for the underlying inflammatory process and the response to treatment.

3.3.1 EBC pH

EBC pH was considered as a promising biomarker of EBC because it is held in a narrow range, is controlled by lower airway source fluid [53], and its acidification has been reported in asthma, COPD, and cystic fibrosis [54–60]. The airway acidification may be an adjunct marker of airway inflammation and can result from gastro-esophageal reflux, inhaling acidic aerosols or acidifying gases, and

from direct acid formation due to disorders at the airway epithelial surface [61, 62]. In cystic fibrosis, the lack of functional cystic fibrosis transmembrane conductance regulator (CFTR) causes airway epithelial Toll-like receptor 5 and subsequent NF-KB signalling which leads to decreased transport of the antioxidant GSH and HCO_3^- which increases the oxidation and acidity of the airway surface liquid [60]. Since many enzymes are pH sensitive, altered airway pH can cause a broad range of effects such as increasing the activity of inducible nitric synthase enzyme which induces the production of NO from L-arginine [62].

The measurement of EBC pH or airway acidification is very challenging and complicated by poor reproducibility [57, 63]. The pH of raw EBC samples is unstable and is profoundly affected by carbon dioxide, the major volatile component of EBC. One strategy is to deaerate and remove carbon dioxide from the EBC with an inert (carbon dioxide-free) gas such as argon or nitrogen. However, even after 20 min of deaeration, EBC samples may still contain an unpredictable and unstable amount of carbon dioxide, which may bias pH readings. To improve the reproducibility of pH readings and standardize the carbon dioxide effect on EBC pH, a carbon dioxide gas standardization method was developed [64, 65]. In this method, carbon dioxide is bubbled into an EBC sample in short intervals of 1 s each which causes a rapid but stepwise increase of the carbon dioxide partial pressure in the EBC sample. After each bubbling period, EBC pH and carbon dioxide partial pressure are measured simultaneously using a blood gas analyzer and a correlation plot between the EBC pH and carbon dioxide partial pressure generated. This correlation allows the calculation of pH at a carbon dioxide partial pressure of 5.33 kPa, the physiological partial pressure of carbon dioxide in the alveoli. Although more reliable and convenient methods need to be developed for EBC pH measurement, this method currently provides the most reproducible EBC pH values.

3.3.2 Arachidonic Acid Derivatives in the EBC

Arachidonic acid is a polyunsaturated omega-6 fatty acid present in the phospholipids of cell membranes that is metabolized through multiple pathways by multiple cell types. In addition to their sensitivity to arachidonic acid metabolites, airway epithelial cells have abundant arachidonic acid and novel cyclooxygenases (COX) and lipoxygenases [66]. Arachidonate metabolites can also be generated and have potent biologic effects on other airway cells including leukocytes, smooth muscle, nerves, mucus glands, and platelets. Because of the transcellular feature of arachidonic acid metabolism and function, ALF is the critical medium for these actions because it contains significant amounts of arachidonic acid and its derivatives. Methods used to detect arachidonic acid derivatives in the EBC include GC/MS, liquid chromatography/mass spectrometry (LC/MS), radioimmunoassay (RIA), and enzyme immunoassay (ELISA).

Arachidonic acid can be released by the activation of enzyme phospholipase A2 (PLA2) and further metabolized by COX, 5-lipoxygenases (5-LO), and cytochrome

P450 to form other biologically active compounds prostaglandins, thromboxanes, and leukotrienes [67–70]. Although leukotrienes are mainly synthesized in leukocytes, non-leukocytes can take up leukocyte-derived leukotrienes through transcellular biosynthesis [71]. As immune modulating lipid mediators, leukotrienes can promote constriction of airway smooth muscle, increase microvascular permeability, stimulate mucus secretion, decrease mucociliary clearance, and increase recruitment of T cells, eosinophils, and mast cells in the airway [72, 73]. 8-Isoprostane, a prostaglandin-F2-like compound, belongs to the F2 isoprostane class and is produced in vivo by the free radical-catalyzed peroxidation of arachidonic acid [74]. Elevated EBC concentrations of arachidonate metabolites such as 8-isoprostane, leukotrienes, and prostanoids have been correlated with parameters of oxidative stress, respiratory infections, and inflammation [75–77]. Increased concentrations of 8-isoprostane in the EBC have been demonstrated in multiple lung diseases such as asthma [78, 79], COPD [80, 81], interstitial lung disease [82], and cystic fibrosis [83, 84]. In children with recurrent wheezing episodes, LTB4, LTE4, and nitrites were higher in the EBC when compared to healthy controls [85]. Elevated leukotrienes and prostanoids in the EBC have also been correlated with parameters of inflammation in the lungs [75–77]. Changes in the EBC may reflect sustained changes or acute changes. In response to a pollen nasal allergen challenge with allergic rhinitis, the concentrations of 8-isoprostane and cysteinyl-leukotrienes increased within 2 h of the challenge and remained elevated at 24 h [86].

3.3.3 Oxygen and Nitrogen Reactive Species and Redox-Relevant Molecules in EBC

Investigations of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are among the many interests of EBC biomarkers for several lung diseases. Formation of multiple RNS starts with NO and NO metabolites have been measured extensively in the EBC from subjects with a variety of pulmonary diseases including asthma, COPD, and cystic fibrosis [4, 87–89]. NO can also rapidly react with superoxide anion ($O_2^{\bullet -}$) to form highly reactive $ONOO^-$ which can cause the nitrosation of either tyrosine or tyrosine residues in proteins to form 3-nitrotyrosine. Nitrotyrosine can then be measured in the EBC by enzyme immune assays or HPLC and mass spectrometry [90–92]. NO can also react with thiols, such as cysteine (Cys), GSH, or protein thiol residues, to produce S-nitrosothiols which can be measured by a colorimetric assay [93]. The end-products of NO metabolism are nitrite (NO_2^-) and nitrate (NO_3^-) which can be reliably measured in the EBC by colorimetric, fluorometric, and chemiluminescent assays as well as ion, gas, or liquid chromatography [94, 95]. Although the measurement of NO-related redox products is relatively straightforward, the regulation of NO metabolites in different airway diseases is not yet fully understood and is not always consistent. For example, elevated nitrate/nitrite contents are found in asthmatic patients, which is postulated to be associated with increased NO metabolism due to increased expression of inducible

NO synthase [96]. In contrast, nitrate/nitrite levels are not elevated in the EBC from COPD patients despite inflammation [97, 98]. However, increased 3-nitrotyrosine levels were observed in the EBC of both asthmatic and COPD patients. Since 3-nitrotyrosine is produced from $ONOO^-$, this suggests increased RNS stress in both of these airway inflammation diseases. When compared to controls, the EBC from patients with idiopathic diffuse parenchymal lung disease had increased 3-nitrotyrosine as well as 8-isoprostane [99].

The ROS H_2O_2 can be released from both inflammatory and structural cells including neutrophils, eosinophils, macrophages, and epithelial cells and is measurable in the EBC [4, 88]. However, H_2O_2 is unstable in the EBC and samples should be freshly collected or rapidly frozen after collection. Common methods used to measure H_2O_2 include spectrophotometric, fluorometric, or chemiluminescent assays and a concentration of ~200 nM is associated with different pulmonary pathologies [100, 101]. H_2O_2 and other ROS can result in the metabolism of polyunsaturated lipids to form the stable by-product malondialdehyde [102, 103] which has been measured by HPLC methods to be in a 10 nM concentration range in the EBC [104, 105]. Increasing reactive oxygen and nitrogen species or their derivatives in the EBC have all been used as indicators of oxidative stress or inflammation in the respiratory tract.

In addition to ROS/RNS, the ALF also contains significant antioxidant compounds such as Cys and GSH. Although the GSH concentration in the bronchoalveolar lining fluid is in the micromolar range, the GSH concentration in the EBC is in the nanomolar range suggesting an ~1,000 dilution of GSH in the EBC pool when compared to the bronchoalveolar lavage fluid [106–108]. GSH is unique among thiol-based antioxidants in that it is only a tripeptide composed of glutamate, Cys, and glycine. Furthermore, GSH has the ability to scavenge both reactive oxygen and nitrogen species. Upon oxidation of two GSH moieties, two hydrogens are donated to form the GSH disulfide (GSSG). GSH reductase then reduces the GSSG back to GSH using NADPH as the electron donor. When compared to healthy children, GSH was decreased while the lipid peroxidation product malondialdehyde level was higher in the EBC of children with asthma, indicating a pulmonary imbalance in the level of oxidants and antioxidants in children with asthma [109]. When subjects with or without an alcohol use disorder were compared, both the lavage fluid and the EBC demonstrated an ~80 % decrease in GSH, increased GSSG, and oxidation of the thiol/disulfide redox potential by ~40 mV [106, 107]. This suggests that changes in the EBC can be representative of physiological changes in the ALF.

3.3.4 Other EBC Analytes

EBC proteins are another group of biomarkers monitored in airway diseases and systematic cytokine profiling may be useful in diagnosis and the decision tree for determination of therapeutic treatments. In the EBC, the cytokines IL-1β, IL-2,

IL-4, IL-5, IL-6, IL-8, IL-10, IL-17, IFN-γ, TGF-β, and TNF-α have been reported to be in the ~50 pg/mL range [110–112]. Assuming an estimated ALF cytokine level in the order of 50 ng/mL, the dilution of different cytokines in the EBC 10^{-3} is generally accepted [47, 113]. However, cytokine detection in EBC is often at the lower limits of detection for ELISAs and these values are further complicated by the absence of a gold standard for dilution of the EBC to the bronchoalveolar lavage.

Cytokines can also be grouped based on the type of T-lymphocytes with which they are associated. For cytokine analysis, a shift in the ratio of Th1/Th2 T-lymphocytes and cytokines is usually accompanied by varied immune response in pathological pulmonary conditions. Examples of such approach have been reported in determining IFN-γ(Th1)/IL-4(Th2) ratio [111, 114]. Systematic approaches, such as proteomic analysis of EBC, have been previously used and may provide a more detailed overall view about cytokine profile in the EBC. However, EBC is challenging for proteomic studies because of low protein concentrations. Proteome analysis of low-abundance proteins depends on the complexity of the protein mixture, the power of the resolution, and the sensitivity of the separation and identification methods. Although proteomic analysis has been used with EBC, the majority of the proteins detected were keratins, a family of fibrous structural proteins present in the outer layer of human skin [115–118]. To detect low-abundance EBC cytokines present in the pg/mL range, advanced techniques such as immunoaffinity depletion and selective target enrichment may be required for proteomic analysis [119, 120]. In proteomic analysis of EBC from non-smokers plus healthy smokers, COPD without emphysema, and subjects with pulmonary emphysema associated with α(1)-antitrypsin deficiency, profile analysis by LC–MS/MS identified several potential biomarkers that distinguished those with lung disease [121]. In this analysis, distinguishing features included several inflammatory cytokines (IL-1α, IL-1β, IL-2, IL-12, IL-15, IFN-α and -γ, and TNF-α); Type I and II cytokeratins; two isoforms of surfactant protein A; Calgranulin A and B, and α1-antitrypsin.

Another common biomarker monitored in the EBC by mass spectrometry is adenosine and adenosine monophosphate (AMP). In a study of cystic fibrosis and children with asthma compared to controls, the EBC AMP-to-urea ratio was elevated in cystic fibrosis patients and the adenosine-to-urea ratio was elevated in asthma [122]. Furthermore, changes in the EBC purine-to-urea ratios correlated with changes in percent-predicted forced expiratory volume in 1 s after cystic fibrosis exacerbation treatments. In additional studies of patients with cystic fibrosis, the ratio of adenosine to urea in the EBC correlated with sputum neutrophil elastase and correlated with the percentage predicted of forced expiratory volume in 1 s in longitudinal, multivariate models [123]. In COPD patients, a similar increase in the EBC adenosine-to-urea ratio was observed with the highest ratios in the most severely affected cohort [124]. In a cross-sectional study of asthmatic children with non-severe asthma and severe asthma, metabolomic profiling of the EBC revealed that the compounds that discriminated between group included adenosine as well as retinoic acid and vitamin D [125].

3.4 Conclusion

Because it contains many potential biomarkers and the collection technique is noninvasive and well accepted by subjects, analyses of exhaled breath or its condensate represent exciting new approaches for investigating lung diseases as well as other pathologies. The potential to collect the sample remotely also provides new opportunities to monitor exposure, the development of pathologies, or therapeutic strategies. However, the key limitation for both the breath and its corresponding condensate as important diagnostic tools is the low concentration range. The measurement of multiple substances concurrently and determination of their ratios would avoid potential artifacts due to correction for dilution. Contamination from the ambient air at the site of collection or that previously inhaled from the surrounding microenvironment is also an ongoing confounding influence that must be taken into account. In addition, the kinetics of uptake and elimination of environmental factors may be different for each compound and further complicate data interpretation [126]. Thus, the steps of identifying the biomarker and validating its application, choosing the appropriate sensor technology, and determining the appropriate normalization factor must be established for each potential biomarker. Since analytes in the breath and condensate may change quickly, developing the most appropriate techniques for collecting the breath or condensate sample is essential. Ideally, the analytical methods should be robust enough to detect small concentration changes in a specific breath or condensate analyte and provide consistent results over time. In addition to the methodological issues, the establishment of different biomarkers as the gold standard for the different pathologies is also made difficult by the significant overlap between the classifications of different lung pathologies and the inability to distinguish between them.

Currently, efforts to address methodological issues include standardization of sample collection and validation of analytical techniques. To establish the reproducibility of analyte measurements in the breath or condensate, more sensitive assays and new molecular detection techniques are necessary. The eNose is gaining in popularity for the analysis of VOCs because it provides a rapid response and is relatively inexpensive. However, additional studies are needed to validate clinical-relevant VOC patterns and profiles, longitudinally assess changes in these profiles, and assess the corresponding influence of different treatment strategies on the profile. The role of technologies such as selected metabolomics and proteomics is emerging and the development of breath or condensate profiles for a particular pathology may prove to be useful in screening and diagnosing lung diseases. The most useful breath and condensate profiles will evolve into robust predictors when system biology approaches are used and analyzed in light of the patient data. Systematic analysis of breath and condensate profiles should provide multicomponent markers with high discriminatory power and the concurrent measure of multiple analytes should limit detection bias and provide patterns of biomarkers that allow investigators to discriminate between different phenotypes within a particular pathology [125]. The development of discriminatory metabolomic and proteomic profiles should also enhance our mechanistic understanding of the disease mechanisms.

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Chapter 4
Volatile Organic Compounds as Exhaled Biomarkers of Inflammation and Oxidative Stress in Respiratory Diseases

F.J. van Schooten, A.W. Boots, A. Smolinska, and J.W. Dallinga

4.1 Introduction

Since ancient times, it is known that the odor of a subject's breath provides important information regarding health status as it reflects the (patho) physiological processes occurring in the body [1]. Every health care taker recognizes the sweet acetone smell of the breath of an uncontrolled diabetes patient or the fishy odor present in the breath of people suffering from a liver disease [2]. These smells are produced by excreted volatile organic compounds (VOCs) and these are a promising potential in facilitating noninvasive clinical diagnostics. It is therefore not surprising that the field of VOCs analysis in exhaled air analysis has developed rapidly over the last few decades and great advances have been made with respect to the technical and statistical analytical aspects. All together, these developments have led to the discovery of new biomarkers in exhaled air that may identify diseases and characterize their underlying biochemical processes.

4.1.1 The Usefulness of VOCs as Biomarker

In order to apply exhaled VOCs as a potential and valid biomarker of disease, certain characteristics should be met including (1) high sensitivity and specificity, (2) fastness and accuracy, (3) methodological simplicity, (4) interpretive simplicity, (5) thorough validation, and (6) pathophysiological link to disease [3].

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An ideal biomarker of disease should recognize an illness already at an early stage and distinguish it from any other illness within a short processing time. Although current advances in sampling and analyzing techniques have already enabled fast breath sampling, their translation into ready-to-use clinical devices has not yet taken place [3]. However, unique VOC profiles have already been shown to discriminate healthy persons from patients suffering from a variety of pulmonary diseases with high sensitivity and specificity (see also Sect. 4.1.2) [4–10].

Characteristics 3 and 4 imply that ideal biomarkers should excel in simplicity in a methodological and interpretive way. Methodological simplicity of breathomics is guaranteed by the combination of its noninvasive character with a low degree of discomfort for the patient and a high clinical accessibility [11, 12]. However, the generated volatile biomarkers do not yet yield easy-to-interpret results as their validation and clinical usefulness are still hampered by the multiplicity of approaches regarding breath sampling, analysis, and normalization [3, 12].

Characteristic 5 implies validation of biomarkers outside the study population they were initially selected in [18]. The usefulness of unique VOC profiles as a biomarker for a variety of diseases including asthma and chronic obstructive pulmonary disease (COPD) has already been shown using such validation [4, 6].

Finally (characteristic 6), a good biomarker should display a clear mechanistic relationship with the disease it was designed for. Unfortunately, this is a difficult requirement to meet within breathomics as compounds originating in breath may become biochemically altered before excretion. In other words, their volatile characteristics do not necessarily reflect the primary processes underlying the disease. Nevertheless, already more than half of the VOCs included in the discriminating profiles designed for asthma, cystic fibrosis (CF) were identified as products formed during oxidative stress, a process discussed in Sect. 4.1.3.1.

4.1.2 VOC Profiling with Relation to Pulmonary Disorders

Applying a single VOC as biomarker is a priori hampered in its use since it is not expected that one compound has enough information to describe the complex and heterogeneous processes including chronic diseases. Consequently, it is anticipated that the pathological processes are the best grasped by studying not individual compounds but by exploring the total amount of exhaled VOCs called the volatome [3]. Certain profiles of VOCs within the volatome give a more sensitive and specific discrimination between various conditions due to its reflection of changing environmental exposures and the formation of compounds during biochemical processes. Recent studies applying volatome analysis have revealed that distinct VOC profiles are capable of discriminating healthy controls from pulmonary patients with a high sensitivity and specificity [4, 7, 8, 10]. Our group has shown that correct classification of asthmatic children and CF patients was possible with high sensitivity and specificity using a VOC profile of only eight and ten VOCs, respectively [4, 5]. For the smoking-related disease COPD, six VOCs were needed to discriminate patients

from healthy controls with 100 % sensitivity and 81 % specificity [6]. Various independent research groups have shown that a combination of VOCs can correctly classify patients with lung cancer with a sensitivity and specificity ranging from, respectively, 71 % to 100 % and 80 % to 100 % [9, 13–15]. Finally, patients suffering from pulmonary tuberculosis can be distinguished from healthy controls with 96 % sensitivity and 79 % specificity using a unique exhaled VOC profile [10].

4.1.3 Oxidative Stress and Inflammation

VOCs represent important endogenous processes including oxidative stress and inflammation. Until now, breathomics is hardly used to study damaging endogenous processes that underlie a variety of (chronic) diseases. However, a few studies have already demonstrated that breathomics can be used to identify the presence of oxidative stress and inflammation.

4.1.3.1 VOCs and Oxidative Stress

Oxidative stress is defined as an imbalance between the formation of highly reactive oxygen species (ROS) and the protection against these species by antioxidants [16]. Oxidative stress may result in increased oxidative damage and has been associated with the pathophysiology of various chronic diseases including sarcoidosis [17], idiopathic pulmonary fibrosis [18], and COPD [19]. A key process resulting from oxidative stress is lipid peroxidation, i.e., the oxidation of fatty acids present in, for example, cell membranes [20]. Volatile products formed during lipid peroxidation include ethane, pentane, hexanal, octanal, nonanal, propanol, and butanol [21, 22]. Some of these VOCs are reported to be elevated in the breath of COPD compared to controls [23, 24]. However, the analysis of single compounds derived from lipid peroxidation in exhaled air is hampered by a relatively low sensitivity and specificity [3]. Our group currently attempts to investigate the availability and significance of these compounds as markers of oxidative stress in the headspace of in vitro systems mimicking this process (Boots et al. unpublished results).

4.1.3.2 VOCs and Inflammation

Most chronic diseases are characterized by inflammation, a process designed to protect the host against damage inflicted by exogenous sources [25]. Since inflammation and ROS are tightly intertwined and capable of inducing one another, VOCs representative for inflammatory processes are often compounds generated during oxidative processes [20, 25]. Additionally, inflammation is associated with typical inflammatory volatiles including nitric oxide (NO), nitrate, and sulphur-containing compounds [26].

Since all organisms generate VOCs as part of their normal metabolism, it implicates that breathomics can also be applied to examine the production of volatile compounds by specific bacteria. Indeed, certain infections are for a long time known to be accompanied by a distinct smell in vivo as well as in vitro [26, 27] and recent studies have shown the usefulness of VOC analysis in evaluating bacterial growth in both in vitro cultures and human samples [28, 29]. This application of VOC profiling encloses the potential of developing a marker of the presence or absence of specific microbes in both environmental and clinical settings [27].

4.2 Breath Sampling

Exhaled air comprises a mixture of dead space air and alveolar air. The dead space air consists of roughly 150 mL air from the upper airway where no gaseous exchange between blood and breath air is facilitated [8, 30]. Its composition strongly resembles the inhaled air. Alveolar air originates from the lower airways where gaseous exchange between blood and breath takes place, resulting in a mixture of inhaled breath and endogenous compounds. Therefore, the concentration of the endogenous compounds is relatively high in alveolar air compared to dead space air.

In principle, there are three ways to sample exhaled air: (a) upper airway collection by sampling dead space air only, (b) lower airway collection by sampling alveolar air only, and (c) mixed air collection by sampling whole breath (mixture of dead space air and alveolar air) [31]. Some breath tests, including nitric oxide (NO) measurements, mainly use the dead space air since their compounds of interest (e.g., NO) are directly released into both the dead space and the alveolar air. Endogenous produced VOCs are ideally measured in alveolar air or in mixed air, its concentration being slightly higher in alveolar than in mixed air, depending on the fraction of dead space air present in mixed air. Whether this dilution may constitute a problem mainly depends on the sensitivity of the analytical instruments used. Whereas for electronic noses the concentration of VOCs may be an issue, in GC–MS a dilution of 10–20 % is no issue at all. An advantage of sampling whole air is that it is technically the easiest and therefore less sensitive for mistakes. It increases the ease of use for the physician and decreases the degree of discomfort for the patient. The most reliable way to separate alveolar from dead space air is to apply a CO_2-controlled valve that uses the end-tidal CO_2 concentration as a marker for the transition of dead space into alveolar air [32].

Sampling may be performed online as well as offline. Online sampling requires that the analytical instrument is brought to the patient (or other way around), which is not to be a problem when handheld devices are used, but may become a burden when larger instruments are applied. Offline sampling, as required for instance for GC–MS analyses, gives delayed results. On the other hand offline sampling, for example collecting exhaled breath in polycarbonate bags followed by transfer of the samples to carbon sorption tubes is an established way to concentrate the samples and it allows the analysis of the sample to be executed under well-defined laboratory conditions. When measuring a high number of samples collected during a prolonged

period of time, it is possible to perform the measurements in a short period of time, thus diminishing the instrumental variation. Moreover, results of a GC–MS analysis may be available after 60 min, when required.

4.2.1 Technologies to Analyze Breath for VOCs

4.2.1.1 Electronic Nose

Recent advances in the field of chemical sensors have facilitated the development of these nonselective sensors, better known as electronic noses or E-noses. E-noses contain a series of nonspecific sensors capable of binding or reacting with VOCs present in complex gas mixtures. Various sensor principles can be applied including polymer-based sensors and metal-oxide-semiconductor (MOSFET) devices. Polymer-based sensors demonstrate volume changes upon contact with exhaled VOCs, thereby changing the conductance of the polymer [32]. MOSFET devices are transistors used for amplifying or switching electronic signals, based on the principle that VOCs upon entering the device will be charged either positively or negatively, affecting the electric field of the sensor [33]. Ultimately, both sensor principles rely on internal changes due to their interaction with molecules from gaseous mixtures, thereby generating a specific profile called a breath-print. These breath-prints represent the total mixture of exhaled VOCs present in breath and can be used to develop pattern-recognition algorithms for various exposures or chronic diseases [34–38]. The main drawback of the E-nose is that the sensors implemented are not selective for single VOCs and are not capable of analyzing (identifying) individual components. Furthermore, a large number of VOCs can act on a single sensor, resulting in low sensitivity and specificity with regard to classification or prediction of the analyzed gas mixtures.

4.2.1.2 Proton Transfer Reaction Mass Spectrometry

Determining the content of complex gas mixtures such as breath with proton transfer-reaction mass spectrometry (PTR-MS) is based on chemical ionization of the target molecules by a proton-transfer reaction with H_3O^+ [39, 40]. The thus generated protonated molecular ions of the components are mass-detected [41]. An advantage of this technique is that samples can be easily analyzed as no preconcentration or separation processes are necessary, as is often the case for other analysis techniques [31]. It is suitable for real-time measurements, which is of special importance in situations where rapid and sudden changes of VOC concentrations are expected [42]. Unfortunately, PTR-MS is also a limited technique that is restricted to detect compounds with a proton affinity higher than that of water [41, 43]. Moreover, PTR-MS cannot differentiate isomeric and isobaric ions since they are all detected at the same nominal mass.

4.2.1.3 Selected Ion Flow Tube Mass Spectrometry

Selected ion flow tube mass spectrometry (SIFT-MS) is an analytical technique for the simultaneous real-time quantification of several gases. This method is based on the formation of reactant ions (precursors) by electron impact or microwave discharge in a carrier gas in a separate ionization region. In short, the exhaled gases are led through a flow tube where they react with a precursor ion, usually H_3O^+, NO^+, or O_2^+ [31]. The ions produced during this reaction can be mass-dependently detected [44]. Main advantage of this technology is the very short response time of 20 ms, which enables real-time measurements and a high sensitivity [44]. However, in analogy with PTR-MS, SIFT-MS does also not allow identification of the whole breath-print as it can only detect gases for which positive precursor ions are selected [45]. Moreover, using only mass-to-charge ratios of chemically ionized molecular ions to identify specific VOCs present in breath remains very difficult. Therefore, this method is not suitable for pinpointing specific VOCs and/or underlying metabolic processes to exposure or disease status.

4.2.1.4 Ion Mobility Spectrometry

The ion mobility spectrometry (IMS) technique separates (molecular) ions according to their mobility as they move through the so-called drift tube filled with a purified gas such as air or nitrogen [46, 47]. As the different ions present in the sample are forced through the tube by means of an applied electric field, they will display different velocities based on their characteristics. Separating the ions can further be optimized by changing the drift length, drift gas, electric field strength, temperature, and pressure [46, 47]. Combining this technique with gas chromatography improves its applicability.

4.2.2 Gas Chromatography–Mass Spectrometry

The most commonly applied methodology to date to accurately measure trace gases in complex mixtures such as exhaled air is based on gas chromatography–mass spectrometry (GC–MS) [6, 48]. This method comprises a gas chromatograph (GC) that separates the different compounds in the mixture and a mass spectrometer (MS) that not only detects the separated volatile compounds but also identifies them based on their total mass spectrum. Various detectors are used of which the time-of-flight (TOF) spectrometer is the most widely applied [31, 46, 49]. GC–MS is proven to be highly sensitive and robust, two characteristics known to add to a high degree of reproducibility [31, 46]. Figure 4.1 displays a classic example of a chromatogram (here called breathogram) obtained via GC–MS analysis of exhaled air.

GC-TOF-MS is at present the only technique that can not only detect specific VOCs linked to exposure or disease status but is also able to identify these VOCs.

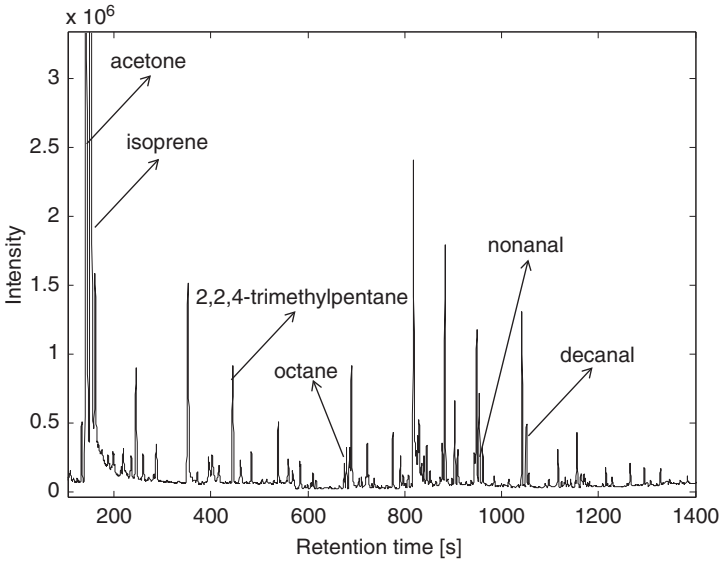

Fig. 4.1 An example of a breathogram recorded via GC–MS with some names of VOCs. The area-under-peak is related to the concentration of the compound presented by this peak

Consequently, the GC-TOF-MS method is currently state-of-the-art, capable of pinpointing specific volatile biomarkers to underlying metabolic processes that might contribute to damage related to exposure or disease. Therefore, it has been suggested that only the GC-TOF-MS method will be able to assist in the diagnosis of exposures and diseases in the near future as it is shown to be an excellent screening tool for new biomarkers [4–6, 31, 50]. This major advantage outweighs the minor drawbacks of this method, including the fact that it is rather time-consuming and cannot take place in situ [42].

4.3 Data (Pre-)processing and Analysis of GC–MS Data

4.3.1 Pre-processing of Raw Breathomics Data

In order to obtain breathomics data usable for multivariate statistical analysis (MSA), the raw data produced by GC–MS have to be properly pre-processed. The main aim of data pre-processing is to remove experimental artifacts and inter-sample variations which usually obscure the biologically meaningful changes and/or lower the power of statistical analysis. Pre-processing of breathomics data involves at least three successive steps: noise reduction, background (baseline) correction, and alignment [8, 30].

Noise reduction is usually the first step of data pre-processing. It minimizes the degree of high frequency noise generated by the detector or other instrumental noise sources. It can be achieved by applying transform functions such as wavelets or Fourier transformations [51].

Distortion in the baseline may affect not only statistical analysis but also alignment and quantification of the compounds. Therefore, proper baseline correction is important. It is usually done in an automated manner. Multiple methods have been proposed based on different types of polynomial-fitting algorithms [52], asymmetric least squares (ALS) [53], B-splines, B-splines with penalization (i.e., P-splines) [54] or the use of orthogonal basis of the background spectra [55].

The last step of pre-processing is alignment, i.e., removing the shifts in peak positions by rectifying the retention times across all samples. Nowadays many methods and packages are commercially or freely available for spectral alignment, including Dynamic Time Warping (the oldest warping techniques), Parametric Time Warping [56], Correlation Optimized Warping (COW) [57], MetAlign [58–60], MZmine [61], Isotope Cluster-Based Compound Matching [62], and PyMS [63]. The differences are mainly technical.

4.3.2 Normalization and Scaling

An intermediate step between data pre-processing and MSA is normalization and scaling. Normalization of the individual peaks in breathomics data aims to remove the overall variations between measured samples (i.e., chromatograms), the so-called size effect. Normalization usually involves a multiplication of each measured sample by a constant. Different ways of computing this constant exist [64–66]. Scaling of breathomics data may be useful and/or necessary, since the amount of compounds in exhaled breath can vary in many orders of magnitude. Moreover, the higher the abundance of a compound, the larger the variations are exhibited by this compound. Thus it is important to scale the compound levels before MSA to avoid spurious influence of high abundance compounds. This is particularly true in nontargeted analysis. An overview of different scaling methods can be found elsewhere [67].

4.3.3 Multivariate Analysis (Supervised, Unsupervised)

After data pre-processing, the MSA is performed to find trends in breathomics data and to extract only that information that is relevant to study possible biomarkers in the exhaled air. A proper MSA requires a good statistical validation [68] as well as trustworthy biological interpretation of the results. Typically, most of the multivariate statistical methods used in the -omics field, such as genomics, proteomics, and metabolomics, can also be utilized in breathomics. Generally, all multivariate

statistical methods can be divided in unsupervised and supervised algorithms. Typically, unsupervised methods are used as first step in MSA. They include the Principal Component Analysis [69, 70] and Hierarchical Cluster Analysis [70]. Unsupervised analysis is performed to explore and visualize the data. Moreover it allows finding trends, detecting outliers, and clustering the input data into classes based on statistical properties of the data only. It permits a simplified representation of the information in the breathomics data. In unsupervised statistical methods a priori knowledge (e.g., class information such as sick and healthy) is not used in the learning procedure of the algorithm. This way it is often not possible to find a specific pattern related to a given class of, for example, the diseased patients. Furthermore, due to highly complex breathomics data, unsupervised methods are very often not powerful to extract class-related information (e.g., disease related). Therefore, an unsupervised approach is very often followed by supervised learning methods such as partial least squares discriminant analysis (PLS-DA) [71], discriminant analysis [72, 73], random forests [74], or support vector machines [75, 76]. In supervised techniques a priori knowledge, e.g., class information, is provided as a dummy variable with group membership (i.e., healthy and disease) for each sample. A very important part of supervised learning is validation. In order to avoid the risk of overfitting (i.e., that the statistical model is too closely fitted to the data) the data should be divided in a training set (used for constructing the statistical model), a validation set (utilized to tune the statistical model), and an independent test set (used to assess the performances of the statistical model). Validation by an independent test set delivers the means to establish a reliable predictive performance of the statistical model and ensures that selected VOCs of interest are indeed truly descriptive of the underlying exposure or disease studied [68].

4.4 Future of VOCs Profiling in Pulmonary Disorders

4.4.1 Current Issues of VOCs Profiling in Monitoring Disease

As indicated in the introduction, recent studies have proven that distinct VOC profiles can discriminate healthy controls from patients suffering from inflammatory lung diseases with high sensitivity and specificity [4–6]. Regarding lung cancer, a combination of VOCs can differentiate patients from healthy controls and diseased controls with high sensitivities and specificities [9, 13–15, 77–79]. Moreover, it has recently been observed that exhaled VOC markers are able to distinguish patients with pulmonary tuberculosis from healthy controls [10] and from non-tuberculosis diseased controls [80]. Interestingly, pilot studies have revealed that breathomics can also be used to diagnose non-pulmonary diseases, including breast, ovarian, and hepatocellular cancer, and coronary heart diseases, with fair sensitivity and specificity [13–15, 77, 81, 82]. Also inflammatory conditions and cancer from

internal organs as liver and the gastrointestinal tract can be monitored [83, 84]. Breathomics is recently shown to be useful in assessing occupational or environmental exposures [85, 86]. The profiling of VOCs already fits the biomarker prerequisite of displaying a rather noninvasive character that combines a low degree of discomfort for the patient with a high clinical accessibility [11, 12]. Biomarkers should be thoroughly validated before being clinically applied to determine the real value of their predictive and/or discriminatory capacities. By performing such a validation test in one or more separate validation set(s), the real value of specific biomarkers can be evaluated outside the test set in which they were initially selected [87]. However, the current differences used by research groups in breath sampling and analysis hamper interlaboratory validation and thus clinical usefulness of volatile biomarkers [12].

4.4.2 Biochemical Background of VOCs and Changes in Relation to Disease

A good disease biomarker relates to the biochemical or molecular processes underlying the disease it was designed for. Until now, this relationship has merely been the focus in studies exploring the use of individual VOCs to predict or classify various diseases. Especially products of lipid peroxidation have been considered as possible volatile biomarkers. For instance, higher exhaled pentane levels are reported in patients with acute asthma [86], cystic fibrosis [24], acute respiratory distress syndrome (ARDS, [88]), ventilator-associated pneumonia (VAP, [89]), obstructive sleep apnoea [86], and lung cancer [82, 90]. Other volatile lipid peroxidation products studied as possible biomarkers include ethane, hexanal, octanal, nonanal, propanol, and butanol [81, 86, 90, 91]. Interestingly, more than half of the number of VOCs included in the discriminating profiles designed for asthma, CF, and COPD by our group were identified as hydrocarbons and/or possible lipid peroxidation products as well [4–6]. Moreover, due to their low solubility in blood, lipid peroxidation products such as ethane and pentane are exhaled within a few lung passages and can therefore serve not only as a pulmonary but also a systemic marker of inflammation and oxidative stress [8, 91]. For instance, ethane and/or pentane are enhanced excreted in the breath of patients with sepsis, systemic inflammatory response syndrome (SIRS), ischemic heart disease, myocardial infarction, cardiopulmonary bypass, and allograft rejection following organ transplantation [92–96].

Ideally, a biomarker should not only be linked to exposure or disease in general, but also to different stages of the disease including development, severity, and progression. Interestingly, this has already been shown for exhaled ethane as this VOC is higher not only in asthmatics compared to healthy controls but also severe compared to mild asthma [97]. Moreover, a recent study by Phillips et al. suggests that VOC patterns can be used to distinguish between active and nonactive

pulmonary tuberculosis albeit with a relatively low accuracy ranging from 65 to 85 % [98]. However, associating volatile biomarkers with the physiology of either exposure or disease is still in its infancy due to the fact that the (patho) physiological meaning of specific VOCs is often not known yet. Consequently, more research regarding the exact identity and biological role of individual VOCs is needed. Unfortunately, this area of research is hampered because compounds originating in breath can very well be biochemically altered before their excretion, implying that the volatiles detected do not necessarily display a direct relation to exposure or disease. Therefore, more studies in clinical as well as in vitro settings are necessary to elucidate the biochemical origin, physiological meaning, and exhalation kinetics of selected VOCs. Nevertheless, even without this mechanistic knowledge, volatile compounds can already be valuable as a predictive tool in a clinical setting.

4.4.3 Potential Future Applications

Until now, breathomics has merely been applied for diagnostic purposes and a future goal lies within the development of easy-to-use devices for the point-of-care use that can detect volatile biomarkers specific for various diseases.

Interestingly, recent developments have been made regarding the screening for lung cancer as it was shown that low-dose CT scanning could significantly reduce lung cancer mortality [99, 100]. Since there are some difficulties in generalizing these results to the community, it has once again been suggested to combine this screening method with other new testing techniques such as VOC measurement to develop a successful screening algorithm for lung cancer [99].

The analysis of exhaled air, however, encloses far more intriguing promises including elucidation of the (clinical and pathological) heterogeneity observed in several chronic diseases, the determination of the pathogens responsible for occurring (respiratory) infections and monitoring of the treatment efficacy.

4.4.4 Respiratory Infections

The occurrence of detrimental respiratory tract infections embodies a great burden not only for patients and the health care system but also for the economy [94, 95]. Especially the sudden worsening of symptoms also referred to as acute exacerbations (AE), in various chronic lung diseases including COPD and CF is known to largely depend on such microbial infections. Fast treatment of exacerbations is often hampered by a poor standardization of both the definition and detection of AE in most lung diseases [101, 102]. Recognizing AE appears to be difficult for both the patients and care takers as exacerbation profiles vary enormously between individuals. Current diagnostics include clinical criteria such as changes in symptoms and lung function but they often occur when an exacerbation is already manifested.

Additionally, sputum induction or bronchoscopy accompanied by sampling broncho-alveolar lavage fluid (BALF) can be applied for qualitative and quantitative laboratory microbiological analysis in order to detect the exact microbial cause [103–105]. However, the usefulness of these techniques is seriously diminished by their invasiveness and by the long-duration of more than 48 h before results become available. To date it is still not possible to predict or quickly diagnose the occurrence and microbial cause of an exacerbation and thus prevent severe lung damage and deterioration in the long term. Therefore, there is an urgent need for noninvasive fast diagnostic tools that will contribute to a rapid diagnosis of AE in general and of the underlying microorganism(s) in particular, enabling an earlier start of appropriate antibiotic therapy and a more favorable health outcome. Analysis of exhaled air, combined with the adequate interpretation of selected VOC levels, might provide such a new diagnostic tool for early identification and closer monitoring of patients with high-risk profiles for AE. Monitoring high-risk patients and early identification of the worsening of pulmonary symptoms might allow adequate therapeutic interventions with oral antibiotics and/or physiotherapy to prevent AE and thus improve the quality of life in these patients [106, 107].

4.5 Summary and Perspective

In conclusion, it can be stated that breath analysis holds the promise to be of huge interest in clinical practice. It has already been proven that for some diseases breath profiles function as quality biomarkers and thus serve as a sensitive and noninvasive methodology. However, large (prospective) cohort studies are necessary in order to validate such selected biomarkers. Future research regarding more specific and highly sensitive sensors may provide the means for these biomarkers to be highly cost effective and very simple to use [35, 38]. At present, a wide range of sensors is under research including metal-oxide sensors and polymer-based sensors. Both sensors are capable of registering the absorption of specific VOCs by means of either resistance or acoustic variations. At present, such sensors are already capable of accurately detecting low concentrations of volatiles and are under development to be used as bedside tests. At the same time, parallel approaches comprise the detection of disease-related VOCs by means of miniaturized GCs coupled to highly sensitive and specific detectors such as the MS. Improved sensitivity and specificity followed by implementation of these technologies into small “foolproof” handheld devices in combination with an easy-to-apply breath collection procedure and advanced signal processing modules might introduce breathomics right into clinical practice.

Conflict of Interest Statement On behalf of all authors I declare that none of us has any relevant financial interests or conflicts related to this manuscript. (Volatile organic compounds as exhaled biomarkers of inflammation and oxidative stress in respiratory diseases.)

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Chapter 5
Pulmonary Infections—Oxidant Injury and Role of Antioxidants

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Infections of the respiratory system caused by infecting microorganisms like bacteria, viruses, fungi, and protozoa may occur through three persuasive routes: tracheobronchial tree, pulmonary vasculature, and via direct spread from infection in the mediastinum, chest wall, or upper abdomen. Once the microbe gains entry into the tissue, the moist, natural aerobic environment of lungs provides a favorable field to flourish, making the respiratory tract susceptible to infections. The micro-organisms, however, need to overcome a large network of pulmonary defenses [1].

5.1 Pulmonary Infection

Some of the bacterial species which cause infection in the lungs are *Streptococcus pneumoniae*, *Haemophilus influenza*, *Staphylococcus aureus*, *Pseudomonas* spp., *Acinetobacter* spp., *Mycobacterium tuberculosis*, *Legionelia* spp., and others. Infection is characterized by infiltration with polymorphonuclear neutrophils and histiocytes, as well as by tissue destruction, necrosis, cavitation, and formation of lung abscesses.

Viral infections caused by influenza, parainfluenza, adenovirus, coxsackie, echovirus, varicella, vaccinia, and measles viruses sometimes lead to viral pneumonia. It is characterized by alveolar wall thickening and infiltration of lymphocytes due to secretion of proteinaceous exudative material. Rhinovirus, a small non-enveloped single-stranded RNA virus is also associated with respiratory tract infections. In case of viral lung infection, huge invasion of macrophages and neutrophils generate the reactive oxygen species (ROS) which then become important players in the disease pathogenesis. Fungal infection can be similarly caused by inhalation of

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spores which may later cause latent infection by the conidia. Various forms of fungal infections involving the airways include histoplasmosis, coccidioidomycosis, blastomycosis, cryptococcosis, and aspergillosis. Other organisms like nocardia, candidia, yeast, protozoa, and some tapeworms may also cause respiratory infections.

5.2 Oxidant Injury and Pulmonary Infection

Oxidant injury is caused by the oxygen-derived products, free radicals like superoxide anion, hydrogen peroxide, and hydroxyl radicals which are normally produced inside the cells during cellular processes and electron transport chain. It is highly reactive and may disturb cell structure and function resulting in cell injury and death. There are many enzymes which scavenge these oxygen intermediates. Lungs constitute one of the major target organs of oxygen injury because of maximum exposure of the cells of the airways to oxygen and a large surface area of blood supply [2–4].

The ROS are produced mainly by two organelles of the cell i.e., mitochondria and endoplasmic reticulum. ROS are produced not only during molecular reduction of oxygen in electron transport chain but also by other mechanisms like the respiratory burst in phagocytes, damage of cell component by ionizing radiation, and also as byproducts of various enzymes like nitric oxide synthase (NOX), xanthine oxidase (XO), and uncoupled endothelial nitric oxide synthase (eNOS). So ROS has beneficial roles in the physiological function of cells in response to factors such as the shear-stress and immunological defense system, thereby preventing damage from foreign pathogens.

Mitochondrial ROS (mROS) are superoxide molecules derived from oxygen produced at different sites of mitochondria. The mROS on one hand can cause damage to the cell, while on the other hand, can also help in the regulation of physiological functions like adaptation to hypoxia, regulation of autophagy, immunity, differentiation, and longevity of cell. Production of mROS may serve as an alarm of the cell, suggesting a change in the extracellular environment that has been induced by stresses like hypoxia, starvation, infection, and growth factor stimulation. A correlation exists between the gravity of stressor and quantity of mROS induced, implying increased production of mROS to increased stress, leading thereby to cell damage/cell death. Because of the dual role of mROS in the cell, it may be difficult to use them as targets of therapy. The effects of antioxidants on mROS also vary with changes in environmental conditions. The molecular targets of mROS for cellular adaptation during stress in different environmental conditions need further studies [5].

5.3 Oxidative Injury by Microorganisms

Pulmonary oxidant stress is an important characteristic of acute lung injury (ALI) [6]. Reactive oxygen intermediates (ROI) produced by the cells take part in host defense against infections caused by bacteria and fungi. They could bring about

destruction of proteins, deoxyribonucleic acids, and lipids. So, it is important to calibrate the ROI for effective antimicrobial defense while averting inflammation and injury. Production of ROS like superoxide anion, and its derivatives hydrogen peroxide and peroxynitrite, have been found to be associated with lung injury caused by influenza viruses [7]. In the earlier studies, pyran polymer-conjugated superoxide dismutase (SOD) when administered to virus-infected mice brought about reduction in mortality [8]. The peroxynitrates can also cause the oxidation of antioxidants like glutathione reductase, SOD, and glutaredoxin, leading thereby to lung injury. Oxidant injury was also found to be boosted in the mitochondrial membrane with an increment of mitochondrial generation of ROS in tissues distant from the lungs [9]. This study also revealed that oxidant injury and metabolic stress contributed directly to disease development.

5.4 Mycobacteria

Tuberculosis caused by *Mycobacterium tuberculosis* (Mtb) is also found to be associated with oxidative stress. Tuberculosis involves the poor antioxidative defense, damaging the host tissue. Mtb has the ability to survive during the redox status of the host, and also the ability to use protective enzymes like SOD, catalase (KatG), alkyl hydroperoxidase (AhpC), and peroxiredoxins [10]. Mycobacteria induce the production of ROS production by mononuclear and polymorphonuclear phagocytes. Chemotherapy given to patients of pulmonary tuberculosis showed an improvement in the level of oxidative stress [11]. The production of ROS is also associated with the productions of DNA lesions using apurinic/apyrimidinic endonuclease IV (End) and exonuclease III (XthA), a 39R59 exonuclease [12]. Pulmonary and extra pulmonary tuberculosis are associated with decreased levels of blood glutathione, glutathione peroxidase, and glutathione reductase and negatively correlated with carbonyl protein content [13]. Since γ-glutamylcysteinylglycine or glutathione (GSH) protects against oxidative stress, it may have potential therapeutic implications [14]. Also, ergothioneine (ERG) and mycothiol (MSH), have been reported in protection against oxidative stress in mycobacterium [15].

Mycobacterium tuberculosis is one of the aerobic bacteria which can survive under oxidative stress by various mechanisms. It has the ability to persist inside macrophages of the host. One of the mechanisms is F420-dependent anti-oxidant mechanism. This system in methanogenic archaea acts as an active enzyme cofactor. It has been observed that the F420-deficient mutants (by the inactivation of fbIC gene) are very sensitive to oxidative stress. The *fbiC* gene (Rv1173) encodes an 856-amino-acid polypeptide FO synthase in the F420 biosynthetic pathway. The inhibition of the F420 biosynthesis pathway or Fqr-class proteins may act as a mechanism to potentiate the action of bactericidal agents [16]. To study as to how the mycobacterium is able to evade the host immune system, state-specific models, based on readily available gene expression data, can be created in silico. In such models, the metabolic adaptations of *M. tuberculosis* can be characterized by the

differential gene expression data with a metabolic network model [17]. More molecules are reported to be involved in protection of the microbes against oxidative stress as seen in mycoredoxin-1 and mycothiol deletion strains of *Mycobacterium smegmatis* [18]. The survival of pathogen i.e., *M. tuberculosis* inside the host may also be possible by oxidation-sensing regulator (MosR), a transcriptional repressor by upregulating expression of rv1050 (a putative exported oxidoreductase) [19].

Heme oxygenase-1 (HO1) has a role in cytoprotection and is found to be expressed in large numbers in the plasma of patients with tuberculosis, acute respiratory distress syndrome, chronic obstructive pulmonary disease, and asthma. The expression of HO-1 therefore has been suggested to be used in prognosis of lung disease [20]. A signal transduction pathway in the Early Secreted Antigenic Target of 6 kDa induction of IL-8 expression in lung epithelial cells which has been identified might be important to understand the innate immune responses to tuberculosis and the pathogenesis of lung injury in tuberculosis [21].

There are reports on the role of serine proteases of the Mtb that provide resistance to acid and oxidative stress. Rv3671c, a putative serine protease is held responsible for persistence of *Mycobacterium tuberculosis* in the hostile environment of the phagosome [22]. The periplasmic domain of Rv3671c is a functional serine protease of the chymotrypsin family and its activity was found to increase upon oxidation. On similar lines, another periplasmic protease of Mtb might have a special role in imparting resistance to acid and oxidative stress [23]. This transmembrane serine protease MarP is important for pH homeostasis in Mtb.

5.5 Bacteria

Bacterial infection leads to the exposure of bacteria-derived lipopolysaccharides, composed of oligo, polysaccharide, and lipid A endotoxin to the host tissue. Lungs are very sensitive to endotoxins; acute endotoxemia directs accumulation of macrophages in the target tissues. The macrophages and neutrophils that reach the infected site are activated by LPS releasing reactive oxygen and nitrogen species contributing to injury and organ failure [24].

Pseudomonas aeruginosa causes acute and chronic infections of the human lung, causing tissue injury. A siderophore (iron bound to pyochelin) secreted by the organism to acquire iron, may actually function as an efficient catalyst for hydroxyl radical ($HO^{\bullet}$) formation. Due to exposure of superoxide ($O_2^{\bullet -}$) and H_2O_2, siderophore can augment injury to pulmonary artery endothelial cells [25]. Therefore, the presence of ferripyochelin at sites of lung infection by *P. aeruginosa* might promote $HO^{\bullet}$ mediated damage to airway epithelial cells resulting in tissue injury.

Lung infection caused by *Bordetella pertussis* shows a stimulated innate resistance (StIR) event which is also mediated by the generation of ROS [26].

There is evidence of inflammation and oxidative injury in bronchopulmonary dysplasia, a neonatal chronic lung disease. The inflammation associated with dysplasia is caused by the exposure of bacterial lipopolysaccharides. Bacterial LPS

bring about increased expression of cytokines regulated by Nox-dependent signaling pathways [27, 28]. The inflammatory mediators help in attracting neutrophils and macrophages to the lungs to combat infection. Neutrophils, after stimulation produce ROS like hypochlorous acid (HOCl) by the help of myeloperoxidase enzyme. The levels of glutathione sulfonamide (GSA) (fourfold) and other neutrophil oxidant biomarkers (twofold) were reported to be significantly higher in culture positive aspirates. GSA is a stable oxidation product of GSH that is formed by condensation of the amine group of the γ-glutamyl residue with the oxidized cysteine. This has led to the recommendation of GSA as a marker of detection of bacterial growth in lung infection [29].

Nontypeable *Haemophilus influenza* (NTHI) is a major cause of acute sino-pulmonary infections, responsible for exacerbations of COPD. During *H. influenza* infection, many stimuli that include reactive oxidants, bring about induction of Nuclear erythroid factor-2 (Nrf2), a basic leucine transcription factor. It detaches from its inhibitor Keap1 present in the cytosol and moves toward nucleus where it binds at the promoter region of antioxidant response elements (AREs) and help in protection against oxidant injury [30]. Also, during infection, *H. influenza* can withstand the effect of ROS produced by the cells, has various molecular mechanisms to protect from the stress [31].

5.6 Oxidant Injury by Viruses

The involvement of oxidative stress during viral infection has been also reported [32]. The addition of environmental contaminant like cadmium induced oxidative stress leading to imbalance in the redox state with reduction in GSH. However addition of antioxidants like GSH derivative (GSH-C4) or the GSH precursor, *N*-acetyl-L-cysteine (NAC) result in the inhibition of viral replication as studied in Madin Darby Canine Kidney [33].

Human immune-deficiency virus (HIV) patients are under constant oxidative stress as reflected in alterations in levels of ascorbic acid, tocopherols, carotenoids, selenium, SOD, and glutathione in various tissues. Elevated levels of hydroperoxides and malondialdehyde in serum are also indicative of oxidative stress during HIV infection. The oxidative stress contributes to HIV disease pathogenesis, viral replication, inflammatory response, reduced immune cell proliferation, loss of immune function, apoptosis, chronic weight loss, and increased sensitivity to drug toxicities [34]. A chronic infection with HIV is known to be associated with an incidence of pulmonary complications including hypertension, vasculopathy, lymphocytic alveolitis, and interstitial pneumonitis, not attributed to either opportunistic infections or presence of the virus. A transactivator, Tat (transactivator of transcription) protein is required for expression of full-length of viral genes, and it influences the expression of cellular inflammatory genes. The Tat-dependent transactivation of genesis known to require specific mediators that include the transcription factor, NF-κB, are known to be sensitive to changes in cellular oxidant burden [35].

The HIV-1 transgenic mice demonstrate significant oxidative/nitrosative stress in the lungs upon administration of endotoxin. This suggests that the pulmonary complications in HIV-1 infections could be due to alteration of the lung proteins by oxidative stress [36]. HIV-related proteins and alcohol together cause dysfunction in the lung epithelium. This is a significant observation as the alveolar barrier gets affected and addition of thiol antioxidant results in improvement in transgenic mice [37]. Human immunodeficiency virus 1-infected individuals display systemic oxidative stress and glutathione deficiency. The master transcription factor nuclear factor (erythroid-derived 2)-like 2 is known to regulate the expression of antioxidant and phase II-metabolizing enzymes by activating the ARE that protects cells and tissues from oxidative stress [38].

Respiratory syncytial virus (RSV) infects the lower respiratory tract in children. It has been found to generate ROS in vitro and oxidative injury in lungs in vivo [39]. Oxidative stress induced by RSV is due to inhibition of antioxidant enzyme expression leading to an imbalance of ROS production and airway antioxidant defenses [39]. There is an increment in lipid peroxidation products and decrement in GSH/GSSG ratio in RSV infected cells [40]. RSV is also one of the primary causes of lower respiratory tract infections in most parts of the world during the first year of life. Infiltrating lymphocytes present in bronchoalveolar lavage fluid (BALF) have been observed in mice infected with a lethal dose of influenza A/PR8/34 virus which demonstrated the role of oxidative stress during lung infection [41].

It has been reported that resveratrol treatment reduced the number of infiltrating lymphocytes and RSV lung titers which finally led to reduced inflammation. Furthermore, resveratrol might help to decrease the IFN-γ levels in BALF of RSV-infected mice by attenuating airway responses to methacholine. Resveratrol inhibited the TIR-domain-containing adapter-inducing interferon-β (TRIF) signaling pathway, controlled Toll-like receptor 3 (TLR3) expressions and also induced M2 receptor expression followed by RSV infection [42]. A significant fall in SOD 1, SOD 3, catalase, and GST expression was found but SOD 2 expression was found to be elevated [40]. It was also observed that there was a decrement in the activity of SOD, catalase, glutathione S-transferase, and glutathione peroxidase in murine lungs and in the airways of children with severe bronchiolitis. It was associated with reduced levels of Nrf2 expression in the lungs of viral infected mice [43].

The role of antioxidants in the RSV infection has been analyzed from the effect of butylated hydroxyanisole (BHA), an antioxidant, in the RSV infected BALB/c mice; a fall in malondialdehyde and 4-hydroxynonenal content in bronchoalveolar lavage of infected mice indicated the reduction in lung oxidative stress. BHA treatment caused a drop in clinical illness and body weight loss with the neutrophil recruitment to the lung and pulmonary cytokine and chemokine production after the infection. Along with these findings, there was a reduction in RSV-induced airway hyper reactivity [44].

Increased production of superoxide, increased activity of xanthine oxidase, oxidized glutathione, malondialdehyde, and decreased production of oxidized glutathione are some of the characteristics associated with respiratory viral infections [33].

Rhinoviruses infection is associated with an increase in levels of intercellular adhesion molecule (ICAM-1), an important molecule which is used as receptor for entry in the pulmonary epithelial cells. Increased levels of superoxide formation are associated with increased ICAM-1 expression in rhinovirus infection [45]. An increased involvement of oxidative stress with adenovirus-induced lung infection is reported as the cause of bronchiolitis obliterans in post-transplant patients. The association of post infectious bronchiolitis obliterans with oxidative status in the lungs of children has also been reported [46].

5.7 Sepsis and Pulmonary Infection

Sepsis results from serious infections caused by various pathogenic organisms like bacteria, viruses, and fungi which lead to multiple organ failure. Oxidative stress has been shown to be associated with sepsis. In a comparative study on the effects of oleanolic acid with dexamethasone on inflammation and apoptosis in lung and distal organs in experimental murine sepsis, oleanolic acid was associated with lower induced nitric oxide synthase (iNOS) expression and higher SOD levels than in the dexamethasone treated group [47]. There are reports on the improved renal and pulmonary function in rats with sepsis, treated with potent antioxidant NAC [48]. ROS elevate vascular barrier dysfunction through Ca^{2+} signaling in the sepsis-induced ALI [49]. Previous studies had also revealed that pulmonary oxidative stress generated in murine sepsis-induced ALI was primarily dependent upon neutrophil iNOS among different isoforms of NOS [50–52]. Similarly in severe viral infections, the level of inducible nitric oxide was found to be higher in mice that were infected with H5N1 and 1918 viruses, in comparison to a seasonal H1N1 virus in lung tissue; the level was moderate in mice that were deficient in iNOS ($NOS2^{-/-}$) in comparison to wild-type control [51]. Additionally, this study also showed the delay in weight loss and death in 1918 virus-infected mice in contrast to control ones when treated with NOS inhibitor, NG-monomethyl-L-arginine [53].

5.8 Antioxidants in the Therapy of Infections

Antioxidants are substances that inhibit the oxidation of other molecules by terminating the chain reaction of free radicals and removing their intermediates via self oxidation [2]. There are several types of antioxidants such as glutathione, vitamin A, vitamin C, vitamin E, as well as enzymes like catalase, SOD, and various peroxidases. Antioxidant enzymes play an important role in defense against oxidative stress in the lung and in the pathogenesis of chronic respiratory diseases. Extracellular SOD, an important antioxidant enzyme, is found in the lungs, it controls pulmonary inflammation and injury by promoting bacterial phagocytosis [54].

The role of Nrf2-mediated antioxidant system to defend the lungs from oxidative injury and inflammation has also been shown in in vitro and in vivo studies. The in vitro study reports augmentation of NF-κB activation and induction of its target inflammatory gene in Nrf2-deficient macrophages vs. the wild type, when these macrophages were examined with poly (I:C) and/or cigarette smoke extract. There was also an enhancement in antioxidant genes in the lungs of wild-type mice as compared to Nrf2-deficient mice after cigarette smoke exposure in the in vivo study [54]. Mortality was found to be higher in cigarette smoke-exposed Nrf2-deficient mice when these mice were infected with influenza virus [55]. Neu-164 and Neu-107, inhibitors of myeloperoxidase enzyme, exhibiting strong antioxidant activity, were found to reduce acute inflammation and oxidative stress triggered by cigarette smoke-induced inflammatory cells through scavenging the ROS [56]. The drugs ketamine, propofol, and ketofol are routinely used for sedation, but a recent report highlights their role on oxidative stress and anti-inflammatory processes in lung tissue in a rodent model of endotoxemia [57]. In the sepsis (LPS mediated) induced ALI, ketamine infusion led to reduction in the levels of TNF-α, IL-1β, IL-6, NF-κB, and COX-2 mRNAs in lung tissue. Propofol was involved in lessening the levels of circulating TNF-α and IL-1β in lung tissue, whereas it led to augmented nitrate/nitrite levels. The third drug, Ketofol, reduced the levels of COX-2 mRNA and the nitrate/nitrite level in lung tissue. However, a recent report suggests that the treatment with antioxidants in excess might be deleterious as it may result in greater oxidative stress [58].

5.9 Anti-Oxidants and HIV Infection

HIV-1-related proteins inhibit Nrf2-mediated antioxidant defenses and thereby disrupt the normally tight alveolar epithelial barrier. Nrf2-RNA silencing dampened the activity of Nrf2/ARE, decreased the expression of the tight junction proteins zonula occludens-1, occludin, and claudin-18, increased paracellular permeability of alveolar epithelial monolayers derived from wild-type rats, and therefore reproduced the effects of HIV-1 transgene expression on the epithelial barrier. In contrast, upregulating Nrf2 activity, either by plasmid-mediated overexpression or treatment with the Nrf2 activator sulforaphane, increased the expression of ARE-dependent antioxidants, including NAD(P)H dehydrogenase, quinone 1, and glutathione, improved the expression of tight junction proteins, and restored the ability to form tight barriers in alveolar epithelial cells from HIV-1 transgenic rats. Taken together, these new findings argue that HIV-1-related proteins downregulate Nrf2 expression and/or activity within the alveolar epithelium, which in turn impairs antioxidant defenses and barrier function, thereby rendering the lung susceptible to oxidative stress and injury. Furthermore, this study suggests that activating the Nrf2/ARE pathway with the dietary supplement sulforaphane could augment antioxidant defenses and lung health in HIV-1-infected individuals [38].

5.10 Respiratory Viral Infections

Modulation of ROS production and oxidative stress contributes one of the therapeutic approaches in virus-induced lung infections. Use of small molecules like thiols, polyphenols, and antioxidant mimetics show effects on viral-induced ROS production and oxidative stress. Also, compounds like triterpenoids, sulforaphane, and isothiocyanates, increase endogenous antioxidant enzyme levels in RSV infection by stimulating Nrf2-dependent gene expression [39]. There may also be suppression of excessive superoxide production from NADPH oxidase 2 (Nox2), which is the primary enzymatic source of superoxide in mammalian inflammatory cells, because it markedly alleviates lung injury and virus replication caused by influenza A virus. So Nox2 oxidase inhibitors could be useful for suppression of virus-induced lung disease [8]. Mice lacking a functional phagocyte NADPH oxidase (Cybb tm1 mice) or treated with the metalloporphyrin antioxidant manganese (III) tetrakis (*N*-ethyl pyridinium-2-yl) porpyhrin (MnTE-2-PyP) show heightened inflammatory infiltrates in their airways in response to pulmonary influenza infection. Raising the resting threshold of lung-resident antigen-presenting cells by modulating homeostatic negative feedback loops may therefore provide generic protection against viral infectious disease, irrespective of the infective strain.

The treatment of lung infections caused by viruses like RSV, and bacteria like *Pseudomonas aeruginosa* may include resveratrol (3,5,4′ trihydroxystilbene) which is a natural polyphenolic compound that has antioxidant property. It helps in the attenuation of inflammatory response in bacteria infected cells. In vitro studies in A549 cells observed that resveratrol treatment significantly reduced ROS generation, human beta-defensin-2 expression, ICAM-1, increased glutathione peroxidase levels (also the markers of apoptosis), suggesting resveratrol as a protective therapeutic agent in lung infection [42, 59]. Long pentraxin 3 (PTX3) is a newly discovered acute phase protein produced at the sites of infection and inflammation by tissue cells, macrophages, monocytes, and dendritic cells. PTX3 plays an important role in preventing infection of certain fungi, bacteria, and viruses in the lung [60]. Another drug, roflumilast is also found to attenuate the RSV infection in human differentiated bronchial epithelial cells [61]. It impeded the damage of ciliated cells and lowered the escalation of MUC5AC, CLCA1, IL-13, IL-6, IL-8, TNFα, and ICAM-1. Furthermore, it also inversed the decrement of Nrf2, HO-1, and GPx mRNA levels [61].

Lung infection caused by H1N1 Influenza A is found to be associated with uncontrolled inflammation and oxidative stress. FABP5 which belongs to the family of fatty acid-binding proteins (FABPs), acts as an anti-inflammatory mediator during lung infection. FABP5 are small, highly conserved, cytoplasmic proteins that bind long-chain fatty acids and other hydrophobic ligands. They increase peroxisome proliferator-activated receptor gamma (PPAR-γ) activity resulting in reduced inflammation which shows the involvement of FABP5 in controlling the oxidative damage and inflammation during lung infection, making it a future therapeutic drug. Treatment with recombinant human thioredoxin-1 is also found to increase

the survival rate of murine model of influenza pneumonia. So thioredoxin-1 acts as an antioxidant and anti inflammatory molecule.

Interestingly, inhalation of recombinant human catalase (rhCAT) exerted protective effect by improving pathological process and by reducing the viral titer in lungs of mice exposed to influenza H1N1 viral pneumonia. Moreover, rhCAT also improved the serum ROS scavenger capacity [62]. Use of rhCAT had a limitation of its elimination from blood, hence, it was modified by coating with one of the active polymer, polyethylene glycol monomethyl ether (PEGrhCAT). The pharmacokinetics in mice revealed that it had longer half life than native rhCAT, treatment with PEGrhCAT was found to be more effective than with the native form. As the PEGrhCAT caused reduction in viral replication, lung injury levels, and ROS production, the molecule can be used as an adjuvant therapy to promote efficacy of anti viral drugs [63]. The use of hydrogen gas in saline has been used as therapeutic and prophylactic potential for the treatment of injury caused by inflammation and oxidative stress.

5.11 Natural Products as Anti-oxidants

The nutritional status plays a major role to maintain an optimal immune system. The active ingredients of the naturally derived agents may affect various domains suggesting an interdependence of optimal immune system and oxidative stress. Consumption of gold kiwi fruit leads to the reduction of plasma lipid peroxidation in those infected with upper respiratory tract infection symptoms. It may be possibly attributed to the diet-derived antioxidants which control ROS generation [64].

Pretreatment of a heteropolysaccharide, RIWP, isolated from *Radix isatidis* enhanced murine alveolar macrophage survival by inhibiting the production of ROS and lipid peroxidation after stimulation with lipopolysaccharide. Upon treatment, the murine alveolar macrophages exhibited diminished generation of nitric oxide, prostaglandin E2, tumor necrosis factor-α and IL-6, and the mitochondrial membrane potential also returned to normal conditions [65].

There are reports on the therapeutic roles of some Chinese herbal medicine for the treatment of tuberculosis. The effects of *Radix Ranunculi Ternati*, *Radix Sophorae Flavescentis*, *Prunella Vulgaris* L., and *Stellera Chamaejasme* L. extracts have been found to have the capability to enhance cell-mediated immune response in a multi-drug resistant tuberculosis model [66]. Kampo (Traditional Japanese Herbal) medicine, Hochuekkito (TJ-41), have been used since they possess a property to inhibit influenza virus replication by the regulation of interferon gamma [69]. The levels of GM-CSF and an antimicrobial peptide, defensin, are found to increase after TJ-41 treatment, so defensin might play a role in inhibiting virus replication [67].

Dioscorin, a Chinese herbal medicine possessing the effective antioxidant and anti-trypsin activities inhibited H_2O_2, a potent ROS engaged in lung and bronchial epithelium injury. The results of the study also suggested that the inhibition was

relayed by attenuation of H_2O_2 alteration on G2/M cell cycle arrest, induction of IκB, and reduction of NF-κB along with the inhibition of IL-8 secretion, and less changes in adhesion molecule expressions in H_2O_2-injured A549 cells [68].

In addition to the medicinal herbs, Wen-Pi-Tang extract also serves as a natural medicine to cure the lung injury caused by influenza virus. This virus is known to generate the xanthine oxidase (XO) activity of the lungs resulting in a higher level of oxygen-free radicals. Wen-Pi-Tang extract diminished the XO activity [69]. Another Chinese medicinal herb, *Magnolia officinalis* also possesses antioxidant activity [70]. Magnolol, the active compound of this herb, reduced the lipid peroxidation intensity in plasma, liver, and lung of rats with sepsis [70]. A Chinese herbal formula, Qing-Fei-Tang, was found to attenuate the oxygen-free radicals that were generated after stimulation of healthy human leukocytes with opsonized zymosan. It also inhibited the release of slow reacting substance of anaphylaxis from guinea pig lung when challenged with antigen. In addition, this herbal remedy re-established the loss of saturated fatty acids in sputum and showed an improvement in lung inflammation [70].

5.12 Conclusions

Different pathogens which infect the lungs cause pulmonary inflammation and oxidative stress. The activated neutrophils, macrophages, and eosinophils induce the production of singlet oxygen and hydrogen peroxide, which damage the surrounding tissues and enhance production of ROS. In the presence of different infections, the host uses a variety of antioxidants that signal through different pathways, bring about enhanced transcription factor generation leading to control of the infection/inflammation/injury.

The challenge for future research lies to establish the antioxidants which can be used in the most efficient manner for lung infection-associated oxidative stress, and which of the wide range of current oxidative stress markers can we employ? There is an intensive ongoing search for markers which would identify the patients who are most likely to encounter adverse outcomes from various lung infections. There exists an enormous potential for not only the synthetic drugs, but also of natural compounds with reduced side effects as antioxidants.

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Chapter 6
Oxidative Stress in Tuberculosis

Indu Verma, Surinder K. Jindal, and Nirmal K. Ganguly

6.1 Introduction

Tuberculosis (TB) constitutes a huge global health burden, largely borne by the developing countries. As evidenced by tuberculin skin positivity, one third of the world's population is infected by *Mycobacterium tuberculosis* (Mtb), the causative agent. There were an estimated 8.7 million new cases and 1.4 million deaths from TB, including 0.43 million deaths in human immunodeficiency virus (HIV)-positive patients in the year 2011; India alone accounted for about 3.1 million TB prevalence and 0.3 million deaths from TB, including TB with positive HIV infection [83]. It is important to investigate the pathogenesis and immunology of TB to improve the disease management and control strategies. In spite of the numerous advances that have taken place in recent years, the mycobacterium remains enigmatic and continues to pose a challenge.

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6.2 Pathogenesis and Immunology of Tuberculosis

Tuberculosis, like other infectious diseases, represents a constant state of struggle between the host and the tubercle bacillus. Pathogenesis of TB can be described in various stages starting with the inhalation of Mtb, finally resulting in formation of granulomas which may either liquefy to produce primary TB or contain the Mtb to arrest the further progression [22]. The host cell-mediated immunity (CMI) tends to contain the disease with the help of activated macrophages. On the other hand, Mtb uses various strategies to withstand the potential bactericidal host defences and evolve as a successful pathogen causing active disease. Thus, the relative strengths of these opposing mechanisms finally determine the disease progression or arrest.

TB is controlled almost entirely by CMI involving macrophages whose function is coordinated by T lymphocytes. The activated alveolar macrophages, dendritic cells (DCs) and probably also the alveolar epithelial cells engulf the inhaled mycobacteria; the bacilli keep on replicating in the macrophages and DCs which finally burst to release the mycobacteria. These are engulfed by other macrophages and recruited mononucleus cells from the neighbouring blood vessels. The inflammatory response spreads to the lymph nodes to initiate CMI and the T-cell activation.

The activated immune system is able to control the spread of TB in the majority of infected individuals. Both the innate and adaptive immune systems are involved in this process. The innate immunity consists of a complex interplay of various receptors, macrophages and dendritic cells, natural killer (NK) cells and neutrophils. A large number of proinflammatory and anti-inflammatory cytokines and chemokines bridge the innate and adaptive immunity [71]. The adaptive immunity involves CD4+/CD8+ T cells, regulatory T cells and memory T cells [19, 29, 75]. There is some recent evidence to support the role of B cell-mediated humoral immunity in protection against Mtb infection in mice [1, 48].

6.3 Oxidative Stress in Tuberculosis

Granuloma formation in TB represents a component of host defence as it results in the containment of infection thus preventing the spread of tubercle bacilli within the same host as well as between the susceptible hosts. However, intracellular bacilli can be released from the granulomas due to cell death/necrosis caused primarily by oxygen free radicals produced by macrophages and other cells [4, 78, 81]. Oxidative stress results in cellular damage due to the oxidation of amino acid residues on proteins, forming protein carbonyls [16], as well as oxidation of protein [11], ultimately resulting in protein fragmentation [5].

On the other hand, both nitrogen intermediates and oxygen radicals may also play an important role in the suppression of infection through mycobacterial inactivation/killing. The pathogenesis of tuberculosis is finally determined by the balance between various mechanisms, such as (1) the generation of reactive oxygen free

radicals to kill the intracellular bacilli, (2) the antioxidant mechanisms employed by mycobacteria to escape the killing by free radicals in phagocytic cells and (3) antioxidant mechanisms by host cells to prevent the tissue damage. This chapter is focused on the mechanism of generation of oxidative stress in TB patients, contribution of oxidative stress to progression/pathogenesis of disease, pathogen and host antioxidant mechanisms and role of antioxidants in the therapy of disease.

Despite the various host antioxidant mechanisms, the accumulation of free radicals results in cellular and systemic oxidative stress [59]. Various studies have reported the increased levels of markers of oxidant-mediated tissue damage in the peripheral circulation of humans with active tuberculosis [62, 63]. Oxidative stress has also been implicated in the pathogenesis of lung fibrosis and lung dysfunction in tuberculosis patients, even following antimicrobial therapy [36, 41]. Besides the elevated levels of various by-products of free radical generation, depletion of various antioxidants, e.g. ascorbic acid and glutathione, has also been reported in TB patients [47, 79], further aggravating the oxidative stress in these patients. These studies clearly show the association between excessive oxidative stress and active TB. Recently, a significant correlation between high oxidant concentration and low concentration of antioxidants with varying bacillary load as well as severity of disease has been shown; it was also suggested that antioxidants supplementation may prove beneficial as well as may help in fast recovery of TB patients [54]. In another study, it has been demonstrated that the oxidative stress index significantly increased in untreated TB patients and decreased in TB patients on anti-tubercular therapy (ATT) with antioxidant supplementation. Hence, oxidative stress index can be considered as a novel marker in TB patients [21].

In vitro, Mtb has been shown to be susceptible to hydrogen peroxide (H_2O_2)-induced damage [53]. This reaction often referred to as 'oxidative burst' is further aggravated by chlorination in macrophages and neutrophils that increases the toxicity of reactive oxygen species (ROS). At low concentrations, ROS are important for normal cellular functions by acting as signalling molecules in immunological response, blood circulation and endocrine functions [32]. During oxidative stress, the excess of ROS causes cell injury by oxidising various macromolecules including proteins, lipids and DNA, thus assuming an important role in the pathogenesis of various diseases [17].

6.3.1 Role of Alveolar Macrophages

Alveolar macrophages represent host's first line of defence against *Mycobacterium tuberculosis*. In the macrophages, Mtb is exposed to both oxygen-dependent and -independent mechanisms. In oxygen-dependent mechanisms, macrophages use ROS and reactive nitrogen intermediates (RNIs) to kill Mtb. Reactive oxygen and nitrogen intermediates have been widely reported as major antimicrobial molecules produced by host during tuberculosis [41]. The rapid release of ROS and RNI in response to phagocytic stimuli is referred as respiratory burst/oxidative burst. In

response to these ROS, the host cells mount antioxidant responses. It is the balance between these two opposing responses that maintains cellular homeostasis. The production of ROS in excess of antioxidant capacity of host results in the oxidative stress.

6.4 Hydrogen Peroxide

H_2O_2 produced by macrophages via oxidative burst was the first ROS identified as effector molecule that mediated mycobactericidal effects of mononuclear phagocytic cells [81]. The role of ROS to kill Mtb in human TB is yet to be documented. Various studies have shown that Mtb infection results in macrophage accumulation and H_2O_2 production in lungs and ascitic fluid [68, 74]. The H_2O_2 release was not found to be TB specific and there are contradictory reports regarding the ROS-mediated killing of Mtb [13, 20]. Several studies have highlighted the role of oxygen free radicals in host protection as well as various strategies explored by Mtb to avoid ROS and reactive nitrogen species (RNS) [40, 42, 78]. Comparison of murine knockout models lacking active NADPH oxidase (NOX) components with the wild-type strain indicated conflicting data among different laboratories. Different studies have shown that Mtb growth was enhanced in the absence of active NOX [2, 20], whereas in another study, no difference was observed between knockout and wild-type mice in their ability to control Mtb infection [38]. The high incidence of tuberculosis in people with NADH oxidase genetic defect suggests the role of ROS in Mtb persistence [42]. On the contrary, a study in guinea pigs has demonstrated that microbicidal activity of macrophages for various strains of Mtb and *M. bovis* was not related to intensity of H_2O_2 generation and respiratory burst [58]. A possible role of H_2O_2 in intracellular communication during formation/dissociation of granulomas in the BCG-induced granulomatosis has been reported in a recent study [52].

Both systemic and tissue oxidative stress were progressive and correlated with the loss of antioxidant mechanisms, suggesting the benefit of antioxidant treatment of patients with tuberculosis [59]. Thus, it can be stated that besides ROS production by host cells in response to mycobacterial infection, the host as well as the pathogen's antioxidant mechanisms may also play an important role in the pathogenesis of tuberculosis.

6.5 Reactive Nitrogen Intermediates in TB

In response to mycobacterial infection, parallel with $O_2^{\bullet -}$ radicals, another major antimicrobial pathway through inducible NO synthase (iNOS or NOS2) leads to increased production of NO [13, 14], which further react with each other to produce highly reactive $OONO^-$ free radicals. The results of several studies using murine

models provide that NO is capable of killing mycobacteria [13, 14] and that iNOS-deficient mice were highly susceptible to TB infection [38, 46]. The presence of increased iNOS protein and mRNA levels in bronchoalveolar lavage (BAL) specimens from active pulmonary TB patients also suggests the role for NO in TB [56]. In our own laboratory, we saw a significant depression of the respiratory burst response in the patient group compared to that in healthy controls [39]. On the other hand, the RNIs and citrulline levels were significantly higher before therapy and returned to normal after 3 months of anti-tubercular treatment; supporting their role in the microbicidal activity of activated macrophages [39].

Antimycobacterial effect of macrophages is known in the absence of oxidative burst suggesting the role of other mechanisms. RNIs such as nitric oxide (NO), nitrate radical (NO_2) and nitrate (NO_3) are potent effector molecules of macrophage-mediated extracellular and intracellular cytotoxicity against various microorganisms, including the mycobacteria [12]. Their role in human TB infection is however controversial. There are experimental data to suggest the putative antimicrobicidal role of NO and related RNI produced by human macrophages as well as the demonstration of high level expression of nitric oxide synthase in macrophages obtained from BAL fluid of patients of active pulmonary TB [8, 56].

6.5.1 Antioxidant Strategies by Mycobacterium tuberculosis

Mycobacterium tuberculosis, in spite of being an aerobic bacterium, is also an intracellular pathogen that initially resides inside a membrane-bound phagosomal compartment of macrophages—an environment subjected to oxidative and nitrosative stress. However, the mycobacterium has evolved a number of protective mechanisms to cope with oxidative stress, responsible in the pathogenesis of tuberculosis. The presence of various protective enzymes, such as the catalase, superoxide dismutase (SOD), peroxidase–peroxynitrite reductase complex and thioredoxin–thioredoxin reductase system, causes neutralisation of oxygen and nitrogen free radicals. It has also been shown that the existence of specialised mechanism and pathways, e.g. those involved in DNA repair, protects the Mtb against the damage caused to various macromolecules by oxidative stress [26].

Catalase peroxidise (KatG) is the enzyme system utilised by mycobacteria to detoxify H_2O_2. *Mycobacterium tuberculosis* has one catalase KatG that has catalase, peroxidase and peroxynitritase activity [45] and has been shown to play a role in the virulence of Mtb. SOD is another protective enzyme which is a metalloprotein and is involved in the dismutation of oxygen free radicals into H_2O_2 and molecular oxygen. There are two different types of SODs in Mtb; SOD A which is an iron containing enzyme (FeSOD) and SOD C, a copper and zinc containing enzyme (CuZnSOD). In various studies, both SOD A and SOD C have been shown to play a role in protecting Mtb against ROS [25, 61]. Deletion of sec A2 gene that encodes a protein required for secretion of SOD A results in enhanced ROS production [65].

Mycothiol is a low-molecular-weight thiol in mycobacteria and functions like glutathione, which is not produced by mycobacteria. Sensitivity of mycothiol (MSH) mutants to oxidative stress suggests the role of mycothiol in maintaining the redox balance in mycobacterial cells in response to oxidative stress [9, 10].

An orthologue for bacterial alkyl hydroperoxidases (AhpC) is encoded by mycobacterial *ahpC* gene [20]. It is a non-heme peroxiredoxin (Prx) utilised to detoxify organic peroxides by reducing them into less reactive alcohol derivatives [85]. Mycobacterial alkyl hydroperoxide reductase (AhpC) has been shown to protect the bacilli against both oxidative and nitrosative stress [50]. Similarly, another Prx system in Mtb is thioredoxin reductase (TPX) that has been shown to be highly effective in protecting the bacilli from host oxidative stress and has been recently shown to be involved in virulence [34]. *Mycobacterium tuberculosis* is also known to contain two methionine sulphoxide reductases (MSRs) that are involved in the reduction of methionine sulphoxide to methionine and to protect against ROS and RNS [43].

F_{420} is a redox active enzyme cofactor which derives its name from the intrinsic 420 nm absorption; various F_{420}-dependent enzymes are known to be involved in oxygen detoxification [67]. Various mycobacterial species including *M. leprae* are known to contain F_{420} as well as unique F_{420}-dependent glucose-6-phosphate dehydrogenase (FGD). In *M. smegmatis*, a non-pathogenic mycobacterial strain, FGD has been demonstrated to have a role in combating the oxidative stress [33]. Recently, it has been reported that F_{420}-deficient mutant of Mtb is hypersensitive to oxidative stress as well as bactericidal agents thus confirming the role of F_{420}-dependent antioxidant mechanism in the pathogenesis of tuberculosis by protecting the Mtb against the oxidative stress produced by host cells [31].

6.5.2 *Sensing of Oxidative Stress by* Mycobacterium tuberculosis

In bacteria, the oxidative/nitrosative stress is sensed by various transcription factors such as OxyR and SoxR. However, in Mtb, the prototypical homologue of SoxR is absent whereas OxyR is non-functional due to a number of mutations [24]. Two-component signal transduction systems are also used by many bacteria to allow them to respond rapidly to changes in their environment. These systems consist of a membrane-bound sensor histidine kinase, which in response to environmental stimulus undergoes auto-phosphorylation on a conserved histidine residue followed by transfer of phosphoryl group to a conserved aspartate residue on the downstream response regulator which is thereby activated as either a positive or a negative transcriptional regulator.

The genome of Mtb has been shown to encode 11 complete pairs of two-component signal transduction systems [18]. Some of these sensors, such as heme-based DosS and DosT, are unique to mycobacteria, whereas others, such as the

WhiB proteins and anti-σ factor RsrA, are unique to actinobacteria [6]. Recent studies have demonstrated the role of a family of Fe-S cluster containing proteins called WhiB proteins that are putative transcription factors and have been suggested to play a functional role in Mtb similar to OxyR and SoxR in other bacteria [28]. Additionally, the DOS R-S/T two-component heme sensor system implicated in the virulence of Mtb is the most well-characterised signalling system in Mtb that is known to sense various gases including carbon monoxide (CO), nitric oxide (NO) and oxygen (O_2) [40].

6.5.3 Host Antioxidant Mechanisms

The antioxidant capacity of host cells is maintained by various intracellular and circulating proteins/non-protein molecules and phase II detoxification enzymes. The induction of expression of these enzymes requires binding of specific inducers to the antioxidant response element (ARE) that is present in the promoter regions of genes encoding phase II enzymes. The transcriptional activation of ARE-dependent phase II antioxidant enzymes is brought about by a nuclear erythroid 2 p45-related factor 2 (Nrf2), a redox-sensitive transcription factor. Under normal physiological conditions, Nrf2 is present in the cytoplasm whereas it translocates to nucleus under oxidative stress leading to transcriptional activation of phase II antioxidant enzymes including NAD(P)H dehydrogenase quinone I (NQO1) and glutamate-cysteine ligase—an enzyme involved in synthesis of an antioxidant tripeptide glutathione [51, 84]. In extrapulmonary tuberculosis in guinea pigs, there is progressive oxidative stress, particularly due to the defect in host antioxidant defences that can be restored by antioxidant therapy. These investigators also showed that the therapeutic strategies that reduce oxidative stress-mediated tissue damage may prove to be beneficial as adjunct therapy to ATT [59].

The host antioxidant defence mechanism, in response to oxidative stress, could be executed either directly or indirectly by a number of protein/non-protein molecules. In direct activity, the antioxidants scavenge the peroxidants ROS and prevent the initiation of ROS-mediated reactions. These include SOD, catalase, glutathione peroxidase (GPx) and Prxs. GPx and Prx are thiol containing compounds that use glutathione (GSH) and thioredoxin (Trx), respectively, as substrates. Besides these direct enzymatic antioxidants, vitamin C (ascorbic acid), vitamin E (alpha tocopherol), beta carotene and lipoic acid are some of the non-enzymatic antioxidants.

Indirectly, antioxidants facilitate the excretion of oxidised reactive metabolites, e.g. the aldehydes, quinines and peroxides. Alternatively they can also participate in the synthesis of thiol molecules that serve as substrate for various direct antioxidant phase II detoxification enzymes, e.g. glutathione-*S*-transferase, NADPH quinone oxidoreductase (NQO1), glutathione cysteine ligase (GCL), glutathione synthetase (GS), gamma glutamyltranspeptidase (GGT), UDP glucuronyltransferase (UGT), Trx reductase and heme oxygenase. Another non-protein antioxidant is a thiol tripeptide molecule glutathione that is present in both oxidised (GSSG)

and reduced (GSH) forms in all mammalian cells and is involved in the maintenance of redox potential by eliminating the free radicals [7]. Earlier it has been reported that in the whole blood of Mtb-infected guinea pigs, reduced-glutathione (GSH) levels were markedly decreased as the infection progressed which was reflected by a significant decrease in the GSH/GSSG ratio [59]. These authors also demonstrated an overall increase in the total glutathione expression in lung lesions which was indicative of the host response to oxidative stress as a consequence of Mtb infection [59].

Heme oxygenase (HO) is a key stress-response enzyme that degrades heme molecules, thereby releasing free iron, carbon monoxide (CO) and biliverdin that have anti-inflammatory and antioxidant properties [30, 77]. Increased expression of HO-1 has been observed in the plasma of individuals with a variety of other pulmonary pathologies, including acute respiratory distress syndrome, chronic obstructive pulmonary disease and asthma [55]. Epiphanio et al. [27] reported that HO-1 contributes to the establishment of malaria infection by decreasing the host inflammatory response to pre-erythrocytic stages, while Shiloh et al. [70] reported that HO-1 and CO induce the 'dormancy regulon' of Mtb and may contribute to latency in tuberculosis infection. Recently, Andrade et al. [3] reported the increased plasma levels of HO-1 in pulmonary TB patients. Considering the intracellular localisation of HO-1, the increased levels of this enzyme could be derived from injured tissues, thus suggesting the strong association of HO-1 with both bacterial burden and disease severity in the lung. HO-1 levels have been suggested [3] as a potentially useful parameter for distinguishing active from latent or treated pulmonary tuberculosis. A positive correlation between HO-1 levels and plasma IL-10 levels and negative correlation with TNF-levels were also reported [3].

6.6 Drug-Resistant Tuberculosis

Antibiotic resistance has been shown to get accelerated by ROS induced by antibacterial stimulation [82]. In a study on the significance of oxidative DNA damage in drug-resistant *Escherichia coli* and *Mycobacterium tuberculosis*, the ROS damage was suggested to play a critical role [82]. Correlation between resistance to first-line anti-tubercular drugs (in particular isoniazid) and reduced NO susceptibility has been shown in clinical *Mycobacterium tuberculosis* isolates from 50 sputum-smear- and culture-positive patients [35].

In other studies the mutation rate of Mtb in response to oxidative stress was not increased in strains with deficient catalase and peroxidase activity (DIC). Interestingly, however, there were mutations in unusual locations similar to those seen in clinical isoniazid-resistant strains [57].

Mtb engulfed by macrophages are shown to survive and grow by inactivation of ROS and RNIs in the presence of several Mtb gene products [86]. For example, KatG, lipoarabinomannan, SOD proteins and others protect Mtb by one or the other mechanisms [6, 49].

6.6.1 Role of Antioxidants in Therapeutic Strategies for TB

Vitamin C, an essential nutrient for some mammals, has potent antioxidant as well as pro-oxidant properties. There are conflicting reports suggesting either beneficial or nil effect of vitamin C in the treatment of TB [73]. Vitamin C could sterilise Mtb cultures in vitro [60]. Correlation between the high vitamin C content of some medicinal plant extracts and their activity against Mtb has been shown in a recent study [76]. A breakthrough study has reported the pro-oxidant activity of vitamin C against drug-susceptible, multi-drug-resistant (MDR) and extensive drug-resistant (XDR) Mtb [80]. The sterilising effect of vitamin C on Mtb in this study was observed to be mediated by high iron concentration, ROS production and DNA damage. These authors proposed for further studies on the benefits of a high vitamin C diet in TB-treated patients and on the development of bactericidal drugs based on ROS production.

There is a potential role of various food supplements and other products which enhance the activity and production of NO and other free radicals. Arginine-rich food (pea nuts) in a subgroup of HIV+/TB patients showed significantly better cure rate of anti-TB treatment [66]. On the other hand, low initial levels of NO in exhaled air were associated with a poor cure rate. Some of the novel copper complexes, studied for their SOD and antioxidant activities, have been found to promote DNA damage in the presence of oxidants and demonstrate anti-tuberculosis activity [37]. Supplementation of anti-tubercular treatment with micronutrients, vitamin E and selenium was shown to reduce oxidative stress in TB patients [69].

Oxidative stress has been blamed for hepatotoxicity caused by several first-line anti-TB drugs such as INH, rifampicin and pyrazinamide. Herbal drugs like curcumin, silymarin and *N*-acetyl cysteine (NAC) in in vitro model of human hepatocellular carcinoma cell lines (HepG2) showed hepato-protective effect during treatment of HepG2 with ATT drugs [72]. Such observations may support the adjuvant role of antioxidant drugs with anti-TB treatment. TB patients show lower iNOS expression and NO production, which however tend to increase during anti-TB treatment [23].

The Nrf2 pathway, which is frequently targeted therapeutically to control inflammatory disease conditions related to oxidative stress, has not been investigated in the context of tuberculosis [15, 44]. Several naturally occurring compounds such as isothiocyanates and thiols have been shown to induce this pathway and increase antioxidant and anti-inflammatory responses. NAC is one such compound that is known to induce Nrf2 translocation and activation of antioxidant gene transcription in treated HepG2 cells [15]. It is an FDA-approved drug that is used in an aerosol form (Mucomyst®) as a mucolytic agent in patients with cystic fibrosis or chronic airway disease and is one of the most commonly used ROS scavenger/antioxidant drugs currently used clinically and in preclinical trials [64].

Currently, NAC is being tested in a human clinical trial to counteract oxidant-mediated liver toxicity associated with tuberculosis drug therapy. In a recent report, the treatment of Mtb-infected guinea pigs with NAC partially restored blood

glutathione concentrations, and serum total antioxidant capacity, while it decreased spleen bacterial counts, lung and spleen lesion burden and the severity of lesion necrosis [59]. These data suggest that the progressive oxidative stress during experimental tuberculosis in guinea pigs is due in part to a defect in host antioxidant defences, which can be partially restored with antioxidant treatment. Therefore, the therapeutic strategies that reduce oxidant-mediated tissue damage may potentially be beneficial as an adjunct to therapy in the treatment and prevention of tuberculosis in humans [59].

In conclusion, the toxic oxygen and nitrogen species are shown to play an important role in the pathogenesis of TB and damage to different organs. There is some evidence to suggest the beneficial use of antioxidants such as vitamin C and NAC in treatment of TB and anti-TB drug toxicity.

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Chapter 7
Oxidative Stress in COPD

Peter J. Barnes

Abbreviations

4HNE	4-Hydroxy-2-nonenal
FEV_1	Forced expiratory volume in 1 second
GSH	Glutathione
GST	Glutathione-*S*-transferase
H_2O_2	Hydrogen peroxide
HDAC	Histone deacetylase
IL	Interleukin
MDA	Malondialdehyde
NF-κB	Nuclear factor-κB
NOX	NADPH oxidase
Nrf2	Nuclear erythroid-2-related factor 2
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TGF	Transforming growth factor

7.1 Introduction

Chronic obstructive pulmonary disease (COPD) is an increasing global health problem, which is now the third leading cause of death worldwide [55]. It currently affects around 10 % of the population of over 45 years but this rises to 50 % in heavy smokers and it has been estimated that the cumulative lifetime risk of

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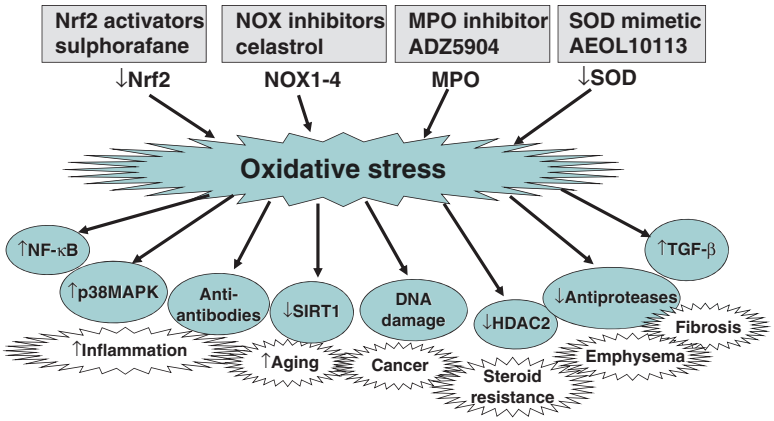

Fig. 7.1 The many effects on increased oxidative stress in COPD. Oxidative stress may be increased in COPD by a reduction in the transcription factor Nrf2, activation of NADPH oxidases (NOX), myeloperoxidase (MPO) and reduced superoxide dismutase (SOD), which may be triggered by inflammatory stimuli. Oxidative stress is a key mechanism that drives the development and progression of COPD through activation of the pro-inflammatory transcription factor nuclear factor-κB (NF-κB), p38 mitogen-activated protein kinase (MAPK), generation of autoantibodies to carbonylated proteins, reduced expression of sirtuin-1, DNA damage, reduced histone deacetylase (HDAC)-2 expression, reduced activity of antiproteases and increased release of transforming growth factor (TGF)-β. Oxidative stress in COPD may be reduced by several approaches (shown in *grey boxes*)

developing is now over 25 % [34]. The increase in COPD globally is greatest in low income countries, where indoor air pollution, such as exposure to biomass smoke, is as common as cigarette smoking as a risk factor [77, 78]. The major aetiological factor driving this disease is likely to be increased oxidative stress in the lungs following long-term exposure to cigarette smoke or the combustion products of biomass fuels [50]. Oxidative stress arises as a result of endogenous antioxidant defences being impaired and/or overwhelmed by the presence of reactive oxygen species (ROS). COPD is characterized by chronic inflammation and fibrosis of the small airways and destruction of the lung parenchyma (emphysema) [39, 58]. A striking feature of COPD is its failure to resolve when exposure to cigarette smoke has stopped [33], which has led to the suggestion that other endogenous factors, such as autoimmunity or persistent infection, may also be driving the disease [4]. Oxidative stress appears to drive many of the pathogenetic mechanisms involved in COPD and its progression (Fig. 7.1).

7.2 Persistent Lung and Systemic Oxidative Stress in COPD

There is evidence for persistent oxidative stress in COPD patients, particularly during acute exacerbations. Alveolar macrophages from COPD patients are more activated and release increased amounts of ROS in the form of superoxide anions

and hydrogen peroxide (H_2O_2) [79]. Similarly, activated peripheral blood neutrophils from COPD patients release increased amounts of ROS, particularly during exacerbations [65]. Lung tissue from COPD patients shows increased lipid peroxidation, as measured by 4-hydroxy-2-nonenal (4HNE) [73]. There is an increased concentration of the volatile product of lipid peroxidation ethane in exhaled breath of COPD patients and this is correlated with disease severity [69]. COPD patients have increased concentrations of H_2O_2, malondialdehyde (MDA), 4HNE and 8-isoprostane in exhaled breath condensate [10, 22, 28, 62] and these are further increased during exacerbations [11, 70]. The increased markers of oxidative stress remain elevated in ex-smokers, indicating that they are derived from endogenous oxidative stress, presumably reflecting persistent lung inflammation [62]. Increased oxidative and nitrative stress result in the formation of peroxynitrite, which is increased in exhaled breath condensate of patients with COPD [68]. This may also be reflected by an increase in tyrosine nitration, as a result of peroxynitrite, in induced sputum and lungs of patients with COPD [42, 75]. Oxidative stress is also increased in skeletal muscle of patients with COPD and may contribute to muscle weakness [8, 9].

In contrast, levels of the endogenous antioxidant glutathione are lower in BAL fluid from COPD patients with frequent exacerbations compared to those with stable COPD [31, 72]. Extracellular superoxide dismutase (SOD3) polymorphisms are more frequent in COPD and their expression is increased in sputum of COPD patients, although there is reduced expression around small airways [74, 88].

7.3 Source of Oxidative Stress in the Lung

The lung is particularly vulnerable to injury from environmental oxidative stress due in part to its anatomical structure. It is constantly exposed to sources of endogenous oxidative stress generated by mitochondrial respiration and inflammatory responses to bacterial and viral infections within the lung. The environmental sources of airborne oxidative stress include oxidant gases, ultrafine particulate material and nanoparticles from industrial pollution and vehicular-exhaust fumes. However, the most important risk factor in causing COPD in the Western world is cigarette smoking, whereas in developing countries inhalation of combustion products from enclosed cooking fires (biomass fuels) being an important additional risk factor [77, 78].

Whilst exposure to cigarette smoke can drive the onset of COPD, once the disease has become established, cessation of smoking does not stop the continued presence of oxidative stress and progression of disease [33, 54]. The continued presence of oxidative stress most likely arises from endogenous sources such as activated neutrophils and macrophages as well as lung epithelial cells. Indeed lung epithelial cells of COPD patients produce oxidative stress derived from mitochondrial respiration [86]. Other sources of intracellular ROS include the cytoplasmic ROS generating enzymes, such as NADPH oxidase (NOX) and the xanthine/xanthine oxidase system as well as the myeloperoxidase (MPO), levels of which are all elevated in COPD patients [48, 52].

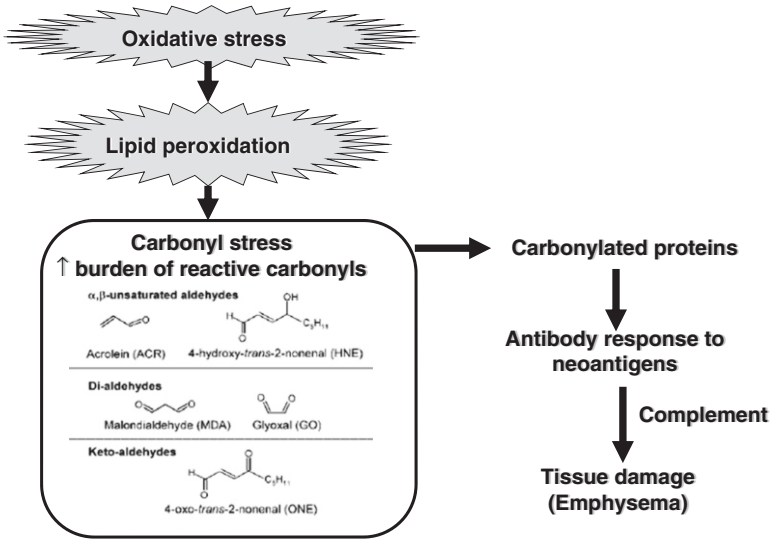

Fig. 7.2 Oxidative and carbonyl stress in COPD. Oxidative stress causes lipid peroxidation resulting in the formation of reactive aldehydes, such as 4-hydroxy-2-nonenal. These carbonylate proteins result in the formation of neo-antigens that may then elicit an antibody response, which causes tissue damage (such as emphysema) through the fixation of complement

Superoxide radicals that are produced endogenously are relatively weak oxidizing agents but are rapidly converted to other more damaging ROS (Fig. 7.1), such as the hydroxyl radical and H_2O_2, or the very powerful and damaging peroxynitrite radical formed when in the presence of nitric oxide [68]. Similarly MPO, released from activated neutrophils which accumulate in the lungs of COPD patients, produces very destructive hypochlorous acid. Hypochlorous acid chlorinates tyrosine residues in proteins, with the formation of 3-chlorotyrosine, which is increased in sputum of COPD patients [66]. However, in healthy cells intracellular antioxidant defences are able to efficiently mop up these damaging ROS, thus limiting their cellular effects.

7.4 Carbonyl Stress in COPD

ROS generation may result in the formation of reactive carbonyls through lipid peroxidation and glycoxidation of sugars, leading to the formation of several aldehydes that result in protein carbonylation (Fig. 7.2) [64]. This accumulation of reactive carbonyls and subsequent protein carbonylation has been commonly referred to as 'carbonyl stress'. It is predominantly associated with chronic disease and with ageing [25]. Unlike other post-translational modifications, protein carbonylation is non-enzymatic and targets specific peptide residues, such as lysine, arginine, cysteine and histidine. Protein carbonylation is increasingly recognized as a major driver of

the underlying pathology associated with many chronic diseases. It is present in both smokers and COPD patients [51]. Increased levels of free carbonyls, such as MDA and 4HNE, products of lipid peroxidation, have also been detected in the lungs of COPD patients [73]. Levels of carbonyl stress are correlated with disease severity as measured by the decline in forced expiratory volume in 1 second (FEV_1). Like many post-translational protein modifications, protein carbonylation can modify protein function, disrupting normal cell function and physiological mechanisms.

7.5 Antioxidant Defences in the Lung

Because the lung is constantly exposed to both external and endogenous sources of oxidative stress, it has evolved a number of efficient antioxidant defensive strategies, of which glutathione (GSH) plays an important part. Moreover, up to 20 % of all glutathione produced is found within the mitochondria in order to neutralize endogenous ROS production as a by-product of metabolism [12]. Protecting the exposed surface of the lung from the environment is the epithelial lining fluid, which contains several antioxidants that include ascorbic acid (vitamin C), α-tocopherol (vitamin E) and uric acid. Larger molecules, such as albumin and mucin, can also act as sacrificial antioxidants due to the presence of exposed sulfhydryl groups. Several epidemiological studies have shown a clear association between reduced levels of the antioxidants in the lung, such as α-tocopherol and ascorbic acid, and deteriorating pulmonary function in COPD [40]. No studies to date have shown that dietary supplementation with antioxidants leads to clinical improvement [85]. However, a 10-year follow-up study did find that it reduced the risk of developing chronic lung disease by 10 % [2] and lowered carbonyl stress levels in the systemic circulation [26].

The exposure of airway epithelial cells from healthy subjects to acute oxidative stress triggers increased GSH synthesis through up-regulation of the expression and activity of a key enzyme in GSH synthesis, glutamylcysteine ligase [80]. However, this enzyme is reduced around the central bronchial epithelium and in alveolar macrophages from smokers and patients with COPD [38], suggesting a defective regulatory mechanism. Similar differential responses between COPD and control subjects were apparent with other GSH-dependent antioxidant enzymes, glutathione-*S*-transferase P1 isoenzyme (GSTP1), glutathione-*S*-transferase M1 (GSTM1) and glutathione peroxidase [84]. A genetic deletion mutation in GSTM1 is associated with the development of emphysema in smokers and increased susceptibility to developing COPD [17]. Similarly, genetic polymorphisms in the GSTP1 and epoxide hydrolase have been associated with increased risk of emphysema [29].

Transforming growth factor (TGF)-β expression is increased in COPD and inhibits the expression of the antioxidant enzymes Mn-SOD (SOD2) and catalase in airway smooth muscle cells [61]. Both these enzymes, which are critical for neutralizing mitochondrial-derived ROS, are under the control of the transcription factor FOXO3. Moreover, a deficiency in FOXO3 activity has previously been

associated with COPD [41]. Gene polymorphisms for SOD2 associated with reduced enzyme expression have also been associated with COPD [71]. Similarly, polymorphisms in SOD3 have also been linked to both reduced lung function in COPD [24] and protection against the development of COPD in smokers when SOD3 activity is enhanced [89]. Over 200 cellular antioxidant and detoxification enzymes are under the control of the transcription factor nuclear erythroid-2-related factor 2 (Nrf2), which regulates gene expression through binding to antioxidant response elements (ARE) within the promoters of the many antioxidant and cytoprotective genes [19]. COPD patients have reduced expression of Nrf2 responsive genes due to reduced Nrf2 activity [56, 57] and this may be the consequence of increased acetylation of Nrf2 as a reduction of histone deacetylase-2 (HDAC2) activity.

7.6 Oxidative Stress and Inflammation in the Airways

At least 50 different cytokines and chemokines are secreted in the lungs of patients with COPD [5]. Many of the intracellular signalling pathways triggering and/or driving the release of these inflammatory mediators are sensitive to oxidative stress as they incorporate redox-sensitive molecular targets, such as the transcription factor nuclear factor-κB (NF-κB), and signalling molecules such as Ras/Rac, Jun-N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) and protein tyrosine phosphatases. Oxidative stress can activate the NF-κB pathway at many levels and NF-κB expression and activation are increased in COPD and correlates with airflow limitation [30]. Moreover, ROS itself also act as an intracellular second messenger as inflammatory stimuli induce micro-oxidative bursts which are essential for cellular activation. Carbonyl stress in the form of electrophilic carbonyls can also impact on many different signalling pathways. As with oxidative stress, this is propagated through the targeting of critical cysteine residues in susceptible signalling molecules [37].

Oxidative stress also activates TGF-β signalling pathways, which themselves induce oxidative stress [36, 53]. This is enhanced by the inhibitory effect of TGF-β on Nrf2 activity and reduced expression of endogenous antioxidants [60].

Oxidative stress increases the expression of matrix metalloproteinase-9 (MMP9), a key elastolytic enzyme involved in emphysema, and further enhances elastolysis through oxidative inactivation of α1-antitrypsin and secretory leukoprotease inhibitor, thereby enhancing the activity of neutrophil elastase [82, 87].

7.7 Corticosteroid Resistance Due to Oxidative Stress

The ability of corticosteroids to repress pro-inflammatory gene expression is also impaired in COPD as a result of oxidative stress [6, 7]. Oxidation, carbonylation and nitration reduce the activity and expression of HDAC2, which is essential for

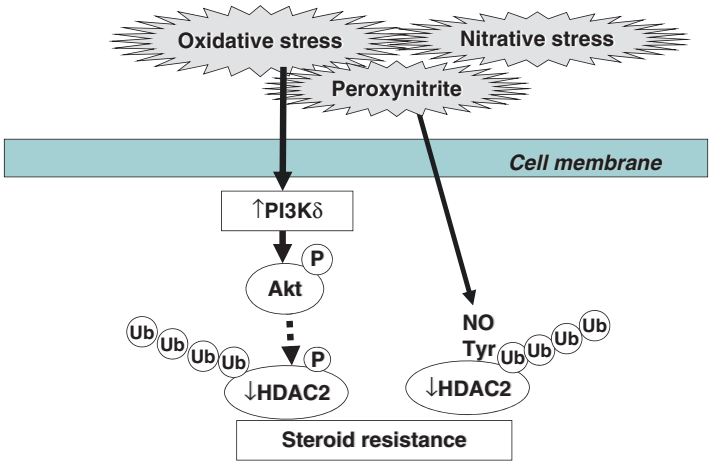

Fig. 7.3 Oxidative stress induces corticosteroid resistance in COPD. Oxidative stress activates phosphoinositide-3-kinase-δ (PI3Kδ) which phosphorylates Akt and subsequently histone deacetylase (HDAC)-2, which is inactivated and ubiquitinated, so that it is degraded by the proteasome. Oxidative and nitrative stress form peroxynitrite which nitrates tyrosine residues (NO-Tyr) on HDAC2, resulting in its inactivation and ubiquitination. Reduced HDAC2 activity and expression prevent corticosteroids from switching off multiple activated inflammatory genes in COPD lungs

the suppression of activated inflammatory genes and the anti-inflammatory actions of corticosteroids (Fig. 7.3) [45, 46]. The reduction of HDAC2 after oxidative stress may be due to nitration of tyrosine residues at the catalytic site and at the C terminal end of the molecule, leading to its ubiquitination and destruction by the proteasome [67]. Oxidative stress also activates the enzyme phosphoinositide-3-kinase-δ (PI3Kδ), which results in phosphorylation and inactivation of HDAC2 [83].

7.8 Oxidative Stress and Accelerated Ageing in COPD

Patients with emphysema appear to have acceleration of the normal lung ageing process and therein increasing evidence for cellular senescence in COPD [44]. This appears to be due, at least in part, to a reduction in endogenous anti-ageing molecules as a result of oxidative stress. Sirtuin-1 is a protein deacetylase that is linked to anti-ageing effects through genomic stabilization and there is a marked reduction in its expression in lungs and peripheral macrophages of COPD patients that is related to disease severity [63]. Oxidative stress reduces sirtuin-1 enzyme activity and expression and this is associated with increased acetylation of NF-κB and increased expression of MMP9, a key enzyme involved in elastin degradation in emphysematous lung [63].

7.9 Oxidative Stress and Autoimmunity in COPD

Accumulating evidence has shown that there is an autoimmune component in COPD [23, 49]. Until recently, a mechanistic link between exposure to oxidative stress and developing autoimmunity in COPD was not established. However, autoantibodies against carbonyl-modified self-proteins, as a result of oxidative stress, are elevated in COPD serum, and increase with disease severity [51]. Since these autoantibodies are complement fixing they could contribute to parenchymal lung destruction. Carbonyl-modified proteins are highly immunogenic and can result in autoimmunity. Carbonyl-modified proteins are recognized by the innate immune system through pattern recognition receptors that are expressed on antigen-presenting cells, such as macrophages and dendritic cells whereupon these potent immunogens are processed and re-expressed in association with MHCII, thereby facilitating the activation of an acquired immune response [3]. Indeed, COPD patients exhibit a strong type 1 immune response in the lower airways with the pulmonary accumulation of Th1 cells and dendritic cells in the small airways of COPD patients, expressing increased amounts of MHCII [39]. However, it is not yet certain whether this autoantibody response to oxidatively modified protein epitopes in COPD is destructive, protective or simply a bystander effect. However, the autoantibodies against carbonyl-modified protein were of a potentially destructive IgG1 isotype and evidence of corresponding immunoglobulin (IgG) and complement (C3) deposition have been observed in COPD [32, 51].

Besides oxidative stress creating the essential neo-antigens, it also helps to drive the influx of immune cells necessary to recognize and process these neo-antigens. Increased oxidative stress in the lungs causes the release of CCL20 and CCL2, which in turn triggers the recruitment of dendritic cells, monocytes and lymphocytes. Th17 cells may orchestrate this response and there is evidence for their activation in COPD lungs. Interleukin (IL)-18 concentrations are increased in COPD patients and IL-18 signalling is enhanced by oxidative stress [43].

7.10 Oxidative Stress and DNA Damage

Oxidative stress causes direct damage to DNA. There is an increase in the expression of 8-hydroxy-2-deoxyguanosine, a biomarker of oxidative damage of DNA in lung of normal smokers and patients with COPD, presumably reflecting the oxidative stress of cigarette smoking [14]. There are normally efficient molecular mechanisms for DNA repair and apurinic/apyrimidinic(AP) sites are common lesions in DNA during the course of repair of oxidative bases. In lung of normal smokers an increase in AP sites reflects active DNA repair, whereas in lungs of COPD patients there is no such increase, indicating a defect in DNA repair in COPD. In addition nuclear expression of the DNA repair protein Ku86 was significantly reduced in COPD compared to normal smoker lungs, indicating a defect on double-stranded

DNA repair in COPD. Furthermore, loss of Ku86 was also found in a mouse model of COPD and in human bronchiolar epithelial cells after exposure to oxidant stress. Knock down of Ku86 mimicked the defect in AP response to oxidative stress. This defect in COPD repair as a result of oxidative stress may account for the increased prevalence of lung cancer in patients with COPD compared to smokers without airway obstruction [1].

7.11 Therapeutic Implications

As discussed above, oxidative stress is a major driving mechanism for the pathophysiology of COPD, so reducing oxidative stress is an important therapeutic strategy [13]. This may be achieved by exogenous antioxidants or by enhancing endogenous antioxidants. *N*-acetylcysteine is a mucolytic agent that also has antioxidant effects through increasing glutathione concentrations but in a large clinical trial was ineffective in reducing exacerbations or disease progression in COPD patients [27]. However, patients not treated with inhaled corticosteroids did obtain some benefit in reducing exacerbations. Carbocisteine, another mucolytic therapy that has antioxidant effects, showed a small reduction in exacerbations in untreated COPD patients [90]. A related drug erdosteine also has some evidence of clinical benefit in COPD patients, although the effects and the studies are small [15]. One of the problems with glutathione-based antioxidants that have a thiol structure is that they are inactivated by oxidative stress, which prompted a search for alternative antioxidant molecules.

Dietary antioxidants include vitamin C, vitamin E, resveratrol and flavonoids such as quercitin, but so far improving dietary antioxidant intake has not been shown to improve lung function of clinical features of COPD [85]. Other antioxidants include SOD mimetics, such as AEOL 10113 [16], and nitrone spin-trap antioxidants, such as NXY-059 [18]. However these antioxidants either have had toxicological problems or were discontinued for other reasons. There are several NOX isoenzyme inhibitors now in development and some of these are entering clinical trials [21, 47]. There is a need to block mitochondrial sources of ROS so that cell-permeable drugs may be necessary. MPO may contribute to the oxidative stress produced by neutrophilic inflammation and a selective MPO inhibitor, the 2-thioxanthine ADZ5904, reduces oxidative stress and reduces the development of emphysema in guinea pigs exposed to cigarette smoke [20]. However this drug has been discontinued for unknown reasons.

Perhaps the most encouraging approaches to antioxidant therapy lie with the use of new NRf2-activators, which activate multiple antioxidant genes and address the defect on Nrf2 response to oxidative stress that appears to occur in COPD cells. The Nrf2 activator sulforaphane denitrosylates HDAC2 and increases its activity in alveolar macrophages from COPD patients and in cigarette smoke-exposed mice, but is ineffective in $Nrf2^{-/-}$ knock-out mice, indicating that its effects are mediated via Nrf2 activation [57]. Sulforaphane, which occurs naturally in cruciferous vegetables

such as broccoli, reacts with Cys residues of the associated protein Keap1 in the cytoplasm to form thioacyl adducts so that Nrf2 translocates to the nucleus to switch on antioxidant genes. A clinical trial of sulforaphane in COPD patients is currently in progress. A synthetic triterpenoid bardoxolone methyl (CDDO) is effective in a cigarette smoke-exposed mouse model of COPD [81], but a phase 3 clinical trial in renal disease was terminated due to adverse effects and increased mortality [76]. Dimethyl fumarate (BG-12) is also an Nrf2 activator and has been effective in phase 3 clinical trials in multiple sclerosis, although side effect such as flushing, nausea and diarrhoea is reported [35]. However, these Nrf2 activators may lack specificity and there is a search for drugs that act on the pathways leading to defective Nrf2 function in COPD, such as the PI3Kδ-HDAC2 pathway [59].

7.12 Conclusions

Elevated levels of ROS are found in COPD and these may be associated with increased inflammation, airway remodelling, autoimmunity, corticosteroid resistance and cellular senescence. In addition systemic oxidative stress may also be a causal link in many COPD co-morbidities such as cardiovascular diseases and metabolic syndrome as well as skeletal muscle wasting. Local oxidative stress may promote the development of lung cancer through DNA damage and impaired DNA repair. Following the initial environmental exposure to ROS, the subsequent intracellular sources of oxidative stress may be important to understanding the pathophysiology of this disease. The disappointing clinical effects of existing antioxidants in COPD studies indicate the need to develop novel more potent antioxidants targeted to the correct intracellular compartment, such as the mitochondria. Combinations of antioxidants, targeting different cellular compartments, may prove more effective than monotherapy. In a similar manner, combining antioxidants with anti-inflammatory drugs, bronchodilators, antibiotics and statins may complement or, in the case of corticosteroids, improve/restore their efficacy.

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Chapter 8
Oxidative Injury Caused by Cigarette Smoking and Air Pollution

Andrew J. Ghio

8.1 Introduction

Oxidative stress is pivotal to the biological effects, tissue injury, and disease observed following exposures to both cigarette smoke and air pollutants. The specific component(s) in both cigarette smoke and air pollutants which participates in the biological effect, tissue injury, and disease and the cellular and molecular mechanism pathways which are involved are yet to be defined with certainty.

8.2 Cigarette Smoking

Cigarette smoke is comprised of between 4,000 and 7,000 constituents [87, 95]. Among these are numerous oxidant compounds; a puff of cigarette smoke was quantified to contain 10^{17} free radicals in the tar phase and 10^{15} in the gas phase [22, 86]. In a burning cigarette, temperatures in the combustion zone (800–950 °C) result in a complete pyrolysis of tobacco. Immediately downstream, a rapid drop in temperature (to 200–600 °C) and a lack of oxygen allow for an incomplete combustion. Subsequently, a complex aerosol is generated during smoking which includes condensed liquid droplets (the particulate fraction or tar) suspended in a mixture of volatile/semi-volatile compounds and combustion gases (the gas fraction). Polyphenols and semiquinones can be identified among the compounds in the

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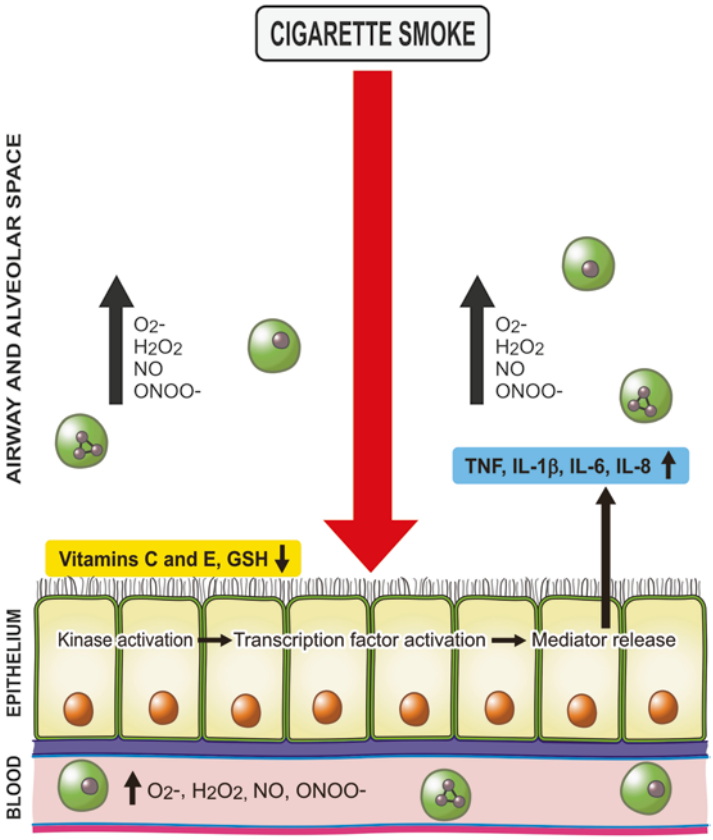

Fig. 8.1 Schematic of the oxidative stress after cigarette smoke exposure. Cigarette smoke will include oxidant compounds including superoxide (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite ($OONO^-$). There is depletion of antioxidants (e.g., vitamins C and E and thiols) in the lung lining fluid. The oxidative stress culminates in a release of inflammatory mediators. Activated phagocytes (green cells in the blood and airway and alveolar space) further increase the oxidant burden with a generation of O_2^-, H_2O_2, NO, and $OONO^-$

cigarette tar while superoxide, epoxides, peroxides, nitric oxide (NO; 500–1,000 ppm), nitrogen dioxide, peroxynitrite ($ONOO^-$), and peroxynitrates are in the gas phase. Smoking one cigarette exposes the human respiratory tract to an enormous particle burden; between 15,000 and 40,000 μg particulate matter (PM).

Smoking produces a shift in the balance between oxidants and antioxidants, creating an oxidative stress (Fig. 8.1). Oxidants in cigarette smoke directly injure cells and tissues, inactivate defense mechanisms, and initiate inflammation which furthers oxidative stress following the initial exposure. It is difficult, if not impossible, to determine whether oxidants included in cigarette smoke or those produced from the ensuing inflammatory response are primarily responsible for the observed oxidative stress in smokers. The endpoints of oxidative stress which have been quantified with smoking are numerous and diverse (Table 8.1). Measurements

Table 8.1 Endpoints of oxidative stress elevated in cigarette smokers

Generation of O_2^- and H_2O_2
Generation of nitrite/nitrate
Levels of superoxide dismutase, catalase, myeloperoxidase, and cytochrome P450 and their activities
Exhaled breath ethane/alkanes, thiobarbituric acid-reactive substances, and other indices of lipid peroxidation
Concentrations of oxidized proteins
Total, reduced, and oxidized glutathione
Activity of glutathione peroxidase, glutathione transferase, and glutathione reductase
Prostanoids (F2-isoprostanes and PGF2 alpha), hydroxyeicosatetraenoic acid products (HETEs), F(4)-neuroprostanes, 7-ketocholesterol, 24- and 27-hydroxycholesterol, low-density lipoproteins, and other cholesterol oxidation products
Concentrations of uric acid and allantoin
DNA damage (8-hydroxy-2′-deoxyguanosine and 8-oxo-2′-deoxyguanosine)
Histopathology (e.g., 4-hydroxy-2-nonenal and 8-hydroxydeoxyguanosine)
Immunohistochemistry for specific proteins, nitrotyrosine
Gene expression microarray analysis
Trolox-equivalent antioxidant capacity
Antioxidant reducing capacity
Total radical trapping parameters

have been obtained in cells and their fractions, whole blood, serum, plasma, cord blood, sputum, exhaled breath, breath condensate, lavage fluid, lavage cells, urine, and tissues (e.g., lungs, vasculature, brain, muscle, testicles, and pancreas).

Oxidant generation with cigarette smoke exposure. Oxidants, including hydrogen peroxide, can be directly measured in the particulate fraction of cigarette smoke [125]. Metal in cigarette smoke is also included in the particulate fraction, but concentrations appear to be insignificant [40]. There is little evidence to support the assertion that iron and copper, introduced into the body by smoking, catalyze Fenton-type reactions in either the lung or any tissue.

Phagocytes (e.g., macrophages and neutrophils) in smokers are elevated in number, and also generate reactive oxygen and nitrogen species at increased rates both in the lung and systemically [51]. In vitro studies confirm that phagocytes collected from cigarette smokers spontaneously release increased amounts of oxidants such as O_2^- and H_2O_2 compared to those from nonsmokers [111]. Hydrogen peroxide is elevated in bronchoalveolar lavage fluid and in exhaled breath condensate collected from cigarette smokers [27] and some portion of this was demonstrated to be attributable to the elevated number of macrophages in the lower respiratory tract of smokers and their increased release of O_2^- [100]. Cell sources of O_2^-, other than NADPH oxidoreductase, can also be increased (e.g., xanthine oxidase) [52].

Cigarette smoking is associated with lipid peroxidation with conversion of polyunsaturated fatty acids to hydroperoxides, endoperoxides, aldehydes (e.g., malondialdehyde), and alkanes (e.g., ethane and pentane). Levels of these end products are increased in smokers including thiobarbituric acid-reactive products (in sputum, blood, and lung components), isoprostanes (in blood, urine, and breath condensate), 4-hydroxy-2-nonenal adducts, and breath alkanes [75, 76].

Cigarette smoking depletes antioxidants [48]. Concentrations of ascorbate and vitamin E are decreased among smokers [33]. Smokers have 15–20 % lower serum concentrations of ascorbate than do nonsmokers but, after smoking cessation, values normalize [72]. Glutathione metabolism appears to be particularly provoked by smoking. Despite glutathione being acutely depleted in cell and animal models and smokers [14, 118], levels of reduced glutathione are elevated in bronchoalveolar lavage fluid of chronic smokers [17]. It has been proposed that such an increase of lung glutathione in smokers may be an attempt, albeit insufficient, to counter excess oxidants with cigarette smoke exposure [70]. Exogenous antioxidants appear to have a capacity to prevent some portion of the biological effect and injury following smoking [16]. Pretreatment with antioxidants decreases lipid peroxidation following exposure to cigarette smoke [23]. There are studies which suggest that vitamins C and E diminish production of oxidants by inflammatory cells and improve pulmonary function in smokers [73, 111]. Supplementation with *N*-acetylcysteine (NAC) also diminishes the in vitro cytotoxicity after smoking [102].

In addition to oxygen-based free radicals, cigarette smoke is a source of reactive nitrogen species and presents a nitrosative stress. Nitric oxide, abundant in cigarette smoke and generated by inflammatory cells, has potent antioxidant and anti-inflammatory actions but also contributes to oxidative reactions [90]. NO reacts with thiols to produce nitrosothiols associated with biological effects [37]. Nitrosothiol levels have been shown to be higher in breath condensate collected from smokers compared with subjects who do not smoke [25]. NO in cigarette smoke can react with O_2^- to form peroxynitrite [81] which decreases antioxidant capacity and augments oxidative stress [112]. Nitric oxide and peroxynitrite can cause the nitration of tyrosine to form nitrotyrosine products of proteins measurable in body fluids and tissues [82]. However, it must be pointed out that NO levels in smokers were reported to be normal or even lower than in nonsmokers [24]. Fractional exhaled nitric oxide (FE_{NO}) was reported to be decreased in smokers [98]. Such reduced NO production was postulated to possibly elevate oxidative stress since this molecule can function as an antioxidant as well as being a prooxidant [126].

In addition to an elevated oxidant burden in the lung, there is an increased systemic oxidative stress in smokers [62]. Plasma trolox-equivalent antioxidant capacity and total glutathione are decreased in cigarette smokers [11]. Peripheral blood neutrophils from smokers release more oxidants than those isolated from nonsmokers [107]. The proposal that oxidants in cigarette smoke, whether in the particulate or the gas phase, pass through the pulmonary alveolar wall into the blood to induce a disseminated systemic oxidative stress is improbable as such radicals would quickly react with molecules in the lung [122].

Oxidative stress and disease after cigarette smoke exposure. The World Health Organization lists smoking as one of the ten greatest contributors to global death and disease and, in many countries, is the most important risk factor for numerous diseases (Table 8.2). Oxidative stress resulting from an imbalance between oxidants and antioxidants is proposed as the basis for diseases following exposure to cigarette smoking.

Table 8.2 Diseases associated with cigarette smoking

Chronic obstructive pulmonary disease (COPD)
Interstitial lung disease (e.g., idiopathic pulmonary fibrosis)
Pneumonia
Tuberculosis
Influenza
Coronary artery disease, rhythm disturbances, and sudden death
Heart failure
Hypertension
Cerebrovascular disease
Peripheral vascular disease
Cataracts
Gum disease and dental caries
Raynaud's disease
Gastroesophageal reflux and peptic ulcer disease
Cancers of the nasal and oral passages, pharynx, larynx, esophagus, stomach, pancreas, kidneys, bladder, breast, cervix, and lung; myeloma and leukemia

Lung disease follows exposure to cigarette smoke with the increased burden of oxidants, either caused directly by the cigarette smoke or generated by inflammatory cells, injuring lung cells and depleting antioxidant defenses [15]. Exposure to cigarette smoke increases cell lysis and epithelial permeability; these effects are inhibited by antioxidants (e.g., glutathione) [63]. Among patients with chronic obstructive pulmonary disease (COPD), there are increased numbers of activated inflammatory cells in the lungs relative to healthy subjects; these cells release greater quantities of O_2^- and H_2O_2 [56, 91]. A correlation has been demonstrated between O_2^- release by peripheral white blood cells and bronchial hyperreactivity in patients with COPD, supporting a role for oxidants in lung disease after smoking [91]. Oxidants have also been demonstrated to mediate mucous hypersecretion and impaired mucociliary clearance which can contribute to injury in COPD [96].

Exposure to cigarette smoke causes systemic diseases [13, 77, 113]. For example, the vascular disease caused by cigarette smoking is associated with oxidation of low-density lipoproteins and their deposition in the vasculature with resultant dysfunction [49]. A depletion of systemic antioxidants has been documented in atherosclerosis.

Addressing the cell and molecular mechanism of disease after exposure to cigarette smoke, oxidative stress is postulated to initiate a series of cellular reactions that include activation of kinase cascades and transcription factors, release of inflammatory mediators, initiation of inflammation, and cell injury/apoptosis [89] (Fig. 8.2). Consequently, oxidative stress is the initiating factor in the pathway by which cigarette smoke exposure leads to disease. Key among the redox-sensitive transcription factors coordinating the inflammatory response to cigarette smoke are NF-κB (pro-oxidative) and Nrf-2 (anti-oxidative). The activation of these transcription factors is observed in both the lung and extra-pulmonary tissues [59].

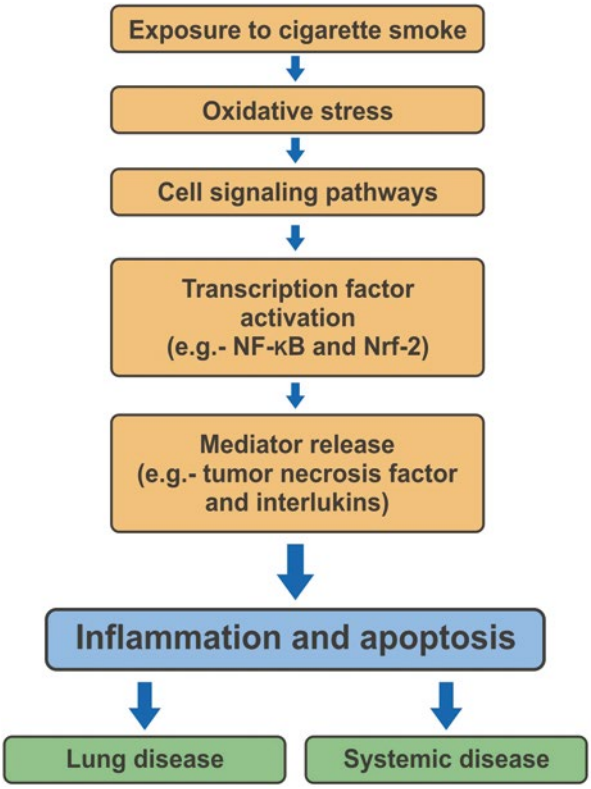

Fig. 8.2 Biological effect after exposure to cigarette smoking follows the generation of an oxidative stress. Oxidant generation activates MAP kinases and transcription factors resulting in a release of inflammatory mediators, inflammation, and apoptosis

Lung injury due to smoking (e.g., COPD and cancer) frequently does not diminish after smoking cessation and can rather progress in ex-smokers [97]. The basic cellular and molecular events underlying the biological effects of cigarette smoke and reasons for persistence of injury despite cessation of the exposure are not fully appreciated. Moreover, smoking cessation also does not eliminate the increased oxidative stress in the respiratory tract suggesting that retained particles may continue to participate in oxidant generation [71]. To explain this incongruity, as well as the observation that tissue injury in smokers is particle-related (Fig. 8.3a), it is proposed that tar disrupts iron homeostasis, increasing the availability of the metal and allowing it to participate in oxidative stress. Retained particles in the lung effectively complex the host iron (Fig. 8.3b). Humic-like substances, included in the particle, complex iron in vitro and accumulate the metal in vivo [44]. Both the humic-like substance and its iron complex generate free radicals, and some portion of this oxidant generation is metal-dependent. Following complexation of the metal by functional groups in the humic-like substance, the complexed iron contributes to electron transport, an increased catalysis of oxidants, and continued oxidative stress, despite smoking cessation. In support of a role for disrupted iron homeostasis in disease after exposure to cigarette smoke, there is increased lavage

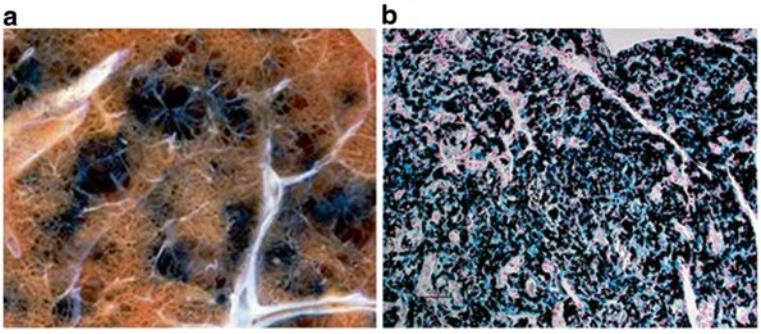

Fig. 8.3 Cigarette smoke exposure, iron, and lung injury. Lung collected at autopsy shows a correlation between the retention of cigarette smoke particles and destruction of lung parenchyma (i.e., bullous formation in this emphysematous patient) (**a**). A photomicrograph demonstrates that iron accumulates in the lung of a smoker and this appears to be particle-associated (**b**; Perls Prussian blue stain with the iron staining blue; magnification of about ×100)

iron concentration in smokers [108]. Accumulation of this metal in macrophages, proportional to the frequency and duration of cigarette smoking, has also been described among smokers [116].

8.3 Air Pollutants

Air pollutants are numerous but those considered significant include the particulate matter (PM), ozone, nitrogen oxides, and sulfur oxides (while measurable, atmospheric concentrations of carbon monoxide are extremely low). An oxidative stress is associated with exposures to all these pollutants.

Air pollution particles. PM in the atmosphere is a temporally and spatially shifting combination of particles that vary in size and chemical composition. These particles originate from both anthropogenic and natural sources. Anthropogenic contributions including organic carbon are greater in the urban environment. Approximately 40 % of particle mass in an urban setting can be attributed to fossil fuel use.

Exposure to air pollution particles is recognized to cause significant, adverse health effects in humans [84]. Epidemiological studies consistently demonstrate an association between increased levels of ambient air pollution particles and measures of human morbidity and mortality [32, 60, 83]. The target tissues for PM include the pulmonary and the cardiovascular systems; biological effects parallel those of cigarette smoking, with lung and cardiac disease predominating (Table 8.3).

Similar to exposure to cigarette smoke, production of oxidants is proposed as a unifying mechanism for the biological activity of PM [12]. Again, paralleling cigarette smoke, there is both direct generation of oxidants by the PM and an interaction between the particles and the host proteins. The PM produces an oxidative stress in both acellular and living systems. In acellular systems, air pollution particles

Table 8.3 Diseases associated with air pollution particles

Chronic obstructive pulmonary disease (COPD)
Pneumonia
Tuberculosis
Coronary artery disease, rhythm disturbances, and sudden death
Cerebrovascular disease
Cancer of the lung

directly generate oxygen-derived free radicals. The direct generation of oxidants by air pollution particles from a variety of ambient and emission sources is demonstrated using an assay for thiobarbituric acid-reactive products [43]. Hydroxyl radical production by particles is detected by salicylate hydroxylation [31], and this is confirmed by a sensitive fluorescence method [2]. Hydroxyl radical formation is eliminated under anaerobic conditions and in the presence of catalase while superoxide dismutase and deferoxamine decrease it. Electron spin resonance (ESR) has also shown oxidant generation by total suspended particulates collected from playgrounds of elementary schools [55], and in particles from a wildfire [64].

In vitro exposures of cells to air pollution particles result in oxidant generation in a wide range of cell types, including phagocytic, epithelial, and endothelial cells. Dusts collected from sewage incineration, a power station, and factories led to concentration-dependent increases in release of superoxide and hydrogen peroxide by alveolar macrophages [9]. Following incubation with diesel exhaust particle (DEP), macrophages released O_2^- as measured by chemiluminescence [80]. Similarly, polymorphonuclear granulocytes also increased generation of oxidants after exposure to ambient PM [54]; this effect was closely associated with the organic fraction of the particle and not altered by metal chelators. In contrast, epithelial cells exposed to an oil fly ash showed a concentration- and time-dependent induction of intracellular oxidants, as measured using dihydrochlorofluorescein, that was associated with metal components [30]. The same pulmonary epithelial cell line also generated intracellular oxidants following exposure to $PM_{2.5}$ [45]. Particles were found to produce a concentration-dependent induction of intracellular O_2^- using human aortic endothelial cells [69]. Flow cytometry showed that diesel particles increased MitoSOX red intensity specific for mitochondrial O_2^-. Further demonstration of oxidant production by the same particle was provided with increased protein carbonyl content and up-regulation of heme oxygenase-1 (HO-1) which could be inhibited by pretreatment with the antioxidant NAC. Finally, a comet assay demonstrated DNA strand breakage, reflecting oxidant exposure, following exposure of respiratory epithelial cells to PM [103]. Both coarse and fine PM fractions (defined as that PM with an aerodynamic diameter of 2.5–10 and 0.1–2.5 μm, respectively) elicited generation of hydroxyl radical and 8-hydroxy-2′-deoxyguanosine. This investigation demonstrated that many cell types responded to a range of air pollution particles by producing oxidants. The nature of the interaction between the cell and the particle that results in oxidant production remains unexplained.

In vivo oxidant production following exposure to different air pollution particles has been confirmed. ESR revealed in vivo production of free radicals in the lungs of animals exposed to an oil fly ash [57]. The soluble fraction of this specific particle, which contained high concentrations of vanadium (V), nickel (Ni), and iron (Fe) compounds, was responsible for both the production of 4-pyridyl-1-oxide-*N*-*tert*-butylnitrone (4-POBN) adducts detected by ESR (consistent with alkyl-type carbon-centered free radicals produced during lipid peroxidation) and the neutrophilic lung injury. Following intratracheal instillation of the same oil fly ash particle in rats, both the particle and individual component metals (vanadium, nickel, and iron) induced an increase in acetaldehyde in lung lavage fluid, further supporting the occurrence of in vivo, metal-catalyzed oxidative stress following air pollution particle exposure [74].

A time- and dose-dependent production of oxidants (i.e., O_2^- and H_2O_2) is shown to follow animal exposure to concentrated ambient air pollution particles [93, 94]. In vivo detection of oxidant production was delineated using chemiluminescence sensors and measurement of thiobarbituric acid-reactive substances in the lung. Indices of oxidative stress and biological effects in the animals were inhibited by NAC. ESR with spin trap also detected oxygen radicals in bronchoalveolar lavage fluid after intratracheal exposure of rats to DEP [4]. Finally, thiol antioxidants were shown to inhibit prooxidant effects of the DEP [117]. These data support the occurrence of in vivo oxidative stress in animals exposed to air pollution particles.

Oxidative stress after air pollution exposure of humans has been quantified using 8-hydroxy-2′-deoxyguanosine levels in urine, blood, and oral and nasal cells [92, 115]. Another study showed increased CO levels in the exhaled air of human subjects after exposure to DEP, which was attributed to induction of heme oxygenase-1 (HO-1) expression, likely to reflect an oxidative stress [78].

The components in air pollution particles that have been associated with oxidant generation include organic compounds and metals. Bacterial endotoxin was also proposed to contribute to oxidative stress after PM inhalation, but studies either do not support this relationship [6] or do not measure any index of oxidant generation. On the other hand, it may rather imply an affiliation since endpoints of biological activity (e.g., inflammation) may correspond to endotoxin content [8]. Air pollution particles contain a wide variety of organic substances including polycyclic aromatic hydrocarbons (PAH) and nitro-PAH, olefins, aldehydes, ketones, nitro-compounds, and quinones [19, 110]. These organic molecules are most commonly found in the smaller size fractions and combustion products of ambient air pollution particles [67]. In vitro oxidant generation by ultrafine particles (defined as PM with an aerodynamic diameter of less than 0.1 μm) correlates with the concentration of organic compounds [67]. The basis for this relationship between organic compounds and oxidant generation following exposure to ambient air pollution particles is postulated to involve the following (Fig. 8.4):

1. A quinone-based radical that directly involves redox-cycling with hydroquinone and semiquinone structures [61]. Using ESR, samples of PM collected from sites around the United States were found to have large quantities of radicals with

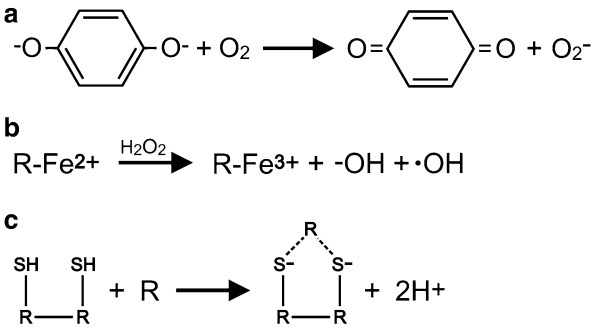

Fig. 8.4 Oxidant generation by organic components in air pollution particles. Potential pathways of oxidant generation by organic compounds in particulate matter include cycling of quinone with hydroquinone/semiquinone structures (**a**), direct redox-cycling by the organic compound following complexation of a metal (**b**), and depletion of thiols following reaction with quinones (**c**)

characteristics similar to semiquinones [28, 106]. Furthermore, organic compounds included in ambient air pollution particles may be converted to redox-cycling quinones through a number of different host proteins [29, 79].

2. Direct redox-cycling by the organic compound following complexation of a metal [53, 88].
3. A reaction between quinones and thiol-containing compounds with depletion of protective nucleophiles [21, 119].

There can also be a generation of oxidants following an interference of normal mitochondrial electron transfer by organic compounds [68, 119]. Several organics are postulated to support inappropriate electron transport between complexes I and III, suggesting PM redox-cycling with a capacity to disrupt the Q cycle; this might result in increased formation of O_2^- [35, 119]. Therefore, one consequence of exposure to organic compounds leading to abnormal electron transport would be disruption of normal mitochondrial function. This was documented following in vitro exposure to PM with organic compounds including a depolarization of the inner membrane of the mitochondria, changes in the mitochondrial permeability transition pore, and disordered ATP synthesis [119]. These studies support a role for organic components of particles in generating oxidative stress in cells, tissues, and living systems following exposure to PM.

Many types of air pollution particles include transition metals which contribute to oxidant generation [26, 104]. Metal analysis of air pollution particles sequestered on PM filters from sites around the United States revealed that Fe was that transition metal present in the highest concentration [43]. In atmospheric particulates, quantities of Fe can regularly be found in concentrations approximately tenfold higher than all others and therefore this metal may assume greater importance. Despite the variety of geographic locations, the concentrations of individual metals in ambient air pollution particles correlated well with each other [43]. Metals which exist in more than one stable valence state can catalyze an electron transfer and therefore demonstrate a capacity to directly generate oxidants [41, 114]

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^-$$

$$Fe^{2+} + O_2^- + 2H^+ \rightarrow Fe^{3+} + H_2O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + -OH$$

In catalyzing the Fenton reaction, ferrous iron (Fe^{2+}) reduces hydrogen peroxide (H_2O_2) with the formation of hydroxyl radical and oxidation of ferrous iron to ferric iron (Fe^{3+}). Hydrogen peroxide and reductants are also required to drive such oxidant generation and these are available in the respiratory tract. In addition to this direct production of oxidants, metals react with thiols to diminish protective capacity and potentially affect an oxidative stress in this manner.

Oxidant production by PM in an acellular system correlated positively with concentrations of Cr, Mn, Fe, Co, Ni, and Cu [85]. Deferoxamine and hydroxyl radical scavengers inhibited oxidant generation implicating hydroxyl radical production. The direct hydroxylation of salicylate after in vitro exposure to air pollution particles supported metal-dependent $^{\bullet}OH$ production [31]. The soluble fraction of residual oil fly ash, which is abundant in metals, was shown to be capable of generating metal-dependent hydroxyl radicals in a cell-free system [18]. In other investigation, malondialdehyde formation was measured after incubation of ambient PM collected from Washington, DC [7]; the metal chelator deferoxamine inhibited this aldehyde formation supporting metal participation in oxidant generation.

In vitro exposure of rat alveolar macrophages to residual oil fly ash increased cellular production of oxidants, and this was inhibited by the addition of either the metal chelator deferoxamine or hydroxyl radical scavengers [42]. Addition of individual metals to the alveolar macrophages, as both soluble sulfates and those complexed to the surface of a latex bead, increased the oxidative burst. Incubation of alveolar macrophages with dusts from sewage sludge incineration, an electric power station, and factories led to a release of hydrogen peroxide by alveolar macrophages exposed to particles [9]. The release of hydrogen peroxide correlated best, in descending order, with the content of Fe, Mn, Cr, V, and As, in the dusts. Respiratory epithelial cells exposed to an oil fly ash showed a concentration- and time-dependent induction of intracellular oxidants triggered by metal components [30]. Finally, after exposure to an air pollution particle collected in Denver, alveolar macrophages generated oxidants which correlated with Fe in the PM [127]. It may be concluded that both parenchymal and nonstructural cells in the lung show evidence of metal-catalyzed oxidative stress following their in vitro exposure to PM.

ESR investigation supports the occurrence of lipid peroxidation in the lung, initiated by metal-catalyzed hydroxyl radical following in vivo exposure to an air pollution particle [57]. The oxidative capacity of PM-associated metals was also demonstrated in human investigation. Instillation of metal-rich ambient particle into the lungs of healthy subjects resulted in airway inflammation characterized by increased oxidants and cytokine production, as well as infiltration [101]. Studies using particles collected in Utah Valley also suggested a role for metals [38].

The closure and reopening of a steel mill impacted the composition of these particles; when the steel mill was operating, the PM contained significantly higher concentrations of metals. Aqueous extracts from the PM with higher concentrations of metals presented an oxidative stress in vitro and increased IL-8 and IL-6 release by respiratory epithelial cells [36]. Human exposure to the same aqueous extracts with higher concentrations of metals caused neutrophilic inflammation in the lower respiratory tract and increased lavage IL-8 and TNF-α levels [39]. This investigation supports a potential participation of metals in oxidant generation after PM exposure. Metals might be involved by directly supporting electron transport producing oxidants. However, it should be noted that protective mechanisms against such metal-catalyzed oxidant generation are extensive and direct generation is unlikely. Furthermore, studies show that metals without the capacity for such electron transfer (e.g., zinc) still present an oxidative stress and their inclusion in PM also enhances oxidant generation [1, 46]. The mechanism may involve depletion of antioxidants.

PM exposure is not simply the result of "bystander injury" with organic and metal components supporting electron transport and generation of destructive oxidants. Cells are not passive victims but produce oxidants in response to air pollution particles. Cell exposures to particles are associated with activation of NADPH oxidases (NOXs), which constitute an important source of oxidant generation in both the alveolar environment and in the vasculature. NOXs comprise a family of enzymes that are multi-subunit enzyme complexes generating O_2^-. In phagocytic cells, the oxidase consists of two membrane-bound subunits (gp91phox also known as NOX2 and p22phox), as well as three cytoplasmic subunits (p40phox, p47phox, and p67phox), and a small GTPase Rac1/2 [5]. Following recruitment of cytoplasmic subunits to the membrane where they interact with gp91phox and p22phox, the protein is assembled and activated to generate large amounts of O_2^- [10]. Non-phagocytic cells contain one or more alternate NOX proteins (NOX1–5) which may be similarly activated [3, 50]. After exposure of mice to concentrated ambient particles from Manhattan, aortic expression of NADPH oxidase subunits rose and was paralleled by elevation in O_2^- generation [124]. Recently, participation of NADPH oxidase, and its oxidant generation, in the biological activity of PM has been demonstrated in mice using inflammatory endpoints, adiposity, and insulin resistance [58, 121].

The generation of an oxidative stress is fundamental to the response of cells, tissues, and living systems to air pollution particles. This is comparable to cigarette smoke and the proteins involved can be identical. PM exposure introduces an oxidative stress which leads to a series of reactions including phosphorylation-dependent cell signaling (ERK, p38, and Jun kinases) [3, 20, 47, 69, 99, 105, 109], transcription factor activation [65, 66, 120], and an increased expression of pro-inflammatory mediators. The final product is an inflammation (pulmonary and systemic), and apoptosis; if the response is prolonged, fibrotic and neoplastic injuries can result.

Ozone. Ozone (O_3) is a secondary air pollutant formed in the atmosphere through a photochemical reaction requiring sunlight, hydrocarbons, and nitrogen dioxide. Relative to rural regions, O_3 concentrations are higher in trafficked/urban areas; indoor levels are increased relative to the outdoor values. Ozone reacts and depletes thiol compounds. After exposure to ozone at atmospheric concentrations

(60–120 ppb), some percentage of the human population (10–15 %) will demonstrate decrements in respiratory function. In addition to this airway hyperreactivity, respiratory tract inflammation can be observed after O_3 exposure. Those with preexisting lung disease can suffer exacerbations of their condition following ozone exposure. More recently, cardiovascular disease and elevated rates of mortality were reported to be associated with ozone levels.

O_3 depletes antioxidants and causes lipid peroxidation to affect an oxidative stress [123]. Exposure increases ozonides, aldehydes, organic and hydrogen peroxides, and organic radicals which may further promote oxidative damage. Ozone exposures initiate the same cell and molecular pathway of inflammation common to cigarette smoking and air pollution particles with activation of oxidant-sensitive MAP kinases and transcription factors culminating in the release of pro-inflammatory mediators [34].

Nitrogen and sulfur oxides. Oxides of nitrogen include nitric oxide (NO), nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), nitrogen tetraoxide (N_2O_4), and nitrogen pentoxide (N_2O_5). Since these various forms occur together and are interconvertible, NOx is used to describe their combined presence. NO and NO_2 are primary pollutants for which motor vehicles are the major contributors. Indoor cooking with fossil fuels, gas appliances, and cigarette smoke also constitutes a significant source. NO in the atmosphere reacts with oxygen to form NO_2, which reacts with water to yield a solution containing a mixture of nitrous and nitric acids (HNO_2 and HNO_3, respectively). Regarding health effects, NO_2 is considered to be the most important NOx; 500 ppm or greater of NO_2 precipitates pulmonary edema, while decreased exposure can result in broncholitis obliterans and pneumonitis. Human exposures to nitrogen dioxide have some potential to produce an inflammatory reaction in the lung.

NOx exposures cause an oxidative stress in the lung. Lipid peroxidation in cells in animals exposed to NO_2 has been confirmed; antioxidants can protect against cell and tissue damage induced by NO_2 [123].

SO_2 reacts with O_2 to produce sulfate; both SO_2 and SO_4 concentrations are associated with acute bronchoconstriction and increased rates of morbidity and mortality in humans. Asthmatics appear to be more sensitive to the effects of SO_2 exposure. Effects of SO_2 are observed at significantly higher levels relative to the low levels of ozone exposure. Exposures are associated with oxidative stress but there is far less evidence to support this relationship, relative to PM and ozone [123].

Conflict of Interest The author reports no conflict of interest.

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Chapter 9
Air Pollution and Oxidative Stress in Allergic Airway Diseases

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9.1 Introduction

The increase in the prevalence of allergic diseases to epidemiological proportions is of great concern both in developed and developing countries [1]. This dramatic rise in prevalence of allergic diseases includes asthma, rhinitis eczema, and potentially life-threatening allergies to certain foods, drugs, or other substances. The *World Allergy Organization White Book on Allergy* estimates that about 30–40 % of the world's population is affected by one or more allergic conditions. What is of greatest concern is that this increase is especially affecting children and young adolescents and that the severity and complexity of these diseases are increasing. The increase in prevalence of these diseases contributes to patient morbidity and mortality as well as to increased cost in the form of medications, hospitalizations, health care utilization, and school/work absenteeism. Among the various factors that are considered to contribute to this rise in prevalence of allergic diseases are the hygiene hypothesis, change in lifestyles, urbanization, environmental pollution, climate change, and reduced biodiversity. Of these factors, the hygiene hypothesis, clime change, and environmental pollution have been attracting attention as important contributing factors [2].

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9.2 Air Pollution and Allergic Airway Diseases

While the precise mechanisms underlying this increase in prevalence of allergic diseases and asthma are not fully elucidated, epidemiological research suggests a causative relationship between air pollution and the increase in incidence of these diseases as well as in the exacerbation of allergic airway diseases. This involves two aspects: the impact of the pollutants on the airways and the host susceptibility. The pollutants include ozone, nitrogen dioxide, and particulate matter, produced by traffic- and industry-induced pollution. Experimental studies have elucidated some of the cellular and molecular mechanisms on how these pollutants induce adverse effects in allergic diseases [3, 4]. Air pollution-induced oxidative stress aggravates airway inflammation by inducing the production of proinflammatory mediators, enhancing bronchial hyperresponsiveness, stimulating bronchospasm, and increasing mucin secretion. Additionally, recent gene–environmental interactions demonstrate the host susceptibility to air pollutants.

Air pollution has a considerable impact on allergic airway-related morbidity and mortality. In a study done in Mexico city, traffic-related air pollution was shown to have adverse effects on the respiratory symptoms and pulmonary function in asthmatic children [5, 6]. In the same study, the researchers found that PM2.5, NO_2, and ozone concentrations were significantly related to an increased incidence of asthma exacerbations. In a prospective cohort study of 8,111 adults in six US cities, fine-particulate air pollution, or a more complex pollution mixture associated with fine-particulate matter, contributed to excess mortality [7]. Exposure to ozone at concentrations found in ambient air was found to be associated with a reduction in lung function and induction of respiratory symptoms including cough and shortness of breath [8–11]. NO_2 concentrations in ambient air were found to be associated with cough, wheezing, and shortness of breath in children. Moreover, urban air pollution may have lasting adverse effects on lung development in children and diminished lung function in adults [12–15] and reduced exposure to PM10 has been shown to attenuate age-related decline in lung function after 11 years [16, 17].

Ozone is one of the most common air pollutants shown to be a trigger for asthma exacerbations in children, even at levels below the US Environmental Protection Agency (EPA) standards of 120 ppb (1 h average) and 75 ppb (8 h average) [18–21]. Studies have shown that among asthmatic children aged 6–18 years, there was a 20 % increase in general hospitalizations and a 19 % increased risk for ICU admissions for each 22 ppb increase in ozone [22]. Particulate matter, such as diesel exhaust particles (DEPs), is another important environmental pollutant that leads to an increased risk for asthma development and exacerbation [23–26]. Diesel exhaust contains small particles that range in size from nanoparticles to coarse particles consisting of a carbonaceous core with a large surface area to which chemicals are adsorbed. These include organic compounds such as polycyclic aromatic hydrocarbons (PAHs), nitro derivatives of PAHs, oxygenated PAH derivatives (ketones, quinones, and diones), heterocyclic compounds, aldehydes, and aliphatic hydrocarbons. PAHs and their oxygenated derivatives (e.g., quinones) can generate reactive oxygen species (ROS) in target cells.

The processes by which DEPs exert their influence on the airway cells have been partially elucidated [27–29].

Endotoxin, also known as LPS, is a component of Gram-negative bacteria and derived from animals and agricultural activities that have been associated with asthma exacerbations and increased prevalence of asthma in early childhood [30, 31] and exposure to house dust LPS has been shown to have a synergistic effect [32]. A meta-analysis has shown that LPS exposure correlated positively with wheeze in infants and toddlers [33]. Furthermore, both LPS and ozone can enhance sputum neutrophilia, as LPS acts via the toll-like receptor 4 (TLR4) on the cell surface on macrophages. Experimental animal models suggest that at least some ozone responses are mediated through TLR4 [34–36]. Toll-like receptors recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [37] and are critical in initiating inflammatory responses to a variety of stimuli, leading to the production of proinflammatory mediators such as IL-1β, TNF-α, and IL-6. Hyaluronic acid (HA), a glycosaminoglycan that is a component of the airway epithelial extracellular matrix [38, 39], is present on the apical surface of airway epithelial cells; its low molecular weight form is an endogenous ligand for TLR4 [40, 41]. In the airway epithelium, low molecular weight fragments can be generated by ROS-induced depolymerization of hyaluronan [42, 43], and by hyaluronidase activity associated with upregulation of TNF-α in concert with IL-1β [44]. Several groups have shown that low molecular weight fragments of HA have proinflammatory actions [45] and that they are increased in bronchoalveolar lavage fluid after ozone challenge via CD44 [39] and TLR4 [38] signaling mechanisms. Also studies have shown that normal volunteers, allergic nonasthmatics, and mild allergic asthmatics had increased HA in their respiratory tract lining fluid after ozone exposure [46].

Airway eosinophilia and increased sensitivity to allergen have been shown to be enhanced by ozone [47–49]. In a study of allergic asthmatics and normal volunteers, only the allergic asthmatics had an increased cell surface expression of TLR4 on macrophages in induced sputum and increased levels of the proinflammatory cytokines IL-1β, IL-6, and IL-8 in the respiratory tract lining fluid after ozone exposure [46]. Compared to normal volunteers, allergic asthmatics showed increased immune signaling involving the NF-κB network [50], in concert with the concept that asthmatics have increased innate immune activation after ozone exposure. Ozone exposure has also been linked to the frequency of hospital admissions [51, 52], worsening of symptoms, and need for rescue medication [26] as well as asthma attacks, respiratory infections, and reductions in peak flow rate [53]. Future work will need to focus on mechanisms explaining this enhanced innate immune response in asthmatics.

9.3 Oxidative Stress and Susceptibility to Air Pollution

Oxidative stress is considered to be a toxic byproduct of aerobic metabolism and a factor involved in tissue damage. Compounds involved in oxidative stress, such as H_2O_2, act as key molecules in signal transduction. Many types of stimuli, including

allergens, infections, various chemical mediators, and growth factors, can induce a transient increase in intracellular ROS—in particular H_2O_2—mainly through activation of NADPH oxidase immediately after the exposure of the stimuli to cells. Dynamic changes in intracellular levels of H_2O_2 may lead to various intracellular signaling events. Under low levels of oxidative stress, appropriate antioxidant defense systems act through the activation of Nrf2. If a higher level of oxidative stress occurs, inflammatory cellular responses are induced by the activation of AP-1 and NF-κB leading to the secretion of proinflammatory cytokines and chemokines. The highest level of oxidative stress causes serious cytotoxic effects, including apoptosis and necrosis. Thus, the maintenance of intracellular ROS in a proper range is crucial in the management of various inflammatory diseases.

As air pollutants promote the production of radical oxygen species in the airways, genetic polymorphisms may further amplify airway inflammation and hyperreactivity in response to environmental agents. A number of intracellular antioxidant enzymes including NQO1, GSTM1, GSTP1, and HO-1 regulate cellular and mucosal oxidant stress [42–46]. These enzymes are regulated by the transcription factor Nrf2. Cells that encounter oxidative stress activate Nrf2 binding to the antioxidant response element (ARE), leading to the transcription of a broad range of antioxidant genes. This cellular response is designed to defend against the harmful effects of oxidative agents. The GSTM1, or glutathione-*S*-transferase Mu1, null genotype has been associated with increased response to environmental agents. Studies have demonstrated an increased risk of acute exacerbation of asthma in response to ozone exposure in subjects with the null genotype [47]. Dillon et al. demonstrated that subjects with the GSTM1 null genotype have an increased inflammatory response with elevated levels of IL-1β and TNF-α in the sputum to inhaled LPS (at 20,000 endotoxin units) [54]. Thus genotypic differences may be an important factor in explaining why some people are more susceptible to endotoxin exposure than others. Subjects with the null genotype for GSTM1 and GSTP1 codon 105 variants had enhanced nasal allergic responses in the presence of DEPs demonstrating that GSTM1 and GSTP1 can enhance the effect of air pollutants on allergic inflammation [55, 56]. Subjects with the GSTMI null genotype have increased neutrophil influx and increased IL-8 production into the airways following exposure to ozone [57, 58].

NO_2 exposure is associated with increased emergency room visits, wheezing, and medication use among children with asthma [59, 60]. Controlled exposure studies of asthmatics have found that NO_2 can enhance the allergic response to inhaled allergens [61, 62]. The authors also identified the impact of diesel-engine traffic on asthma wheezing and bronchodilator usage.

Among adults with asthma, exposure to traffic loads has been associated with lung function and health status [63]. This causal relationship between worsening asthma and diesel traffic pollution was clearly demonstrated by McCreanor et al. [64], where walking along Oxford Street (more diesel exhaust) induced asymptomatic but significantly greater reductions in FEV1 (up to 6.1 %) and forced vital capacity (up to 5.4 %) than did walking through Hyde Park. This effect was greater in subjects with moderate asthma than in those with mild asthma and accompanied

by increases in sputum myeloperoxidase and airway acidification in exhaled breath condensates (EBC). A sub-analysis was performed among moderate and severe asthma patients, and revealed that EBC biomarkers were correlated with PM10 concentration.

Three birth cohort studies from Germany, Holland, and Sweden followed children until the age of 4 or 6 years and suggested a positive relationship between traffic-related pollution and physician-diagnosed asthma [65–67]. A Japanese cohort study also reported an association between NO_2 levels and asthma incidence [68]; the authors studied the effects of air pollution on the prevalence and incidence of asthma among 2,506 children over a period of 4 years, and found that children living less than 50 m from heavily trafficked roads were more likely to develop asthma. They also reported a possible link between asthma development and increased concentrations of PM10.

9.4 Effect of Air Pollutants at Cellular Level and Oxidative Stress

Ohtoshi et al. [69] reported that exposure to DEP in vitro stimulates human airway epithelial cells to produce cytokines relevant to airway inflammation. Bayram et al. [70] reported that exposure to DEP in vitro induced bronchial epithelial cells (BECs) to release interleukin-8 (IL-8), granulocyte–macrophage colony-stimulating factor (GM-CSF), regulated upon activation, normal T-cell expressed, and secreted (RANTES), and soluble intercellular adhesion molecules (ICAM)-1. BECs from asthmatic patients constitutively released significantly greater amounts of cytokines than did those from non-asthmatic individuals. DEPs upregulated expression of the ICAM-1 gene in human BECs [71]. Research has also demonstrated that DEP-induced IL-8 production was regulated at the transcriptional level [72]. Additionally, DEPs have been observed to induce eotaxin gene expression in BECs [73], although another report refutes this [74]. DEPs induced dose-dependent activation of nuclear factor (NF)-κB in human BECs, as identified using an electrophoretic mobility shift assay [73]. Studies using reporter assays with normal and mutated IL-8 promoters indicated that IL-8 gene transcription was induced via NF-κB activation. Hashimoto et al. [75] reported that DEP-induced activation of p38 mitogen-activated protein kinase (MAPK) plays an important role in the production of IL-8 and RANTES. Other reports demonstrated the importance of other intracellular signal transduction pathways, such as mitogen-activated protein kinase kinase (MEK)-1 [76] and c-Jun N-terminal kinase (jnk) [77], in DEP-stimulated human BECs and macrophages. DEP has been shown to have a direct impact on airway epithelial cells not only enhancing the release of proinflammatory cytokines but also upregulating co-stimulatory molecules like CD86 and HLA-DR and also enhancing antigen presentation [78]. Zhang et al. [79] investigated the effects of DEPs on expression of fra-1, a heterodimeric partner of activator protein-1, in a murine lung epithelial cell line and found that DEPs markedly upregulated expression of fra-1 but not fra-2.

Overexpression of fra-1 downregulated c-Jun, nuclear factor-like 2 (Nrf2) enhanced activator protein-1, and ARE mediated reporter gene expression, respectively. fra-1 induction by DEPs may play a role in the selective regulation of expression of genes involved in alveolar epithelial cell injury and repair. Blanchet et al. [80] reported that PM2.5 and DEPs induced the expression and secretion of amphiregulin, an epidermal growth factor receptor (EGFR) ligand in BECs. Amphiregulin secretion was mediated by activation of the EGFR and extracellular signal-regulated kinase/MAPK pathways. Exposure to diesel exhaust (PM10, 300 μg/m^3) enhanced EGFR expression and phosphorylation of tyrosine 1173 [81]. These findings suggest that EGFR plays a key role in the bronchial response to diesel exhaust fumes. Long-term DEP exposure may lead to an airway remodeling process in asthmatics [82]. More profound adverse effects have also been reported, such as effects on the cardiovascular system [83].

DEP-induced IL-8, ICAM-1, GM-CSF, and RANTES expression is inhibited by antioxidant agents such as *N*-acetyl cysteine (NAC) and pyrrolidine dithiocarbamate, so ROS may be involved in this induction [73, 84]. DEP-induced NF-κB activation was completely inhibited by pretreatment with NAC [84]. DEP-induced activation of MAPK pathways was also blocked by NAC and pyrrolidine dithiocarbamate [75]. These observations suggest that DEP-induced activation of signal pathways and transcription factors is a result of ROS, derived both primarily and secondarily from DEPs. Bonvallot et al. [85] found that the ability of DEPs to induce GM-CSF expression was almost completely eliminated after washing the DEPs, suggesting the importance of adsorbed chemicals. While exposure to DEPs induces proinflammatory gene in human airway epithelial cells, benzene-extracted components had effects that mimicked those of DEPs on the release of several cytokines (IL-8, GM-CSF, RANTES), and NF-κB activation [86, 87].

The role of ROS generated directly and indirectly by exposure to DEPs has been well studied. The ROS play an important role in proinflammatory reaction in airways. Nuclear erythroid 2 P45-related factor Nrf2 is a key transcription factor that regulates host antioxidant and contributes to regulate airway inflammation and exacerbation of allergic inflammation induced by DEPs. C57BL/6J Nrf2−/− mice exposed to low-dose DEPs for 8 weeks showed significantly increased AHR and lymphocyte and eosinophil counts, together with increased IL-12, IL-13, and thymus and activation-regulated chemokine concentrations in bronchoalveolar lavage fluid than wild-type mice. In contrast, expression of antioxidant enzyme genes was significantly higher in wild-type mice than in Nrf2−/− mice [88]. These results strongly suggest that DEP-induced oxidative stress and host antioxidant responses are regulated by Nrf2. Furthermore, the responsiveness of the Nrf2-directed antioxidant pathway acts as a major determinant of susceptibility to allergen-mediated asthma [89]. These findings suggest that the synergistic effects of the oxidative stresses caused by DEPs and allergens contribute to the major pathways underlying exacerbation of allergic asthma.

It is important to develop methods of identifying susceptible individuals within a large population and to evaluate whether measurement of airway inflammatory biomarkers in EBC would be an appropriate method of assessment.

9.5 Conclusion

Epidemiological studies have shown that air pollutants are involved in the pathogenesis of allergic airway diseases such as asthma and rhinitis, both in terms of their development and exacerbation. Air pollution-induced oxidative stress is increased in allergic airway diseases like asthma, and this can be a critical contributor to asthma development and can initiate various intracellular signaling pathways that lead to a break in immune tolerance and exaggerated allergic inflammation. Recent increases in the incidence of asthma may be attributed not only to increased oxidative stress in the environment but also to host susceptibility. Controlling oxidative stress is critical for effectively managing asthma. Development of a safe and rapid method of screening individuals for their susceptibility to air pollution is also an important strategy.

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Chapter 10
Pulmonary Fibrosis and Oxidative Stress

Corrine R. Kliment and Tim D. Oury

Abbreviations

BALF	Bronchoalveolar lavage fluid
ECSOD	Extracellular superoxide dismutase
IPF	Idiopathic pulmonary fibrosis
MBD	Matrix binding domain
MPO	Myeloperoxidase
NAC	*N*-acetylcysteine
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

10.1 Introduction

The lung is continually exposed to higher oxygen levels than other tissues. Furthermore, exogenous oxidants, as well as pollutants, can augment oxidant production and activate inflammatory cells to generate additional reactive oxygen and nitrogen species. The lung protects itself against these oxidants with protective antioxidants and antioxidant enzymes. Various disease states in the lung involve

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dysregulation of this balance through excessive oxidant production or decreases in antioxidants. Idiopathic pulmonary fibrosis (IPF) is a lung disease characterized by progressive fibrosis of the alveolar interstitium [1, 2]. Reactive oxygen species (ROS) and markers of oxidative stress are evident in human IPF [3, 4] and levels of ROS negatively correlate with pulmonary function in IPF and may predict disease severity [5].

10.2 Idiopathic Pulmonary Fibrosis

10.2.1 Diagnosis and Pathological Findings

IPF is an interstitial lung disease characterized by severe and progressive fibrosis of the alveolar interstitium. In the United States, the prevalence of IPF is estimated to be 42.7 per 100,000 and the disease incidence to be 16.3 per 100,000 [6]. Patients develop symptoms of dyspnea (shortness of breath) and nonproductive cough with presentation between 50 and 70 years of age. IPF is slightly more common in males than females [2] and has a dismal prognosis with a 5-year mortality rate between 50 and 70 % [1, 7].

From the time of IPF diagnosis, there is a mean survival of 3–5 years [1, 7]. A diagnosis of IPF is made from a thorough history and physical exam, chest radiography, pulmonary function tests, high resolution computed tomography (CT), and lung biopsy [1, 7]. Patients typically present with a history of greater than 3 months of dyspnea and a nonproductive cough. On physical exam, bilateral dry inspiratory crackles may be appreciated at the lung bases. Chest radiography shows ground glass opacities and CT analysis shows irregular thickening of the alveolar septa. As fibrosis of the lung progresses, the normal lung architecture becomes distorted under the tension of the fibrosis. This change is often described as a “honeycomb” appearance of the lung [8].

The gold standard of diagnosis of IPF is still pathologic examination of lung tissues. Histologically, IPF has a pattern of usual interstitial pneumonia (UIP), which is characterized by areas of immature and mature fibrosis (temporal heterogeneity) and alveolar inflammation with intervening areas of normal tissue architecture [8]. On H&E staining, myofibroblastic foci are present, which are light-staining areas of spindle-shaped mesenchymal cell expansion among collagen and matrix deposition (Image 10.1). These foci are randomly dispersed throughout the lung and are a marker of active disease [8] with myofibroblasts that are producing collagen and matrix components. Inflammation is also present and is assessed through bronchoalveolar lavage and interstitial microscopy, which shows the presence of macrophages, neutrophils, eosinophils, mast cells, and lymphocytes [2, 8]. The role of these cell types in the disease process is unclear.

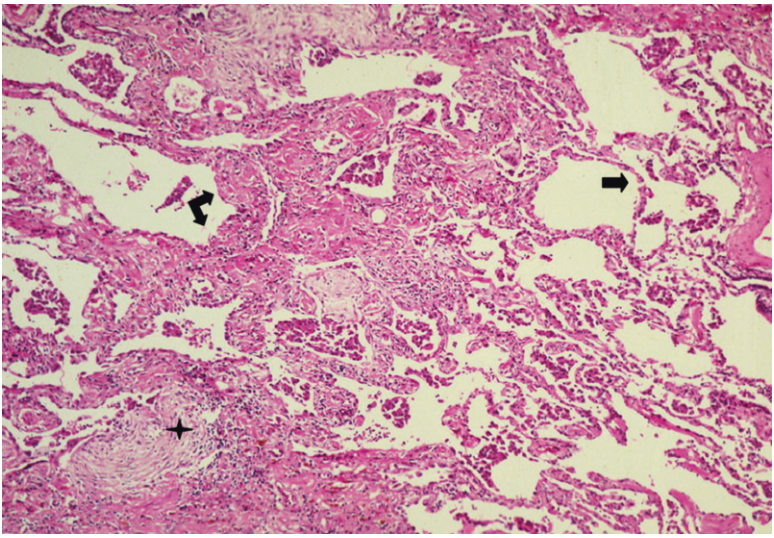

Image 10.1 H&E staining of human lung with pulmonary fibrosis demonstrating temporal heterogeneity with areas of normal thin lung parenchyma (*black arrow*) and collagen deposition in areas of fibrosis (*double headed arrow*). The fibroblastic foci are also present representing an area of active remodeling and myofibroblast activity (*star*). Image courtesy of Dr. Tim Oury

10.2.2 Pathophysiology

Pulmonary fibrosis can occur in various situations: due to unknown stimuli (idiopathic); environmental/occupational exposure, i.e., asbestos, silica; induced by pharmacological agents, i.e., bleomycin; radiation exposure; and associated with other primary diseases such as collagen vascular diseases or familial forms [2, 7]. These variations of IPF differ by their proposed pathogenic factors. The underlying processes of pulmonary fibrosis are currently thought to involve the presence of persistent stimuli or injury, aberrant wound healing, and dysregulated repair/remodeling of the lung that results in fibrosis (Table 10.1).

Epithelial injury is thought to be one of the initial steps in the pathogenesis of pulmonary fibrosis. Furthermore, analysis of UIP lung biopsies revealed a significant loss in type I epithelial cells in fibroblastic foci and areas of lung deterioration, along with increases in epithelial apoptosis markers [39]. Experimental animal models also support a role for apoptosis of alveolar epithelial cells modulated by the Fas–Fas ligand system in the pathogenesis of pulmonary fibrosis [40–42]. After epithelial cell death, the basement membrane of the alveolar surface is left denuded and exposed. Epithelial wound healing or re-epithelialization is a concerted effort by various cells types to restore the lung after an injury or cell death. Mesenchymal cells, such as fibroblasts and myofibroblasts, promote new matrix synthesis to form

Table 10.1 The various types of pulmonary fibrosis and proposed pathogenesis

Type of pulmonary fibrosis	Stimuli	Proposed pathogenic factors
Idiopathic	Unknown	Aberrant wound healing [9, 10]; profibrotic proteins, i.e., TGF-β [11–14]; oxidative stress [15–17]; initial inflammation [18–20]
Environmental, occupational	Asbestos; silica; paraquat	Particle transition metals, i.e., free iron, and oxidative stress [21, 22]; inflammation [23–25]; profibrotic proteins, i.e., TGF-β [22, 26]
Pharmacologic agents	Bleomycin; amiodarone	Agent-induced alterations in oxidants/antioxidants [27–29]
Radiation-induced	Radiation therapy to the chest	Oxidative stress [30]; loss of antioxidants [31]; profibrotic proteins, i.e., TGF-β [32, 33]; inflammation [34]
Hereditary familial IPF	Genetic mutations	Surfactant protein C and A1 [35], TERT and TERC genes [36]
Collagen vascular diseases	Collagen abnormality	Autoimmune tissue injury and aberrant matrix deposition [37, 38]

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a suitable scaffold upon which epithelial cells can repopulate lost cells. This process is thought to be disrupted and dysregulated in the development of IPF. Transforming growth factor-β (TGF-β) is a profibrotic protein released primarily by alveolar epithelial cells and macrophages within the lung. TGF-β has multiple functions including inducing the expression of collagens, proteoglycans, and matrix components by fibroblast/myofibroblasts, is chemotactic to macrophages and fibroblasts, and can lead to the development of fibrotic lesions [11, 13, 43, 44].

While the pathogenesis of IPF remains unclear, inflammation and oxidant/antioxidant imbalances within the lung are believed to be involved [5, 45]. The role of inflammation has not been delineated in human disease and is a controversial issue, which is likely one reason why many investigators consider pulmonary fibrosis to be primarily a disease of abnormal wound repair. Therapeutic studies that target inflammation, such as corticosteroids, have failed to show clinical benefits [46–48]. However, several studies have highlighted associations between the presence of inflammatory cells and disease prognosis. Neutrophilia and eosinophilia are seen in the bronchoalveolar lavage fluid (BALF) of 70–90 % and 40–60 % of IPF patients, respectively [7]. This increase in inflammatory cells has been associated with a worse prognosis and mortality in some clinical studies [18, 49, 50]. Immune activation and inflammation have been shown to play an important role in fibrosis models [25, 51].

Inflammatory cells can damage the lung through the release of oxidative species, proteases (i.e., matrix metalloproteinases [MMPs], elastase), peroxidases (i.e., myeloperoxidase [MPO]), cytokines, and growth factors [52, 53]. These findings collectively suggest that inflammation, while not necessarily the primary mechanism of IPF pathogenesis, may contribute to a profibrotic environment by affecting

the wound repair process, level of oxidative stress, and the extent of remodeling. Studies support that chemotactic factors and neutrophils are present in IPF [20, 54] and increase the likelihood of disease progression and lack of response to immunosuppressive agents [1, 18, 55]. In IPF, inflammatory cells may also release exaggerated amounts of ROS [17].

Inflammatory cells may impact IPF pathogenesis by enhancing the oxidative imbalance in the lung. Studies show increased ROS production in leukocytes from the serum and evidence of enhanced oxidative stress in the plasma and BALF of IPF patients [3, 4]. Levels of oxidative stress have been shown to negatively correlate with aspects of pulmonary function in IPF patients and may provide information about disease severity [5]. Given this evidence, free radicals are thought to play an important role in IPF pathogenesis, potentially through both direct and indirect mechanisms.

10.2.3 Oxidative Stress in the Lung

Oxidative stress is frequently defined as the imbalance of oxidant production and antioxidant defenses, where oxidants dominate and lead to cellular dysfunction and tissue damage. When considering oxidative stress, the lung is somewhat unique due to its exposure to relatively higher oxygen tensions than other tissues. The oxygen pressure of inhaled air is 20 kPa (150 mmHg). Pressures in venous blood flow are around 6 kPa (45 mmHg) and may be as low as 0.13 kPa in some tissues, while the oxygen at the alveoli of the lung is ~13.3 kPa (100 mmHg) [56]. Thus, the lung is constantly facing relatively high oxygen tensions, which may augment oxidative insults. Under normal conditions, the ability of the lung to maintain an oxidative balance and a nontoxic pulmonary environment is likely due to a combination of mechanisms including protective antioxidants, low metabolic demands, and low levels of transition metals. Unregulated production of ROS and reactive nitrogen species (RNS) in the lung and other tissues can lead to an imbalance in oxidants relative to antioxidants leading to oxidative and nitrosative stress, respectively.

10.2.4 Reactive Species

ROS are formed from one-electron reduction of diatomic oxygen and subsequent one-electron reductions to achieve more reactive oxygen by-products, such as superoxide radical, hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) either spontaneously or through enzyme catalysis [57–59]. Hydroxyl radical can be formed from the reaction of H_2O_2 with transition metal ions or the breakdown of peroxynitrite and is one of the most reactive radicals produced in biological systems. It acts on local substrates at diffusion-limited rates of between 10^9 and 10^{11} M^{-1} s^{-1} [57], with a half-life of only milliseconds. The reactivity of superoxide and hydrogen peroxide is approximately 10^{1-3} M^{-1} s^{-1} [60].

In vivo, other mechanisms for the generation of free radical and reduction–oxidation products include electron leak from mitochondrial metabolism, enzymatic reactions (oxidases and peroxidases), and release from activated leukocytes through the NADPH oxidase-catalyzed oxidative burst [58]. The mitochondrial respiration chain is extremely active and generates superoxide when electrons leak from the energy-producing system. Enzymatic production of reduction–oxidation products can occur through the reaction of xanthine oxidase with hypoxanthine in the presence of O_2, producing superoxide, xanthine, urate, and H_2O_2. Xanthine oxidase has been shown to be upregulated with lung injury and is dependent on iron stores for its increased activity [61–63].

Activated leukocytes (neutrophils, macrophages, eosinophils) are another primary source of reduction–oxidation products through enzymes such as NADPH oxidase, MPO, and eosinophil peroxidase. These oxidases catalyze one- or two-electron reductions to form superoxide and H_2O_2, respectively, that contribute to the killing of microbes, intracellular and extracellular signaling [64, 65], and potential damage to host tissues when released from these cells [58].

NADPH oxidases are important sources of reduction–oxidation products in many other noninflammatory cells in the lung. Indeed, several recent studies indicate that ROS production by NADPH oxidases plays a central role in the pathogenesis of pulmonary fibrosis and inflammation [25, 66–68]. The Nalp3 inflammasome has been shown to have a central role in animal models of pulmonary fibrosis and is activated by reduction–oxidation by-products of NADPH oxidase in the lung [25]. Recent studies found that NADPH oxidase-4 plays a central role in ROS production and myofibroblast activation [66], cells known to be important in matrix remodeling and the progression of pulmonary fibrosis [66–69] further highlighting the active role ROS play in the pathogenesis of pulmonary fibrosis.

MPO is a second enzyme found in neutrophil azurophillic granules that aids in bacterial killing, by utilizing H_2O_2 and chloride ions to produce toxic hypochlorous acid (HOCl) or bleach. MPO is a highly cationic enzyme allowing it to localize to cell surfaces, such as endothelial and epithelial surfaces, through interactions with glycosaminoglycan chains. HOCl can participate with hydroxyl radical in the fragmentation of extracellular matrix (ECM) components such as hyaluronan and glycosaminoglycan side chains like heparan sulfate [70–72] (see below). MPO has also been shown to catalyze the metabolism of nitric oxide ($^{\bullet}$NO) making it unavailable for modulating vascular tone [73]. Eosinophil peroxidase is the eosinophil equivalent of MPO and has similar biological activity.

10.2.5 Nitrogen

An excess of various nitrogen-containing species within a system leads to nitrosative stress. Nitric oxide ($^{\bullet}$NO) is an important nitrogen species produced by nitric oxide synthase (NOS) enzymes through metabolism of L-arginine. There are three NOS enzymes, two of which are constitutively expressed: endothelial NOS (eNOS), neuronal NOS (nNOS), and one inducible NOS (iNOS/NOS2). Twenty times more $^{\bullet}$NO can be

produced by iNOS than the other enzymes [58]. iNOS activity is induced by external stimuli such as bacterial lipopolysaccharide [74]. Nitric oxide mediates the relaxation of smooth muscle cells in the cardiovascular and pulmonary systems. However, in addition to these beneficial effects of •NO, it can contribute to pathologic processes, especially when produced in large quantities. Notably, nitric oxide has been shown to be important in the pathogenesis of pulmonary fibrosis, especially when produced by iNOS [75, 76]. Covalent reactions can occur between •NO and NO-derived species with biological molecules such as proteins, DNA, lipids, and amino acids, which can modify the function of these molecules [77].

The radicals produced by reduction of oxygen can react with freely diffusible nitric oxide to form additional radical species, in effect inactivating nitric oxide, which is a potent signaling molecule (i.e., the inactivation of nuclear factor-kappa B [NF-κB] [78]) and vaso-relaxant [74]. Peroxynitrite anion ($ONOO^-$) can be formed through the diffusion-limited reaction of superoxide with nitric oxide (•NO) [79] or by reactions between hydrogen peroxide and nitrite [80]. The antioxidant enzyme superoxide dismutase (SOD), which is highly expressed in the lung, acts to keep superoxide levels low, thus preserving nitric oxide function [81]. Peroxynitrite is a powerful oxidant that can modify tyrosine residues producing nitrotyrosine and is also a potent oxidizer of thiols. At a physiologic pH, the protonated form of peroxynitrite (peroxynitrous acid) will decompose into hydroxyl radical and nitrogen dioxide. One-electron reduction reactions of •NO will form nitrite, nitrogen dioxide, and nitrate. Notably, MPO can catalyze nitrite-dependent nitration of tyrosine residues which can further promote nitrosative tissue damage [82].

A study by Saleh et al. provides support for the involvement of nitrosative stress in IPF lungs. They found that the lungs of IPF patients had increased NOS expression and nitrotyrosine modifications of proteins, suggesting that the IPF lungs were exposed to elevated nitrosative stress compared to healthy controls [83]. Animal studies further suggest that •NO signaling stimulates increased production of remodeling proteins, such as TGF-β and MMP enzymes in pulmonary fibroblasts [75]. iNOS-null animals that were exposed to silica inhalation developed significantly less pulmonary fibrosis than wild-type mice, supporting a profibrotic role for •NO produced by iNOS in the lung [76].

10.2.6 Oxidative Stress and IPF: Evidence from Patient Populations

The underlying pathogenesis of pulmonary fibrosis is currently thought to involve the presence of persistent stimuli or injury, such as oxidative stress, and dysregulated repair of the lung that results in fibrosis. Several studies have found evidence of increased oxidative stress in IPF. Reduction–oxidation products and free radicals of oxygen metabolism are difficult to measure directly in tissues; thus, many clinical studies have utilized biological markers of oxidative reactions for assessment, such as modified carbonyls, proteins, DNA, and lipids.

Bronchoalveolar lavage is a technique used to sample the epithelial-lining fluid of the lung. 8-Isoprostane, a product of free radical-mediated lipid peroxidation, is increased in the BALF of IPF patients [84], as well as in exhaled breath condensate along with hydrogen peroxide [85]. Exhaled ethane, a second marker of lipid peroxidation, is increased in patients with interstitial lung disease [86] and mirrored the patients' PaO_2 levels and clinical course, as patients with significantly elevated levels of ethane died or rapidly deteriorated. Oxidized proteins with carbonyl modifications are increased in the BALF of non-smoking IPF and sarcoidosis patients [87]. Increased carbonyl-modified proteins have also been shown in systemic sclerosis, IPF, eosinophilic pneumonia, and allergic alveolitis [88]; however, proteomic studies reveal that more low molecular proteins are altered in IPF BALF [89]. These oxidative markers negatively correlated with pulmonary function in all of these studies. The role of oxidative stress and IPF phenotype was investigated by Bocchino et al. by evaluating the relationship between oxidative stress status and IPF phenotype in primary human fibroblasts from IPF and non-fibrotic control lungs. They established that the IPF phenotype displayed increased ROS presence, increased alpha smooth muscle actin and type I collagen, and resistance to cell death [90]. In vitro studies suggest that exposure of alveolar and bronchial epithelium to H_2O_2 leads to increased TGF-β expression and epithelial-to-mesenchymal cell changes, such as increased alpha smooth muscle actin expression, decreased epithelial markers, and increased ECM production [91].

Antioxidant enzyme status is also altered in patients with IPF. Glutathione levels in alveolar epithelial-lining fluid are decreased in IPF lungs [16, 45]. Markart et al. show that IPF patients appear to compensate for oxidative stress with increased expression of Nrf-2, a redox-sensitive antioxidant transcription regulator, and significant increases in low molecular weight antioxidants during fibrotic phases [92]. Notably, these antioxidants are insufficient to counterbalance the oxidative stress. Kinnula et al. report significant decreases in extracellular superoxide dismutase (ECSOD) in fibrotic regions of UIP lungs [93], which suggests that oxidative stress would be increased in these areas. The loss of antioxidants in the lung and abnormal cellular signaling for antioxidant expression may have a role in IPF pathogenesis.

10.2.7 Oxidative Stress and Animal Models of IPF

Animal models of pulmonary fibrosis have offered opportunities to further evaluate the role of oxidative stress in alveolar injury, inflammation, and fibrosis development. The stimuli commonly used to initiate pulmonary fibrosis in these animal models are bleomycin (intratracheal, subcutaneous, or intraperitoneal administration), asbestos, silica, FITC, and adenoviral TGF-β, which are administered intratracheally or via an inhalation chamber. Bleomycin forms a complex with redox-active iron, molecular oxygen, and DNA, resulting in DNA strand breaks [94]. Bleomycin also produces superoxide and hydroxyl radicals that can damage

cell membranes, lipids, and proteins [95]. Asbestos-induced pulmonary fibrosis studies show increased superoxide production directly through transition metal reactions and indirectly through oxidative bursts from recruited neutrophils and macrophages exposed to asbestos [24, 96].

The NADPH oxidase (NOX) family, as discussed previously, consists of oxidoreductase genes that have roles in the generation of ROS and promotion of pulmonary fibrosis. NOX4 expression is upregulated by TGF-β and results in H_2O_2 production by fetal lung mesenchymal cells [66]. Hecker et al. have shown that NOX4 is involved in myofibroblast differentiation, contractility, and ECM production in response to TGF-β [66]. NOX4 expression is also increased in fibroblastic foci of human lungs with IPF. Its role in fibrogenesis is also thought to be mediated via cell apoptosis via oxidative stress. Carnesecchi et al. have shown that NOX4-deficient mice develop less fibrosis in a bleomycin mouse model, shown to be mediated through decreased TGF-β-induced epithelial apoptosis [69].

Epithelial apoptosis is thought to significantly contribute to the pathogenesis of pulmonary fibrosis [40–42]. Recent studies have found that oxidative stress plays a key role in regulating apoptosis of these cells in models of pulmonary fibrosis. Specifically, oxidative modification of Fas has been shown to enhance apoptosis [97, 98] and these studies further highlight the importance of localized oxidative stress leading to oxidative modification of specific proteins that contribute to disease pathogenesis.

It has also been demonstrated that antioxidants can prevent bleomycin- and asbestos-induced pulmonary fibrosis. Examples include studies showing protection against pulmonary fibrosis with *N*-acetylcysteine (NAC) and desferoxomine administration [29], treatment with lecithinized SOD, ECSOD over-expression [99], as well as protection when there is decreased ROS production in NADPH oxidase knockouts or knockdowns [67, 100]. In contrast a lack of SOD exacerbates bleomycin-induced fibrosis [101]. In bleomycin- and asbestos-induced pulmonary fibrosis, ECSOD protects by limiting both inflammation and fibrosis development [101–103]. Importantly, ROS produced by asbestos fibers have been shown to directly activate profibrotic TGF-β in the lung [22, 26], which is important in fibrosis development. Furthermore, antioxidant treatment with SOD was able to inhibit asbestos fiber-induced activation of latent TGF-β. These studies indicate that ROS can directly contribute to profibrotic activation of TGF-β, and that antioxidants may be useful in preventing this effect.

Similar to the asbestos model, intratracheal instillation of silica results in an acute accumulation of inflammatory cells (neutrophils, macrophages, lymphocytes, and occasional eosinophils) in the alveolar spaces and interstitium [104–106], damage to epithelial cells [107, 108], and subsequent collagen deposition [109–111] and fibrosis development [111, 112]. Models of radiation-induced pulmonary fibrosis have also shown a role for ROS as treatment with MnSOD has been shown to inhibit fibrosis [113, 114]. The absence of ECSOD promotes fibrosis in various inhalation injury models and treatment with SOD-mimetic agents, such as TBAP, is also protective [101, 103, 115–117].

Many studies have identified several potential mechanisms through which the presence of oxidative stress in the lungs can lead to increased inflammation and fibrosis. ROS can alter inflammation through the activation of NF-κB and activator protein-1 (AP-1) [118, 119]. These redox-sensitive transcription factors can bind to promoter regions in DNA and control the gene expression of a host of genes including those controlling pro-inflammatory cytokines, growth factors, and apoptotic signals [118]. NF-κB activation occurs in alveolar epithelial cells after asbestos exposure [23, 120]. Oxidative stress is also evident in the fibro-proliferative response. In vitro, H_2O_2 can stimulate the proliferation of cultured human fibroblasts [121]. Furthermore, fibroblasts isolated from IPF lungs are capable of inducing apoptosis in epithelial cells in vitro [122], further promoting the cycle of alveolar damage and abnormal repair. Hydrogen peroxide, as a diffusible factor, can lead to increased epithelial cell death, as well [53]. Finally, oxidative species can lead to direct degradation of the ECM and can control proteolytic degradation through the activity of MMPs and tissue inhibitors of MMPs [123, 124].

10.3 Molecular Targets of Oxidants in IPF

10.3.1 Extracellular Matrix

The ECM is critical for maintaining a strong structure that can withstand mechanical stretch and recoil of the lung as well as providing the architectural support for normal epithelial growth. The ECM of the lung is composed of several major components including collagens, elastin, fibronectin, proteoglycans, hyaluronan, and laminin [125, 126]. Pulmonary fibrosis is characterized by often drastic changes in the ECM, which can be the result of excessive matrix deposition (an increase in collagen deposition [2, 127, 128]), impairment in ECM degradation and resolution, or a combination of these two. Thus, ECM changes become very complex over the pathogenic course of IPF. Figure 10.1 depicts the potential changes that may occur in a normal lung and after epithelial injury to alveoli.

Heparan sulfate proteoglycans comprise a membrane-bound core protein with attached sulfated polysaccharide side chains [129]. Polyanionic proteoglycans, like heparan sulfates, have the ability to bind highly cationic proteins and transition metals. The binding of transition metals makes them potential sites for metal-catalyzed redox chemistry within the body. Furthermore, cationic proteins such as MPO and eosinophil peroxidase bind to the ECM and are sites of additional radical production such as HOCl from Cl^- and hydrogen peroxide [71, 72].

Hyaluronic acid, a glycosaminoglycan, and syndecan proteoglycans are prominent in the lung and are highlighted here, representing potential ECM targets for ROS. These components can function in various ways within the ECM: (1) bind and localize soluble and insoluble ligands, i.e., growth factors, TGF-β, FGF, cytokines; (2) act as soluble paracrine or autocrine factors when the ectodomain is shed;

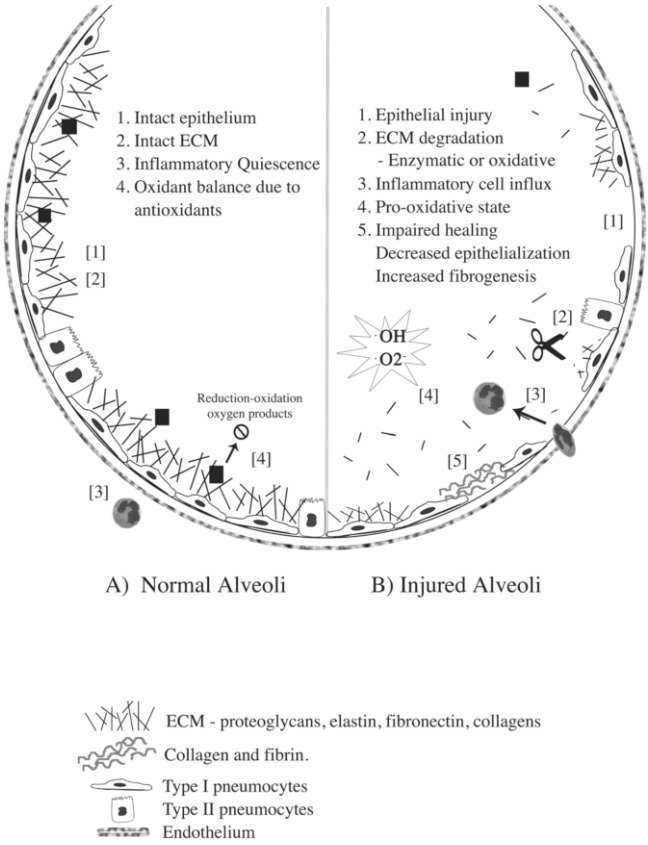

Fig. 10.1 Within normal uninjured alveoli (**a**), several processes create a homeostatic environment. The epithelium remains intact (1) along with an intact extracellular matrix (2) that binds and localizes many proteins such as growth factors and cytokines. Because there is no alveolar injury, inflammatory cell recruitment is not necessary (3) and oxidants/antioxidants are in balance. When lung injury occurs (**b**), the epithelium becomes denuded (1) and the ECM is degraded by enzymatic and oxidative mechanisms (2). The cellular injury, ECM degradation products, and cytokines cause inflammatory cell influx (3) and oxidative stress (4). This complex environment results in impaired epithelialization and dysregulated matrix deposition (5) (Reproduced from Free Radic Biol Med. 2011 May 1;50(9):1075–80)

(3) maintain receptor abilities for internalization of ligands; and (4) facilitate leukocyte migration and trafficking [130]. Syndecans are also linked to the actin cytoskeleton and have key roles in controlling cellular migration, proliferation, and homeostasis. Known ligands for syndecans that are of importance with regard to inflammation and fibrosis include TGF-β1 and 2, HGF, VEGF, PDGF-AA, FGF, cytokines, and chemokines such as IL-8, MCP-1, and TNF-α [130–133]. Syndecans can also bind other ECM components such as fibronectin and laminin and can bind enzymes such as neutrophil elastase, tissue plasminogen activator, and ECSOD. Syndecan-1 and -4 can bind elastase in dermal wound fluids protecting them from their inhibitors

and modulating the proteolytic potential of the microenvironment [134]. They can also bind MPO, which may promote increased oxidative stress, as described above.

The ECM can be degraded in two primary ways: enzymatic cleavage (MMPs, hyaluronidases, heparanase) or oxidative cleavage, which can transform the ECM into soluble effector molecules. Heparanase is an endoglycosidic enzyme that cleaves HS side chains [135] and is capable of cleaving syndecan-1 [136]. Syndecan core protein ectodomains can be shed from the cell surface through proteolytic cleavage of the juxtamembrane region. Matrilysin or MMP7 is a protease that binds to heparan sulfate [137] and induces shedding of the syndecan-1 ectodomain in a model of acute lung injury induced by bleomycin [138]. Indeed, MMP7 is believed to play a central role in the pathogenesis of pulmonary fibrosis [139, 140]. Consistent with this, MMP2 and MMP9 can also shed syndecan ectodomains in vitro [141]. This shedding can be regulated by tissue inhibitors of MMPs, such as TIMP 3 [142].

The role of oxidative stress in ECM dysregulation can be highlighted by recent studies of hyaluronic acid and the heparan sulfate proteoglycan syndecan-1 [143–145]. Hyaluronic acid shedding has been shown in the lungs of IPF patients [146]. Shedding of syndecan-1 is also significantly elevated in the BALF of IPF patients. In both the asbestos and bleomycin mouse models of pulmonary fibrosis, syndecan-1 is shed into the BALF during the inflammatory and fibrotic phases of injury.

Notably, the absence of ECSOD in the lung results in exaggerated syndecan-1, heparan sulfate, and hyaluronic acid fragmentation or shedding [143–145, 147]. Combined with data that demonstrates oxidants can directly lead to shedding and fragmentation of these ECM components in vitro, these findings suggest that oxidants are directly contributing to fragmentation and shedding of the ECM components in models of pulmonary fibrosis. The importance of oxidative fragmentation/shedding of high molecular weight hyaluronan to low molecular weight species is highlighted by studies demonstrating that high molecular weight hyaluronan has anti-fibrotic and anti-inflammatory activity, but low molecular weight hyaluronan has pro-inflammatory and profibrotic activity in the lung [148, 149]. Shed syndecan-1 can also promote fibrosis in several unique ways. Shed syndecan-1 induces neutrophil chemotaxis (which can be inhibited by ECSOD in vivo and in vitro), inhibits alveolar re-epithelialization, and stimulates fibrogenic TGF-β release [144]. These studies show the important role that oxidative stress has in modulating the ECM and how the oxidative by-products can promote fibrosis in the lung.

Studies also indicate that oxidative fragmentation of HS side chains can occur through hypochlorite species generated by MPO [70–72, 150] and through hydroxyl radicals generated by xanthine oxidase [151, 152]. This is particularly important in sites of inflammation and neutrophil influx. Potential oxidative reactions can occur to the core protein itself or the polysaccharide side chains. Protein backbone oxidation is more complex and significant cleavage occurs only with very ROS such as hydroxyl radicals [153]. Oxidation and cleavage of the core protein can occur through hydrogen abstraction from a central α-carbon and subsequent reaction with oxygen to form a peroxyl radical [154]. This radical can undergo conversion to an α-C alcohol and the peptide bond can be cleaved by hydrolysis or to an alkoxyl

species resulting in cleavage of the peptide bond. Polysaccharide side chain fragmentation can occur through hydrogen abstraction from any of the C–H bonds on the sugar residue creating a C-centered radical, called an α-hydroxyalkyl radical (•C(OH)RR′) [153]. This radical can then be converted to a peroxyl radical in the presence of oxygen and undergo chain hydrolysis or can undergo β-scission of the glycosidic bond which would fragment the chain [153, 154].

10.3.2 Matrix Metalloproteinases

ECM degradation and turnover are also regulated by the activity of MMPs and their tissue inhibitor counterparts (TIMPs). MMPs are matrix-degrading proteinases (currently a total of 22) shown to be upregulated in models of pulmonary fibrosis [124, 155]. The majority of MMPs are synthesized as proenzymes and activated by proteolysis of a cysteine-zinc pro-domain, called a "cysteine switch" [123, 156]. ROS are also capable of activating MMPs, increasing their transcription, and deactivating proteases [156–158]. Thus, oxidants may play a significant role in upregulated activity of MMPs in pulmonary fibrosis. The substrates of MMPs are ECM components and soluble factors and include, but are not limited to, the following: (1) MMP1, 8, and 13 are collagenases targeting collagens I, II, III, VII, X, gelatin, and pro-TNF-α; (2) MMP2 and 9 are gelatinases targeting type IV and V collagen, gelatin, elastin, fibronectin, pro-TGF-β, and pro-TNF-α; (3) MMP3, 10, and 11 are stomelysins that target proteoglycans, laminin, fibronectin, gelatin, and pro-TNF-α; and (4) MMP7 (matrilysin) targets proteoglycans, collagens, laminin, decorin, gelatin, and fibronectin [123]. Tissue inhibitors of metalloproteinases (TIMPs 1–4) are extracellular or membrane-bound enzymes that bind tightly to MMPs to inhibit their degradative activity [123].

In IPF patients, MMP2 and 9 and TIMPs 1 and 2 are elevated in areas of alveolar damage and at disrupted basement membranes [159]. McKeown et al. report increases in MMP3, 7, 8, and 9 in BALF from IPF patients with levels higher in patients with earlier mortality [160]. Rosas et al. report increases in MMP1 and 7 in serum, BALF, and lung tissue in IPF patients, suggesting they may be blood biomarkers for IPF [140]. MMP7-null mice are also protected from bleomycin-induced pulmonary fibrosis [139]. Animal studies show similar results with increases in MMP2 and 9 in the fibrotic phase of bleomycin-induced fibrosis [124]. Cabrera et al. report that an over-expression of MMP9 diminishes bleomycin-induced fibrosis [161]. In asbestos-induced fibrosis, MMP9 and MMP2 are important during the inflammatory and fibrotic phases of disease pathogenesis, respectively [124]. Inhibitors of MMPs have also been successful in protecting against asbestos-induced pulmonary fibrosis [124]. While there are differences in findings related to the role of MMPs in pulmonary fibrosis pathogenesis, the balance of MMPs and TIMPs is likely to play important roles during the course of fibrogenesis in the lung.

10.3.3 Antioxidants in the Lung

The lung expresses a variety of antioxidant resources to protect against oxidative stress within the tissue. These antioxidant defenses include low molecular weight antioxidants (glutathione, vitamins—vit. E, uric acid, etc.); metal-binding proteins (transferrin, lactoferrin, etc.); thiol-containing proteins with redox regulatory activity (thioredoxin, peroxiredoxin, and glutaredoxins); enzymes that degrade H_2O_2 (catalase and glutathione peroxidases); mucins; detoxifying enzymes (glutathione-S-transferases); and SODs. These antioxidants create a homeostatic system that functions to scavenge oxidative species and radicals that can damage cellular and matrix components. Several of these antioxidants have been shown to be important in IPF and are highlighted below.

Catalase is an important scavenger of H_2O_2 expressed within the alveolar epithelium and inflammatory cells of the lung. H_2O_2 has been shown to be an activator of pulmonary fibroblasts from IPF lungs and catalase can inhibit this activation [53]. A recent study reports that catalase administration to asbestos-treated wild-type mice protects against pulmonary fibrosis development, by inhibiting the production of H_2O_2 through Rac1 GTPase-stimulated NADPH oxidase [162, 163]. Similarly, in a rat model of asbestosis, extended administration of polyethylene glycol (PEG)-linked catalase for 20 days decreases fibrosis and collagen deposition in the lung [163]. It should be noted that patients with acatalasemia do not have pulmonary fibrosis; thus, the loss of catalase itself is not causative in pulmonary fibrosis.

Glutathione and alpha-tocopherol are important low molecular weight antioxidants found in the lung. Glutathione has been shown to be decreased in the epithelial-lining fluid [16] and in fibrotic lesions of IPF lungs. Furthermore, one study reports that the administration of oral NAC can increase glutathione levels in BAL fluid [164], sputum [15], and within alveolar epithelial cells to reduce oxidant production [165], suggesting that antioxidants can be exogenously administered and alter the environment of the lung. Administration of aerosolized glutathione to a small number of IPF patients resulted in a shift in the lung's oxidant–antioxidant balance toward the later [166]. In a recent study, alpha-tocopherol, commonly known as vitamin E, was reported to be elevated in the BAL fluid of IPF patients and was emulated during fibrosis in the bleomycin animal model [92].

SOD was first described by Fridovich and McCord in 1969 [167, 168]. There are three SOD enzyme isoforms including intracellular CuZn SOD (SOD1), mitochondrial manganese MnSOD (SOD2), and extracellular CuZn SOD (SOD3). ECSOD was identified by Marklund et al. in 1982 and is highly expressed in the lung [169, 170]. When the epithelium of the lung is exposed to oxygen or noxious stimuli, extracellular antioxidants have a critical role in preventing oxidative stress.

ECSOD has been implicated in the pathogenesis of pulmonary diseases involving oxidative stress [102, 171, 172]. ECSOD is an active extracellular scavenger of superoxide free radicals by catalyzing the dismutation of superoxide into hydrogen peroxide and oxygen. This occurs at a rate constant of $>10^9\ M^{-1}\ s^{-1}$ [172]. ECSOD is highly expressed in the vasculature [173] and functions to preserve nitric oxide

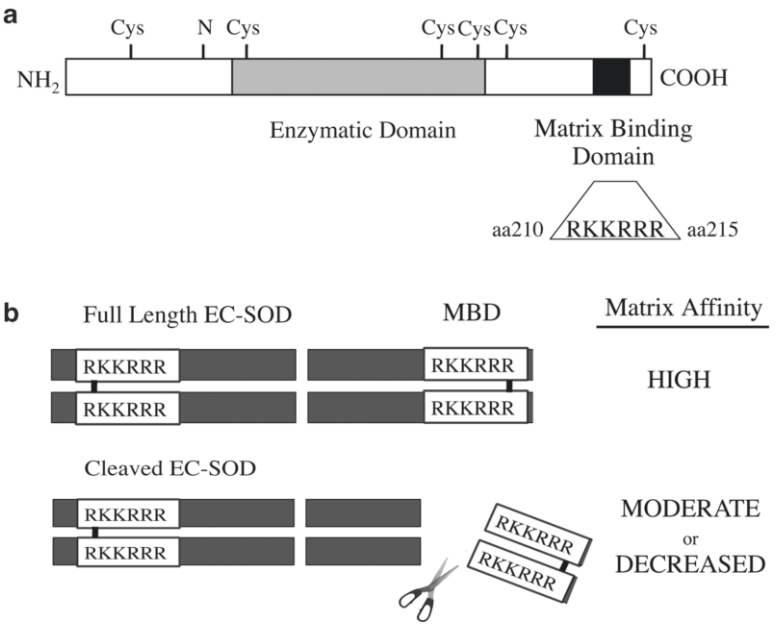

Fig. 10.2 Schematic of ECSOD structure and heparin affinity. (**a**) The ECSOD monomer contains an enzymatic functional domain (*grey*), a unique matrix binding domain (MBD) at the carboxyl terminus (*black*) which is composed of arginine (R) and lysine (K) residues, variable free cysteine residues that can participate in disulfide bonding (Cys), and an N-linked glycosylation site. The positively charged MBD makes the site suitable for binding to highly negatively charged heparin species in the ECM. (**b**) ECSOD tetramer affinity for the matrix can be regulated by proteolytic removal of the MBD (Reproduced from Free Radic Biol Med. 2011 May 1;50(9):1075–80)

(NO) bioavailability within various organ systems [81, 174–176] by removing superoxide that can deplete NO. Figure 10.2 depicts the structure and functional domains of ECSOD. This enzyme is highly expressed in the lung and localizes to cell surfaces by binding to heparan sulfate species [172, 177–180] and type I collagen [147, 171] through its matrix binding domain (MBD).

Enzymatic cleavage of any of the four MBDs will decrease or abolish the affinity of ECSOD for the matrix and cell surfaces (Fig. 10.2b). ECSOD has three heparin affinity types: no affinity (type A), moderate affinity (type B), and high affinity (type C). Trypsin or endoproteinase treatment of ECSOD, which targets lysine residues, can abolish or weaken the matrix binding affinity of ECSOD [177]. This supports the important role of the cluster of basic amino acids in the C-terminus of ECSOD.

ECSOD has been shown to play an important role in several models of pulmonary fibrosis including bleomycin-, asbestos-, and radiation-induced fibrosis. Knockout mice lacking ECSOD throughout their tissues have significantly more lung fibrosis, acute lung injury, and inflammation dominated by a neutrophil influx due to bleomycin and asbestos intratracheal administration [101, 103, 117].

ECSOD distribution in the lungs of wild-type mice also changes, as it is lost from the parenchyma where it normally resides and increases in air spaces in fibrosis models [117, 181, 182], and after hyperoxia [115]. ECSOD appears to be exerting its anti-inflammatory and anti-fibrotic effects by inhibiting oxidative degradation of matrix components, as discussed above.

10.4 Therapeutic Approaches: Controlling Oxidative Stress

10.4.1 Treatment

Despite recent studies and advances in the understanding of the pathogenesis and clinical course, there are currently no effective therapies for IPF, aside from lung transplantation, which comes with its own complications. Several options are available, such as anti-inflammatory agents; however, there are very few to no clinical studies that show improvements in progression-free survival, functional capacity, or quality of life [183]. While corticosteroids have been used over the last 50 years, Flaherty et al. report that fewer than 20 % of patients have improvement with steroid therapy [48, 184]. Chronic, low dose prednisone may be a maintenance therapy in responsive patients, but is not recommended for all IPF cases [7, 183].

Immunosuppressive/cytotoxic agents, such as azathioprine, which impairs leukocyte proliferation [183], and cyclophosphamide, an alkylating agent, are used in patients who are non-responsive to steroids and have shown favorable results in 15–20 % of IPF cases [1]. Raghu et al. completed a study of high dose prednisone therapy versus high dose prednisone plus azathioprine and found no significant differences in clinical measures, such as forced vital capacity (FVC) and diffusion capacity of carbon monoxide (DLCO), with either therapy [185]. Cyclophosphamide has shown no survival benefit in studies [46, 186] and has a profound side-effect profile [183, 186] which limits its utility.

Finally, anti-fibrotic agents, such as colchicine [187], have been tried, but have been unsuccessful in humans. Colchicine functions by decreasing collagen formation through fibroblasts and macrophages [183] and its metabolites have the ability to scavenge free radicals; however, this also leads to the production of secondary radicals [188, 189]. While colchicine showed promise in vitro and in animal models [190, 191], it has shown no survival or lung function benefit in clinical IPF [47, 192] thus having limited clinical utility in IPF therapy.

Amidst the grim outlook of current therapies, the IPF research community continues to find new molecular targets and therapeutic options, some of which have antioxidant activity. Pirfenidone is a pyridone molecule that has anti-inflammatory and anti-fibrotic effects in both in vitro and in vivo pulmonary studies. It can also scavenge hydroxyl and superoxide free radicals [193–195]. It was successful in abrogating bleomycin-induced fibrosis in animal models [196, 197], by decreasing

TGF-β expression and subsequent collagen deposition. In a Phase II open-label trial, pirfenidone was effective in both improving 1-year survival to 78 % (compared to 70 % reported in other studies) and stabilizing or improving lung function (diffusing capacity (DLCO) and FVC) in patients with advanced IPF [198]. Pirfenidone is not currently approved in the United States.

Many studies have focused on carnosine, NAC, and SODs. Administration of carnosine, a free radical scavenging peptide, in mice decreases inflammatory and fibrotic markers in bleomycin-induced fibrosis [199] and is available for human administration. NAC, one of the most highly studied thiol-containing agents, scavenges H_2O_2 (hydrogen peroxide), $^{\bullet}OH$ (hydroxyl radical), and HOCl and promotes glutathione synthesis [164]. The IFIGENIA trial (a randomized, double-blinded, placebo-controlled trial) reported that the addition of high dose NAC to the standard therapy of prednisone and azathioprine can significantly slow IPF progression compared to standard therapy alone when evaluated on FVC, DLCO, and a composite physiologic index [200, 201]. One study reported that aerosolized NAC resulted in improved oxygen saturation and CT image changes in IPF patients; however, it had no effect on pulmonary function or quality of life [202]. It remains unclear if there is a survival benefit from NAC therapy. A recent study from the IPF Clinical Research Network analyzed the safety and efficacy of combination therapy with prednisone, azathioprine, and NAC with a randomized double-blind, placebo-controlled trial that demonstrated increased mortality and hospitalization during interim analysis with combination therapy as compared with NAC alone or placebo [203].

Antioxidant mimetics may be another potential therapeutic strategy. A recent review discusses these and additional antioxidant mimetics in detail [204]. Small-molecular-weight SOD mimetics, such as metalloporphyrins, have been effective in limiting radiation-induced lung injury, oxidative stress, inflammation, and bleomycin-induced pulmonary fibrosis in animal models [116, 205, 206]. The metalloporphyrins have several distinct antioxidant actions including scavenging superoxide (SOD-like activity), hydrogen peroxide (catalase activity), and peroxynitrite and inhibiting lipid peroxidation [204] and are not readily metabolized in vivo [204, 205]. While it is hoped that novel antioxidant therapies will provide therapeutic benefit to patients, it is clear that oxidative stress is just one component of pulmonary fibrosis.

Thus, combination therapies of antioxidants with other anti-fibrotic agents may be a more rationale approach to future therapeutic investigations (Table 10.2).

Lung transplantation is the only current option that prolongs survival in IPF patients. Considerations for lung transplantations should be made early on in the disease course, as the wait-list time is around 46 months, during which time many patients with advanced disease die prior to transplant [7, 183]. The 5-year survival post-transplant is approximately 40 % [207]. Lung transplantation is also associated with increased pulmonary and systemic oxidative stress during the post-transplant period [208].

Table 10.2 Therapeutics in IPF

Therapeutic	Mechanism of action	Clinical or laboratory results
Corticosteroids [7, 48, 183, 184]	Inhibition of inflammation	Benefit in 20 % of patients
Azathioprine [185]	Suppression of leukocyte proliferation	Positive effect in 15–20 % of steroid nonresponders
Cyclophosphamide [46, 186]	DNA alkylation	No effect on patient survival
Colchicine [188–192]	Decrease collagen synthesis; scavenge free radicals	Protective in bleomycin animal model
		No benefit in human studies
Pirfenidone [193–198]	Anti-fibrotic, TGF-β regulation; scavenge free radicals	Protective in bleomycin animal model
		Increased 1-year survival and lung function
Carnosine [199]	Scavenge free radicals	Protective in bleomycin animal model
N-acetyl-cysteine [200–202]	Scavenge free radicals; promote glutathione expression	

10.5 Final Discussion

The current belief in IPF pathogenesis is that cellular injury, which is often repetitive, acts as the inciting event for fibrosis development and oxidative imbalance within the lung. Causes of the oxidative stress include, but are not limited to, the cellular injury, transition metal exposure, inflammation, or drugs that participate in reduction–oxidation reactions. The importance of oxidative modifications to the ECM and how they alter cellular responses in human IPF remains an open and under-investigated area. Recent studies show that ECM degradation products do have biological function and may add to the progression of pulmonary fibrosis (Fig. 10.3). While there is not sufficient evidence that the ECM products are initiating or causative factors in IPF, they appear to promote a profibrotic environment.

The current literature on the pathogenesis of tissue fibrosis focuses primarily on the roles of epithelial, mesenchymal, and inflammatory cells. Specifically, the role for oxidative shedding of matrix components and the effects of the shed species during tissue injury remain unclear. ECSOD is the most abundant antioxidant enzyme in the extracellular space of many tissues where it is localized through binding to matrix components such as heparan sulfates. Novel evidence is available that supports the importance of oxidative stress in the ECM, such as with syndecan-1 or hyaluronic acid, and that antioxidants, like ECSOD, have primary roles in protecting the matrix and preventing detrimental downstream consequences (Fig. 10.3).

In addition to scavenging oxidants once they are produced, it may also be possible to directly inhibit their production from their source. Novel strategies to inhibit oxidant production by any of these complex enzymes or transition metal systems may provide ideal targets for therapeutic intervention for IPF patients.

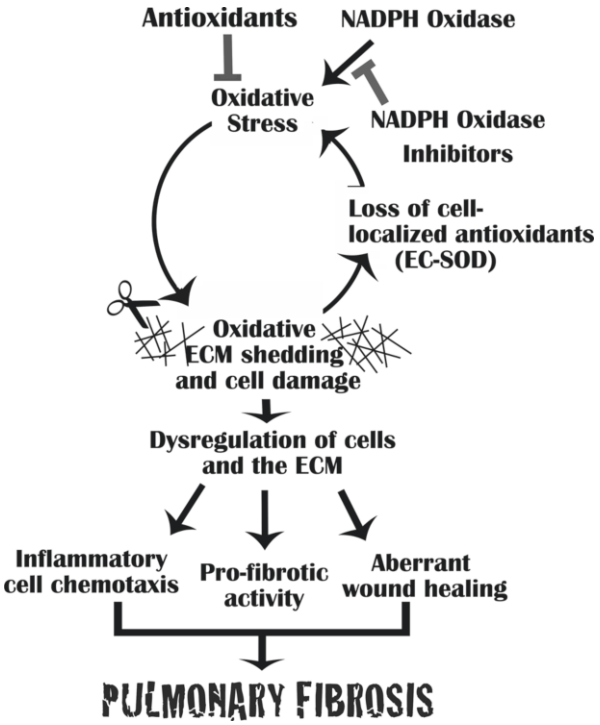

Fig. 10.3 Oxidative stress can accompany or be caused by various stimuli in the lung, i.e., repetitive cellular injury, transition metal or particulate exposure, noxious drugs, inflammation, and enzymatic activity such as NADPH oxidases. This oxidative imbalance can result in degradation of ECM components and a loss of protective antioxidants. This leaves the tissue susceptible to increased inflammation, profibrotic signals, and aberrant wound healing—all of which may contribute to the progression of pulmonary fibrosis (Reproduced from Free Radic Biol Med. 2011 May 1;50(9):1075–80)

This review highlights the important role that oxidative stress has in the pathogenesis of IPF and emphasizes the importance of the ECM in the pathogenesis of pulmonary fibrosis. Oxidative degradation of the ECM may prove to be a good therapeutic target given that (1) intact ECM is critical for appropriate wound healing; (2) ECM degradation by-products are biologically active, and (3) the ECM localizes many cytokines, growth factors, and enzymes shown to potentiate fibrosis. Additional investigations into antioxidant therapeutics are necessary to elucidate their full potential, especially with regard to ECM degradation. The clinical arena of IPF needs more effective therapies and while antioxidants alone may not be the complete answer, combination therapies that include antioxidants, or inhibitors of oxidant generation, may contribute to future effective therapies for this disease.

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Chapter 11
Oxidative Stress in Sarcoidosis

Sahajal Dhooria and Dheeraj Gupta

Sarcoidosis is a multisystem disorder characterized by the formation of non-caseating granulomas. These granulomas form as a result of a heightened helper T-cell type 1 (Th1) response orchestrated by a number of cytokines [1]. The exact etiology is not clear but genetic predisposition, the type of inciting antigen, role of microbes, environmental and occupational factors are some aspects in its pathogenesis which have received the attention of investigators the world over [2]. In the past two decades, the role of oxidative stress in the pathogenesis of diffuse parenchymal lung diseases (DPLDs) including sarcoidosis has been highlighted. Oxidative stress has been shown to play an important part in the evolution of idiopathic pulmonary fibrosis (IPF) [3]. Evidence of increased oxidative stress has also been found in sarcoidosis. Several mechanisms have been proposed by which the oxidative stress translates into lung injury in sarcoidosis [4, 5].

The following text elaborates on the evidence of occurrence of oxidative stress in sarcoidosis and its proposed role in pathogenesis. The possible role of antioxidants to counteract oxidative stress in this disease and the clinical implications of the current knowledge on this subject have also been discussed.

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11.1 Markers of Oxidative Stress in Sarcoidosis

11.1.1 Serum Markers (Markers of Systemic Oxidative Stress)

11.1.1.1 Lipid and Protein Peroxidation Products

Malondialdehyde (MDA) is the end product of lipid peroxidation. Significantly higher levels of MDA have been found in the serum [6, 7] and erythrocytes [7] of sarcoidosis patients as compared to controls. MDA levels were also found to be significantly higher in patients with active disease as compared to those with inactive disease [6]. Both serum and erythrocyte MDA levels are strong predictors of the presence of disease even after adjusting for oxidative stress and lipid parameters [7]. Levels of oxidized low density lipoprotein (oxLDL) are also higher in sarcoidosis patients, more so in patients with active disease [6].

11.1.1.2 Oxygen-Free Radicals and Enzymatic Oxidants/Antioxidants

Serum and erythrocyte superoxide anion concentrations are higher in patients with sarcoidosis [7]. Serum total hydroperoxide levels as measured by spectrophotometry are increased in clinically stable patients not treated with steroids, as compared to steroid-treated patients and healthy volunteers [8]. But they do not correlate with the diffusing capacity of carbon monoxide (DLCO), partial arterial oxygen tension (PaO_2), Medical Research Council (MRC) dyspnea scale, or chest X-ray stage [8].

Significantly lower superoxide dismutase (SOD) activity has been found in both the serum and the erythrocytes of sarcoidosis patients as compared to controls [7]. Paraoxonase (PON1) is a hydrolase located on high density lipoprotein (HDL) that protects both low density lipoprotein (LDL) and HDL from oxidation [9]. It thus plays a role in preserving HDL's capacity to function as an anti-apoptotic molecule as oxidized HDL loses its anti-apoptotic function [10]. Reduced levels of PON1 have been reported in diseases known to have increased oxidative stress including diabetes, hypercholesterolemia, and cardiovascular disease [11, 12]. Low levels of serum PON1 pointing towards a decreasing antioxidant activity have been found in sarcoidosis patients with active disease as compared to controls as well as to patients with inactive disease [6].

11.1.1.3 Non-enzymatic Low Molecular Weight Antioxidants

Serum vitamin C, uric acid, and reduced glutathione levels are decreased in the blood of sarcoidosis patients as compared to matched controls [13]. NADPH levels are reduced in erythrocytes of female sarcoidosis patients [14].

11.1.1.4 Total Oxidant Status, Total Antioxidant Status, and Prooxidant–Antioxidant Balance

Total oxidant status (TOS) gives the summation of the effect of all the oxidant molecules in the serum. It can be assessed by an assay based on the conversion of ferrous to ferric ions [15]. It is significantly higher in the serum and erythrocytes of sarcoidosis patients than in controls [7]. Prooxidant–antioxidant balance (PAB) values are higher while total antioxidant status (TAS) values are significantly lower in sarcoidosis patients as compared to controls [7, 13].

A significant positive correlation exists between serum angiotensin converting enzyme (ACE) levels and serum MDA and TOS levels while a significant negative correlation is found between serum ACE and serum TAS levels [7].

To summarize, markers of oxidative stress and total oxidant activity are increased, while antioxidant molecules and total antioxidant activity are found to be decreased in the sera of sarcoidosis patients. There is emerging evidence on oxidative markers being correlated with disease activity.

11.1.2 Markers in Respiratory Tract Samples (Markers of Local Oxidative Stress)

11.1.2.1 Lipid and Protein Peroxidation Products

8-Isoprostane (8-IP) is a PGF2α isomer produced in vivo by free radical-induced peroxidation of arachidonic acid [16]. Levels of 8-IP are elevated in bronchoalveolar lavage (BAL) fluid in sarcoidosis [17]. They are correlated negatively with the number of BAL fluid lymphocytes. 8-IP levels are also elevated in the exhaled breath condensate (EBC) of sarcoidosis patients as compared to controls [18]. In another study, they were shown to be elevated in active sarcoidosis while not significantly increased in patients with inactive disease, and were correlated with serum ACE levels [19]. In fact, EBC 8-IP concentrations are highest in patients with stage-3 disease [20]. Moreover, if 8-IP levels are below detection levels, there is a greater than three times increased chance of an early remission [20]. Unfortunately, complete remission is not associated with a consistent decrease of EBC 8-IP; however treatment with steroids reduces the levels of this molecule irrespective of remission [20].

Ethane, an end product of lipid peroxidation of omega-3 fatty acids like linolenic acid, is expired as a gas [21]. Elevated concentrations of exhaled ethane, representing oxidative stress have been found in asthma and chronic obstructive pulmonary disease [22, 23]. Exhaled ethane was found to be elevated in a mixed population of various interstitial lung diseases including IPF, cryptogenic organizing pneumonia, collagen vascular disease-associated-ILD, and sarcoidosis [24]. It correlated with tracer uptake on gallium-67 scintigraphy [24].

Metal-catalyzed generation of reactive oxygen species (ROS), for example through Fenton reaction leads to oxidation of proteins which are measured as the total carbonyl content in the BAL fluid [25]. BAL fluid protein carbonyls are significantly elevated in sarcoidosis [25, 26], although normal levels were found in one study [27]. Also, the total carbonyl content correlates significantly with absolute eosinophil numbers in BAL [25]. Besides this, albumin, immunoglobulins, α-1 antitrypsin, and complement C3 are also found oxidized in the BAL of sarcoidosis patients [28].

11.1.2.2 Oxygen-Free Radicals and Enzymatic Oxidants/Antioxidants

There is an increased ex vivo production of hydrogen peroxide by alveolar macrophages extracted by BAL in sarcoidosis patients [29]. However, it does not correlate with ACE levels, the results of gallium-67 scans, or the percent of lymphocytes in the BAL [29]. Hydrogen peroxide levels are also significantly elevated in the EBC of sarcoidosis patients [30].

Alveolar macrophages of patients with both active and inactive sarcoidosis produce higher amounts of superoxide anions than of healthy subjects [31]. However, the amount of superoxide produced on stimulation with phorbol myristate acetate is higher in patients with high intensity lymphocytic alveolitis than in patients with inactive disease [31]. In another study, alveolar macrophages from patients with high intensity alveolitis had a reduced superoxide anion release after in vitro stimulation [32]. This apparent paradox is explained by the possible constant in vivo activation of the cells with subsequent reduced ability to respond after additional stimulation in vitro [32].

Haem oxygenase-1 (HO-1) is an inducible microsomal enzyme that catalyzes the conversion of haem into carbon monoxide and biliverdin [33]. It plays a cytoprotective role against oxidative stress [34]. Cytokines like interleukin-1 and tumor necrosis factor-α (TNF-α) induce HO-1 as a protective function against oxidant injury. There is a decreased expression of HO-1 in fibrotic lung disorders like IPF [35], while it is shown to be increased in the induced sputum in granulomatous lung diseases like stage I–III sarcoidosis and chronic beryllium disease [36]. There is intense HO-1 immunoreactivity in alveolar macrophages and weak to intense activity in the granulomas of sarcoidosis, while it is weak to negative in fibrotic areas of the lung [37]. Manganese SOD, an important enzymatic oxidant is also upregulated in sarcoid granulomas [38].

Peroxiredoxins are another group of antioxidant enzymes that can reduce hydrogen peroxides and organic peroxides [39]. They are shown to express in alveolar macrophages of sarcoidosis patients [40]. Thioredoxin, a class of small redox proteins is highly expressed and locally produced by sarcoid granulomas [41, 42]. It might activate T cells by acting as a local inducing factor for interleukin-2R expression [42]. It may also serve as a marker of ongoing cell regeneration and inflammation [41].

11.1.2.3 Non-enzymatic Low Molecular Weight Antioxidants

The concentrations of ascorbic acid, α-tocopherol, and retinol are increased while glutathione concentrations are unaltered in the BAL fluid of sarcoidosis patients [43]. This increase may represent an adaptive response to oxidative stress [43].

Thus, there is an increase in lipid peroxidation products, oxidative enzymes, and oxygen-free radicals in the respiratory secretions in sarcoidosis. At the same time, the levels of antioxidant molecules are also elevated, possibly as a mechanism to counteract the heightened oxidative stress.

11.2 Proposed Role of Oxidative Stress in Pathogenesis

The characteristic finding in the BAL of sarcoidosis patients is a predominance of lymphocytes and usually a normal level of neutrophils and eosinophils. Neutrophils are found to increase in late or advanced sarcoidosis [44]. Furthermore, in newly diagnosed patients with sarcoidosis, BAL neutrophilia [45, 46] and eosinophilia [46] indicate an unfavorable prognosis. These cells are potent producers of ROS, which are released upon stimulation of NADPH-oxidase.

Immune cells at the site of lesions of sarcoidosis produce ROS-like superoxide anion, which are released into blood and taken by erythrocytes through the anion channels [5]. SOD converts superoxide anions into hydrogen peroxide which contributes significantly to TOS [15]. The increased levels of superoxide and hydrogen peroxide lead to increased lipid peroxidation resulting in increased levels of MDA. Hydrogen peroxide can also catalytically inactivate SOD [47].

It is possible that the relationship between ROS production and lipid peroxidation is different in different subgroups of sarcoidosis patients. In patients with active lymphocytic inflammation, alveolar macrophages are constantly stimulated and release superoxide anion [4]. These are the patients who suffer from acute disease with intense inflammation which has a favorable prognosis. These patients show lower levels of 8-IP, thus reflecting less of chronic lipid membrane damage. This happens possibly because of the rapid scavenging of ROS by naturally occurring antioxidants [4]. Membrane lipid peroxidation (as reflected by levels of 8-IP) is greatest in a different subgroup of patients, who have radiological stage-3 disease, with lesser lymphocytic [4] and more of eosinophilic inflammation [18]. Moreover, there is a negative correlation between the number of BAL fluid eosinophils and DLCO [18].

ROS play a role in inflammation and fibrosis in multiple ways. First, they promote inflammation by activating the transcription factors, nuclear factor-κB (NF-κB), and activator protein-1 through histone acetylation/deacetylation [48]. NF-κB, a potential biomarker for oxidative stress [49] is increased in alveolar macrophages [50] and blood monocytes [51] of active sarcoidosis patients. These transcription factors induce pro-inflammatory cytokines like IL-8 [48], as well as promote free radical formation, thus propagating both inflammation and oxidative stress [52].

Second, ROS play a part in the proteinase–antiproteinase balance. They can activate [53] or inactivate metalloproteinases [54], as well as inactivate their inhibitors [55]. Third, ROS increase the release of transforming growth factor-β (TGF-β) from human alveolar epithelial cells, thus promoting fibrosis [56]. Fourth, ROS and reactive nitrogen species (RNS) mediate the induction of plasminogen activator inhibitor-1 (PAI-1), which plays an important role in the development of lung fibrosis [57].

An association between ACE and oxidative stress parameters [7] points to a possible link between oxidative stress and differentiation of monocytes to macrophages leading to granuloma formation.

Calcium oxalate is present in sarcoid granulomas, sequesters iron, and ferritin [58]. There is a possible link between the oxidative stress mediated by accumulated iron and the influx of alveolar macrophages, giant cell formation, and a granulomatous response in the lung [58].

Enzymatic and non-enzymatic antioxidants are increased in the lung tissue as a response to oxidative stress while their systemic levels are decreased possibly due to increased consumption and accumulation in the lung. It is possible that there is a state of relative deficiency of antioxidant molecules, despite the higher than normal levels [43]. Antioxidants like HO-1 may be important in the defense of alveolar macrophages in the inflammatory, but not in the fibrotic stage of the disease [37].

11.3 Role of Exogenous Antioxidants in Sarcoidosis

There are many antioxidant compounds that can be administered exogenously to counteract increased oxidative stress in various conditions. A few have been tried in sarcoidosis. No conclusive benefit of any such treatment has been shown.

Quercetin is a dietary antioxidant. The highest concentrations are found in the flower buds (capers) of the plant Capparis spinosa. Other sources are buckwheat, blueberry, and cranberry. Supplementation with quercetin increases the total plasma antioxidant capacity in healthy volunteers [59]. Its antioxidant capacity is several times that of various endogenous antioxidants like glutathione and vitamin E [52]. Moreover, it accumulates in the lungs [60], and acts as an anti-inflammatory agent by reducing levels of TNF-α and IL-8, known to be elevated in sarcoidosis [13]. Total plasma antioxidant capacity is enhanced while MDA levels are reduced significantly by quercetin supplementation in sarcoidosis patients [52]. Also, the ratios TNF-α/IL-10 and IL-8/IL-10 are reduced by quercetin in patients with sarcoidosis [13, 52]. The baseline level of oxidative stress and inflammation is a major determinant of the beneficial effect of antioxidant supplementation [52].

Pentoxifylline is a phosphodiesterase inhibitor which is shown to induce a dose-dependent suppression of the spontaneous TNF-α release from alveolar macrophages in sarcoidosis [61]. Besides its anti-inflammatory properties, it also has mild capacity to scavenge oxygen-free radicals in vitro [62]. Although a post hoc

analysis of a small study suggests that pentoxifylline reduces flares and has steroid-sparing effects, no definitive conclusions can be drawn regarding the efficacy of pentoxifylline in pulmonary sarcoidosis [63].

N-acetylcysteine (NAC) has antioxidant, mucolytic, and anti-inflammatory properties [64]. It has been used in the treatment of various pulmonary disorders like IPF [65]. NAC exerts a dose-dependent inhibitory effect on IL-8 and MMP-9 release and ICAM-expression by BAL macrophages and lymphocytes from patients with sarcoidosis [64]. No clinical trials or experimental studies have assessed its therapeutic efficacy in pulmonary sarcoidosis.

11.4 Clinical Applications and Questions To Be Answered

There are many potential clinical applications of the knowledge that has been and is being generated on the role of oxidative stress in sarcoidosis. Certain questions need to be answered before the measurement of oxidative markers becomes a useful clinical tool. Do markers of oxidative stress, local (BAL and EBC), or systemic (serum), truly reflect the severity of disease? Whether a "high oxidative stress disease" is a distinct phenotype that behaves differently from "low oxidative stress disease"? Do increased levels of ROS and lipid peroxidation products represent different pathology or stages of disease? As mentioned earlier, high superoxide levels associated with lymphocytic inflammation were seen in reversible disease while high 8-IP levels associated with eosinophilic inflammation were observed in chronic irreversible disease. Could high HO-1 levels point to an active granulomatous process, while low levels represent fibrosis?

Regarding implications on therapeutics, the role of antioxidants to treating sarcoidosis is not clear. It is evident from many of the above-described observations that steroids reduce oxidative stress in addition to the reduction in the level of inflammation. Whether antioxidants have any additive effect when used in conjunction with steroids is a moot question to be answered. Can antioxidants slow down progression at a stage when steroids are either not warranted or have failed to show benefit? A whole gamut of antioxidants awaits clinical testing in well-planned clinical trials in carefully selected patients.

Conflict of Interest S.D.: Conflicts of Interest—none, financial disclosures—none. D.G.: Conflicts of Interest—none, financial disclosures—none

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Chapter 12
Asbestos Fibers: Mechanisms of Injury

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12.1 Introduction

The adverse clinical consequences of asbestos fiber inhalation are well described (Table 12.1) [1]. There are two aspects of exposure to the asbestos fiber that alter the development of these asbestos-related diseases. The first effector of risk for disease is dose. Fibrotic lung disease (asbestosis), malignancy, and pleural changes are all dose-dependent. Despite efforts by investigators to show that asbestos-induced fibrosis must be present to provoke lung cancer or to show that pleural plaques substantially increase the risk of lung cancer, the risks for each independently increase with exposure—these three clinical features occur in parallel [2–5]. There is no timeline which allows the physician to predict if, or when, one will occur before the other. Each manifestation can occur singularly or together. Statistical evidence showing one or the other event leads to malignancy has been very difficult to sort out and has been a substantial source of disagreement among epidemiologists who have been interested in understanding this issue.

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Table 12.1 Asbestos related diseases

Asbestosis
Benign pleural disease
Bloody exudative effusions
Pleural plaques
Diffused pleural thickening
Mesothelioma
Lung cancer

The second aspect associated with the development of disease attributed to asbestos exposure is time—that is not only the time from first exposure to the current exposure in those working, but also the total time from first exposure to asbestos regardless of the working status. It is not sufficient to address the impact of the years of fiber inhalation. In former workers, additional years following ceasing employment (without exposure) can be relevant. The three manifestations described above—mesothelioma, fibrosis, and lung cancer—are impacted by this notion of latency, that is, the time from first exposure to the development of disease; however, the manifestation most impacted is mesothelioma.

Mesothelioma is the most sensitive and specific marker of the adverse health effects attributed to asbestos [6]. It is sensitive because this tumor can develop from lesser asbestos fiber exposures (in the presence of a substantial [usually more than 30 years [latency]]) and specific as the great percentage of those with this disease can provide a history of workplace or environmental asbestos exposure.

Several recent publications lead one to realize that the implications of asbestos exposure are even more disconcerting as we learn more about the consequences of its inhalation. The first report answers the question "Is the world-wide rate for mesothelioma declining from rates recognized in 1995?" [7]. In seven countries, the mortality rate increased (in five, in a statistically significant manner). The mortality rates were essentially no different in 24 countries (in five, rates declined but were not statistically different from the 1996 rates). In the U.S., for example, the permissible exposure limit for asbestos and current standard for exposure was established in 1986 (although less stringent rules were in place prior to this) and recent rates of importation and utilization of this fiber has dramatically lessened to less than 1,000 metric tons yearly (from more than 700,000 metric tons/year in the 1950s) [8, 9]. Exposures to asbestos continue in renovated or demolished buildings or as a result of continuing the importing policies of brake pads, asbestos fittings, and washers. As an example, there is clear evidence that the decline in asbestos utilization in the U.S. has been dramatic, yet there has been no change in the U.S. mesothelioma rate from 1995 to 2006. Countries which banned asbestos ($n=50$ as of 2009) and countries with the greatest decline in asbestos utilization from 1970 to 1985 showed the greatest annual rate of mesothelioma decline in the 1995–2006 period, yet, overall, when comparing 1996–2005 data, the annual rate of mesothelioma deaths in 31 countries showed no statistically significant decline. In this report, one can only conclude that even though current exposures are trending downward, it appears that the latency period remains the driving force for the continued development of this disease.

The second report helps explain why the changes that have been made in many countries are yet to alter the frequency of this disease. Using the relative risk for mesothelioma in workers who had stopped working (and therefore, exposure to asbestos)

Table 12.2 Selected manuscripts of cross-sectional studies by decade showing changes in the prevalence of asbestosis in the U.S. over time

1965
In a population of 121 asbestos workers with a 40-year latency of asbestos exposure, 94.2 % had a radiologic diagnosis of asbestosis
Selikoff IJ et al. The occurrence among insulation workers in the United States. Ann NY Acad Sci 1965; 132:139–155
1979
In 359 present and retired shipyard workers with ≥10 years of exposure, 44 % had parenchymal interstitial disease
Polakoff PL et al. Prevalence of radiographic abnormalities among northern California shipyard workers. Ann N Y Acad Sci 1979; 330:333–9
1988
In 1016 workers in the sheet metal industry employed) 35 years, parenchymal interstitial fibrosis (consistent with asbestosis) was found in 33.1 %
Selikoff IJ, Lilis R. Radiological abnormalities among sheet metal workers in the construction industry in the United States and Canada: relationship to asbestos exposure. Arch Environ Health 1991; 46:30–36
1998
In electricians with >20 years of union membership, the prevalence of small opacities was 2.1 %
Hessel PA et al. Lung health among electricians in Edmonton, Alberta, Canada. J Occup Environ Med 1998; 40: 1007–12
2009
Follow-up from 1988 study. 2181 sheet-metal workers who had a negative CXR in the initial study were re-tested from 1986 to 2004. 5.3 % had CXR changes consistent with asbestosis. Of cases, 91.3 % worked ≥29 years. Workers beginning after 1970 had no disease
Welch LS, Halle E. Asbestos-related disease among sheet-metal workers 1986–2004: radiographic changes over time. Am J Ind Med 2009; 52:519–22

between 3 and 15 years ago as a comparator, the authors showed that the risk for mesothelioma in those still working vs. those who had ceased employment more than 30 years ago was not different [10]. A second report is a British case-control study comparing cases with mesothelioma to workers in different jobs. Among all tradesmen, carpenters were at the highest risk for disease development. Of consequence, the lifetime risk for mesothelioma was determined when asbestos exposure occurred prior to the age of 30years, even if the exposure lasted for less than 10 years. Increasing the exposures for a greater duration beyond the age of 30 years did not significantly add to the risk of development of the disease [11].

Of these clinical manifestations associated with asbestos fiber inhalation, United States federal standards have been developed to protect workers from asbestosis. There is no intent in the standard to diminish the number of workers with lung cancer, pleural plaques, or mesothelioma. The implication is that the protective effect of the standard will lessen the number of cases of asbestosis and in that way lessen the other manifestations. Cross-sectional reports suggest that the implementation of this standard has dramatically altered the number of cases of asbestosis (Table 12.2), although, as noted above, there has been no measurable impact on the mesothelioma rate. The scene in the developing world is quite alarming. As an example, in India, several industrial hygiene surveys report very high levels of asbestos, even though

there is no direct reporting of mesothelioma in the National Cancer Registry in association with asbestos [12].

There is very strong epidemiologic evidence linking chronic exposure to asbestos and lung cancer, mesothelioma, and pulmonary fibrosis, yet the underlying biological and chemical mechanisms that support this linkage are not as well described. The basic process involves fiber deposition in the lung (with fiber clearance, sequestering of the fiber into the interstitium, or transmission of uncleared fibers into the pleura). Uncleared fibers begin the acute inflammatory response and evolve into chronic inflammation with continuous inflammatory cell infiltrates, reactive oxygen species (ROS) formation, cytokine release, and ultimately genotoxicity with DNA damage affecting cell replication and differentiation. The interaction of the ROS with the pulmonary milieu plays a ubiquitous role in the overall destructive process of the uncleared asbestos fiber, but numerous other processes occur to lead to disease. This leads to the question: What are the important features of the asbestos fiber and what are the biologic and chemical events that occur in the lung in association with this fiber that place a worker at life-long risk for the development of asbestos-associated diseases?

12.2 The Asbestos Fiber

Fibers can be identified and counted by phase contrast optical microscopy, scanning electron microscopy, polarized light microscopy, and transmission electron microscopy. Each of these technical approaches has strengths and weaknesses in fiber identification and visualization. Furthermore, although there is a considerable agreement of what defines a fiber, disagreements remain. For example, the World Health Organization (WHO) considers fibers suitable for counting if the particle is >5 μm in length with length to diameter ratio of at least 3:1 (known as WHO fibers) [13]. The National Institute for Occupational Health (NIOSH) has recommended that a fiber be defined as any particle >5 μm in length with a length to diameter ratio of 5:1 and a diameter <3 μm [14]. Fiber counting using different microscopic techniques and different definitions yield very different outcomes [15].

Overall, reports on the relationship between fiber dimensions and asbestosis show that the severity of pulmonary fibrosis, length of exposure, and type of exposure are broadly proportional to the number of asbestos fibers or asbestos bodies found in the parenchymal lung tissue [16–19]. In general, fibers exceeding 20 μm in length are associated with asbestosis, and fibers longer than 10 μm in length are the most carcinogenic. Inhalation of short amosite fibers <5 μm in length produced virtually no fibrosis in rats compared with long amosite fibers with 11 % >10 μm that produced extensive interstitial fibrosis at 12 months [17]. There is some evidence that fibers less than 5 μm in length can also promote pulmonary fibrosis and malignancy, especially when administered as a lung overload condition, as can occur in dust clouds [20].

There is national and international agreement that exposure to asbestos fibers causes lung and pleural cancer, as well as interstitial lung disease (asbestosis) [21, 22].

Despite the perspective that the relationship of fiber characteristics and diseases appears to be the best understood of all the inhaled particles recognized to cause disease [23], individuals with asbestos exposure may present with a series of illnesses that are not obviously related. For example, it is not intuitive for the clinician to recognize that an exposure to an environmental agent that causes parenchymal fibrosis also has the potential to induce pleural malignancy. Although this link has been recognized epidemiologically, it has not been well explained physiologically. Even now, when there are some insights into the mechanisms of fibrosis associated with the persistence of fibers and their make-up, there is no proven hypothesis which describes how fibers leave the lung, enter the pleural space, and induce any of the pleural effects described in Table 12.1. The link between these clinical manifestations could be attributed to the number of fibers in the parenchyma as well as the duration that the fibers have remained in the lung, the shape and dimensions of the fibers (specifically length and diameter), the composition of the fiber—particularly the characteristics of the fiber surface (important in biopersistence), and the interactions between the pulmonary milieu and the fiber which affects the way that the fiber is handled (the genetic background of the host in association with the effects of environmental agents [e.g., cigarette smoke, the presence of other fibrogenic dusts]). Animal reports show that once fibers deposit in the parenchyma, they are no longer able to be cleared by the efficient muco-ciliary escalator of the airway, and are dealt with by the substantially less effective phagocytotic properties of macrophages [24].

The lung has the ability to respond differently to different particles; witness the different histologic features resulting from coal and silica exposures. Over time, an understanding of the relationship between the shape and dimensions of the different asbestos fibers and their pathogenicity has evolved. The only serpentine fiber is chrysotile. This accounts for 95 % of the asbestos previously used for industrial purposes in the U.S. The most often widely used amphibole fiber is crocidolite, but this group also includes tremolite, amosite, anthophyllite, and actinolite. Chrysotile fibers are soft, curly, and break easily while the amphiboles are firm and sharp.

Different types of asbestos fibers provoke a different (lesser or greater) response. Authors have commented that the use of the word “asbestos” to include both serpentine and the amphibole fibers has made it more difficult to understand the relationships between the fiber characteristics and disease [25]. As an example, not only are their shapes and sizes different but also chrysotile contains just trace amounts of iron while crocidolite can contain as much as 36 %. The elemental composition of the fiber plays a role in its biochemical reactivity in the lung [26]. In a sense, this statement is borne out by the comprehensive review relating exposure to the asbestos fibers with different characteristics and disease [27]. The authors performed a meta-analysis of the mesothelioma risk based on fiber exposure recognized in the work environment in 15 epidemiologic studies and for the lung cancer risk in 11 epidemiologic studies. Fiber exposure was defined by the type of fiber (chrysotile or crocidolite), fiber length (either >5 and <10 or >10 m), and the fiber diameter (<0.2, <0.4, >0.2 m and all widths). Based on fibers 10 μm or longer, the risk for mesothelioma associated with exposure to chrysotile and crocidolite fiber exposure was very different. The best estimate for chrysotile potency to induce mesothelioma was approximately between 0 and 1/200th that of crocidolite in inducing mesothelioma. Crocidolite was

approximately ten times more potent as an inducer of lung cancer compared to chrysotile when the fiber was thin (width <0.4 μm and length <0.2 μm), but the potency for crocidolite was less, yet still more than chrysotile, when comparing the lung cancer rates following exposures to wider fibers. Others have cited the carcinogenicity of longer amphiboles to be two orders of magnitude greater than that of chrysotile [28].

This relationship between amphibole fibers and mesothelioma was verified in a case-control study. Lung samples from 69 male mesothelioma cases and 57 controls matched for age (all were under 50 years of age) and gender were evaluated and the mineral fiber content identified, fibers sized, and the number of fibers counted by electron microscopy. Exposure to amphibole fibers contributed most to mesothelioma. The presence of amosite and crocidolite fibers accounted for 80 % of the cases, with tremolite adding another 7 % (all amphiboles). Because chrysotile has a much shorter biopersistence, its contribution was more difficult to estimate [29].

An understanding of the way that the different fibers in the lung are handled is incomplete. Churg and Wright addressed this in a 1994 review [30]. First, differences in the amounts of the types of fibers deposited in the parenchyma are due to clearance rates and not deposition rates. Second, although exposures in most industrial settings are greater to chrysotile fibers compared to amphiboles, amphiboles persist in the lung in disproportionately large amounts and chrysotile in disproportionately small amounts. The process of leaching (loss of magnesium content) with gradual fiber dissolution is well recognized in vitro, yet has not been sufficiently proven in the human lung. Finally, the half-time for clearance of amphibole fibers is thought to be years or decades, while the great majority of chrysotile fibers are cleared in weeks to months, although in some instances the fibers are sequestered in the interstitial space and persist [31]. Paradoxically, in some studies of fiber persistence of mesothelioma, chrysotile fibers were recognized to be the major source of asbestos exposure, yet such fibers may be identified in only a minority of cases of mesothelioma, while amphiboles such as tremolite, which are only a very small fraction of the exposure and often considered contaminants to chrysotile exposures, are the main fiber found in the lung [32, 33]. It appears that the majority of chrysotile fibers are metabolized in a relatively short period, yet the persistence of these fibers, particularly if they are sequestered in the lung (i.e., in the interstitium) appear to have the potential to contribute to disease. This general lack of biopersistence of chrysotile fibers in the lung is the most likely explanation for its relative lack of virulence compared to amphibole fibers [34].

In the early 1980s, Stanton et al. published animal work showing that mesothelioma rates in asbestos-exposed animals were fiber size dependent. Specifically, if fibers were long (>4 μm) and thin (<0.25 μm) in diameter there was substantially more disease compared to fibers shorter and thicker [35]. Recent work has validated this conclusion in lung cancer. From 1940 to 1973, the North and South Carolina asbestos textile mills employed over 6,000 individuals. Chrysotile was the predominant fiber used. When the development of lung cancer in this population was reviewed, the authors showed that lung cancer mortality was more strongly associated with those exposed to long, thin fibers [36]. To complicate this further, cigarette smoking alters fiber clearance. When the fiber burden in the airway mucosa of

cigarette smokers with heavy occupational asbestos exposure was compared to a similarly exposed group of matched non-smokers, the amount of chrysotile fibers was higher by approximately 50-fold ($p < 0.006$) and the concentration of amosite fibers was increased approximately sixfold ($p < 0.02$) in smokers [37].

An analysis of the fiber content in lung biopsy or autopsy specimens from residents of Quebec with asbestos-induced lung disease was recently reported. Of particular interest in this report was the ability to relate work history to lung fiber content. Although the asbestos mines in Quebec contain nearly exclusive amounts of chrysotile with minimal amphibole contamination, 85 % of the workers presented chrysotile fibers in the lung, while 76 %, 64 %, and 43 % had tremolite, amosite, and crocidolite, respectively. Half of the fibers were short, 30 % were thin and only 20 % corresponded to the WHO definition of fibers cited above. Mean years away from asbestos exposure for those with asbestosis was 17 years, 29 years with mesothelioma, and 19 years with lung cancer. Although the number of chrysotile fibers declined disproportionately more than amphiboles over time, chrysotile particles (many of lesser dimensions than necessary to be classified as a fiber) were still observed in the lungs of workers 30 years or more after last exposure and exceeded the level found in unexposed populations [38]. With such information, even though the mechanisms of metabolism and clearance of chrysotile fibers in the lungs are recognized to occur, the role of chrysotile as an agent which may induce illness cannot be discounted.

Despite the work cited above, others have reported that the relationship between fiber types and size, and the pulmonary (i.e., fibrosis and lung cancer risk) and pleural (pleural inflammatory changes and mesothelioma) milieu is not clear-cut [39]. It appears that fibers alone can cause disease. As an example, asbestos fibers can directly interfere with chromosomal segregation during mitosis and damage DNA [40]. Certainly, the pulmonary milieu can be changed to alter the virulence of the asbestos fiber. Cigarette smoking increases the lung manifestations of asbestos-related disease, again suggesting that the interaction between the fiber and the lung milieu (in this example in the presence of cigarette smoke), and not entirely the fiber itself (with its potentiating characteristics of length, diameter, aspect ratio, and type), is the culprit in these diseases [41].

12.2.1 How Fibers Cause Disease

Asbestosis is defined by the American College of Chest Physicians as bilateral diffuse interstitial fibrosis of the lungs caused by the inhalation of asbestos fibers [42]. Most patients with clinically recognized asbestosis present with dyspnea and dry cough, and physical examination typically reveals inspiratory rales at the lung bases. Functional changes on pulmonary function testing in the fully developed case of asbestosis are restrictive indices with a decreased diffusing capacity for carbon monoxide. Histologic examination of the lung in milder cases of asbestosis may show characteristic changes, yet the concomitant spirometric changes are not yet measurable [41]. The typical radiographic finding is a

Table 12.3 Mechanisms of disease induced by the asbestos fiber

The acute inflammatory response
The chronic inflammatory response
Fibrosis
Transformation into malignancy
Development of pleural abnormalities

lower zone reticulonodular infiltrate on plain films. Computed tomography features appear to be very similar if not identical to those seen in usual interstitial pneumonia, i.e., peripheral bands, lines, thickened interlobular septa, and honeycombing, with disease most severe at the lung bases [43, 44].

The microscopic pathology of asbestosis reflects the end product of the lung's response to substantial fiber exposure over a protracted period of time. The histologic hallmarks of this disease are (1) interstitial fibrosis and (2) the presence of asbestos bodies within the pulmonary parenchyma. Although we address asbestosis as the end product of a chronic inflammatory response, relatively few inflammatory cells are recognizable. Inflammation, when it can be recognized, occurs at the site of fiber deposition along the airways and alveoli. The histologic features of the disease begin with relatively homogeneous fibrosis of the alveoli adjacent to the bronchioles in the peripheral aspects of the lower zones of the lung. Then, depending on the stimulus for progressive fibrosis, fibrosis can extend towards the hilum and encompass surrounding bronchioles. Fibrosis which may also develop in the walls of the respiratory bronchioles and alveolar ducts is strictly not asbestosis, and is best described as bronchiolar wall fibrosis. This is another characteristic response to asbestos exposure [45].

The metabolic processes in the lung multiply the effects associated with the effects of the deposition of the asbestos fiber in the lung (Table 12.3). Recurrent asbestos fiber exposure interacts with the pulmonary milieu and generates ROS and other oxidants, induces an influx of inflammatory cells—initially macrophages and neutrophils, but with time fibroblasts, perpetuates a self-generating release of a large number of cytokines and growth factors. As an example, inflammation and fibrosis as well as expression of genes linked to cell proliferation and antioxidant defense occur in a dose-related fashion after inhalation exposures to asbestos fibers [46]. Although each of the following may be, in a sense a separate process, these events cannot be separated and contribute to the pathology resulting from asbestos fiber inhalation. These processes include oxidative stress (perhaps the most inextricably linked part of the process), inflammation, fibrosis, and genotoxicity [47].

12.3 Inflammation and Fibrosis

Research performed in the 1980s served as the starting point for understanding the effects of asbestos fiber inhalation and the acute inflammatory pulmonary effects. A series of lung pathology follow-up studies of young rats that had undergone a

singular 1-h nose-only exposure to chrysotile fibers showed acute inflammatory changes. After 2 days, unlike infectious inflammation where the primary cell is the neutrophil or lymphocyte, the primary changes at the bifurcation of the alveolar duct was a dramatic thickening of the epithelial and interstitial layers, with a tenfold influx of alveolar macrophages (AMs) on the bifurcation and a threefold increase of macrophages in the interstitium. The features of inflammation are most prominent at the site of fiber deposition. After 1 month, the number of type I and II epithelial cells remained increased, and the interstitium was collagenous and even thicker. Alveolar macrophages are more prevalent and now cells reflecting localized fibrosis, i.e., myofibroblasts, and smooth muscle cells are identifiable. No further follow up of these abnormalities was provided, leaving the authors to ponder whether fibrosis would continue or resolve, and what role serial exposures would play in further development of fibrosis [48]. Further studies of this model (this time with a 5 h exposure to asbestos and intraperitoneal injection of [3]thymidine at 19, 24, and 48 h, 8 days and then 1 month post-exposure with sacrifice 4 h post-injection) with determination of the cell mitotic activity by the uptake of [3] thymidine, revealed the most activity within the first 48 h with a return to normal at 8 days and an unchanged level at 1 month. The increased uptake correlated pathologically with increased numbers of bronchial-alveolar epithelial and interstitial cells. The enhanced mitotic activity of the cells was thought attributable to the fibers present or factors released by stimulated macrophages attracted to the areas of fibers [49]. Histologically, the early stage of asbestosis is characterized by discrete foci of fibrosis within the walls of the respiratory bronchioles and alveolar duct bifurcations where there is an accumulation of asbestos bodies [19]. Inhalation of asbestos fibers triggers the accumulation of AMs with an inflammatory reaction, followed by more diffuse pulmonary involvement characterized by the loss of alveolar epithelial type I and II cells, fibroblast proliferation, and eventually collagen deposition. Macrophage ingestion of asbestos fibers triggers a fibrogenic response from fibroblast proliferation through release of growth factors such as transforming growth factor β (TGF-β) and platelet-derived growth factor (PDGF). These growth factors, in addition to numerous inflammatory cytokines such as tumor necrosis factor-α (TNF α) and interleukin-1β (IL-1β), collectively promote collagen deposition found in asbestosis [50].

Histology, cell counts, biochemical markers of inflammation, and the extent of cellular proliferation were determined in the lungs of a rat model following a "lesser" and "greater" airborne exposure for 20 days to chrysotile asbestos. Rats were sacrificed at varying times afterward. No effects were found in the "lesser" exposure group, while focal histologic changes of cellularity and fibrosis and a increasing number of neutrophils based on the time of sacrifice relative to end of exposure was recognized in animals with the "greater" exposure. In another group of rats with the same exposure, but sacrificed following a 20-day delay, assessment of DNA synthesis by pre-morbid injection of antibody to 5-bromo-2′-deoxyuridine (BrdU) labeled cells were measured in the interstitium of the lung parenchyma, the bronchi and bronchioles, and the cells of the visceral pleura. Like the data described in the manuscript above, a significant increase of DNA synthesis in all three areas

occurred in rats sacrificed 5 days after ceasing exposure (initially), but not later on (at 20 days or in the group sacrificed 40 days after initiating exposure). The suggestion is that for chronic inflammation to develop, continuous or "chronic" exposure is necessary. Intriguingly, increased gene expression of manganese-containing superoxide dismutase, an enzyme which protects lung cells from hyperoxic lung injury, occured in animals sacrificed at all three times, and led the authors to suggest that this was a marker of chronic inflammation [51].

In a sophisticated study which addressed the role of fibers vs. mediators in the development of inflammatory changes, investigators began with sex-mismatched chimeric and naïve female mice and provided 3, 9, or 40 days of asbestos exposure. The female chimeric mice received a total body irradiation and then received bone marrow from another population of male mice, using the sex chromosome as a specific marker. At the time of sacrifice of groups, lung histology, bronchoalveolar fluid (BALF) cell counts, and measurement of levels of numerous mediators in BALF were measured to assess inflammatory activity. Not surprisingly, there was less asbestos-induced inflammation in mice which had received irradiation and bone marrow transplant. This effect was most exaggerated in the mice who had received the longest asbestos exposure. Using markers on donor cells, the loss of the natural bone-marrow-derived stem cells following whole-body irradiation substantially lessened the number of inflammatory cells in the lung with the associated lessening of release of inflammatory mediators [52]. The need for bone marrow stem cells to propagate fibrosis reflects the systemic inflammation induced by asbestos.

In summary, the amount of inflammatory response triggered by ingestion of asbestos fibers is primarily related to the dose and length of the inhaled fiber. High doses of inhaled asbestos fibers over short periods promote an acute alveolar macrophage predominant inflammation, whereas low doses over prolonged exposure periods promote neutrophil-predominant chronic inflammation. The ways that acute inflammation becomes chronic inflammation, and in some instances, fibrosis and even, malignancy, is complex and not well understood. As noted in the earlier studies cited above, acute inflammation is a time-limited process. Chronic inflammation is not time-limited and reflects on-going tissue damage in a lung with underlying injury. In the example of asbestos, the failure to clear and the inability to metabolize the fiber (in particular, amphibole fibers) drives the process. The histology reflects the continuing influx of inflammatory cells with an uncontrolled release of cytokines and growth factors and the consequences of such an event. The result of the attempt to get rid of the lung of the foreign body and repair previously injured tissue is a proliferative response with even more disordered tissue. Finally, this increases susceptibility to malignancy by causing DNA damage.

Chronic inflammation, with its associated developing fibrosis, has the potential to dramatically alter how fibers are removed from the lung. The effectiveness of the process described below depends on the integrity of the cells lining the airway, the presence of intact and unobstructed lymphatic vessels, and the relative lack of interstitial inflammation and fibrosis. Using principles of fluid dynamics, Miserocchi

et al. explained how fibers are translocated from the airway into the interstitium and from there into the pleural space [53]. First, fibers in the alveolar lining fluid reach the interstitium through phagocytosis by type I alveolar lining cells which allow a "pass-through" into the interstitium by combined osmotic (through active sodium absorption) and hydraulic (the interstitial pressure is less than the airway) pressure gradients. Macrophages become "frustrated" by their inability to phagocytize the long fibers; the result being the release of mediators reflecting the heightened metabolic activity of these cells [54]. Alveolar epithelial cell (ACE) injury also damages fibroblasts and myofibroblasts and perpetuates the inflammatory response in the interstitium with the laying down of increased amounts of extracellular matrix; the start of or the perpetuation of the underlying pathologic process of asbestosis. Second, asbestos fibers can exit the lung through lymphatic vessels. In a normally functioning lung, very fine fibers can be cleared in 24 h [55]. The lymphatic circulation inevitably drains into the blood and, in that way, fibers may be dispersed to all organs [56]. Fibers in lymphatic vessels and in the blood can enter the pleural space dragged by water flux gradients. Third, movement of fibers from the lung parenchyma into the pleural space can occur directly. If there is an inflammatory response in the lung (such as asbestos-induced alveolitis), the interstitial pressure is raised and this can drive fibers in the lung parenchyma through minute pores in the visceral pleura into the pleural space. In this context, it is understandable how not only fibrotic lung disease and lung cancer are the end-products of asbestos-induced fibrosis, but how malignant mesothelioma can be included as an inflammatory-induced malignancy.

12.3.1 Reactive Oxygen Species

An important mechanism for the development of inflammation and fibrosis attributable to asbestos fiber inhalation is the formation of ROS. Although not as clearly defined as ROS, reactive nitrogen species are also important messengers of toxicity. Three separate mechanisms for ROS production have been implicated in the development of asbestosis. These include fiber surface reactivity due to iron homeostasis, cellular release from AMs, and mitochondria-derived ROS released from both inflammatory cells such as lung epithelial cells [50]. Asbestos inhalation elicits an AM response to phagocytize and clear the fibers, but this response results in ROS production by a Ras-related C3 botulinum toxin substrate 1 (Rac1) dependent mechanism as well as by the release of inflammatory cytokines and growth factors. After ingestion by the AM, the asbestos body becomes a fibrous structure with asbestos in its core surrounded by mucopolysaccharides and iron-rich proteins such as ferritin and hemosiderin that are redox active [57]. Only a small proportion of the total fiber burden of the lung ever becomes coated, probably not more than 10 %, and the proportion of coated fibers increase with fiber length [17]. The purpose and function of coated asbestos fibers is to reduce their cytotoxicity since coated fibers are less cytotoxic to alveolar macrophages than uncoated fibers. The surface of

asbestos fibers deposited in the lungs acquires iron that is redox active and cycles between reduced and oxidized forms. Additionally, alterations in iron homeostasis in the lung have been observed. The asbestos body generates the highly reactive hydroxyl radical (HO^+) from hydrogen peroxide (H_2O_2) which can lead to alteration in antioxidant enzymes and DNA damage in target lung epithelial, AM, and mesothelial cells [20, 58].

The cytotoxic effect of asbestos on mesothelial cells was shown to occur after phagocytosis of crocidolite fibers which causing increased intracellular oxidation, breakage of DNA strands, apoptosis, and cell-cycle arrest; phagocytosis was considered as an independent variable for toxicity [59].

There are a large number of cytokines which play a role in the inflammation process as it relates. In their review of inflammation and mesothelioma, Miller and Shukla [54] identified TNF-α, TGF-β, platelet-derived growth factor (PGDF), insulin-like growth factor (IGF), interleukin-6, interleukin-8, vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF).

As an example, in vivo activated AMs release mediators of inflammation such as TNF-α. This cytokine, as well as others, contribute to the ultimate response of malignancy. Yet, in vitro, asbestos is very toxic to human mesothelial cells and these cells do not transform into malignant cells, but die. When TNF-α is added to human mesothelial cell culture in vitro, the response is an expression of TNF-alpha receptor through the NF-κB-dependent mechanism on the human mesothelial cells. Instead of cell death when asbestos was added, when TNF-α is present, there was cell damage, but resistance to cell death. Taking this a step further, the investigators showed that through cytogenetic techniques, many of the surviving AMs had chromosomal injury. They postulated that these AMs with genetic injury are susceptible to malignant transformation to mesothelioma [60].

Galffy et al. showed high IL-8 levels in the pleural fluid of malignant mesothelioma patients compared to those with congestive heart failure. Follow up in vitro studies showed that IL-8 directly promoted malignant mesothelioma cell growth, but not mesothelial cell growth [61].

Interleukin 6 (IL-6) is a key mediator in the pathway of chronic inflammation and fibrosis. Asbestos fibers and asbestos-induced oxidative stress stimulates IL-6 expression and secretion in pulmonary type II-like epithelial cells and in normal human bronchial epithelial cells. The extent of this process depends on the intracellular redox-oxidative state. Intracellular OH^- scavengers such as *N*-acetylcysteine (a precursor of glutathione) lessened IL-6 secretion by the asbestos fiber or hydrogen peroxide (H_2O_2). The presence of the asbestos fiber and H_2O_2 stimulate DNA-binding activity to the nuclear factor-kappa B (NF-κB), and NF-IL-6-recognized sites in the IL-6 promoter, the result being IL-6 induction. This can be blocked by another OH^- radical scavenger, tetramethylthiourea. The chronic inflammatory changes build towards fibrosis. Using the measurement of [3H]thymidine incorporation to determine mitotic changes, adding recombinant IL-6 stimulated lung fibroblast growth. Furthermore, elevated IL-6 levels were found in bronchoalveolar lavage fluids from patients diagnosed with lung fibrosis and work-related histories of long-term asbestos exposure [62].

12.3.2 Mitochondrial Reactive Oxygen Species

Another factor associated with the inflammatory process is the generation of ROS from the mitochondria of key target cells. In the case of inflammatory cells, recent animal studies using murine models have established a prominent role for AM mitochondrial H_2O_2 production in mediating the fibrogenic response of asbestosis [63–66]. Observations from these studies include the recognition that:

1. Alveolar macrophages exposed to asbestos produce H_2O_2. This may be inhibited by catalase or through mitigation of AM mitochondrial oxidative stress.
2. Ras-related C3 botulinum toxin substrate 1 (Rac1) has been localized in the AM mitochondria of patients with asbestosis. Rac1 augments AM mitochondrial H_2O_2 production.
3. Knockdown of the complex III iron–sulfur protein in the mitochondrial electron transport chain reduces asbestos-induced AM H_2O_2 production.
4. Deletion of Rac1 in the AMs of asbestos-exposed mice shows reduced oxidative stress and pulmonary fibrosis.

The observations from these studies demonstrate that ingestion of asbestos fibers triggers H_2O_2 production in AM through the transfer of electrons from complex III to Rac1. Mitochondrial ROS production is also found in other important target cells, such as lung epithelial and mesothelial cells. Higher levels of mitochondrial ROS production and oxidative stress trigger DNA damage, p53 activation, cell-cycle blockade, and cell death. It has been speculated that Rac1 may be a possible biomarker for the presence of pulmonary fibrosis related to asbestos [67].

12.3.3 Epithelial Cell Apoptosis

Asbestos-induced AM and AEC mitochondrial ROS production promotes AEC apoptosis that appears to be important for myofibroblast differentiation, collagen deposition by myofibroblasts, and ultimately pulmonary fibrosis. The two mechanisms by which cells undergo apoptosis include the extrinsic (death receptor related) and intrinsic (mitochondria-regulated) death pathways. Diverse stimuli, including ROS, deoxyribonucleic acid (DNA) damage, and asbestos activate the intrinsic death pathway by increasing the permeability of the outer mitochondrial membrane; reducing the mitochondrial membrane potential and releasing apoptotic proteins, including cytochrome c. Considerable in vitro and in vivo data show that asbestos can induce both lytic cell death and apoptosis. Apoptosis is a regulated, ATP-dependent process characterized by membrane blebbing, cell shrinkage, nuclear chromatin condensation, and DNA fragmentation. Unlike the inflammatory signaling arising from lytic cell death, apoptosis enables cells with extensive DNA damage to be eliminated without inciting an inflammatory response. Substantial evidence convincingly confirms that AEC apoptosis is important in the pathophysiology of pulmonary fibrosis [50].

Numerous studies have demonstrated findings relating pulmonary fibrosis to apoptosis. Animal models of asbestosis demonstrate and patients with idiopathic pulmonary fibrosis develop significant injury to the alveolar epithelium. The AECs of patients with idiopathic pulmonary fibrosis have shown to have DNA strand-break formation and apoptosis. Asbestos is well described to induce AEC DNA damage and apoptosis. Additionally, murine models have shown that the presence of AEC apoptosis is sufficient for inducing pulmonary fibrosis. Blocking of AEC-targeted apoptosis is protective for the development of pulmonary fibrosis. Prevention of αvβ6 integrin release from lung epithelial cells, a key activator of latent TGF-β, prevents TGF-β activation and pulmonary fibrosis. Although these data firmly implicate AEC apoptosis in the pathophysiology of pulmonary fibrosis following exposure to various agents, including asbestos, future studies are necessary to define the precise molecular mechanisms involved in apoptosis.

12.3.4 p53 Cellular Response

Tumor protein 53 (p53) integrates various signals and initiates cellular responses to include cell-cycle arrest, cell differentiation, apoptosis, and other functions. A normal-functioning p53 response after exposure to DNA-damaging agents prevents the accumulation of cellular mutations. Over half of all human cancers have p53 mutations and p53 null mice have a marked increase in cancer predisposition. p53 is also redox sensitive, and its transcriptional function is linked to oxidative stress, which allows it to mediate the cellular effects including the induction of apoptotic cell death [68, 69]. The precise mechanism of p53 regulation of cellular apoptosis has not been elucidated but p53 activates mitochondrial-related death through gene expression of pro-apoptotic stimuli and suppression of anti-apoptotic genetic expression. High levels of apoptosis due to asbestos fibers may promote a fibrotic response in the form of asbestosis.

12.4 Genotoxicity of Asbestos

Asbestos-induced genotoxicity has been demonstrated in mesothelial and lung epithelial cells and studies show that all forms of asbestos are genotoxic to lung cells. Asbestosis exposure and fiber toxicity is clearly linked to the development of lung cancer and pleural mesothelioma. The development of carcinoma in asbestos exposure may be multifactorial as related to chronic inflammation from asbestosis, the genotoxicity of inhaled asbestos particles, and environmental factors such as cigarette smoking [70]. Asbestos-related bronchogenic carcinoma most often occurs in the setting of alveolitis with thickening of alveolar walls and peribronchial regions of the lung [19]. Animal models of asbestosis have further demonstrated adenoid

proliferation in the respiratory bronchioles in the background of chronic inflammation and fibrosis. In asbestos, workers with greater than 20 years of exposure, it is not possible to separate the mechanisms of carcinogenesis of the lung from those of inflammation or fibrosis—the processes run in parallel. Based on case-control studies, there is an increase in lung cancer cases even in the absence of demonstrable pulmonary fibrosis [71]. The link of asbestosis with lung cancer is substantial as noted by the excess number of deaths due to lung cancer in patients with asbestosis [72]. Currently, the worldwide incidence of asbestos-induced cancer and other diseases is still on the rise because of their long latency periods [7]. A major factor in the development of lung cancer may be the formation of ROS which target mitochondrial and cause mutagenic events [58]. Accumulating evidence have demonstrated that asbestos is genotoxic as assessed using a variety of techniques such as assays of DNA damage and apoptosis, chromosomal damage, aneuploidy studies, sister chromatid exchange, and altered cell ploidy [73]. An additional factor in the development of bronchogenic carcinoma is the high rate of cigarette smoking identified in asbestos-exposed individuals. Similar to the well-established increased risk of lung cancer in patients with idiopathic pulmonary fibrosis, there are numerous reports that show a direct relationship between excess asbestosis cases and lung cancer mortality [2].

12.4.1 Mechanisms of Lung Cancer and Mesothelioma

Many of the processes outlined previously on the development of asbestosis in exposed individuals also apply to lung cancer and mesothelioma [74]. Long latency periods for lung cancer over 20 years and greater than 40 years for mesothelioma suggest a multistep process of acute then chronic inflammation with persistent fiber-induced stimulation with resultant inflammatory cell infiltration, release of cytokines, production of ROS, and DNA damage with disordered cell replication. Importantly, once disordered pulmonary architecture with histologically identifiable inflammatory changes, the fiber clearance process is adversely affected and the inflammatory process has the potential to become heightened. ROS such as superoxide, hydroxyl radical, and hydrogen peroxide play a major role and are catalyzed by iron species on inhaled asbestos fibers. Additionally, there is generation of nitric oxide involved in this inflammatory process [75]. ROS, along with chemokines and cytokines, may cause alterations in growth and differentiation of target epithelial and mesothelial cells. In vitro studies of ROS have demonstrated breaks in DNA in solution and cultured cells. More recent evidence suggests the overall carcinogenic activity of asbestos is encompassed by several processes to include DNA damage caused by reactive oxygen and nitrogen species production, chromosome tangling with associated DNA damage, and adsorption of various carcinogens around asbestos fibers [76]. Asbestos fibers initiate a number of signaling and survival pathways in mesothelial cells and lung epithelial cells and these pathways are up regulated in lung cancers and mesothelioma, where they contribute to tumor development, homeostasis, and resistance to chemotherapy [77].

12.4.2 Tobacco Smoking

Tobacco smoking is a common confounder in human studies involving asbestos workers due to historical rates of smoking in this population [78]. This increase in lung cancer among smokers is partially due to the impairment of asbestos clearance in smokers, which probably accounts for the observation that tobacco smoke augments asbestos pulmonary toxicity [79]. Asbestos fibers can also act as condensation nuclei for aromatic hydrocarbons that result in a more effective transfer and uptake in tracheal epithelial cells. Cigarette smoke exposure increases the retention of short fibers more than the retention of long fibers. An increase in the short fiber load in smokers may play a role in fibrogenesis [80]. In addition, several models have likewise demonstrated that cigarette smoke causes single-stranded breaks in DNA [81].

12.4.3 Reactive Oxygen Species

Asbestos-initiated chronic oxidative stress and initiation of ROS production contributes to carcinogenesis by the promotion of oxidative DNA damage and alteration of redox signaling pathways in exposed epithelial and mesothelial cells [82]. The surface iron associated with asbestos bodies generates hydroxyl radical formation either through a redox reaction or by catalyzing a Fenton-like reaction. The uptake of asbestos fibers can stimulate phagocytic cells such as AMs and polymorphonuclear leukocytes to release a variety of ROS to include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and probably hydroxyl radicals through membrane-associated NADPH (nicotinamide adenine dinucleotide phosphate) oxidase [83]. These ROS contribute to genotoxicity through DNA damage and cell apoptosis with the subsequent development of malignancies. Evidence for ROS causation is demonstrated by several key concepts. Iron chelators and antioxidants prevent asbestos-induced DNA damage and apoptosis, there is a direct relationship between the surface iron on the fibers and DNA-strand break formation, and finally asbestos induces the formation of oxidative DNA lesions [72].

12.4.4 DNA Damage and Apoptosis

Extensive studies have provided details on the molecular mechanisms underlying asbestos-induced DNA damage and apoptosis [41, 68] Apoptosis is a highly regulated physiologic cell death process critical for development, host defense, and prevention of malignant transformation and inflammation throughout the body. Two major mechanisms regulating apoptosis are (1) the intrinsic pathway mediated by the mitochondria (caused by DNA damage), and (2) the extrinsic pathway induced by death-signaling ligands, such as TNF-α or FAS ligands [71, 84]. Several mechanisms, including iron-derived free radicals (ROS) as previously described, the mitochondrial intrinsic death pathway, the extrinsic death receptor pathway, and

altered DNA repair, have been implicated. These mechanisms, along with reactive nitrogen species, act in conjunction to cause apoptosis. Within the intrinsic death pathway, mitochondrial DNA is more susceptible to oxidative damage (such as that caused by asbestos-induced ROS). Studies suggest that failure of normal apoptosis may contribute to cancer formation. Both iron-derived ROS and TNF-α mediate the apoptotic death receptor pathway and increased antioxidant defenses of malignant cells may resist apoptosis. Finally, DNA damage induced by asbestos-derived free radicals activates nuclear transcription factors and activated protein 1 that governs apoptosis, proliferation, and inflammatory changes [85].

12.4.5 p53 Expression

An alteration in p53 expression has been implicated in the pathophysiology of asbestos-associated bronchogenic lung cancer [86]. Asbestos activates both p53 and p21 expression in lung epithelial and mesothelial cells that result in cell-cycle arrest [87]. Increased p53 levels have been detected in the lung cancers of asbestosis patients. Specific p53 point mutations are present in the lung epithelium of asbestos-exposed individuals as well as smokers. Studies performed to examine asbestos-induced whole genome expression profiling confirm that p53 activation plays a crucial role (along with nearly 2,500 other genes) in the regulation of tumor suppression, cell-cycle arrest, apoptosis, and cell survival [88]. As such, p53 plays an important role in the regulation of lung cellular DNA-damage response following exposure to oxidative stress, as occurs with both tobacco smoke and asbestos inhalation. It has been noted that additional research is necessary to determine how p53-dependent signaling alters mitochondria-regulated epithelial cell apoptosis and whether this is a target to prevent malignant transformation due to asbestosis [50].

In summary, current evidence suggests that all forms of asbestos are directly genotoxic to relevant lung target cells, both pulmonary epithelial cells and mesothelial cells. Asbestos-induced genotoxicity can be found as either DNA damage or cell death through apoptosis. Both mechanisms trigger DNA repair mechanisms and complex cellular signaling pathways that ultimately determine cell death. These responses include cell-cycle arrest, transcriptional and posttranscriptional activation of select genes involved in DNA repair, and apoptosis. At the lung tissue level, it is speculated that high levels of apoptosis may promote a fibrotic response/asbestosis, while persistent DNA damage resulting from defects in apoptosis may lead to the formation of either bronchogenic carcinoma or mesothelioma [72].

12.5 Conclusion

The cellular processes of acute and chronic inflammation, fibrosis, and genotoxicity (all with associated mechanisms of ROS-mediated injury) run parallel to the clinical processes of asbestosis, malignant pleural disease, and parenchymal malignancy.

There are common mechanisms for the development of these clinical manifestations in the presence of asbestos exposure. It is not surprising that there remains confusion regarding the requirement that pulmonary fibrosis precede lung cancer, as the processes of fibrosis and the development of pulmonary malignancy are dose-related events with common mechanisms. Yet, the explanation of how the switch is "turned" and how fibrosis becomes malignancy remains elusive.

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Chapter 13
Oxidative Stress and Respiratory Muscle Dysfunction

Kazuto Matsunaga

Abbreviations

AP-1	Activator protein-1
ALS	Amyotrophic lateral sclerosis
COPD	Chronic obstructive pulmonary disease
CMV	Controlled mechanical ventilation
FEV1	Forced expiratory volume in 1 second
HSP	Heat shock protein
MAP kinase	Mitogen-activated protein kinase
MMPs	Matrix metalloproteases
NFκB	Nuclear factor κ-B pathways
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase

13.1 Respiratory Muscles

The respiratory muscles are the mechanical effectors of the breathing system. They are often divided into three major groups: (1) the inspiratory muscles, (2) the expiratory muscles, and (3) the accessory muscles of respiration. The muscles that maintain the patency of the upper airway during the respiratory cycle are sometimes also considered muscles of respiration.

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The diaphragm is the major muscle of inspiration and accounts for approximately 70 % of the inhaled tidal volume in the normal individuals [1]. At functional residual capacity, the diaphragm lies within the chest with a zone of apposition along the chest wall. At total lung capacity, the contracted diaphragm displaces abdominal contents and expands the ribs (chest wall) outward. The innervation of the diaphragm is via the phrenic nerve that originates from cervical nerve roots. The intercostal muscles are thin sheets of muscular fibers that run between the ribs in the costal spaces [2]. There are two sheets of muscle fibers, the external and internal intercostals. The external intercostals function to expand the rib cage during inspiration. The internal intercostals are deeper and function to decrease rib cage size during expiration. Innervation of the intercostalis is via the intercostal nerves originating from the thoracic spine nerve roots. The abdominal muscles (rectus abdominis, internal oblique, external oblique, and transversus abdominis) also serve functions in respiration that mainly assist expiration. The internal and external obliques and transversus abdominis result in an inward movement of the abdominal wall that displaces the diaphragm upward into the thoracic cavity and assists exhalation. The abdominal muscles may also play a minor role in inspiration [3]. If their contraction reduces lung volume below function residual capacity, abdominal muscles can store elastic recoil in the chest wall that assists expansion of the chest wall during the next inspiration.

The accessary muscles of respiration (sternocleidomastoid, scalenes, trapezii, latissimus dorsi, platysma, and pectoralis major and minor muscles) can expand the rib cage and assist inspiration during situations of increased ventilator damaged such as during exercise or during circumstances in which other inspiratory muscles are impaired as in tetraplegia or chronic obstructive pulmonary disease (COPD). It is now clear that some of them function during quiet tidal breathing [4]. The muscles of the upper airways are also considered to be muscles of respiration because they maintain the patency of the upper airway and allow air to flow into and out of the lungs [5]. Some of these also participate in protection of the lower airway during swallowing, a key function in the defense of the respiratory systems.

13.2 Diseases Affecting Respiratory Muscle Dysfunction

The diaphragm is the major muscle of inspiration. It is a dome-shaped structure composed of two muscular leaflets attached to a central tendinous dome. Diaphragm weakness or paralysis can involve either one of the diaphragm leaflets or both. Unilateral diaphragm paralysis is most commonly due to injury to the phrenic nerve. Bilateral disease is most commonly due to diffuse muscle or motor neuron disease such as amyotrophic lateral sclerosis (ALS).

Muscle atrophy is present in numerous pathologies such as cancer, sepsis, collagen disease, and diabetes [6, 7]. Moreover, muscle atrophy can also occur in the absence of disease during prolonged periods of reduced muscle activity [8]. Indeed, it is well established that prolonged bed rest, limb immobilization, or

Table 13.1 Major causes of respiratory muscle dysfunction

Neuropathic causes	Myopathic causes
Trauma	*Muscular dystrophies*
Cardiac or thoracic surgery	Limb girdle
Spinal cord injury	Duchenne and Becker
Radiation injury	*Metabolic myopathies*
Lung cancer	Hyper- or hypothyroidism
Mediastinal tumor	Acid maltase deficiency
Metabolic	*Collagen disease*
Diabetes	Systemic lupus erythematosus
Vitamin deficiency (B6, B12, folate)	Dermatomyositis
Inflammatory neuritis	Mixed connective tissue disease
Idiopathic	*Miscellaneous*
Vasculitis	Amyloidosis
Miscellaneous	Malnutrition
Cervical spondylosis	Mechanical ventilation
Poliomyelitis	Chronic obstructive pulmonary disease
Amyotrophic lateral sclerosis	Idiopathic

diaphragm inactivity via mechanical ventilation can produce muscle atrophy in humans. Increasing evidence indicates that COPD is a complex systemic disease involving more than airflow obstruction [9]. Even though the mechanisms of muscle dysfunction in COPD are still poorly understood, data from human studies clearly indicate that atrophy of skeletal muscles is apparent in COPD [10]. Furthermore, these abnormalities are related to respiratory function, exercise intolerance, quality of life, mortality, and health care resource utilization [11]. Major causes of respiratory muscle dysfunction are listed in Table 13.1.

13.3 Mechanisms of Muscle Atrophy

13.3.1 Models of Muscle Atrophy

Skeletal muscle accounts for 40–50 % of the total body mass in a male with normal body weight. Skeletal muscle turnover is a dynamic process balancing protein synthesis and breakdown. However, many acute and chronic illnesses cause the loss of muscle mass due to net breakdown of muscle proteins [12]. To investigate the mechanisms responsible for muscle atrophy in humans, several animal models have been developed to mimic the various conditions that produce human disuse muscle atrophy [8, 13–18]. Using the rat hindlimb suspension and limb immobilization models, it has been demonstrated that disuse muscle atrophy occurs due to both a decrease in muscle protein synthesis and an increase in the rate of proteolysis [13, 14]. In the hindlimb suspension model, the rate of protein synthesis declines rapidly after

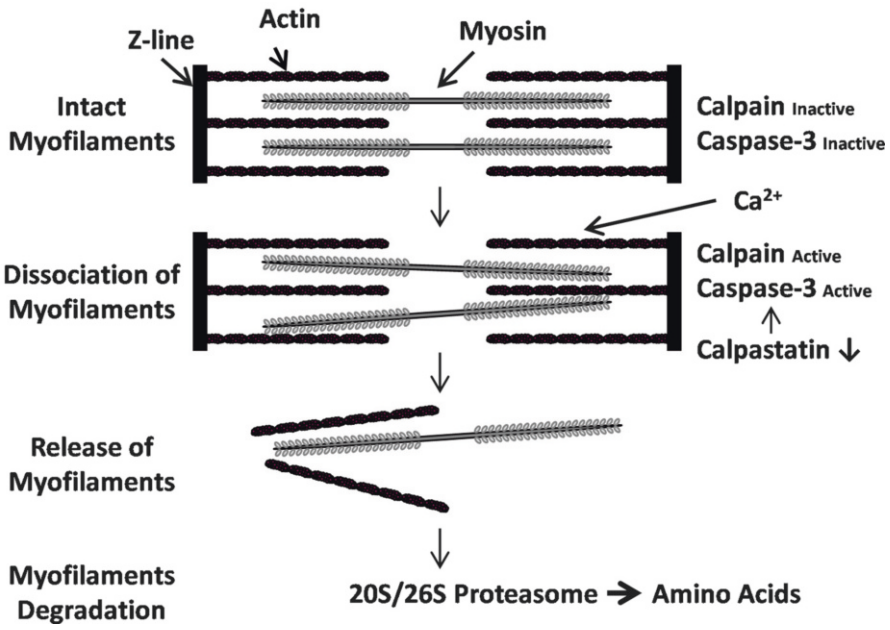

Fig. 13.1 Simplified overview of the proteolytic degradation of myofilaments during disuse muscle atrophy

the onset of muscle unloading [14]. Moreover, the decrease in protein synthesis is followed by a large and rapid increase in proteolysis [8]. These data indicate that a reduction in the activity of skeletal muscle negatively influences muscle mass via alterations in the rates of protein synthesis and degradation that lead to disuse muscle atrophy. Another model used to investigate muscle atrophy is controlled mechanical ventilation (CMV) that unloads the diaphragm. Several animal studies reported that prolonged CMV results in a rapid onset of diaphragmatic fiber atrophy [15–18]. CMV-induced muscle atrophy also occurs as a result of both decreased protein synthesis and elevated proteolysis [17, 18]. Importantly, it is accepted that ventilator-induced diaphragmatic weakness contributes to difficult weaning from CMV.

13.3.2 Proteolytic Pathways in Skeletal Muscles

Several proteolytic systems are involved in the degradation of muscle proteins [12] (Fig. 13.1). The most investigated proteases in skeletal muscles are lysosomal proteases (e.g., calpain) and the proteasome system. Although lysosomal proteases are activated in skeletal muscle causing disuse atrophy, the importance of these proteases appears limited [19, 20]. By contrast, there is evidence that strongly suggests that both calpain and the ubiquitin-proteasome pathways are involved in the muscle protein degradation during muscle atrophy [19, 20]. Moreover, another protease,

caspase-3, may play an important role in atrophy of muscle fibers [21]. The bulk of muscle proteins (50–70 %) exist in actomyosin complexes [22]. While the proteasome system can degrade monomeric contractile proteins such as actin and myosin, this protease does not degrade intact actomyosin complexes [23]. Thus, myofilaments must be released from the sarcomere as monomeric proteins before degradation by proteasome pathways [22, 24]. There is evidence showing that both calpain and caspase-3 are capable of inducing a dissociation of the myofilaments [21–23]. Therefore, activation of one or both of these proteases is required to achieve proteolytic degradation of myofilamants during disuse muscle atrophy (Fig. 13.1).

Calpain is a Ca^{2+}-dependent cysteine protease that is activated during periods of muscle inactivity [23]. Calpain releases sarcomeric proteins by cleaving cytoskeletal proteins (e.g., titin, nebulin) that anchor the contractile elements [20, 25]. Calpain activity is regulated by the cytosolic calcium levels and the concentration of the endogenous calpain inhibitor calpastatin [23]. It has been argued that oxidative stress could play an important role in cytosolic calcium concentration elevations induced by reduced muscle activity [26]. A biological explanation for this thesis is that the oxidant-induced formation of reactive aldehydes reduces the plasma membrane Ca^{2+} ATPase activity [27]. This would retard Ca^{2+} removal from the cell and promote intracellular Ca^{2+} accumulation. Nonetheless, it remains unknown whether this mechanism is solely responsible for the calcium overload in muscle cells mediated by reduced muscle activity.

Caspases are endoproteases that degrade proteins and sometimes cause programmed cell death [28]. In the cell, caspases are expressed as inactive precursors, and activation of caspases can result in events leading to protein breakdown and apoptosis. Recent evidence suggests that caspase-3 may play an important role in muscle protein degradation [21, 29]. Specifically, the activation of caspase-3 promotes the degradation of actomyosin complexes, and the inhibition of caspase-3 activity suppresses the rate of proteolysis [21]. In the case of diabetes-induced muscle atrophy, it seems possible that caspase-3 is activated by the activation of caspase-12 (via a calcium release pathway) and/or activation of caspase-9 (via a mitochondrial pathway) [21]. A key interaction between these caspase-3 activation pathways is that both of these types of signaling can be activated by reactive oxygen species (ROS) [28]. Oxidative stress can promote the activation of the calcium release pathway that activates caspase-3 by increased cellular calcium. Calpain activation can also contribute to caspase-3 activation via this calcium-mediated pathway [30]. The mitochondrial pathway of caspase-3 activation is complex, but ROS can lead to mitochondrial release of cytochrome *C*, resulting in the activation of caspase-9 and subsequent activation of caspase-3 [31]. It is noteworthy that calpastain is a substrate for both caspase-3 and calpain. Therefore, an increase in caspase-3 or calpain activity reduces the calpastain levels in cells and promotes calpain activation [21, 23]. The interactions between the calpain and caspase-3 proteolytic system could play an important role in the regulation of myofilament release in skeletal muscle during periods of muscle inactivity.

In the proteasome system of proteolysis, protein can be degraded by either the 20S core proteasome or the 26S proteasome [32–34]. This pathway plays an important role in ATP-dependent degradation of ubiquitinated proteins [35].

Moreover, there is evidence that the 20S core proteasome can selectively degrade oxidative-stress modified proteins without ubiquitination [32, 33]. The binding of ubiquitin to protein substrates requires the ubiquitin-activating enzyme (E1), specific ubiquitin-conjugating enzyme (E2) and, in many cases, specific ubiquitin protein ligase enzymes (E3). Prior studies reveal that the specific ubiquitin-conjugating enzyme $E2_{14K}$ is a regulator of skeletal muscle ubiquitin–protein conjugation. $E2_{14K}$ interacts with a specific E3 ligase to promote muscle wasting in a variety of catabolic states [36]. Moreover, two ubiquitin E3 ligases, atrogin1 and muscle ring finger-1, have been discovered in skeletal muscle [37, 38]. Importantly, ROS has been shown to up-regulate the gene expression of these key proteasome components [36].

13.3.3 Oxidant Production in Inactive Muscles

It is well established that oxidative stress occurs when oxidant production in skeletal muscles exceeds the antioxidant capacity to buffer oxidants [12, 39]. There is much evidence that oxidative injury occurs during periods of disuse in locomotor skeletal muscles [26, 40–42] and in the unloaded diaphragm during mechanical ventilation [18, 43]. At present, it seems plausible that oxidative stress in inactive muscles may be due to the interaction of at least five different oxidant production pathways: (1) xanthine oxidase (XO) pathway; (2) nitric oxide synthase (NOS); (3) increased cellular levels of reactive iron; (4) NADPH oxidase; and (5) mitochondrial production of superoxide radicals [40].

XO is produced in cells via sulfhydryl oxidation or proteolysis of xanthine dehydrogenase by calcium-activated proteases such as calpain [44]. In the presence of oxygen and purine substances, XO catalyzes the formation of superoxide radicals and uric acid. Superoxide produced by the XO pathway can react with nitric oxide (NO) to form the highly reactive and biologically damaging peroxynitrite ($ONOO^-$) [45]. The production of peroxynitrite and other reactive nitrogen species (RNS) is related to cellular injury due to increased lipid peroxidation and nitrosylation of proteins [46]. NO is synthesized from the amino acid L-arginine by many cell types. Synthesis occurs through NOS of three main types: neuronal NOS (NOS1), which was originally found in neural tissue but is also present in most cell types; endothelial NOS (NOS3), originally described in endothelial cells; and inducible NOS (NOS2) that is predominantly found in inflammatory conditions, but is now recognized to be more widespread. The NOS convert L-arginine into NO and L-citrulline utilizing NADPH. Both NOS1 and NOS3 are calcium activated, and these synthases are expressed in skeletal muscle. There is evidence that NOS activity is increased in immobilized skeletal muscles, resulting in the increased production of NO [40]. In addition, both H_2O_2 and superoxide radicals are capable of promoting the release of iron from ferritin [45]. In reference to iron-mediated oxidant stress in skeletal muscle, immobilization of the rat soleus muscle has been shown to promote

increases in the total muscle iron levels [47, 48]. This increase in muscle iron was related to elevated lipid peroxidation in the immobilized muscles [47]. There is recent evidence of a nonphagocytic and nonmitochondrial NADPH oxidase is found in human skeletal muscle [49]. Numerous factors can increase the NADPH oxidase activity in cells, including the calcium-sensitive protein kinase C-ERK1/2 pathway [49]. Because muscle inactivity results in an increase in intracellular calcium concentration, it seems plausible that the NADPH oxidase activity would increase the superoxide production. However, it is uncertain whether muscle inactivity results in an increased NADPH oxidase activity. It has been estimated that, at physiological levels, 1–3 % of the total oxygen reduced in the mitochondria may form superoxide radicals [45]. In skeletal muscle, mitochondrial production of ROS is greatest during heavy muscle exercise when the ATP requirement is high. In contrast, the mitochondrial-mediated production of ROS is at a low level during periods of reduced muscle activity [50]. These data indicate that mitochondrial contributions to disuse-mediated oxidative injury in skeletal muscle would be minimal.

13.3.4 Antioxidant Defense Systems

Both enzymatic and nonenzymatic antioxidants protect muscle fibers from oxidative injury during periods of increased ROS production [51]. The principal antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase, and catalase. Additional antioxidant enzymes such as peroxiredoxin, glutareoxin, and thioredoxin reductase also contribute to cellular protection against oxidation. In addition, numerous nonenzymatic antioxidants exist in cells (e.g., glutathione, uric acid, bilirubin) [51]. ROS can act through several different pathways of signaling transduction. According to Allen and Tresini, almost half of the ROS effects reported involve members of the mitogen-activated protein kinase (MAP kinase) and nuclear factor κ-B (NFκB) pathways [52], but the effects are not limited to these processes. Recently, Jin et al. studied the role of the ROS activation of NFκB and MAP kinases in the adaptations of muscle cells to oxidative stress and concluded that these pathways are critical cellular responses for maintaining muscle homeostasis through up-regulation of the expression of antioxidant enzymes and other cytoprotective proteins [53]. A single isometric contraction protocol in mouse muscle, which had been shown to increase muscle ROS generation, also increases the activity of muscle antioxidant defense enzymes such as SOD and catalase together with heat shock protein (HSP) 60 and HSP70 content [54], changes which were replicated in studies of human muscle [55]. Supplementation with vitamin C or other antioxidants reduced these adaptive responses, supporting that these adaptations of muscle cells were regulated by ROS [56, 57]. Although many transcription factors are redox sensitive, NFκB and activator protein-1 (AP-1) are now considered to be key factors in the up-regulation of antioxidant enzymes such as SOD and catalase in response to oxidative stress [53, 58, 59].

13.4 Links Between Oxidative Stress and Muscle Atrophy

Several lines of evidence suggest that oxidative stress in inactive skeletal muscle contributes to muscle atrophy. Kondo et al. revealed that immobilization of skeletal muscles is associated with increased ROS production resulting in oxidative injury in inactive muscle fibers and that disuse muscle atrophy could be retarded via antioxidants [48]. This is the first evidence showing that oxidants play a signaling role in the regulation of disuse muscle atrophy. They treated rats with the lipid-soluble antioxidant vitamin-E, which reduced immobilization-induced muscle atrophy [40]. Appel et al. confirmed the ability of vitamin-E to diminish disuse muscle atrophy [60]. Furthermore, the prevention of oxidant stress through the administration of the antioxidant cysteine effectively suppressed protein ubiquitination and myosin heavy chain fragmentation in the gastrocnemius muscle after hindlimb suspension [61]. Importantly, these experiments demonstrated that maintenance of the muscle redox status attenuated the disuse muscle atrophy [61]. However, it should be noted that not all antioxidant interventions are capable of retarding disuse muscle atrophy. Koesterer et al. demonstrated that muscle protein oxidation, indicated by an increased level of protein carbonyls, was associated with hindlimb in rats [62]. Although in vitro experiments demonstrated antioxidant protection against lipid peroxidation induced by different radical-generating systems, the antioxidant supplementation did not attenuate the disuse muscle atrophy associated with hindlimb [62]. Several lines of evidence link ROS to disuse muscle atrophy via the redox control of proteolysis. However, it is still uncertain which ROS pathways are responsible for oxidant production in unloaded skeletal muscles. Although evidence exists that antioxidants can retard muscle atrophy, it is unclear if oxidant production is an absolute requirement for muscle atrophy or simply contributes to the rate of muscle atrophy.

13.5 Mechanical Ventilation and Respiratory Muscle Dysfunction

Mechanical ventilation is a critical component of intensive care medicine. Respiratory failure, neuromuscular diseases, drug overdose, and recovery from general anesthetics are common indications for the use of mechanical ventilation. Clinical problems in weaning patients after prolonged mechanical ventilation have been reported in a large number of individuals, and it is postulated that a common cause of difficult weaning is diaphragmatic force and endurance deficits [63, 64]. Several animal studies have shown that mechanical ventilation for prolonged periods is associated with diaphragmatic atrophy and contractile dysfunction [15–18]. Moreover, several lines of evidence suggest that an increase in protein oxidation is involved in the mechanical ventilation-induced diaphragm atrophy. Shanely et al. have reported that CMV results in increased oxidized proteins in the diaphragm, as indicated by elevated protein carbonyls [17, 18]. A more recent study showed that

CMV-induced oxidative injury does not occur rapidly after the onset of CMV, but is present after 6 h of CMV [43]. CMV-induced protein oxidation was evidenced by insoluble proteins in the diaphragm with molecular masses of 40 and 200 kDa. This work postulated that diaphragmatic actin (40 kDa) and myosin (200 kDa) are strong candidates for oxidation during CMV [43]. In humans, Levine et al. obtained biopsy specimens from the costal diaphragms of 14 brain dead organ donors before organ harvest (case subjects) and compared them with intraoperative biopsy specimens from the diaphragms of eight patients who were undergoing lung surgery (control subjects) [29]. The case subjects had diaphragmatic inactivity and underwent MV for 18–69 h; among the control subjects the diaphragmatic inactivity and MV were limited to 2–3 h. As compared with the diaphragm-biopsy specimens from controls, specimens from the case subjects showed decreased cross-sectional areas of slow-twitch and fast-twitch muscle fibers of the diaphragm [29]. Furthermore, in the case subjects, the decrease in the diaphragmatic glutathione concentration was consistent with oxidative stress, and the increase in active caspase-3 suggested an increased rate of protein release from the myofibrillar lattice within the diaphragm [29].

In critically ill patients undergoing long-term CMV, there are multiple deleterious changes in the human diaphragm, including decreased force-generating capacity, muscle fiber injury, muscle atrophy, and increased expression of ubiquitinated proteins, Nf-κB, and calpain isoforms, all of which have been previously implicated in different aspects of skeletal muscle injury and atrophy responses [65]. Furthermore, this work also showed that the degree of diaphragmatic atrophy was directly proportional to the length of CMV [65]. Collectively, these studies strongly suggest that diaphragmatic dysfunction, injury, and atrophy occur rapidly in critically ill patients during CMV. These findings are consistent with increased diaphragmatic proteolysis during muscle inactivity.

13.6 COPD and Respiratory Muscle Dysfunction

Skeletal muscle dysfunction is of particular interest in COPD. It directly influences exercise performance [66], is associated with poor health status [67] and is an independent predictor of health care utilization [68] and mortality [69]. COPD is a disease characterized by a usually progressive airflow limitation that is not fully reversible, and has potentially significant extra-pulmonary effects [70]. Although a disease of the lungs, extra-pulmonary features of COPD are increasingly recognized as important contributors to morbidity and mortality [71]. Furthermore, the respiratory muscle function plays a key role in the pathogenesis of breathlessness [72] and the maximum inspiratory pressure is an independent predictor of survival in severe disease [73]. Although the muscle dysfunction in COPD patients is characterized by a significant reduction in muscle strength and endurance, cross-sectional studies have revealed that the muscle dysfunction in COPD is complicated, comprising muscle atrophy, fiber type shift, and loss of capillary density. The most commonly studied skeletal muscles are the quadriceps and the diaphragm.

13.6.1 Muscle Dysfunction in COPD

Compared with healthy controls, the quadriceps femoris muscle strength is reduced by about 20–30 % in patients with COPD [66, 74–77]. The degree of the reduction in limb muscle strength correlates with the severity of the disease process [74]. A marked increase in susceptibility to fatigue is also observed, with a more rapid decline in performance during continuous [78, 79] or repeated bouts of exercise [80, 81]. Reduced quadriceps strength in patients with moderate COPD could lead to poor exercise performance, increased dyspnea and worsening quality of life [66]. Furthermore, relatively low quadriceps strength is a powerful predictor of mortality in severe COPD patients [69].

Quadriceps strength improves significantly after pulmonary rehabilitation [82], but did not improve after treatment with bronchodilators in patients with COPD [83, 84]. Concerning the ventilatory muscles, the maximal strength of the diaphragm muscle, as measured by the maximal transdiaphragmatic pressure (Pdimax), remains about 30–40 % lower in COPD patients as compared with control subjects [85, 86]. Also, the inspiratory muscle strength in patients with severe COPD, as measured by the maximal inspiratory pressure, was an average of 59 % of that measured in control subjects [87]. The inspiratory muscle strength is more severely reduced than that of the expiratory muscle, and proximal upper muscle strength is more impaired than distal upper limb strength in patients with moderate COPD [87]. The reduction of arm strength is milder than that of leg strength [77]. The possible reason for these differences is that the ventilatory muscles, especially the diaphragm, have workloads different from the lower limb muscles because they are in a chronically overloaded state due to the increased work of breathing by airflow obstruction and hyperinflation.

The endurance of limb muscles is attenuated by about 30 % in patients with moderate COPD and the poor muscle endurance in such patients correlates positively with the physical activity index, forced expiratory volume in 1 second (FEV1), and resting partial pressure of oxygen in arterial blood (PaO_2) [88, 89]. The reduction of quadriceps muscle endurance in COPD patients has been confirmed using artificial stimulation protocols [90], and is related to the reduced oxidative capacity of the mitochondria and to the development of oxidative stress in the muscle [78].

The reduction in strength can largely be explained by a comparable reduction in the quadriceps cross-sectional area and fat-free mass independent of the airflow obstruction and COPD subtype [74, 91–95]. The magnitude of this loss of mass is greater than that of the whole body weight, which indicates that the loss of muscle tissue precedes the loss of other body tissues in COPD patients [74]. When the limb muscle strength is normalized per cross-sectional area or mass, no differences can be observed between COPD patients and control subjects [74, 91, 96, 97], which indicates that muscle atrophy seems to be the sole cause of the reduced limb muscle strength and endurance in COPD patients. However, when the peak torque, isometric strength, and total work of the leg muscle mass are analyzed, greater muscle mass is needed to generate a given functional output in COPD patients than in

control subjects [98]. The quadriceps twitch force was significantly decreased in severe patients with COPD compared to that in control subjects [99]. The quadriceps muscle twitch force falls more rapidly in COPD patients than in control subjects even when the exercise is performed with similar oxygen consumption conditions, which suggests that the limb muscles of COPD patients are more fatigable than those of healthy individuals. These results suggest that factors other than atrophy might play a role in skeletal muscle dysfunction in COPD patients [98].

Less is known about changes in single diaphragm fibers, and debate exists as to whether their force-generating capacity is altered. Some studies reported no change in fiber size [100], while others observed selective atrophy of type I fibers [101]. The fiber type of quadriceps muscles undergoes redistribution from type I (slow-twitch oxidative) fibers to type IIb (fast twitch glycolytic) fibers in severe COPD patients. The type I fiber proportion declines by 20 %, whereas the proportion of type IIb fibers increases by 10 % in patients with severe COPD [102]. Although the exact functional consequences of this fiber type redistribution remain unclear, the fact that type II fibers are fatigue-prone suggests that an increased proportion of type II fibers might be an important factor in increased leg muscle fatigability and reduced endurance. These shifts in fiber from type I to type II were also observed in vastus lateralis muscles in patients with severe COPD [103], and the proportion of type I fibers correlates with FEV1, FEV1/FVC, and the BMI in patients with moderate to severe COPD [104]. In contrast, the proportion of type I fibers was increased and that of type II fibers was decreased in diaphragm muscles. The overall proportion of type I fibers was increased 20–50 %, and the increase was closely related to lung hyperinflation [105, 106]. The shift in fiber type from type II to type I is also seen in the parasternal intercostal muscles of patients with severe COPD [107]. Functionally, shoulder girdle muscle (pectoralis major and latissimus dorsi) strength and abdominal strength are preserved relative to the quadriceps [74, 76], presumably due to the additional activity of expiratory muscles in COPD and the disuse and deconditioning of quadriceps muscle [76].

Capillary density in peripheral muscles is reduced in patients with COPD [108]. The number of contacts between capillaries and fibers is also reduced [102, 108]. As pulmonary rehabilitation in patients with COPD is associated with an increase in the number of capillary fiber contacts [102], however, the reduction in the muscle capillarization was not detected after a pulmonary rehabilitation program [109].

13.6.2 Involvement of Oxidative/Nitrative Stress in COPD

Concerning the production of RNS in the airways of patients with COPD, the exhaled NO levels are much lower than in asthmatic patients and are not so different from those in healthy subjects in spite of having almost the same degree of iNOS expression in the airways as asthmatic patients [110]. In contrast, 3-nitrotyrosine formation in the sputum cells from the patients with COPD was much higher than that from asthmatic patients [110]. These findings suggest that, in the airways of

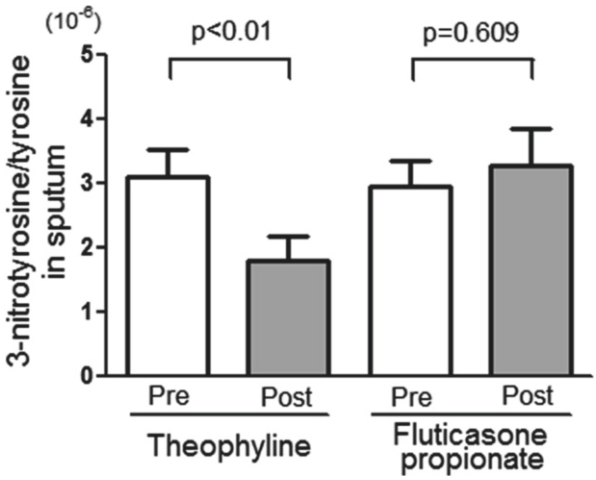

Fig. 13.2 Effect of theophylline or inhaled steroids on the nitration of tyrosine in airway. The ratio of 3-nitrotyrosine/tyrosine in induced sputum was compared before and after treatment with theophylline or fluticasone propionate in COPD patients

COPD, NO production might be the same as that in asthma. NO produced in the airways of COPD might be consumed by ROS such as superoxide anion resulting in the formation of RNS such as peroxynitrite.

The lung inflammation of COPD patients is further aggravated by oxidative stress and excessive proteinases in the lung. Because peroxynitrite is a powerful oxidant, it can cause an imbalance between oxidants and antioxidants. Peroxynitrite can cause the inactivation of antiprotease [111] and the activation of matrix metalloproteinases (MMPs) [112]. These effects of peroxynitrite could also cause an imbalance in proteases/antiproteases. Peroxynitrite, but not NO, stimulates fibroblast-mediated tissue remodeling [113, 114]. Because excessive peroxynitrite is produced in the airways/lung parenchyma of COPD patients [115], RNS may be associated with the development of COPD. We have shown that 3-nitrotyrosine can be a marker of the airway inflammation in patients with COPD [116]. Furthermore, in COPD airways, low-dose theophylline significantly reduces RNS production and neutrophil infiltration in airway to a greater extent than inhaled corticosteroid (Fig. 13.2) [117].

Several studies have demonstrated that increased levels of oxidative/nitrative stress in muscle of COPD [118–122]. Oxidative stress can alter muscle contractility [39], potentially affecting muscle strength, and contribute to muscle fatigue. The administration of antioxidants improves exercise tolerance in COPD patients [123], showing a direct effect of ROS on exercise capacity in such patients. Oxidative stress can also contribute to accelerating protein breakdown [124–127] as a potential mechanism leading to muscle wasting [118, 119]. In quadriceps muscles of severe COPD, the total glutathione concentrations are lower, whereas the levels of 4-hydroxy-2-nonenal (HNE) protein and lipofuscin are significantly higher than those detected in control subjects [96, 119, 127], and protein tyrosine nitration and iNOS protein are increased (Fig. 13.3) [127, 128]. Carbonyl formation and lipid peroxidation also increase in quadriceps muscles of patients with severe COPD [118].

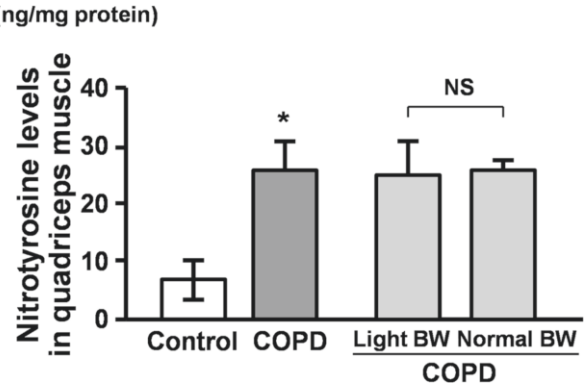

Fig. 13.3 Nitrotyrosine levels in quadriceps muscle of patients with COPD. *BW* body weight. *$p < 0.001$ vs. control

These results suggest that exercising limb muscles might be an important source of ROS products released into the plasma of COPD patients [120]. In contrast, little is known about oxidative/nitrative stress in the diaphragm of COPD patients. In one report, carbonyl and HNE-protein adducts were significantly elevated without an increase in protein tyrosine nitration in the diaphragms of patients with severe COPD [129]. In another report, however, lipid peroxidation was not detected in the diaphragms of patients with moderate and severe COPD [130].

Although indirect evidence supports a strong role for oxidative stress in depressed skeletal muscle strength and endurance, the contribution of oxidative stress to the contractile dysfunction of diaphragm remains under investigation. The levels of uncoupling protein 3 in the skeletal muscle (UCP3) are reduced [131], particularly in the subgroup of patients with low BMI [132] and in the more oxidative fibers [133]. Moreover, the UCP3 levels correlate with the fat-free mass index in the skeletal muscle of COPD patients [132]. As UCP3 is a protein that may protect mitochondria against lipotoxicity, it might prevent ROS-induced oxidative damage in fatty acid.

13.7 Conclusions

Respiratory muscle dysfunction is an important clinical problem. Oxidative stress can contribute to accelerating muscle protein breakdown, and it can also alter muscle contractility, potentially affecting muscle strength and endurance. Importantly, a growing number of studies suggest that antioxidants can serve as therapeutic agents in delaying the rate of muscle atrophy. Further studies are required to identify the precise interactions of muscle dysfunction and oxidative stress in respiratory diseases.

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Chapter 14
Oxidative Stress and Lung Cancer

Aditya Jindal and Navneet Singh

14.1 Introduction

Chronic inflammation has since long been associated with tumorigenesis. This concept has been prevalent in the traditional systems of medicine, notably Ayurveda [1]. However, this was more of an empirical view, without any real understanding of what was happening at the molecular level. In the past few decades, the molecular mechanisms underlying this concept have begun to be understood. The role of oxidative stress as the driver of chronic inflammation has been recognized recently.

14.2 Sources of Free Radicals

The evolution of aerobic respiration led to an overall increased efficiency of energy production. However, its side effect was the generation of free radicals as a by-product of this process. The oxidative enzymes are located on the mitochondrial membranes; electrons are transferred to oxygen during aerobic respiration, resulting in the generation of free radicals or reactive oxygen species (ROS). These include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$), and organic peroxides. Also, during periods of hypoxia, the mitochondria produce nitric oxide (NO), which leads to the production of reactive nitrogen species (RNS). Other reactive species can be further generated, such as reactive aldehydes-malondialdehyde

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(MDA) and 4-hydroxynonenal (4-HNE) [2]. These free radicals then react with macro- and micro-molecules in the cell and lead to derangements in the intracellular milieu, which can progress to tumorigenesis.

Neutrophils are among the most common cells in the lungs. Their enhanced phagocytic activity is due to the oxidative burst, during which process their oxygen consumption increases significantly in order to produce free radicals. These are then used to kill invading microorganisms. However, the excess free radicals produced by them may also cause damage to the normal body mechanisms and structures [3]. There are four major enzymes present in neutrophils which catalyze the formation of free radicals: NADPH oxidase which produces the O_2^- ion, superoxide dismutase (SOD) which forms H_2O_2 from the O_2^- ion, myeloperoxidase which is responsible for the formation of $HOCl^-$ and nitric oxide synthase (NOS) which synthesizes NO [4–10].

Another source of ROS is the autophagy of old and defective mitochondria. These ROS can promote tumorigenesis by affecting cell cycle pathways [11]. ROS/RNS are primarily formed during chronic inflammation. This can occur due to multiple factors such as exposure to physical, chemical, and biological stimuli. Among the factors specific to the lung include asbestos exposure, radiation, industrial toxins, cigarette smoke, and even ambient air pollution [12–14].

Oxidative stress can also occur secondary to chronic infections, though these are characteristically associated with cancers in other organs, such as the liver.

14.3 Types of Free Radicals

The different types of free radicals are produced in varying amounts and have different levels of reactivity. It has been estimated that almost 1–5 % of the total oxygen consumed by the body leads on to the development of the O_2^- anion. It is dissociated into H_2O_2 and water by the enzyme SOD. ROS-induced DNA damage is usually caused by the following reactions—oxidation, methylation, nitration, deamination, and depurination. DNA damage is manifested as strand breaks, point mutations, strand cross-links, and mutations in proto-oncogenes and tumor suppressor genes.

H_2O_2 is less reactive as compared to the other oxygen-derived free radicals. Because it is freely diffusable across the cell membranes, it may be responsible for a greater spectrum of activity than other radicals. The most important free radical involved in the causation of intracellular damage is the $OH^{\bullet}$ ion. It is very unstable, though it is unable to diffuse very far within the cell, it is highly reactive, and can react with any intracellular molecule. Damage to deoxy ribonucleic acid (DNA) leads to the generation of 8-hydroxyguanosine (8-OHG), which on undergoing further hydrolysis leads to the formation of 8-hydroxydeoxyguanosine (8-OHdG). Detection of this molecule strongly suggests the presence of oxidative damage and has been closely correlated with carcinogenesis [15]. One of the major changes it causes in the DNA molecule is the transversion of Guanine: Cytosine to Thymine: Adenine (i.e., G → T transversion). The presence of this transversion is associated with extensive point mutations [16]. Other products of $OH^{\bullet}$ ion damage to DNA include

5-hydroxyuracil, 5-hydroxymethyluracil, 5-hydroxyadenine, 8-hydroxyadenine, and 2, 6-diamino-4-hydroxy-5-formamidopyrimidine [3].

Neutrophils produce additional ROS to the ones mentioned previously, including hypochlorous acid (HOCl). HOCl leads to DNA damage by causing strand cross-links, chlorination of bases, and oxidation of pyrimidine bases. The damage to DNA is sometimes so severe that it can cause cell death. 5-chlorouracil is formed after damage to DNA by HOCl; it also serves as a marker for oxidative damage due to HOCl [3]. MDA and 4-hydroxynonenal are other toxic products formed as a result of lipid peroxidation from ROS and are extremely toxic to DNA and other cellular molecules.

RNS also contribute to oxidative stress. The most important molecule in this context is NO. It is synthesized from the molecule L-arginine by the enzyme NOS. NOS exists in three isoforms—neuronal, endothelial, and inducible. The first two isoforms are calcium dependent and produce physiological amounts of NO; however, the inducible form of NOS is calcium independent and produces excess amounts of NO under inflammatory conditions. NO leads to the formation of peroxynitrite ($ONOO^-$) which leads to the formation of 8-nitroguanine. This molecule, similar to 8-OHdG, serves as a marker for nitrate stress-induced injury. It gets incorporated into DNA and undergoes spontaneous depurination, resulting in the formation of an apurinic site. This leads to a G→C transversion [12].

14.4 Effects of Oxidative Stress

Oxidative stress affects and modulates tumor initiation, progression, invasion, angiogenesis, and metastasis. It acts by causing DNA mutations, epigenetic changes, chromosomal aberrations, protein dysfunction, and modulation of cell signaling pathways and second messenger systems. Oxidative stress also serves as the link between chronic inflammation and cancer.

An important characteristic of tumor promoters is the ability to recruit normal cells and task them to produce free radicals in excessive amounts [17, 18]. Damage to cellular molecules produced by these radicals is essential in tumorigenesis. In fact, tumor promotion can be inhibited in experimental animals by the use of antioxidants which further inhibit the respiratory burst of phagocytes [17, 19].

ROS also enhance tumor cell survival by multiple means. As an example, ROS inhibit phosphatase and tensin homolog deleted from chromosome 10 (PTEN), which is an inhibitor of Akt. Akt is a serine threonine kinase which has multiple actions; it inhibits apoptosis by inactivating proapoptotic molecules such as caspase-9 and promotes tumorigenesis by stabilizing oncogenes and dysregulating cell cycle checkpoints [20–23]. Uncontrolled cell proliferation, which is another characteristic of tumor cells, is also promoted by oxidative stress. This is modulated through pathological activation of cell signaling pathways, specifically the mitogen-activated protein (MAP) kinase/AP-1 and NF-κB pathways [24]. ROS are also involved in tumor invasion, metastases, and angiogenesis. These are mediated by aberrant and uncontrolled signaling by ROS leading to dysregulation of matrix metalloproteinases, intercellular adhesion molecules and angiogenetic factors [25–29].

Resistance of cancer cells to chemotherapy and radiotherapy may also be mediated through ROS/RNS. Various molecular mechanisms of resistance have been identified including the activation of efflux pumps, increased expression of detoxifying enzymes, and resistance to apoptosis [30–33]. A central role of the transcription factor NF-κB has been demonstrated in this regard [2]. Several chemotherapeutic agents used in the treatment of lung and other cancers such as paclitaxel, doxorubicin, daunomycin, vinblastine, vincristine, 5-fluorouracil, cisplatin, and tamoxifen are associated with the activation of NF-κB [34, 35].

14.5 Antioxidants

A variety of antioxidant systems exist in the body to counteract the destructive actions of ROS (Table 14.1). The main antioxidant in this regard is glutathione which is a tripeptide and protects DNA from free radical damage. It can also act by directly detoxifying carcinogens and exporting them from cells. Paradoxically, elevated levels of glutathione are seen in some cancers where they are associated with resistance to chemotherapy [36–40]. Glutathione peroxidase reacts with H_2O_2 and other peroxides and catalyses the reduction of fatty acid hydroperoxides; glutathione reductase is the complementary enzyme and regenerates glutathione from the reduced state [13, 41].

Other antioxidant enzymes include SOD and catalase. SOD acts by converting superoxide anions to H_2O_2 while catalase further converts H_2O_2 to water. Peroxiredoxins also act in a similar fashion and reduce H_2O_2 to water [2]. A recent cohort study evaluated the role of SOD in the prediction of all cause cancer mortality. The result showed that elevated levels of SOD were associated with all cancer mortality. These results demonstrated that elevated SOD levels may occur as a response to oxidant stress. However, elevated levels of SOD were not associated with increased mortality from lung cancer [42, 43].

Ascorbic acid is another antioxidant compound which acts as a free radical scavenger. It is involved in pathways that regenerate other antioxidants. It exists mainly in its reduced state in the body; its oxidation produces dehydroascorbic acid, which is transported intracellularly and is regenerated to ascorbic acid. Similar compounds include α-carotene, β-carotene, cryptoxanthin, lutein, zeaxanthin, lycopene, α-tocopherol, selenium, and vitamin-E [13].

14.6 Oxidative Stress Specific to Lung Cancer

Oxidative stress has also been implicated in the causation of lung cancer. In this context, smoking of tobacco-based products has a special importance. The particulate as well as the gaseous phase of cigarette smoke contain ROS which are relatively stable with long half-lives. These ROS cause direct damage in addition to the

Table 14.1 Summary of trials evaluating supplementation of antioxidants for the prevention of cancer

Name of trial and year	Study design	Intervention	Result
ATBC (1994) [87]	Randomized, double-blind, placebo-controlled trial	29,133 male smokers; alpha-tocopherol or beta-carotene supplementation for the prevention of lung cancer	No reduction in incidence with alpha-tocopherol; higher incidence of lung cancer among those who received beta carotene than placebo
Hennekens et al. (1996) [91] (Physicians' Health Study)	Randomized, double-blind, placebo-controlled trial	22,071 male physicians; beta-carotene supplementation for preventing cancer	No benefit or harm with supplementation
Omenn et al. (1996) [88] [Carotene and Retinol Efficacy Trial (CARET)]	Randomized, double-blind, placebo-controlled trial	18,314 subjects; evaluation of the efficacy of retinol and beta-carotene supplements for the prevention of lung cancer	No benefit of intervention; increased incidence of lung cancer in intervention group; trial stopped early
Lee et al. (2005) [92] (Women's Health Study)	Randomized, double-blind, placebo-controlled trial	39,876 females; supplementation with aspirin, vitamin E, and beta-carotene for the primary prevention of cancer and cardiovascular disease	No benefit with supplementation
Kamangar et al. (2006) [93]	Randomized, double-blind, placebo-controlled trial	29,584 adults; daily vitamin and mineral (retinol, zinc, riboflavin, niacin, ascorbic acid, molybdenum, beta-carotene, vitamin E, selenium) supplementation for the prevention of cancer and mortality from all causes	No benefit with supplementation
Gaziano et al. (2009) [94] (Physicians' Health Study II)	Randomized, double-blind, placebo-controlled trial	14,641 male physicians; evaluation of long-term vitamin E or C supplementation to decrease risk of prostate and total cancer events among men	No benefit with supplementation
Lippman et al. (2009) [95] (Selenium and Vitamin E Cancer Prevention Trial)	Randomized, double-blind, placebo-controlled trial	35,533 men; supplementation of selenium, vitamin E, or both could prevent prostate and other cancers	No benefit with supplementation
Hercberg et al. (2010) [96]	Randomized, double-blind, placebo-controlled trial	6,364 placebo and 6,377 supplemented group; supplementation with combination of antioxidants (vitamin C, E), beta-carotene and selenium to reduce the incidence of cancer and ischemic-cardiovascular disease	Decrease in total cancer incidence and total mortality with antioxidant supplementation which disappeared on follow-up

establishment of a secondary oxidative stress response, which is responsible for further injury and inflammation. In fact, this oxidative stress response has been implicated as the driving force behind the spectrum of smoking induced injury in the lung, culminating in lung cancer [44–48].

One of the most common forms of oxidative stress which has been described extensively is the peroxidation of lipids by ROS. The peroxidation of lipids leads to the generation of multiple products, the more common being MDA, 4-hydroxynonenal (4-HNE), acrolein, and crotonaldehyde. These products react with DNA molecules and lead to formation of DNA adducts, which are mutagenic and may contribute to neoplastic transformation [49, 50]. Acrolein and crotonaldehyde are constituents of cigarette smoke and behave like exogenous ROS, while MDA and 4-hydroxynonenal are formed upon exposure to tobacco smoke [51, 52].

It has been found that cigarette smoking was associated with reduced levels of antioxidants as compared to the antioxidant levels in nonsmokers. The circulating levels of ascorbic acid and carotenoids have been shown to correlate inversely with the number of cigarettes smoked per day [53]. Other studies have shown an increase in the ratio of dehydroascorbic acid to ascorbic acid in smokers as compared to that in nonsmokers [54]. Also, another study demonstrated an increase in the levels of ascorbic acid 4 weeks after smoking cessation [55].

Studies on the levels of antioxidant enzymes, e.g., catalase, glutathione peroxidase, and SOD, in smoker versus nonsmokers have shown divergent results, with some studies showing a decrease and others showing an increase in levels [56–67]. Overall, no satisfactory conclusion can be drawn from these studies if they are interpreted together. The confusion may stem from the fact that cancer patients may have elevated levels of antioxidant enzymes as a compensatory mechanism to oxidative stress.

Oxidant stress may also explain the association between environmental pollutant exposure and lung cancer. Specifically, the main category of environmental pollutants consists of polycyclic aromatic hydrocarbons which are found primarily in effluents from power plants and internal combustion engines. Here also, it is important to mention the role of cigarette smoke, which may have an additive effect, over and above that of environmental pollution, in the causation of lung cancer. The prototype aromatic hydrocarbon is benzopyrene, which is also the best studied. Once activated by metabolic processes in the body, it leads to the formation of benzo(a)pyrene diol epoxide (BPDE), which further reacts with the guanosine moiety of DNS to form covalent BPDE-DNA adducts. These adducts are mutagenic and are associated with carcinogenesis in the human lung [68–75].

The role of oxidant enzymes within the neutrophils is also highlighted by the above example. The conversion of benzopyrene to BPDE is catalyzed by the enzyme myeloperoxidase, contained in the neutrophils. In fact, certain polymorphisms of the gene for this enzyme have been found to be protective in lung cancer, i.e., 463G→A transition in the promoter region of MPO. A 40–70 % decreased risk of lung cancer has been found for the AA genotype in some studies [76–81]. It has also been observed that the maximum benefit of the AA allele was present in smokers, which suggests an associated between chemical carcinogens, oxidative stress,

genomic predisposition, and carcinogenesis [80, 81]. This linkage has been found to be maximum for small cell cancer of lung, which is associated with heavy smoking [76]. So, the conclusion would be that neutrophil-derived oxidant enzymes such as myeloperoxidase mediate the march from chemical carcinogen exposure to carcinogenesis.

Another role of oxidative stress in lung carcinogenesis is seen after exposure to fibrous and nonfibrous particles. The prototype of this group is asbestos. It is a fibrous particle of which various types are available, and some are more carcinogenic than others. For example, crocidolite (blue asbestos) and amosite (brown asbestos) are more mutagenic than chrysotile (white asbestos). Asbestos exposure is associated with the development of lung cancer and malignant mesothelioma. Both oxidative and nitrative stresses have been found to be associated with asbestos. Neutrophil-derived myeloperoxidase is of prime importance in this setting. The role of RNS was demonstrated in a study which showed increased levels of 8-nitroguanine, iNOS, and NF-κB in the bronchial epithelial cells of mice which were exposed to high intratracheal levels of asbestos. Significantly, the immunoreactivities of the mentioned compounds were higher in the group exposed to crocidolite as compared to chrysotile, which could explain the differing carcinogenic potential of these compounds [82].

Evidence accumulated in clinical trials has tended to favor the imbalance between oxidants and antioxidants as being one of the causative factors in carcinogenesis. A recent study compared the levels of urinary 8-OHdG, plasma MDA, red cell Cu-Zn SOD, and glutathione peroxidase in 222 patients of lung cancer with 207 control subjects [83]. It was found that the levels of 8-OHdG and MDA were significantly higher while the red cell SOD and glutathione peroxidase activities were significantly lower in patients than in controls [83]. Similar alterations have also been noted in other studies [84–86].

14.7 Antioxidant Supplementation and Lung Cancer

Considering the exhaustive research on the aetiological role of oxidative stress in lung cancer, it was only logical that the therapeutic benefit of antioxidants be tested in the prevention and treatment of this disease. Indeed, there have been major studies in the last few years on this subject. However, the results were far from expected.

The alpha-tocopherol beta-carotene (ATBC) trial, funded by the National Cancer Institute (USA), was a randomized, double-blinded primary prevention trial to study whether supplementation with either alpha-tocopherol or beta-carotene or both would lead to a reduction in the risk of lung cancer [87]. The results were unexpected; there was no reduction in risk with supplementation. Conversely, there was a 16 % increase in the risk of lung cancer in subjects receiving either beta-carotene alone or in combination with alpha-tocopherol [87]. Similarly, the CARET trial tested the combination of beta-carotene and retinyl palmitate (vitamin A) taken daily against placebo in 18,314 men and women at high risk of developing lung cancer.

The trial was stopped 21 months early because there was evidence of no benefit and substantial evidence of harm; in fact, there were 28 % more lung cancers and 17 % more deaths in the group with supplementation than in the placebo group [88].

A Cochrane review included nine trials of antioxidant supplementation—these included supplementation of vitamin A, C, E, carotenoids, and selenium (Table 14.1). None of the included studies showed a reduction in lung cancer risk; in fact, there was an increased risk of mortality with beta-carotene [89].

Thus, antioxidant supplementation has not lived up to its initial promise of preventing lung and other cancers. Research into this area is still ongoing and it is expected that future studies may reveal compounds which are actually protective against malignancy. In this context, a study published recently has shown that intake of cruciferous vegetables may be associated with a decreased risk of lung cancer. Cruciferous vegetables may act by activating phase II detoxification enzymes for the deactivation of pro-carcinogens [90]. However, the last word on this issue is yet to be said!

14.8 Future Implications

The link between oxidative stress, inflammation, and cancer is likely to be applicable in the context of occurrence and progression of lung cancer since more and more experimental evidence is accumulating in this regard. Although there are no therapeutic options which can exploit this link at present, the future is likely to witness the discovery of anticancer agents which target this link. The potential for research in this direction is enormous.

Conflict of Interest The authors would like to state that there is no conflict of interest regarding this chapter.

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Chapter 15
Pulmonary Arterial Hypertension and Oxidative Stress

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15.1 Introduction

Pulmonary arterial hypertension (PAH) is a complex and multidisciplinary disorder comprising a series of diseases that result from restricted blood flow through the pulmonary arterial circulation [213, 232]. All of these conditions share a common arterial histopathology characterized by medial hypertrophy, eccentric and concentric intimal fibrosis, and plexiform lesions [114, 213]. The pathophysiology of PAH is not completely understood. Many factors have been shown to be involved in the pathogenesis of PAH, including growth factors, pro-inflammatory molecules, vascular tone mediators, genetic mutations, microRNAs (miRs), and oxidative stress [5, 221, 284]. Currently, the treatment for PAH remains limited and the disease is still associated with a poor long-term prognosis [221]. Growing evidence suggests that reactive

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oxygen species (ROS) and oxidative stress play a pathogenic role in PAH and some antioxidants appear to be useful in various forms of pulmonary hypertension (PH) [373].

15.2 Pulmonary Arterial Hypertension

15.2.1 Epidemiology

PAH was previously considered a rare disease with an unknown frequency, but in 2006 a French registry reported a prevalence of 15 per million [158, 232]. The most common cause found in this study was idiopathic pulmonary arterial hypertension (IPAH) accounting for 39.2 % of the cases, followed by anorexigen exposure, connective tissue disease, congenital heart diseases (CHDs), portal hypertension, and HIV infection [158]. The Scottish morbidity record found a prevalence of 52 cases per million in an adult population [273]. In both studies, PAH was more common in the female population [158]. According to the Centers for Disease Control and Prevention (CDC), deaths attributed to PH varied between 11,000 and 16,000 per year between 1980 and 2002 [159].

15.2.2 Diagnosis and Pathological Findings

15.2.2.1 Signs and Symptoms

The main symptoms found in patients with PH are dyspnea on exertion (around 60 % of patients), fatigue, angina pectoris, syncope, palpitations, and lower extremity edema [232]. Clinical signs include accentuated pulmonary component of S2 audible at the apex (90 % of patients with IPAH), early systolic click, mid-systolic ejection murmur, left parasternal lift, right ventricular (RV) S4, and increased jugular "a" wave [232]. In more advanced stages of the disease, other signs may be seen, including a holosystolic murmur that increases with inspiration, increased jugular "v" waves, pulsatile hepatomegaly, hepatojugular reflex, peripheral edema, ascites, low pulse pressure, and cool extremities [232]. These usually indicate right ventricular (RV) failure [230]. The main chest X-ray finding suggesting PH is enlargement of main and hilar pulmonary arterial shadows accompanied by attenuation of peripheral pulmonary vascular markings [213, 230]. Electrocardiographic findings that should raise the suspicion of PH include right axis deviation, signs of RV hypertrophy (tall R wave in RV leads and R/S ratio <1 in V5 and V6), and right atrial enlargement (tall p wave in leads II, III, and aVF and frontal p axis of more than 75°) [213, 230, 232].

Table 15.1 Arbitrary criteria for estimating the presence of PH based on tricuspid regurgitation peak velocity and Doppler-calculated PA systolic pressure at rest (assuming a normal right atrial pressure of 5 mmHg) and on additional echocardiographic variables

	Class[a]	Level[b]
Echocardiographic diagnosis: PH unlikely		
Tricuspid regurgitation velocity ≤2.8 m/s, PA systolic pressure ≤36 mmHg, and no additional echocardiographic variables suggestive of PH	I	B
Echocardiographic diagnosis: PH possible		
Tricuspid regurgitation velocity ≤2.8 m/s, PA systolic pressure ≤36 mmHg, but presence of additional echocardiographic variables suggestive of PH	IIa	C
Tricuspid regurgitation velocity 2.9–3.4 m/s, PA systolic pressure 37–50 mmHg with/without additional echocardiographic variables suggestive of PH	IIa	C
Echocardiographic diagnosis: PH likely		
Tricuspid regurgitation velocity >3.4 m/s, PA systolic pressure >50 mmHg with/without additional echocardiographic variables suggestive of PH	I	B
Exercise Doppler echocardiography is not recommended for screening of PH	III	C

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[a]Class of recommendation

[b]Level of recommendation

15.2.2.2 Diagnosis and Classification

PAH is defined as a mean pulmonary arterial pressure (mPAP) greater than 25 mmHg at rest with a normal pulmonary capillary wedge pressure (PCWP) of 15 mmHg or less and a pulmonary vascular resistance (PVR) greater than 3 Wood units [232]. Screening is crucial in all patients with risk factors for PAH, such as bone morphogenetic protein receptor 2 (BMPR2) mutation, first-degree relative with BMPR2 mutation, history of anorexigen intake (fenfluramine), HIV infection, portal hypertension, CHD with systemic-to-pulmonary shunt, systemic sclerosis, recent acute pulmonary embolism, and sickle cell disease (SCD) [232]. If clinical, radiologic, and electrocardiographic findings raise the suspicion of PH, a Doppler echocardiogram is the screening test of choice, providing an estimate of the RV systolic pressure and RV function, as well as allowing identification of potential cardiac causes of PH [230, 232]. Common echocardiographic findings seen in patients with PAH include enlargement of right-sided chambers, abnormal surface of the interventricular septum, and underfilled left atrium and left ventricle [232]. The European Society of Cardiology (ESC) and the European Respiratory Society (ERS) proposed a series of arbitrary criteria for establishing the presence of PH based on echocardiographic findings that have been shown to correlate with PH on right heart catheterization (RHC) (Table 15.1) [125]. In cases where a tricuspid regurgitation profile cannot be determined by conventional echocardiography, intravenous saline or encapsulated microbubble contrast agents can be administered to enhance the signal [147, 232]. Patients with abnormal echocardiograms, including RV systolic pressure greater than 40 mmHg, should be further evaluated [232].

Table 15.2 WHO clinical classification of pulmonary hypertension (Dana Point, 2008)

1. Pulmonary arterial hypertension (PAH)
   - 1.1. Idiopathic PAH
   - 1.2. Heritable
     - 1.2.1. BMPR2
     - 1.2.2. ALK1, endoglin (with or without hereditary hemorrhagic telangiectasia)
     - 1.2.3. Unknown
   - 1.3. Drugs and toxin-induced
   - 1.4. Associated with
     - 1.4.1. Connective tissue disease
     - 1.4.2. HIV infection
     - 1.4.3. Portal hypertension
     - 1.4.4. Congenital heart disease
     - 1.4.5. Schistosomiasis
     - 1.4.6. Chronic hemolytic anemia
   - 1.5. Persistent pulmonary hypertension of the newborn

1′ Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH)

2. Pulmonary hypertension owing to left heart disease
   - 2.1. Systolic dysfunction
   - 2.2. Diastolic dysfunction
   - 2.3. Valvular disease
3. Pulmonary hypertension owing to lung disease and/or hypoxia
   - 3.1. Chronic obstructive pulmonary disease
   - 3.2. Interstitial lung disease
   - 3.3. Other pulmonary diseases with mixed restrictive and obstructive pattern
   - 3.4. Sleep-disordered breathing
   - 3.5. Alveolar hypoventilation disorders
   - 3.6. Chronic exposure to high altitudes
   - 3.7. Developmental abnormalities
4. Chronic thromboembolic pulmonary hypertension (CTEPH)
5. Pulmonary hypertension with unclear multifactorial mechanisms
   - 5.1. Hematologic disorders, myeloproliferative disorders, splenectomy
   - 5.2. Systemic disorders: sarcoidosis, pulmonary Langerhans cell histiocytosis, lymphangioleiomyomatosis, neurofibromatosis, vasculitis
   - 5.3. Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
   - 5.4. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

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When continuing evaluation of these patients, all causes of PH (including PAH and non-PAH causes) must be considered in order to guide proper management [232]. The revised WHO classification of PH (Dana Point 2008) is shown in Table 15.2 [317]. Although all of the secondary causes of PH should be evaluated before establishing the diagnosis of PAH, excluding chronic thromboembolic pulmonary hypertension (CTEPH) is particularly important because the management of these patients is very different, as some patients may be eligible for surgical treatment

[248], and this condition may coexist in the presence of other risk factors for PAH such as scleroderma [232]. The screening test of choice for ruling out CTEPH is the ventilation/perfusion lung scan, since a normal result virtually rules out this condition [148, 232, 248]. Despite the usefulness of the V/Q scan in patients without underlying lung disease, pulmonary multidetector CT angiography (MDCTA) is now considered the gold standard for the diagnosis of CTEPH because it allows identification of thrombosis, concomitant lung changes, and can aid in the diagnosis of pulmonary embolism in patients with preexisting lung disease [176]. Even though Doppler echocardiography aids in the detection of possible PH, the only way to confirm the diagnosis is through RHC [232, 248]. Once left ventricular or valvular disease (Group 2), lung disease (Group 3), and CTEPH (Group 4) are excluded, a RHC showing a mPAP greater than 25 mmHg and a PVR greater than 3 Wood units with a normal PCWP <15 mmHg confirm the presence of PAH, which means that it remains a diagnosis of exclusion [232].

The most recent classification of PH was established in the fourth World Symposium on Pulmonary Hypertension that was held in Dana Point in 2008 [317]. Patients with PAH should be classified into one of the five groups shown in Table 15.2 [317].

Idiopathic Pulmonary Arterial Hypertension and Heritable Pulmonary Arterial Hypertension: Groups 1.1 and 1.2

IPAH is sporadic and unrelated to any family history or identified risk factor [317]. Heritable PAH is diagnosed when there are mutations of genes that have been identified as having a strong association with the PAH phenotype, such as the *BMPR2* gene, which is present in 70 % of heritable cases. Other mutations that have been identified in patients with PAH are located in the activin receptor-like kinase type 1 (*ALK1*) or endoglin (*ENG*) genes [248, 317]. Some studies have also suggested that mutations in the Smad proteins and caveolin-1 (*CAV1*) genes may also predispose to PAH [9, 18, 28, 259, 316]. It is critical that these patients get involved in a comprehensive program that includes genetic testing, counseling, and discussion of risks and benefits [21, 317].

Drug and Toxin-Induced PAH: Group 1.3

Drug and toxin-induced PAH is further classified depending on the strength of the association between the exposure and the presence of disease, but the main substances that have been found to have a strong association with PAH are anorexigens (aminorex, fenfluramine) and toxic rapeseed oil. Other agents that have been related to PAH include cocaine, phenylpropanolamine, St. John's Wort, chemotherapeutic medications, selective serotonin reuptake inhibitors (SSRIs), and amphetamines [317]. However, further studies are needed to establish the true association of these latter substances.

Associated with PAH: Group 1.4

Associated with PAH (APAH) includes connective tissue disorders, congenital systemic-to-pulmonary shunts, portal hypertension, HIV infection, schistosomiasis, and chronic hemolytic anemia [232].

PAH Associated with Connective Tissue Diseases: Group 1.4.1

The presence of PAH has been well established in systemic sclerosis, with an estimated prevalence of 7–12 % and is associated with poor prognosis in this group of patients [138, 248, 253]. The presence of PAH has also been reported in systemic lupus erythematosus (SLE) and mixed connective tissue disease, but the exact prevalence has not been determined [317]. Other mechanisms may be involved in the induction of PH in these patients, such as left heart dysfunction, lung fibrosis, and primary cardiac involvement, which highlights the importance of determining the true cause of PH with RHC.

PAH Associated with HIV Infection: Group 1.4.2

The presence of PAH in patients with HIV infection is rare, with a prevalence of 0.5 % [28, 317]. Clinical, hemodynamic, and histological findings are very similar to those seen in IPAH patients [28, 317]. Concomitant PAH in patients with HIV significantly worsens their prognosis [243].

Porto-pulmonary Hypertension: Group 1.4.3

PAH associated with an increase in the pressure of the portal circulation is classified as porto-pulmonary hypertension (POPH) [248]. Some prospective studies have shown a prevalence of 5–6 % in patients with advanced liver disease [303]. POPH is also a predictor of poor prognosis, since these patients are usually not eligible for liver transplantation due to the high perioperative morbidity and mortality that have been documented in this population [303]. RHC should be performed to accurately diagnose PAH, since other factors, such as fluid overload and diastolic dysfunction, may elevate the pressure of the pulmonary vasculature in patients with portal hypertension [317].

Congenital Heart Diseases: Group 1.4.4

PAH is a fairly common complication of CHD in patients that have left-to-right shunts [81, 317]. It is estimated that 4–15 % of patients with CHD will develop PAH [81] and the most common anomalies associated with PAH are ventricular septal defects (VSD) [104]. Patients with CHD who develop PAH are classified into four groups: Eisenmenger's syndrome, PAH associated with systemic-to-pulmonary shunts, PAH with small defects, and PAH after corrective cardiac surgery [81, 317]. Eisenmenger's syndrome is the most severe form of PAH in this context, where there is a reversal of the initial shunt to a right-to-left shunt, where deoxygenated

blood is being returned to the systemic circulation and cyanosis ensues along with other potential complications such as blood hyperviscosity, hemostasis, stroke, and endocarditis [81].

Schistosomiasis: Group 1.4.5

Before the Dana Point classification of PH, schistosomiasis was listed under the subgroup of chronic thrombotic or thromboembolic disease. Nevertheless, recent evidence has shown that the obstructive mechanism of schistosoma eggs plays a minor role in the induction of PH in this group of patients, and clinical and pathological findings resemble those of IPAH [248, 317]. Although the exact mechanisms responsible for the induction of PH in patients with schistosomiasis remain largely unknown, the inflammatory response to the schistosoma antigens with the release of cytokines that have also been proven to be upregulated in IPAH, as well as the presence of hepatosplenic disease and portal hypertension likely plays an important role [133]. For these reasons, schistosomiasis is now listed under Group 1 of the Dana Point Classification [248, 317].

Chronic Hemolytic Anemia: Group 1.4.6

PAH has been identified as a complication of many hemolytic anemias including SCD, thalassemia, hereditary spherocytosis, stomacytosis, and microangiopathic hemolytic anemia [317]. Histological findings seen in IPAH have been commonly described in patients with SCD [317]. However, the true prevalence of PAH in these patients remains unknown since most epidemiological studies have defined the presence of PH in terms of echocardiography rather than RHC [317]. Such studies have documented a prevalence of 20–30 % in patients with SCD and 10–75 % in patients with thalassemia [219]. The pathophysiology of PAH induced by hemolysis is not entirely understood, but mechanisms such as inactivation of nitric oxide (NO) by free hemoglobin, depletion of L-arginine in the presence of elevated arginase, and increased endothelin-1 (ET-1) responses have been described [110, 250, 301].

Pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomatosis (PCH) are rare conditions that were included in Group 1 of the most recent WHO classification of PH (Dana Point 2008) [317]. This inclusion was based on the similarities of PVOD/PCH and PAH regarding histologic findings, clinical presentation, risk factors, and potential for inheritance [317]. Nevertheless, they are still considered separate conditions classified as 1′ (Table 15.2) [317].

As discussed above, PAH is a diagnosis of exclusion and both PAH and non-PAH causes of PH may overlap. Therefore, it is crucial to evaluate and classify patients based on their etiology of PH and WHO group (Table 15.2), and confirm that the elevated pressure is limited exclusively to the pulmonary arterial system [90]. This can only be accomplished with a RHC, which remains an indispensable tool in the assessment of patients with PH [90]. Additionally, this test gives further information that is useful to determine prognosis, such as the severity of the hemodynamic impairment and the vasoreactivity of the pulmonary circulation [125]. The diagnostic PH algorithm established by the American College of Cardiology Foundation/American Heart Association Task Force (ACCF/AHA) experts can be found in Fig. 15.1 [125, 232].

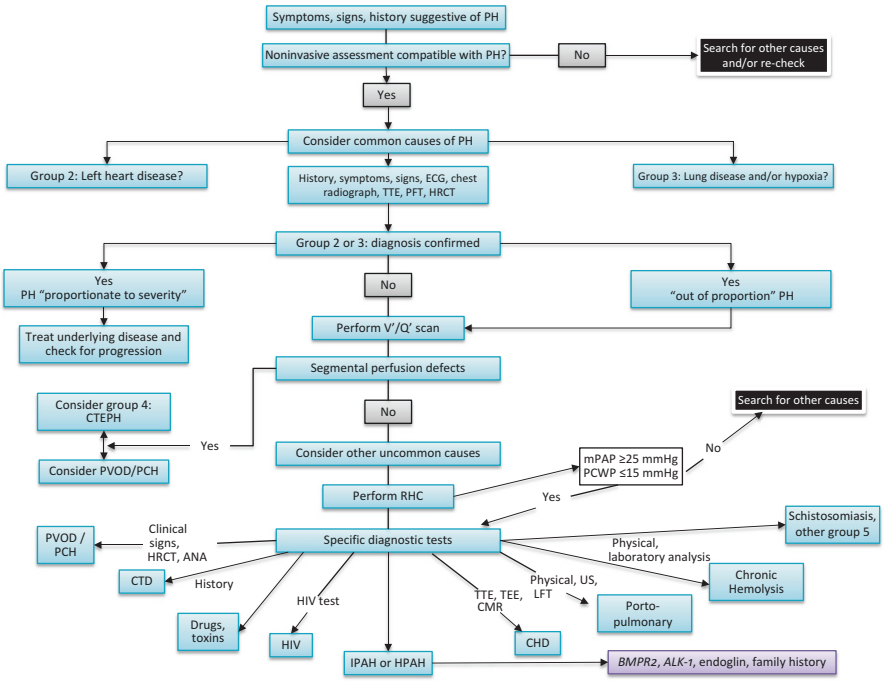

Fig. 15.1 Diagnostic algorithm for pulmonary hypertension. Reproduced with permission from [125]

15.2.2.3 Gold Standard and Pathological Findings

The gold standard for the diagnosis of PAH is the RHC since it is defined by hemodynamic criteria [125, 232]. Lung biopsy in patients with PAH is not recommended, since it has a high morbidity and mortality in this group of patients and is unlikely to change the diagnosis or treatment [125]. Therefore, the natural history of vascular lesions that occur in PAH is not entirely known because biopsies are not regularly obtained in these patients [232]. Arterial abnormalities seen in histological studies of patients with PAH include intimal hyperplasia, inflammation, adventitial proliferation, medial hypertrophy, thrombosis in situ, abnormal muscularization of non-muscular precapillary arteries, and plexiform arteriopathy [232, 284].

15.2.2.4 Prognosis

Despite a better understanding of the pathophysiological mechanisms involved in PAH and the improvement in treatment options, the long-term prognosis remains poor [232]. Data from the French Network on Pulmonary Hypertension Registry revealed a survival rate of 83 % (95 % CI 72–95 %) at 1 year, 57 % (95 % CI 57–79 %) at 2 years, and 58 % at 3 years [158].

15.2.3 Pathophysiology

PH results from an increase in PVR and restriction in blood flow through the pulmonary vascular circulation, finally leading to altered right heart function [232]. Elevation of PVR and decreases in pulmonary vascular compliance cause increased RV afterload, which ultimately results in adaptive RV hypertrophy [221]. If the pressure overload persists, the RV eventually dilates and becomes dysfunctional, leading to increase in RV contraction time, asynchrony, and decreased RV stroke volume [221]. All of the latter changes result in underfilling of the left ventricle (LV) and subsequent reduction in cardiac output [126, 221, 223].

The main cause of elevated PVR is the reduction in luminal cross section due to vascular remodeling, which results from altered cell growth, apoptosis, migration, and production of extracellular matrix [5, 232]. Various stimuli can induce vascular remodeling, including mechanical forces (changes in transmural pressure, stretch, shear stress), inflammatory cytokines, serotonin (5-hydroxytryptamine [5-HT]), hypoxia, growth factors, angiotensin II (AT-II), endothelin-1 (ET-1), increased serine elastase activity, and increased production of ROS [5]. All of these stimuli induce changes in different cells that are responsible for the changes seen in vascular remodeling, mainly endothelial cells (EC) and smooth muscle cells (SMC) [5].

15.2.3.1 Pulmonary Arterial Endothelial Cells

Pulmonary arterial endothelial cells (PAEC) that are exposed to injury caused by the various stimuli mentioned above may become dysfunctional and respond in ways that contribute to vascular remodeling [5]. This remodeling occurs through the release of agents that stimulate proliferation of pulmonary arterial smooth muscle cells (PASMC), such as platelet-derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2) and/or failure to produce factors that suppress proliferation of PASMC, such as apelin [284]. Furthermore, PAEC from patients with IPAH have increased expression of the Tie2 receptor, which results in increased production of 5-HT and subsequent PASMC proliferation (Fig. 15.2) [5, 91, 284]. Moreover, dysfunctional PAEC seen in PH generate less nitric oxide (NO) as a result of uncoupling of endothelial NO synthase (eNOS), which ultimately leads to an increase in the production of ROS, particularly superoxide (Fig. 15.2) [5]. The effect of ROS in pulmonary vascular remodeling is further discussed in the next section. Uncoupling of eNOS is related to low levels of enzymatic cofactors L-arginine and tetrahydrobiopterin (BH_4) [200]. L-Arginine depletion results from the upregulation of arginase, which has been documented both in animal and human EC exposed to different stimuli, including hypoxia, lipopolysaccharide (LPS), shear stress, and inflammatory cytokines [105]. Increased asymmetric dimethylarginine (ADMA) has also been found to be elevated in patients with PH [5, 312]. ADMA is an endogenous analogue of L-arginine and competes for the substrate binding site of eNOS, which can further contribute to the uncoupling of the enzyme [5, 284].

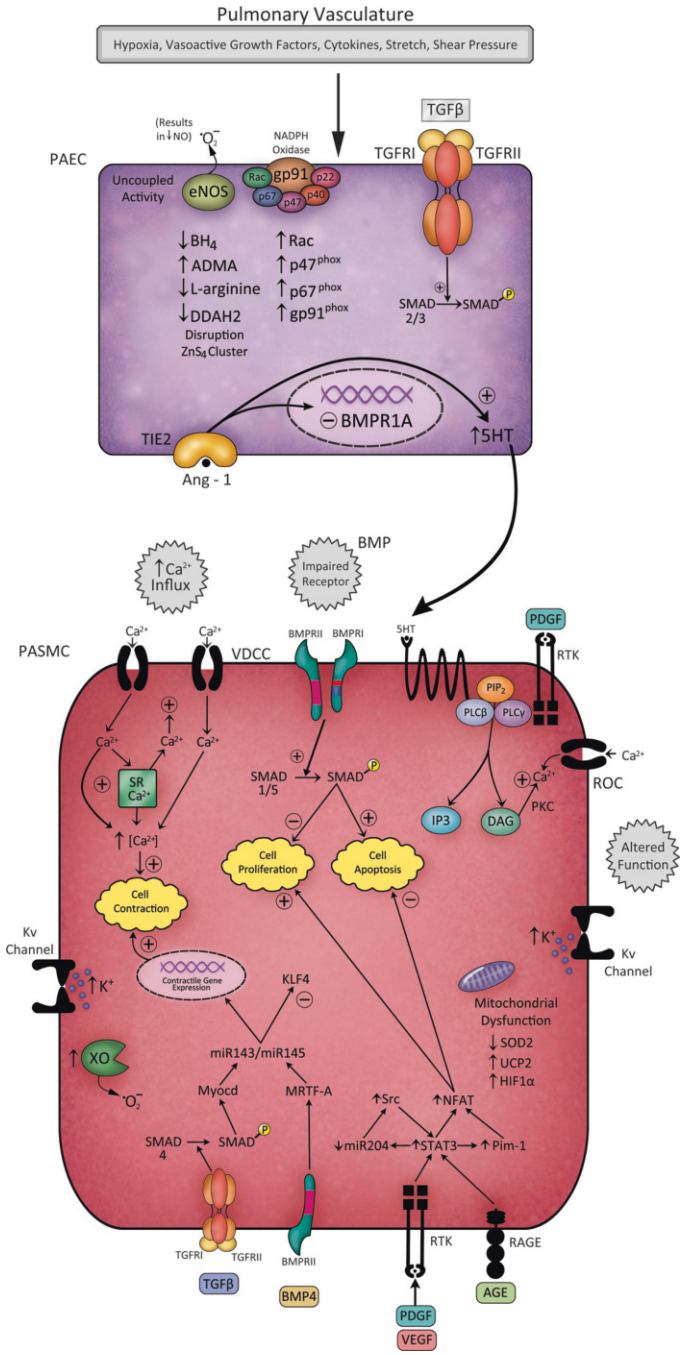

Fig. 15.2 Overview of mechanisms involved in the pathogenesis of PAH. Diverse stimuli result in endothelial dysfunction and abnormal PASMC proliferation. Decreased NO production in PAEC due to eNOS uncoupling attenuates relaxation of PASMC and promotes vasoconstriction. Factors that contribute to eNOS uncoupling include decreased arginine, increased ADMA, enhanced arginase activity, low BH_4, and disruption of the zinc tetrathiolate (ZnS_4) cluster.

ADMA has also been shown to contribute to mitochondrial dysfunction through the increase of uncoupling protein-2 (UCP2), which leads to augmented mitochondrial ROS (mROS) production and decreased ATP synthesis (Fig. 15.2) [5, 329].

In addition to decreased synthesis of the vasodilator NO, dysfunctional endothelial cells also produce lower levels of prostacyclin, and higher levels of vasoactive substances such as ET-1, AT-II, and thromboxane A_2 (TXA_2), and growth factors, namely PDGF, transforming growth factor β (TGF-β), FGF-2, and vascular endothelial growth factor (VEGF) [5, 100, 227, 361]. All of these may stimulate PASMC proliferation in vascular remodeling [5]. Finally, PAEC from patients with PAH seem to have increased glycolytic activity and a highly proliferative response to growth factors, which contributes to the formation of plexiform lesions [5, 284, 382]. PAEC seen in these lesions exhibit increased levels of hypoxia-inducible factor (HIF) subunits (HIF-1α and HIF-1β), which induce VEGF under hypoxic conditions [5, 342].

Elevated expression of VEGF and VEGF receptor 2 (VEGFR2) has been documented in plexiform lesions of patients with PAH [221, 342]. VEGF promotes survival and suppresses apoptosis in PAEC [221, 305]. However, mice and rats exposed to hypoxia combined with the VEGFR2 inhibitor, SU5416, develop PAH [221, 353]. Moreover, VEGF is decreased in the monocrotaline (MCT) rat model of PAH,

Fig. 15.2 (continued) The eNOS uncoupling not only results in lower NO levels but also increases ROS production. Upregulation of NADPH oxidase subunits further contributes to the generation of ROS. Altered function of potassium Kv channels in PASMC leads to membrane depolarization and opening of voltage-dependent calcium channels. Influx of calcium ions stimulates additional release of Ca^{2+} from the SR. Increased $[Ca^{2+}]_{cyt}$ and upregulated membrane receptors (5-HT, ET-1, leukotrienes) decrease apoptosis and stimulate cell proliferation. Increased Ang-1 downregulates BMPR1A in PAEC and enhances 5-HT production, promoting PASMC contraction and proliferation. As a result of BMPRII mutations, PASMC display dysfunctional BMP signaling pathways, which normally inhibit cell proliferation and stimulate cell apoptosis. Mitochondrial dysfunction leads to increased ROS production and is evidenced by the low levels of SOD2, high levels of UCP2, and impaired function of complexes I and II. Increased activity of XO also results in higher production of ROS. Increased expression of the STAT3/Pim1/Src/NFAT axis and suppression of miR-204 also promote cellular proliferation and reduce apoptosis. TGF-β and BMP4 increase the expression of miR-143/miR-145 through the stimulation of Myocd and MRTF-A, respectively. These miRNAs inhibit KLF4 which ultimately results in enhanced contractile gene expression. *PAEC* pulmonary arterial endothelial cells, *eNOS* endothelial nitric oxide synthase, *NADPH* nicotinamide adenine dinucleotide phosphate, *TGF-β* transforming growth factor β, *TGFRI* type I receptor for TGF-β, *TGFRII* type II receptor for TGF-β, BH_4 tetrahydrobiopterin, *ADMA* asymmetric dimethylarginine, *DDAH2* dimethylaminohydrolase-2, *TIE2* tyrosine protein kinase receptor, *Ang-1* angiopoietin, *BMP* bone morphogenetic protein, *BMPR1A* BMP receptor 1A, *BMPRI* BMP type I receptor, *BMPRII* BMP type II receptor, *5-HT* 5-hydroxytryptamine, *PASMC* pulmonary arterial smooth muscle cells, *VDCC* voltage-dependent calcium channel, *PIP2* phosphatidylinositol 4,5-bisphosphate, *PLC* phospholipase C, *IP3* inositol triphosphate, *DAG* diacylglycerol, *PKC* protein kinase C, *ROC* receptor-operated calcium channel, *SR* sarcoplasmic reticulum, *Kv channel* voltage-gated potassium channel, *SOD2* superoxide dismutase 2, *UCP2* uncoupling protein-2, *HIF-1α* hypoxia-inducible factor α, *XO* xanthine oxidase, *RAGE* receptor for advanced glycation endproducts, *AGE* advanced glycation endproducts, *RTK* receptor tyrosine kinase, *PDGF* platelet-derived growth factor, *VEGF* vascular endothelial growth factor, *STAT3* signal transducer and activator, *NFAT* nuclear factor of activated T-cells, *MRTF* myocardin-related transcription factor, *Myocd* myocardin, *KLF4* Krüppel-like factor 4

which correlates with early endothelial injury. Overexpression of VEGF also protects against chronic hypoxia and MCT exposure, and VEGFR inhibition results in initial EC apoptosis with subsequent selection of EC clones that are resistant to apoptosis and form angio-obliterative lesions [221, 353]. Therefore, VEGF appears to play a crucial role in angiogenesis and EC growth after vascular injury. Other factors associated with plexiform lesions are angiopoietin 1, 5-lipoxygenase, survivin, and Ki-67 [5, 129, 131, 375]. However, the exact mechanisms responsible for the formation of plexiform lesions are not completely understood [5].

15.2.3.2 Pulmonary Arterial Smooth Muscle Cells

Many pathologic changes take place in the SMC layer of PAs during vascular remodeling. Proximal vessels usually undergo significant hypertrophy, while smaller resistance vessels commonly show hyperplasia [5, 231, 238]. Matrix protein deposition is also a characteristic feature of the muscular layer of PAs in PAH, where SMC seem to acquire a more synthetic, rather than contractile, phenotype, with larger endoplasmic reticula and Golgi apparatus, and increased production of collagen and elastin [5, 238]. Muscularization of otherwise nonmuscular blood vessels results from differentiation of pericytes into SMC and hypertrophy of SMC precursor cells [5, 284].

Factors that have been identified in the induction of SMC hypertrophy include bone morphogenetic protein 4 (BMP4), TGF-β1, 5-HT, ET-1, inhibition of glycogen synthase kinase 3β (GSK-3β), and activation of p70S6 kinase [5, 174]. Abnormal activation of transcription factors (HIF-1α and nuclear factor of activated T-cells [NFAT]), increased expression of survivin and PDGF, calcium overload, mitochondrial hyperpolarization, and decreased expression of voltage-gated potassium channels (Kv) all contribute to the increased survival and decreased apoptosis of PASMC seen in PAH (Fig. 15.2) [221, 232].

Finally, in vitro studies have shown that PASMC from PAH patients have higher mRNA and protein levels of Notch 3 and HES-5 [221]. Notch participates in vasculogenesis, angiogenesis, and differentiation of vascular SMC [11, 221]. HES-5, a target gene for Notch 3, is exclusively expressed in adult SMC and may be involved in SMC maturation and proliferation [53, 96, 221, 279].

15.2.3.3 Neointima Formation

The formation of a layer of cells and extracellular matrix between the endothelium and the internal elastic lamina occurs in severe PH [5, 387]. The neointima is composed of myofibroblasts that express SM markers such as smooth muscle α-actin and vimentin [5]. These cells lack markers of highly differentiated SMC, such as SM-myosin heavy chain, and do not exhibit EC markers either [5, 387]. The exact origin of these cells is unclear. They may originate in stem cells, transdifferentiation of endothelial cells, migration of SMC from the media, or migration of adventitial fibroblasts [5, 284]. This currently remains a subject of intense study [284].

15.2.3.4 Changes in the Adventitia

PAH is associated with thickening and disorganization of the pulmonary adventitia, with excessive activation of adventitial metalloproteases [232]. In patients with PAH related to collagen vascular diseases such as scleroderma, the adventitia appears markedly remodeled [5]. Activation of fibroblasts by different stimuli can induce a phenotypic change in these cells, altering their structure and functional behavior [5]. An example of this is the induction of a contractile phenotype in fibroblasts by TGF-β1 and TGF-β2 [5, 387]. The activation and proliferation of fibroblasts and myofibroblasts result in thickening of the adventitia in PH, and some studies have shown that these changes precede remodeling of the intima and SMC layer, which suggests that the initial detection of vascular injury might take place in the adventitia [5, 146].

15.2.3.5 Genes and Transcription Factors Involved in PAH

Genes associated with PAH have helped to identify potential mechanisms involved in the pathogenesis of the disease. Studies have shown that approximately 70 % of patients with heritable pulmonary arterial hypertension (HPAH) and 10–20 % of patients with IPAH are heterozygous for a mutation in *BMPR2*, which is a member of the TGF-β superfamily of growth factor receptors [284]. HPAH is inherited in an autosomal dominant fashion with incomplete penetrance and genetic anticipation [232]. The impaired function of the BMPR2 results in a loss of function of the SMAD signaling pathway, causing proliferation and decreased apoptosis of PASMC in response to TGF-β and BMP2 (Fig. 15.2) [232]. On the other hand, BMPR2 impairment in EC results in increased susceptibility to apoptosis, which alters the normal migration and survival of EC needed in angiogenesis and regeneration of damaged blood vessels (Fig. 15.2) [85, 284]. Abnormal BMPR2 signaling has also been associated with increased ET-1 production in human lung microvascular EC [221, 324].

Recently, signal transducer and activator of transcription 3 (STAT3) has been shown to participate in aberrant PASMC proliferation [221, 272]. IL-6, TGF-β, PDGF, VEGF, ET-1, and AT-II can activate STAT3, which in turn increases the expression of Pim1 (Fig. 15.2) [221, 272, 390]. PIM1 promotes the activation of NFAT, increasing cytokine secretion, enhancing PASMC proliferation, and suppressing PASMC apoptosis (Fig. 15.2) [221, 287]. STAT3 has also been implicated in induction of survivin expression through activation of Krüppel-like factor 5 (KLF5) and in downregulation of eNOS expression (Fig. 15.2) [74, 221].

Moreover, studies have shown that mice with deletion of the peroxisome proliferator-activator receptor gamma (PPAR-γ) gene develop spontaneous PAH [136], and mutations in this gene have also been identified in patients with severe PH [5, 12, 284]. PPAR-γ participates in the antiproliferative effect of BMP2 signaling in PASMC, which is BMPR2/PPAR-γ/ApoE dependent [8, 140, 221]. The receptor of advanced glycation end products (RAGE) is an upstream target of PPAR-γ in PAH, and has been shown to activate STAT3 and downregulate BMPR2

and PPAR-γ in PAH-PASMC (Fig. 15.2) [221, 236]. Furthermore, BMP2-mediated survival of PAEC depends on the formation of a nuclear complex between β-catenin and PPAR-γ [8]. One of the transcriptional targets of this complex is apelin, which is reduced in patients with IPAH [8]. Apelin promotes PAEC survival and migration, and suppresses PASMC growth [284]. Apelin-deficient PAEC have increased apoptosis and promote PASMC proliferation [8, 221]. Other genes that have been associated with the PAH phenotype include *ALK1*, *ENG*, and *CAV1* [18, 59, 142, 218, 221].

15.2.3.6 MicroRNAs Involved in PAH

miRs are now of great interest in the study of diseases that display abnormal cell growth, since they are involved in various posttranscriptional regulatory mechanisms [221]. In PAH, only few miRs have been identified as being abnormally expressed [221]. Downregulation of miR-204 in PAH-PASMC was found to correlate with PAH severity and higher cell proliferation [74]. It was shown that downregulated levels of miR-204 enhance a constitutive activation of Src and STAT3, leading to an increase in PASMC proliferation (Fig. 15.2) [74]. Additionally, downregulation of miR-204 appears to upregulate IL-6 secretion, which in turn downregulates BMPR2 and further contributes to the proliferative phenotype of PAH-PASMC [221, 272]. IL-6 is a potent activator of STAT3, which means that these interactions result in a feed-forward loop between miR-204 downregulation and STAT3 (Fig. 15.2) [221].

Src and p53 pathways regulate the organization of miR-145 and miR-143, which are involved in SMC differentiation and proliferation [221, 283]. TGF-β and BMP4 stimulate the expression of myocardin (Myocd) and Myocd-related transcription factor A (MRTF-A), respectively. These factors in turn activate miR-143 and miR-145 transcription, resulting in decreased KLF4 expression and promotion of contractile gene expression in SMC (Fig. 15.2) [83, 221]. Plexiform and concentric lesions seen in patients with PAH display abnormal expression of miR-143/miR-145 and mice exposed to hypoxia show elevated levels of miR-145 [54, 221].

In PAEC, expression of miR-126 appears to be dysregulated specifically in plexiform lesions [36, 221]. This miR plays an important role in neovascularization, EC proliferation, and vascular integrity, and regulates factors involved in apoptosis and modulation of cell cycle arrest [221, 355, 391]. Other miRs that have been found to contribute to the pathogenesis of PAH include miR-150, which is reduced in patients with PAH and is associated with decreased NK cells and B1 cell expansion; miR-210, the miR most highly upregulated by hypoxia [195, 221]; miR-21, which is highly upregulated in hypoxia and appears to participate in abnormal proliferation and migration of PASMC [221]; and miR-17, which is also upregulated in hypoxia, and targets p21 and Janus kinase (JAK1) impairing angiogenic functions of endothelial cells [221]. miRs remain a subject of intense study, since they are regarded as useful biomarkers, prognostic tools, and potential targets for future therapies [221].

15.3 Oxidative Stress and PAH

Several studies have implicated oxidative stress in the pathogenesis of PAH. Oxidative and nitrosative stress are characterized by an imbalance between oxidant and antioxidant production that can lead to downstream cell and tissue damage. Oxidative stress in PAH is associated with increased production of ROS and reactive nitrogen species (RNS), decreased nitric oxide (NO) levels, and mitochondrial dysfunction. Dysregulation of ROS/RNS/NO homeostasis can impair vascular tone and lead to activation of antiapoptotic and mitogenic pathways resulting in cell hyperproliferation and obliteration of the vasculature in PAH.

ROS are produced from oxygen during normal metabolic processes. ROS can be characterized as either free radicals, reactive molecules with one or more unpaired electrons, or nonradicals, molecules which share unpaired electrons between two free radicals [34] (Table 15.3). Hydroxyl radical ($^{\bullet}OH$) is considered the most reactive free radical in biological systems [335]. In the lung, ROS can be generated by alveolar epithelial cells, endothelial cells, alveolar macrophages, neutrophils, and eosinophils. In the pulmonary vasculature, ROS can be produced by complexes in the cell membrane, within mitochondria and peroxisomes, and from within the cytoplasm. The major enzymatic sources of ROS include uncoupled eNOS, xanthine oxidase (XO), nicotine adenine dinucleotide phosphate (NADPH) oxidase (NOX), and mitochondrial electron transport enzymes (Fig. 15.3). RNS are various nitrogen-containing species (Table 15.3) that can alter protein function via S-nitrosylation, tyrosine nitration, and glutathionylation. NO is the predominant source of nitrosative stress and, at high concentrations, can react with ROS to generate other RNS, including peroxynitrite, $ONOO^{-}$.

Table 15.3 Major oxidants

Oxidative stress			
Free radicals		**Nonradicals**	
Hydroxyl radical	$OH^{\bullet}$	Hydrogen peroxide	H_2O_2
Superoxide anion	$O_2^{\bullet -}$	Hypochloric acid	HOCl
Peroxyl radical	$ROO^{\bullet}$	Ozone	O_3
Hydroperoxyl radical	$HOO^{\bullet}$	Lipid peroxide	LOOH
Lipid peroxyl	$LOO^{\bullet}$		
Nitrosative stress			
Nitric oxide	$NO^{\bullet}$		
Peroxynitrite anion	$ONOO^{-}$		
Nitrogen dioxide	NO_2		
Nitrite	NO_2^{-}		
Nitrate	NO_3^{-}		

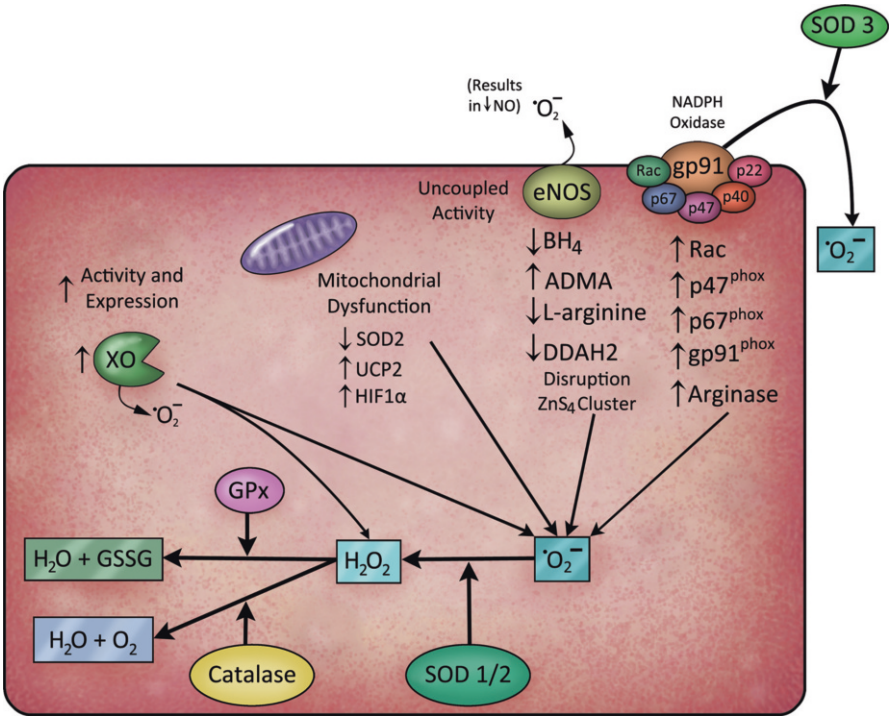

Fig. 15.3 Overview of the mechanisms involved in ROS production and antioxidant mechanisms that counterbalance this oxidative stress. eNOS uncoupling due to decreased arginine, increased ADMA, enhanced arginase activity, low BH_4, and disruption of the zinc tetrathiolate (ZnS_4) cluster results in increased production of superoxide. Upregulation of NADPH oxidase subunits and xanthine oxidase further contributes to the generation of ROS. Superoxide dismutase catalyzes the conversion of superoxide to hydrogen peroxide. Hydrogen peroxide is reduced by catalase and glutathione peroxidase. *XO* xanthine oxidase, *SOD2* superoxide dismutase 2, *UCP2* uncoupling protein-2, *HIF-1α* hypoxia-inducible factor **α**, *BH_4* tetrahydrobiopterin, *ADMA* asymmetric dimethylarginine, *DDAH2* dimethylaminohydrolase-2, *NADPH* nicotinamide adenine dinucleotide phosphate, *SOD* superoxide dismutase, *GPx* glutathione peroxidase, *GSSG* glutathione disulfide

15.3.1 Mediators and Molecular Mechanisms of Oxidative Stress in PAH

15.3.1.1 Nitric Oxide Dysregulation

NO is a gaseous lipophilic free radical and primary pulmonary vasodilator produced and released by the endothelium. In addition to regulating vascular tone, NO attenuates platelet aggregation and inhibits vascular SMC proliferation and migration within the vascular wall [404]. NO is biosynthesized during the conversion of the amino acid L-arginine to L-citrulline by a family of enzymes called nitric oxide synthases (NOS). Three different isoforms of NOS have been identified including

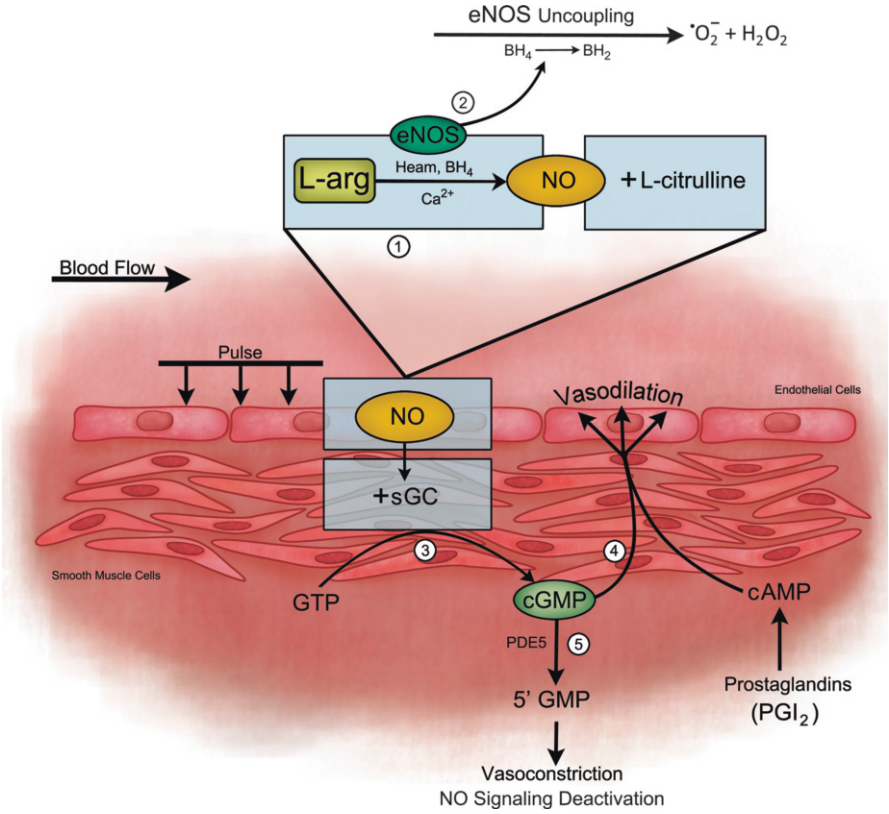

Fig. 15.4 Nitric oxide signaling in PAH. Oxidative stress and nitric oxide (NO) dysregulation in the pathogenesis of PAH. (1) Biosynthesis of NO from the amino acid L-arginine by the enzyme endothelial nitric oxide synthases (eNOS) with L-citrulline as a side product and important cofactors such tetrahydrobiopterin (BH_4), calcium, and heme. (2) Uncoupling of eNOS—when cofactors are limited and there is production of ROS, superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). (3) Binding of NO to its target protein, soluble guanylate cyclase (sGC) and conversion of guanosine triphosphate (GTP) to cGMP resulting in blood vessel dilation (4). (5) Cleavage of cGMP by PDE5 into 5′GMP leading to inhibition of NO signaling resulting in vessel contraction

neuronal NOS (nNOS), inducible NOS (iNOS/NOS2), and endothelial NOS (eNOS). The production of NO by NOS requires NADPH and O_2, as well as the cofactors tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and Ca^{2+}/calmodulin (CaM) [52, 220] (Fig. 15.4).

After release from endothelial cells, NO binds to soluble guanylate cyclase (sGC) in vascular cells and converts guanosine triphosphate (GTP) to cGMP, which leads to activation of downstream cGMP-dependent signaling [77, 270]. cGMP is a transient signaling molecule, as it is rapidly cleaved by phosphodiesterases (PDEs), predominantly PDE5, into 5′GMP, thereby inhibiting NO signaling (Fig. 15.4). Although eNOS-derived NO is primarily responsible for endothelium-dependent vasodilation, iNOS has also been shown to regulate pulmonary vascular tone [111, 113].

In mice, deletion of eNOS results in systemic hypertension [157] and mild PH [326], while eNOS overexpression leads to systemic hypotension [130, 265]. Exposure of eNOS-deficient mice to chronic hypoxia exacerbates PH and right ventricular hypertrophy (RVH) [327] and administration of inhaled NO attenuates hypoxia-induced PH, RVH, and vascular remodeling in rats [192, 297, 300]. In addition, recent findings demonstrate that endothelial-like progenitor cells (ELPC) expressing eNOS reverse MCT-induced PH [395] and attenuate right ventricular systolic pressure (RVSP) and pulmonary arterial muscularization in a lung lobectomy model of PH [366]. Taken together, these findings suggest a critical role for dysregulation of eNOS-derived NO in the pathogenesis of PAH.

While there is general consensus that NO signaling is impaired in PAH, it remains unclear whether this is primarily due to reduced synthesis, decreased bioavailability, decreased responsiveness, or increased consumption of NO. Some studies have demonstrated attenuated bioavailability of NO via hemoglobin and superoxide scavenging [154] or by increased hemolysis in fatal PAH [156].

15.3.1.2 eNOS Regulation

NO synthesis and bioavailability in the pulmonary vasculature are dependent upon the regulation of eNOS [60]. eNOS expression is controlled by two regulatory regions, the positive regulatory domains I and II, and its transcription is regulated by many cofactors acting by complex *cis* and *trans* interactions [309]. Additionally, methylation of nucleotides in those regions specifies vascular endothelial cell expression of eNOS [55]. Following eNOS protein translation, its compartmentalization activity is regulated by phosphorylation of specific serine and threonine residues [42, 43, 69, 194, 252], as well as additional posttranslational modifications (myristoylation and palmitoylation) which allow for eNOS localization to the plasma membrane and subsequent targeting to caveolae [263], where caveolin-1 (Cav-1) regulates intracellular NO signaling [255].

In addition to the Cav-1/caveolae trafficking system [145, 255, 302], the chaperon Hsp90 has also been identified as a regulator of eNOS activity by its rapid binding upon EC activation [386]. One possible mechanism of this regulation is through interaction of eNOS and Hsp90 with CaM. Following VEGF stimulation of EC, there is disruption of the Ca^{2+}/CaM-dependent eNOS/Cav-1 complex and promotion of Hsp90 and eNOS association. The Hsp90/eNOS complex is then triggered for VEGF-activated Akt-dependent phosphorylation of eNOS [49, 336]. Prolonged exposure of cells to Ca^{2+} results in degradation of eNOS and Hsp90, followed by a decrease in NO production [19]. It has also been shown that Hsp90 as an adaptor protein binds eNOS to sGC, allowing cGMP signaling to take place and facilitating responses to NO donors [350, 386].

15.3.1.3 Uncoupling of eNOS in PAH

In addition to impaired NO signaling in the pathobiology of PAH, "eNOS uncoupling" in conditions of substrate/cofactor deficiency or RNS production in the setting of NO excess can lead to decreases in NO bioavailability and increases in

oxidative stress with downstream alterations in vascular tone and aberrant vascular remodeling. eNOS uncoupling can occur in the setting of BH_4 or L-arginine deficiency [196, 200] and results in a shift from NO synthesis to other ROS production with resultant endothelial dysfunction [72] (Fig. 15.4). All three isoforms of NOS contain an oxygenase and a reductase domain, each of which has its own catalytic activity. The oxygenase domain has binding sites for heme and BH_4, while the reductase domain has binding sites for FAD, FMN, and NADPH. Both domains are linked by the binding site for CaM, an important regulator of NOS function.

For the formation of NO from L-arginine, eNOS requires the critical cofactor BH_4, which stabilizes the dimeric structure of eNOS and facilitates binding of L-arginine [73]. When BH_4 levels are insufficient, "eNOS uncoupling" may result with activation of the reductase domain and transfer of electrons to O_2, rather than L-arginine, and production of superoxide ($O_2^{\bullet -}$) [51] (Fig. 15.4). BH_4 can be oxidized by ROS to BH_2, a competitive BH_4 antagonist [130], which shifts eNOS enzymatic activity towards superoxide production [183]. Deficiency of BH_4 in a mouse model led to spontaneous development of PH under normoxic conditions as well as exaggerated hypoxia-induced PH, vascular remodeling, and RVH, which was secondary to reduced NOS activity and increased superoxide production associated with reduced BH_4 levels [183]. Furthermore, overexpression of GTP-cyclohydrolase 1, the rate-limiting enzyme in BH_4 biosynthesis, prevented PH in mice, and exogenous supplementation of BH_4 attenuated MCT-induced PH and muscularization of distal pulmonary arteries in rats [120, 180]. Additionally, the BH_4 analogue, acetyl-7,7-dimethyl-7,8-dihydropterin, improved NO-mediated pulmonary artery dilation and induced eNOS expression in the endothelium of rats with hypoxia-induced PH [196].

Further support for eNOS uncoupling in the pathogenesis of PAH comes from Cav-1-deficient mice that develop PH [222, 396] due to increased superoxide [179] and peroxynitrite production and tyrosine nitration-dependent impairment of protein kinase G (PKG) activity secondary to increased eNOS activity and NO levels [398]. Importantly, PH in Cav-1-knockout (KO) mice can be reversed with NOS inhibition and prevented with BH_4 administration in Cav-1-deficient neonatal mice [376, 377].

Uncoupling of eNOS can also occur in the setting of limited L-arginine availability. Although intracellular concentrations of L-arginine typically far exceed what is necessary for NO production [60], arginase can metabolize L-arginine to L-ornithine and urea, and compete with NOS for substrate. Arginase is upregulated in the lungs of mice exposed to hypoxia [173], as well as in hypoxia-exposed SMC [61], and is increased in EC of PAH patients [381]. Increases in arginase lead to endothelial dysfunction [306, 381], increases in EC and SMC proliferation [205], as well as increases in collagen deposition [186]. Inhibition of arginase decreases SMC and EC proliferation [67], and attenuates pulmonary vascular remodeling in an animal PH model [67]. Increased levels of L-arginine have also been implicated in the development of PAH in patients with SCD [154]. In addition to limiting NO availability, increased arginase and enhanced synthesis of ornithine have also been implicated in SMC remodeling and PH [144, 266].

L-Arginine availability can also be influenced by endogenous methylarginines, specifically L-monomethlyl arginine (L-NMMA) and ADMA, which are produced through posttranslational methylation of amino acids in arginine [14, 372] and compete with L-arginine for the binding site on eNOS [51]. Both L-NMMA and ADMA

are eliminated largely through active metabolism by dimethylarginine dimethylaminohydrolase (DDAH) [204]. Levels of ADMA are increased in animal models of PH [17, 241] and have been associated with increased oxidative stress and endothelial dysfunction [334]. Furthermore, DDAH levels are reduced in animal models of PH [17, 241] and DDAH1 overexpression in mice has been shown to decrease the sustained phase of hypoxic pulmonary vasoconstriction (HPV) via activation of the NO-sCG pathway [24]. Additionally, levels of ADMA are increased in the plasma of patients with pediatric and idiopathic PAH [132, 280] and also have been associated with increased pulmonary vascular pressures in decompensated heart failure patients in the intensive care unit [312].

15.3.1.4 NO Reactions with Other ROS: Formation of RNS

Nitrosative stress has also been implicated in the pathogenesis of PAH. NO is the main RNS produced within cells and can react with other ROS such as superoxide to generate peroxynitrite anion ($ONOO^-$). Peroxynitrite is a potent oxidant that nitrates tyrosine residues and can lead to formation of other extremely reactive RNS such as nitrogen dioxide, nitrosoperoxycarbonate anion, nitrite, and nitrate. These RNS can lead to significant alterations in protein structure and function, lipid peroxidation, nucleic acid damage, and cell death. Nitrotyrosine, a product of tyrosine nitration and marker of peroxynitrite, is upregulated in the endothelium and PASMC of rats subjected to chronic hypoxia [87, 167] and hypoxia-induced peroxynitrite production has been shown to increase proliferation in PASMC [3]. Peroxynitrite-mediated tyrosine nitration has also been shown to inactivate prostacyclin synthase leading to reduced levels of prostaglandin I_2 [401], eNOS uncoupling, as well as inhibition of PKG [4, 397]. In addition, peroxynitrite can activate many signaling pathways involved in cell proliferation including ERK and protein kinase C [3]. Moreover, treatment of newborn rats with a $ONOO^-$ decomposition catalyst, 5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrinato iron(III) (FeTPPS), attenuated chronic hypoxia-induced PH and decreased proliferation in neonatal PASMC [32].

In addition to tyrosine nitration, RNS can also induce S-nitrosylation and glutathionylation of regulatory proteins that may alter protein function and downstream signaling. Notably, NO can induce S-nitrosylation through formation of dinitrogen trioxide that can covalently link NO to free thiol groups on cysteine residues within proteins leading to formation of *S*-nitrosothiols. Several S-nitrosylation targets may play an important role in modulating oxidative stress and vascular remodeling in PAH including eNOS, sGC, hemoglobin, mitochondrial complex I, NOX, and cyclooxygenase (COX)-2 [224]. The functional effects of S-nitrosylation of several of these key proteins promote vasodilation and decrease oxidative stress, although S-nitrosylation of sGC and eNOS may inhibit NO-mediated effects on vascular tone. In red blood cells (RBC), hypoxia impairs S-nitrosylation of hemoglobin and deficiency of *S*-nitrosohemoglobin (SNO-Hb) is associated with exaggerated HPV and increased pulmonary arterial pressures [233]. Furthermore, restoration of

SNO-Hb levels by ethyl nitrite inhalation enhanced vasorelaxation and improved hemodynamics and oxygenation in PAH patients [233]. Although S-nitrosylation-induced vascular alterations appear to be protective in PAH, the role of *S*-nitrosothiols in the pathogenesis of PAH remains incompletely understood.

15.3.1.5 Xanthine Oxidase

Xanthine oxidoreductase (XOR) is a critical source of intracellular ROS. It catalyzes the terminal two steps of purine degradation, from hypoxanthine to xanthine and then to uric acid, with release of $O_2^{\bullet -}$ and H_2O_2 (Fig. 15.3). It primarily exists in cells as a dehydrogenase reducing NAD^+ to NADH, but in the setting of inflammation, oxidation of cysteine residues or limited proteolysis converts xanthine dehydrogenase into xanthine oxidase (XO). XO transfers substrate-derived electrons to O_2, generating $O_2^{\bullet -}$ and H_2O_2. H_2O_2 is a major ROS product of XOR action under normal and pathophysiological conditions [7, 335] and has been shown to regulate many pathways involved in vascular remodeling including proliferation and Ca^{2+} signaling [143, 356, 389]. H_2O_2 has also been shown to contribute to superoxide production and decreased NO via activation of NOX [208, 400], eNOS uncoupling in an NOX-dependent manner [16, 46], and limiting access to BH_4. Furthermore, H_2O_2 has been shown to inhibit the activity of extracellular superoxide dismutase (EC-SOD) in PASMC and treatment with catalase (which catalyzes decomposition of H_2O_2) enhances EC-SOD activity and decreases superoxide levels in a model of persistent pulmonary hypertension of the newborn (PPHN) [363].

XOR is upregulated in the lung and serum of rats exposed to chronic hypoxia and treatment with allopurinol, an XO inhibitor, attenuates hypoxia-induced PH, pulmonary vascular remodeling, and RVH [151, 167]. In addition, XO activity is increased in the plasma of patients with IPAH [124, 321], suggesting a role for XOR-mediated ROS in the pathogenesis of PAH.

15.3.1.6 NADPH Oxidases

ROS produced by oxidases such as NOX are considered a major contributor to oxidative and nitrosative stress in the lungs and pulmonary vasculature [7, 82], and have been shown to play an important role in dysregulation of vascular tone in the setting of hypoxia [118, 211]. The parenchymal family of NOXs includes NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2 and the phagocyte NOX includes gp91phox (NOX2). Only NOX1, NOX2, and NOX4 are found in the human vasculature and generate ROS by electron transfer from NADPH to oxygen to generate $O_2^{\bullet -}$ that can be further converted to H_2O_2 by cellular superoxide dismutases (SODs). For enzymatic function, each NOX requires several adaptor subunits. In endothelial cells, NOX2 is constitutively associated with $p22^{phox}$ and, after stimulation, $p47^{phox}$ is phosphorylated followed by recruitment of $p67^{phox}$, $p40^{phox}$, and Rac1 to the NOX2 complex where it is then able to generate $O_2^{\bullet -}$ [20] (Fig. 15.3).

In the pulmonary vasculature, NOX1, NOX2, and NOX4, as well as the subunits $p22^{phox}$ $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ are expressed in the lung and pulmonary arteries of mice [246]; however, NOX4 is the predominant NOX upregulated by hypoxia in PASMC [245, 246], PAEC [260], and pulmonary artery adventitial fibroblasts [207]. In addition, $p22^{phox}$ and NOX4 have recently been shown to be upregulated in PASMC in a lamb model of pulmonary hypertension of the newborn (PPHN) [362]. Knockdown of NOX4 decreased ROS production and attenuated proliferation in PASMC and pulmonary artery adventitial fibroblasts [207, 245, 246], as well as increased apoptosis in adventitial fibroblasts [207]. In addition, knockdown of NOX4 increased EC-SOD activity as well as attenuated increases in cyclin D1 and NF-κB in PPHN-PASMC [362]. Furthermore, NOX4-derived ROS have been shown to mediate hypoxia-induced decreases in Kv channel current and increase Kv1.5 channel oxidation in PASMC [245].

NOX4 has also been shown to be upregulated by TGF-β in PASMC [328]. TGF-β significantly induced NOX4 expression and ROS in human PASMC in a Smad2/3-dependent manner that was attenuated by diphenylene iodonium, an NADPH inhibitor, knockdown of NOX4 by siRNA, and transfection of dominant negative Smad2/3 plasmids. In addition, TGF-β stimulation induced NOX4-dependent increases in proliferation in PASMC and, furthermore, led to increases in contractile protein expression that was redox- but not NOX4 dependent. Furthermore, NOX4 has been shown to be significantly upregulated in the lungs of PAH patients compared with healthy donor control lungs [246].

NOX1 and NOX2 have also been shown to play a potential role in the pathogenesis of PAH. In a chronic hypoxia-induced PH model in mice, deficiency of NOX2 reduced hypoxia-induced ROS production, pulmonary artery vasoreactivity, and attenuated hypoxia-induced increases in RVSP, pulmonary vascular remodeling, and RVH [211]. Interestingly, in a rat MCT-induced PH model, PASMC isolated from MCT-treated rats had increased expression of NOX1 and enhanced superoxide production. Knockdown of NOX1 reduced superoxide production as well as attenuated MCT-induced increases in SOD2, cyclin D1, and phosphorylation of ERK. Furthermore, knockdown of NOX1 attenuated proliferation and migration of PASMC from MCT-treated rats [348].

NOXs have also been shown to play an important role in the endothelium in response to hypoxia [122, 405]. PAEC exposed to hypoxia-reoxygenation had significant release of H_2O_2 compared with control cells and inhibition of NOX with diphenyliodonium attenuated H_2O_2 production in response to hypoxia-reoxygenation [405]. In addition, acute hypoxic vasoconstriction (HPV) was attenuated in $p47^{phox}$-deficient mice and ex vivo treatment with an NOX inhibitor significantly reduced HPV in isolated perfused rabbit lungs [371]. Although human data on the role of NOX regulation in the pathobiology of PAH is limited, there is strong animal data supporting an important role for NOX-derived ROS in the pathogenesis of PAH. Further study in patients is warranted to elucidate the role of NOX in human PAH and to determine whether NOX represents an effective pathway for therapeutic targeting in PAH.

15.3.1.7 Mitochondria-Derived ROS

Mitochondria are an additional source of ROS production that may play a role in the pathogenesis of PAH [99]. PAH has been reported in patients with genetic alterations in mitochondrial genes [322, 349] and there is growing recognition that metabolic aberrations and mitochondrial dysfunction exist in PASMC and PAEC isolated from patients with PAH [40, 117, 235, 382]. ROS are generated in mitochondria during the electron transport chain when electrons flowing down the redox gradient prematurely react at complexes I and III with O_2 to generate $O_2^{\bullet -}$ [98, 103, 370]. There is also data to suggest that complex II may be a source of mROS generation in the lungs from hypoxic mice and the hearts isolated from MCT-treated rats [267, 292]. Additional ROS can be generated in mitochondria from superoxide by manganese SOD2 that catalyzes rapid conversion of $O_2^{\bullet -}$ to diffusible H_2O_2 (Fig. 15.3), which can serve as a signaling molecule and regulate transcription factors such as HIF-1α [57, 137, 235] and sulfhydryl-rich voltage-gated potassium Kv channels [155], which have been shown to play a critical role in PAH.

Debate exists as to whether hypoxia increases or decreases mROS and, furthermore, whether mROS promote or protect against pulmonary vascular remodeling [98, 368]. Previous work has demonstrated that hypoxia increases mROS, Ca^{2+} influx, and PASMC contractility and that inhibition of the electron transport chain attenuates increases in Ca^{2+} and HPV [56, 106, 290, 359]. In addition, hypoxia-induced increases in mROS have also been shown to enhance PASMC proliferation via opening of mitochondrial K^+_{ATP} channels and overproduction of H_2O_2 [155]. Furthermore, a recent study demonstrates that redox signaling in PASMC in response to hypoxia is dependent upon subcellular mitochondrial compartment location [358].

While supraphysiologic levels of mROS can lead to oxidative damage and cellular dysfunction, mROS are critical regulators of vascular tone and sustained decreases in mROS may lead to upregulation of transcription factors and signaling pathways that promote aberrant vascular remodeling in PAH. Emerging data suggest that mitochondrial function is impaired in PAH and that cellular metabolism is shifted towards glycolysis leading to enhanced cellular proliferation and resistance to apoptosis, similar to cancer cells (i.e., the Warburg effect) [39, 347]. This has been attributed to decreased mROS production, inhibition of Kv channels with subsequent increases in Ca^{2+} signaling, and activation of HIF-1α and NFAT which promote proliferation and suppress apoptosis [40, 41, 240, 369].

Reduced levels of mROS have been found in animals models of PH including the fawn-hooded rat (FHR) that spontaneously develops PAH [40] and MCT-treated rats [235]. Additionally, PASMC isolated from PAH patients have decreased Kv1.5 expression, increased intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$, increased mitochondrial membrane potential, and activation of NFAT [41]. Inhibition of NFAT with VIVIT or cyclosporine restored Kv1.5 expression, decreased $[Ca^{2+}]_i$, and reversed mitochondrial hyperpolarization leading to decreased proliferation and increased apoptosis in PAH-PASMC [41].

In addition, treatment with dichloroacetate (DCA), a pyruvate dehydrogenase kinase (PDK) inhibitor that enhances oxidative phosphorylation, improved mortality and hemodynamics, as well as reversed vascular remodeling and RVH in MCT-treated and chronic hypoxia-exposed rats [235, 239]. DCA was found to reverse MCT-induced vascular remodeling by restoring Kv1.5 expression, depolarizing mitochondria, increasing H_2O_2 production, and inducing apoptosis in PASMC [235, 239]. Furthermore, mitochondrial survivin, a cytoprotective protein that promotes tumorigenesis and inhibits apoptosis in cancer cells [94], has also been shown to be upregulated in MCT-treated rats and in pulmonary arteries of PAH patients [234]. Adenoviral transfection of a dominant negative survivin mutant increased Kv channel current, depolarized mitochondria, attenuated proliferation, and increased apoptosis in PASMC. Intratracheal administration of the survivin mutant in vivo improved hemodynamics and survival and attenuated vascular remodeling in MCT-treated rats [234]. Although conflicting data exists in animal models, mitochondrial-derived ROS clearly play an important role in the pulmonary vasculature and mitochondrial dysfunction is increasingly recognized as contributing to the pathobiology of PAH. Future studies are necessary to evaluate whether mitochondrial-based therapies have efficacy in animal models of PH and patients with PAH.

15.3.1.8 Lipid Peroxidation and Isoprostanes

Lipid peroxidation has recently been recognized as an additional source of ROS during pulmonary vascular dysfunction [251]. Isoprostanes, chemically stable isomers of prostanoids, are formed when ROS products (particularly peroxynitrite) react with unsaturated bonds of membrane lipids such as arachidonic acid [168]. As isomers of prostaglandins (PG), they can act on several cell types within the pulmonary vasculature via specific prostanoid receptors, including the thromboxane A_2 receptor (TP), and PGE_2 and $PGF_2\alpha$ receptors (EP and FP) [109, 169]. In PASMC and EC, isoprostanes can be released in response to stimulation with growth factors (PDGF, TGF-β), pro-inflammatory cytokines (TNF-α, interferon-γ, IL-1β), as well as by ROS (H_2O_2 and $O_2^{\bullet -}$) [168]. This can lead to activation of signaling pathways downstream of prostanoid receptors including RhoA/ROCK, phospholipase C (PLC), and cyclic AMP/protein kinase A [168], resulting in vasoconstriction and release of other vasoconstrictors, including endothelin-1 (ET-1) from endothelial cells and PASMC [167, 388].

Isoprostane levels have been shown to be elevated in the lung in animal models of hypoxia- and hyperoxia-induced PH [166, 178]. In addition, inhibition of the TP receptor has been shown to reduce ET-1 production in PASMC, as well as attenuate RVH and lung smooth muscle-α actin expression in a hyperoxia neonatal rat model [166]. Urinary levels of isoprostaglandin $F_2\alpha$ type-III ($iPF_2\alpha$-III), a stable lipid peroxidation product indicative of oxidative stress [298], are significantly elevated in patients with PAH compared with controls [75, 296], as well as in patients with *BMPR2* mutations regardless of disease status [201]. Furthermore, while urinary levels of $iPF_2\alpha$-III inversely correlate with vasoreactivity to inhaled NO [75],

increased urinary iPF$_{2\alpha}$-III levels directly correlate with hemodynamic and clinical response to epoprostenol [296], and recently have been found to be independently associated with mortality in PAH patients [76]. Although future studies in animal models and patients will be necessary to further elucidate the role of isoprostanes in PAH, emerging data suggest that isoprostanes may play a role in the pathogenesis of PAH and may serve as a possible lipid peroxidation biomarker in PAH patients.

15.3.2 Oxidative Stress and Animal Models of PH

15.3.2.1 Hypoxia-Induced PH Model

Oxidative stress has been implicated in the pathogenesis of PAH in several animal models of PH (Table 15.4). In the chronic hypoxia model of PH, hypoxia has been shown to induce ROS/RNS production with observed increases in lung superoxide [260], phosphatidylcholine hydroperoxide (PCOOH) [151], isoprostanes [178], nitrotyrosine [87, 167], and oxidized glutathione (GSSG) [261]. Hypoxia has also been shown to increase expression of ROS generators including eNOS [112], NOX2 [211], NOX4 [245, 246], XO [151, 167], and, in some studies, mROS [56, 357, 359]. In addition, hypoxia decreases expression of the antioxidant EC-SOD (SOD3) in the lungs of mice [261] and in pulmonary arteries from calves exposed to chronic hypoxia [143]. Furthermore, several studies have demonstrated efficacy of antioxidants (e.g., *N*-acetyl cysteine) [198], inhibitors of ROS-producing enzymes (e.g., allopurinol) [26, 151, 167], peroxynitrite decomposition catalysts [32], and SOD mimetics [351] in hypoxia-induced PH rodent models [151, 199, 260], suggesting oxidative stress contributes significantly to the pathogenesis of hypoxia-induced PH.

In the hypoxia-induced PH model in newborn pigs, increases in oxidative stress were observed after 3 days of hypoxia with increases in isoprostanes in pulmonary resistance arteries [88]. Additionally, NOX1 and p67^{phox} were increased and SOD1 was decreased in pulmonary arteries from pigs raised in hypoxia for 3 or 10 days. Furthermore, inhibition of NOX with apocynin or treatment with an SOD mimetic + polyethylene glycol-catalase attenuated acetylcholine vascular responses of pulmonary arteries from hypoxia-exposed pigs [88].

15.3.2.2 Monocrotaline-Induced PH Model

In the MCT model, increases in isoprostanes [177] and NOX1 [348] have been observed in rats and increased NOX4 expression was reported in mice exposed to MCT [311]. Additionally, while increases in antioxidants SOD, catalase, and glutathione peroxidase have been reported in the lungs [97, 172], decreases in SOD1 and SOD2 have been observed in RV homogenates from MCT-treated rats [292]. Adenoviral overexpression of EC-SOD in MCT-treated rats decreased lung tissue

Table 15.4 Animal models of PH

Model	Phenotype	References
Chronic hypoxia (rodent)	Increased superoxide, phosphatidylcholine hydroperoxide, isoprostanes, nitrotyrosine, and oxidized glutathione (GSSG) in lung	[87, 151, 167, 178, 260, 261]
	Increased NOX4 expression in PASMC, PAEC, and pulmonary artery adventitial fibroblasts	[207, 245, 246, 260]
	Increased lung expression of eNOS, NOX2, NOX4, XO, and mitochondrial ROS (mROS)	[56, 112, 151, 167, 211, 245, 246, 357, 359]
	Decreased lung expression of EC-SOD (SOD3)	[143, 261]
	Allopurinol attenuated hypoxia-induced PH, pulmonary vascular remodeling, and RVH	[26, 151, 167]
	FeTPPS (peroxynitrite decomposition catalyst) reduced lung nitrotyrosine, attenuated vascular remodeling, and normalized pulmonary vascular resistance	[32]
	MnTE-2-PyP (SOD mimetic) attenuated hypoxia-induced PH, RVH, and pulmonary vascular remodeling	[351]
	N-acetyl cysteine inhibited hypoxia-induced PH, RVH, and muscularization of distal pulmonary arteries	[198]
Chronic hypoxia (pigs)	Increased isoprostanes, NOX1, and $p67^{phox}$ in pulmonary arteries; decreased SOD1 in pulmonary arteries	[88]
	Apocynin (NOX inhibitor) and SOD mimetic + polyethylene glycol-catalase attenuated acetylcholine vascular responses of pulmonary arteries from hypoxia-exposed pigs	
Monocrotaline (rats)	Increased isoprostanes in lung; increased superoxide and NOX1 in PASMC; increased NOX4 in lung (mice)	[177, 348, 311]
	Increased SOD, catalase, and glutathione peroxidase in lung	[97, 172]
	Decreased SOD1 and SOD2 in RV	[292]
	Decreased mROS in PASMC	[235]
	EC-SOD overexpression attenuated MCT-induced PH, RVH, and vascular remodeling	[177]
	Resveratrol attenuated MCT-induced PH, RVH, and vascular remodeling	[269]
	Antioxidant EUK-134 attenuated MCT-induced right heart failure	[291]
PPHN (lambs)	Increased H_2O_2 in PAs; increased superoxide in lung and PAs; increased $p22^{phox}$ and NOX4 in lung, PAs, and PASMC; increased $p67^{phox}$ in lung and PAs; decreased EC-SOD in lung and PASMC	[48, 325, 362]
	Recombinant SOD1 enhanced pulmonary vascular responses to inhaled NO	[325]
Neonatal shunt model (lambs)	Increased superoxide, Rac, $p45^{phox}$ in lung, increased eNOS uncoupling	[135]

Sugen hypoxia model (rats)	Increased nitrotyrosine and heme oxygenase 1 (HO-1) in lung; decreased HO-1 in RV	[38, 352]
	Protandim (Nrf2 activator) prevented RV failure and fibrosis	[37]
FHR	Decreased ROS in PAs and PASMC; decreased SOD2 in PASMC; mitochondrial abnormalities, normoxic activation of HIF-1α, and inhibition of Kv1.5 channels in PASMC; metabolism shift from oxidative phosphorylation to glycolysis in PASMC	[40, 293]
	SOD2 overexpression in PASMC restored Kv1.5 expression and inactivated HIF-1α; metalloporphyrin Mn(III)tetrakis (4-benzoic acid) porphyrin (SOD mimetic) improved hemodynamics and exercise capacity, decreased vascular remodeling	[15]
Transgenic BMPR2-mutant mice	Increased isoprostanes and isofurans in lung; increased superoxide and peroxide in VSMC	[116, 201]
$ALK1^{+/-}$ mice	Develop spontaneous PH; increased $iPF_2\alpha$-III and H_2O_2 in lungs; increased eNOS uncoupling	[170]
	Tempol (SOD mimetic) prevented PH and RVH	[170]
ET-1 transgenic mouse	Develop hypertrophic vascular remodeling and impaired vascular relaxation; increased NOX activity and $gp91^{phox}$ expression in mesenteric arteries	[13]
SOD2-knockout mouse	Severe mitochondrial injury; central nervous system and cardiac injury; significant postnatal mortality	[202]
SOD1-knockout mouse	Develop spontaneous PH; increased urinary isoprostanes; increased plasma TBARS; increased superoxide in PAs	[286, 310]
	A-285222 (selective NFAT inhibitor) decreased PH, arterial wall thickness, and vasoreactivity	[286]
	Tempol (SOD mimetic) reversed PH, reduced NFAT activity	
SOD3-knockout mouse	Exaggerated hypoxia-induced PH; increased urinary isoprostanes; increased plasma TBARS	[310, 380]
SOD3 mutation (rats)	Exaggerated MCT-induced PH; increased TBARS and nitrotyrosine in lung	[380]
	SOD mimetic Mn(III)TmPyP attenuated MCT-induced PH and RVH	
SOD3 overexpression	Attenuated and reversed hypoxia-induced PH; attenuated MCT-induced PH; attenuated PH secondary to bleomycin-induced fibrosis	[6, 177, 261, 346]
Caveolin-1-knockout mouse	Develops spontaneous PH; increased eNOS and peroxynitrite in lung; tyrosine nitration of PKG in lung	[376, 396, 397]
	L-NAME and BH_4 reverse PH; mice deficient in both Cav-1 and eNOS are protected from the development of PH	[376, 377]

levels of 8-isoprostane and attenuated RVSP and pulmonary vascular remodeling [177]. Furthermore, several antioxidants [291, 393] and resveratrol [269] have shown benefit in the MCT-induced PH model in rats.

15.3.2.3 SU5416-Hypoxia PH Model

In the Sugen hypoxia model, rats treated with SU5416 followed by exposure to chronic hypoxia had significantly increased expression of nitrotyrosine and heme oxygenase 1 (HO-1) in the lung compared with controls [352], in contrast to the RV where levels of HO-1 were decreased following Sugen hypoxia [38]. Treatment with protandim, a nuclear factor erythroid 2-related factor 2 (Nrf2) activator which induces antioxidant expression (e.g., HO-1, SOD), prevented RV failure and fibrosis; however, it did not attenuate pulmonary vascular remodeling [37].

15.3.2.4 Pulmonary Hypertension of the Newborn Model

Increases in oxidative stress have also been demonstrated in the newborn lamb PPHN model where animals undergo prenatal ligation of the ductus arteriosus [48, 325, 362], as well as a CHD model where a surgical shunt between the aorta and pulmonary artery is created in prenatal lambs [135]. In the PPHN model, newborn lambs that had undergone ductus arteriosus ligation in utero demonstrated increased levels of superoxide, decreased SOD expression/activity, as well as increased $p67^{phox}$ expression in pulmonary arteries [48]. Treatment of PPHN lambs with recombinant SOD1 enhanced pulmonary vascular responses to inhaled NO with greater decreases in PVR, suggesting a critical role for NOX-mediated ROS and potential efficacy of SOD in PPHN [325]. A more recent study demonstrated increased NOX4 and $p22^{phox}$ and decreased EC-SOD in the lungs and PASMC from PPHN lambs [362]. Similarly, in the neonatal shunt model, shunted lambs demonstrated elevated superoxide levels and increased expression of Rac and $p45^{phox}$ in the lung, as well as eNOS uncoupling, further supporting the role of NOX and eNOS in ROS generation in animal models of PH [135].

15.3.2.5 Fawn-Hooded Rat Model

The FHR, a strain in which PAH occurs spontaneously, has provided critical information on the role of mitochondrial dysfunction in the pathogenesis of PAH. The FHR has an autosomal recessive disorder similar to Hermansky–Pudlak syndrome characterized by dysfunction of several organs including systemic hypertension, pulmonary fibrosis, renal disease, as well as platelet and coagulation dysfunction [193]. As described above, PASMC isolated from FHR have decreased ROS, decreased SOD2 expression, as well as marked mitochondrial abnormalities, normoxic activation of HIF-1α, and inhibition of Kv1.5 channels [40]. In addition,

PASMC from FHR demonstrate a shift in metabolism from oxidative phosphorylation to glycolysis despite adequate oxygen [293]. Overexpression of SOD2 in PASMC from FHR restored Kv1.5 expression and inactivated HIF-1α, and treatment of FHR with an SOD mimetic (metalloporphyrin Mn(III)tetrakis (4-benzoic acid) porphyrin) improved hemodynamics and exercise capacity, as well as decreased vascular remodeling [15].

15.3.2.6 Genetic Models of PH

Genetic models have offered the opportunity to further evaluate the role of ROS in pulmonary vascular remodeling and the development of PAH. Several genetically modified mice that develop PH have recently been associated with increases in oxidative stress. Transgenic (TG) mice with a mutation in the cytoplasmic tail of *BMPR2* have increased lung levels of lipid peroxidation products, isoprostanes, and isofurans, and transfection of rat vascular SMC with BMPR2 mutants increases superoxide and peroxide production compared with wild type (WT) BMPR2-transfected cells [116, 201]. Mutations in *ALK1*, which encode an endothelial-specific receptor of the TGF-β superfamily and are associated with hereditary hemorrhagic telangiectasia (HHT) and PAH [141, 142], have also been associated with increased oxidative stress [170]. Mice heterozygous for *ALK1*, that develop PH as they age, have increased ROS in the lungs (iPF$_2$α-III, H_2O_2) at 12 weeks of age secondary to increased eNOS uncoupling, and treatment with tempol, an SOD mimetic, prevents increases in RVSP and RVH in ALK1$^{+/-}$ mice [170]. In addition, TG mice overexpressing ET-1 in the endothelium, that develop hypertrophic vascular remodeling and have impaired vascular relaxation, have enhanced vascular NOX activity and increased expression of gp91^{phox} [13], suggesting these TG mice have increased oxidative stress.

Genetic models of SOD have provided additional insight into oxidative stress and ROS scavenging in animal models of PH. Mice lacking mitochondrial manganese SOD (MnSOD, SOD2) have severe mitochondrial injury with central nervous system and cardiac injury leading to significant postnatal mortality [202]. Mice deficient in intracellular copper-zinc SOD (CuZnSOD, SOD1) or extracellular SOD (EC-SOD, SOD3) have increased oxidative stress as measured by urinary isoprostanes and plasma thiobarbituric acid-reactive (TBARS) levels, and mice deficient for both SOD1 and SOD3 have additional increases in oxidant stress markers [310]. The absence of SOD1 has recently been reported to be associated with the development of spontaneous PH and is dependent on NFAT activation in PASMC [286]. SOD1-deficient mice have elevated superoxide levels and develop significant increases in RVSP under normoxic conditions. Spontaneous PH in SOD1-deficient mice is attenuated by selective inhibition of NFAT as well as tempol, an SOD mimetic, which prevents NFAT activation in SOD1-knockout mice [286]. Although SOD3-knockout mice do not develop spontaneous PH, the absence of SOD3 exacerbates hypoxia-induced PH with significant increases in RV pressures, RVH, and vascular remodeling compared with WT mice [380]. Similarly, a loss-of-function

SOD3 mutation in rats leads to increased TBARS and nitrotyrosine in the lung, as well as exaggerated PH and RVH following MCT, which is attenuated by the SOD mimetic Mn(III)TmPyP [380].

Transgenic overexpression of SOD1 [330] and SOD3 [6, 177, 261] protects against oxidative stress and overexpression of SOD3 has been shown to both attenuate [261] and reverse established PH in response to chronic hypoxia [6], as well as attenuate MCT-induced PH [177], and PH secondary to bleomycin-induced fibrosis [346]. Interestingly, in both the chronic hypoxia-induced PH model and in the bleomycin model of secondary PH, overexpression of EC-SOD in the lung attenuated upregulation of the transcription factor early growth factor-1 (Egr-1) [261, 346]. EC-SOD also decreased TGF-β induction in the bleomycin model [346] and prevented eNOS downregulation in the rat MCT model [177]. Additionally, PAs from EC-SOD knockout mice have enhanced vasoconstriction in response to 5-hydroxytryptamine (5-HT), while PAs from transgenic mice overexpressing EC-SOD have decreased superoxide production and attenuated 5-HT-induced vasoconstriction [210].

The caveolin-1-knockout mouse also provides additional evidence that oxidative and nitrosative stress play a role in the pathobiology of PAH. Mice deficient in Cav-1 develop PH spontaneously with significant increases in PA pressures and RVH compared with WT control mice [396], and restoration of endothelial cell-specific Cav-1 in knockout animals rescues the PH phenotype [254]. The absence of Cav-1 leads to increased activation of eNOS [376], NO-dependent peroxynitrite production, and tyrosine nitration of PKG, which can be reversed by PKG overexpression [397]. Furthermore, inhibition of eNOS with L-NAME [376, 398] or BH_4 treatment [377] prevents PH in Cav-1-knockout mice. Additionally, mice deficient in both Cav-1 and eNOS are protected from the development of PH [398].

15.3.3 Oxidative Stress and Human PAH

Several studies have demonstrated increases in oxidative stress in patients with PAH. As described above, elevated levels of urinary $iPF_2\alpha$-III have been demonstrated in PAH patients [75, 296] and recently have been shown to be independently associated with survival in PAH [76]. Additional studies have demonstrated increased levels of plasma malondialdehyde (MDA) [124, 162] and xanthine oxidase [124, 321], as well as decreased EC-SOD [124] and glutathione peroxidase activity [162] in the plasma of PAH patients. Increases in oxidative stress markers have also been demonstrated in plasma from patients with chronic obstructive pulmonary disease (COPD) and secondary PH [175], and in children with congenital portosystemic venous shunts at risk of developing PH [257]. Furthermore, oxidative posttranslational modification of albumin has been shown in patients with both idiopathic PAH and PAH secondary to SCD [262].

Increases in oxidative stress have also been demonstrated in lung tissue from PAH patients [225]. Immunohistochemical staining demonstrated increased staining for nitrotyrosine and 8-hydroxy guanosine, a marker of oxidative DNA damage, in

lung tissue from PAH patients compared with controls [47]. Levels of the eicosanoid metabolites, 5-oxo-eicosatetraenoic acid (5-oxo-ETE) and 5-hydroxyeicosatetraenoic acid (5-HETE), were also found to be elevated in lung tissue from PAH patients not on prostacyclin and secondary PH patients [47]. In addition, lung tissue homogenates from PAH patients had decreased SOD activity and levels of SOD2 compared with control lungs [47]. Furthermore, SOD and glutathione peroxidase activity were also decreased in airway epithelial cells and lysates from bronchial tissue obtained from explanted PAH lungs compared with controls [225]. Taken together, substantial evidence from animal models and human PAH samples suggest that oxidative stress plays a critical role in the pathogenesis of PAH.

15.3.4 ROS and Mechanisms of Pulmonary Vascular Remodeling

Several mechanisms have been identified by which oxidative stress can mediate the vascular alterations observed in PAH. ROS have been shown to alter the balance of vasoactive mediators, enhance calcium signaling, upregulate growth factors, and induce pro-proliferative signaling pathways, all of which can contribute to enhanced vasoconstriction and pulmonary vascular remodeling in PAH. XO-derived O_2 metabolites have been shown to significantly increase thromboxane B_2 levels 30-fold while only minimally increase PGI_2 levels, leading to enhanced vasoconstriction in isolated perfused rabbit lungs [337]. In addition, peroxynitrite has been shown to inactivate PGI_2 synthase and reduce levels of PGI_2 [401]. ROS have also been shown to upregulate endothelin-converting enzyme-1 [215] and induce ET-1 expression in endothelial cells [66] and, furthermore, ET-1 has been shown to stimulate PASMC proliferation via increases in superoxide production [360]. Additionally, H_2O_2 has been shown to promote eNOS uncoupling leading to decreases in NO and further increases in ROS [46, 400]. Taken together, several studies suggest that oxidative stress leads to an imbalance in vascular mediators with release of potent vasoconstrictors that can overwhelm the effects of endothelial-derived vasodilators and promote enhanced vasoconstriction and vascular remodeling in PAH.

ROS have also been shown to enhance Ca^{2+} mobilization [209] and Ca^{2+} sensitization in PASMC [50, 171, 185], and therefore may play a critical role in enhanced contraction and proliferation of PASMC in PAH. H_2O_2 leads to release of Ca^{2+} from inositol 1,4,5-trisphosphate (IP_3)-gated sarcoplasmic reticulum stores in PASMC [209] via activation of phospholipase C-γ1 [356] and conversion of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and IP_3. Calcium mobilization by H_2O_2 in PASMC [209] and sustained constriction of rat intrapulmonary arteries (IPA) have also been shown to be dependent on ryanodine-sensitive intracellular Ca^{2+} stores [276]. In addition, superoxide has been shown to activate Rho A/Rho-kinase (ROCK) leading to increased phosphorylation of myosin light chain (MLC), Ca^{2+} sensitization, and vasoconstriction in rat pulmonary arteries [185]. Similarly, hypoxia- and ET-1-induced ROS production enhance Ca^{2+} sensitization via activation of Rho A/ROCK signaling in PASMC [50, 171].

mROS production has also been implicated in pulmonary vascular remodeling as discussed above. Numerous studies have demonstrated that hypoxia increases mROS, Ca^{2+} influx, and PASMC contractility [56, 106, 290, 359]. However, more recent studies suggest that decreases in mROS lead to inhibition of Kv channels, membrane depolarization, activation of voltage-gated Ca^{2+} channels, and increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]$) which lead to increased vasoconstriction, enhanced proliferation, and suppression of apoptosis [40, 41, 240, 369].

ROS can also increase expression of several growth factors and enhance pro-proliferative signaling pathways that play a critical role in vascular remodeling in PAH. ROS have been shown to activate latent TGF-β [27] and TGF-β can further induce ROS via induction of NOX4 leading to enhanced proliferation and contraction in PASMC [328]. ROS can also induce PASMC expression of FGF-2 [35] which is upregulated in a lamb model of increased pulmonary blood flow and PH [361]. VEGF expression is also upregulated by ROS in PASMC [31] and is dependent on TGF-β activation of NADPH and ROS generation [226]. In addition, hypoxia has been shown to upregulate VEGF expression in pulmonary artery endothelial cells [212], and both H_2O_2 [249] and hypoxia have been shown to increase PDGF expression in endothelial cells [191].

ROS can also activate signaling pathways and transcription factors that regulate cellular proliferation, growth, and apoptosis leading to enhanced proliferation and growth of PASMC, PAEC, and fibroblasts, as well as matrix deposition in the pulmonary arterial wall. ROS have been shown to activate the G protein Ras leading to recruitment of phosphatidylinositol 3′kinase (PI3K) and activation of downstream signaling pathways involved in cell survival and hypertrophy, including Akt/protein kinase B and ERK1/2 [89, 344]. H_2O_2 has also been shown to upregulate the p38 mitogen-activated protein kinase (MAPK) pathway [343] and induce Src-dependent JNK activation in vascular SMC [389], as well as Src-dependent activation of big MAPK1 (BMK1/ERK5) in fibroblasts [1]. Peroxynitrite can also stimulate proliferation of PAEC and PASMC via activation of the Ras-Raf-MEK-ERK pathway as well as via protein kinase C [3].

ROS have also been shown to modulate key transcription factors that play a role in PAH and that regulate genes involved in the cell cycle and cell growth. H_2O_2 and hypoxia have been shown to upregulate transcription of peroxisome proliferator-activated receptor-γ coactivator-1 protein-α (PGC-1α), a transcriptional coactivator and critical regulator of mitochondrial biogenesis [163]. In PASMC, hypoxia has been shown to induce PGC-1α expression via PI3K/Akt signaling and activate mitochondrial biogenesis via NRF-1 and TFAM [288]. Additionally, knockdown of PGC-1α inhibits hypoxia-induced cyclin expression and proliferation of PASMC [288], suggesting that ROS-induced PGC-1α may play a key role in regulating mitochondrial biogenesis and vascular remodeling in PAH. XO-derived ROS have also been shown to upregulate Egr-1 via ERK1/2 in PASMC, which has been shown to play an important role in animal models of PH [92, 203, 345]. Furthermore, ROS have been shown to induce NFAT expression [181], a critical transcription factor linked to PASMC proliferation and vascular remodeling which plays a key

role in the pathogenesis of PAH [33, 84, 286]. Interestingly, NFAT has recently been linked to the development of spontaneous PH in SOD1-deficient mice suggesting a critical role for NFAT in mediating ROS-induced PAH [286].

15.4 Antioxidants in PAH

Drugs that are currently available for the management of PAH include calcium channel blockers, prostanoids, endothelin-1 receptor antagonists, and PDE5 inhibitors, which lie outside the scope of this review [247]. Even though there have been significant advances in the understanding of PAH pathogenesis and new therapeutic options available for treatment, PAH remains incurable and patients eventually progress to right heart failure and death [247]. Present therapeutic approaches have been developed based on the imbalance in endothelium-derived vasoactive mediators that exists in patients with PAH [247]. Growing evidence of the importance of oxidative stress in the pathogenesis of PAH has led to the identification of new therapeutic targets. Antioxidant strategies for the treatment of PH have been recently classified into four groups: enzymatic ROS scavengers and regulators, small chemical ROS scavengers, inhibitors of ROS generation, and Nrf2 activators [332]. Additional strategies include eNOS uncoupling agents and mitochondria-active agents.

15.4.1 Enzymatic ROS Scavengers and Regulators

Enzymatic ROS scavengers and regulators include SOD, catalase, glutathione peroxidase, glutathione reductase, glutaredoxin, thioredoxin, thioredoxin reductase, peroxiredoxin, and sulfiredoxin. These enzymatic scavengers exist naturally in human cells and act synergistically in order to protect tissues against free radical damage [62].

15.4.1.1 Superoxide Dismutase

SOD is one of the most important enzymatic antioxidants in the body and is ubiquitously expressed [5, 62]. All three isoforms (SOD1, SOD2, SOD3) act by catalyzing the rapid conversion of $O_2^{\bullet -}$ into H_2O_2 (Fig. 15.3) [5]. SOD has been shown to be downregulated in animal models of PH and PAH patients [5], and administration of SOD has been shown to be beneficial in animal models of PH. Steinhorn et al. found that treatment with recombinant human SOD (rhSOD) in sheep with PPHN reduced PVR in vivo and enhanced relaxation responses of pulmonary arteries to exogenous NO ex vivo [325]. Farrow et al. also showed that rhSOD increases eNOS expression and restores its function, decreases generation of ROS, and increases BH_4 in PPHN lambs [115]. The effect of SOD administration in human PAH has not been studied.

15.4.1.2 Catalase

The enzyme catalase is also key in the antioxidant machinery of cells and is of particular importance during high levels of oxidative stress, since it has a very high turnover number [5]. Catalase exerts its antioxidant action by converting hydrogen peroxide into water and oxygen (Fig. 15.3) [5]. Data regarding the role and expression of catalase during PH is variable, with increased activity reported in MCT-treated rats [172], decreased levels in lambs with PH secondary to increased postnatal pulmonary blood flow [313], and no difference reported in humans with IPAH [225]. Studies to evaluate the effect of exogenous catalase in animal PH models have revealed variable results. Goats pre-treated with intravenous catalase and subjected to endotoxin infusions displayed minimal attenuation of PH compared with controls [229]. However, endotoxin-exposed sheep pre-treated with intraperitoneal catalase had attenuated elevation of pulmonary pressures compared to untreated controls [242]. Wedgwood et al. evaluated the effect of catalase on isolated pulmonary arteries from PPHN lambs and found a normalization of the vasodilator responses to exogenous NO [364]. They also demonstrated that intratracheal administration of catalase to PPHN lambs enhanced SOD3 activity and improved oxygenation [363]. Thibeault et al. evaluated the effect of intratracheal injection of liposome-encapsulated catalase in a rat model of hyperoxia, finding reduction in vascular and parenchymal damage caused by oxygen toxicity [340]. The role of catalase in treatment for human PAH is not clear and further studies are needed to determine potential benefit [5].

15.4.2 Small Chemical ROS Scavengers

15.4.2.1 Dietary Antioxidants

Vitamin C

Ascorbic acid is an excellent reducing agent, capable of donating an electron to oxidizing radicals such as hydroxyl, alkoxyl, peroxyl, thiol, and tocopheroxyl [101]. This makes vitamin C a good antioxidant and a substance of interest for the treatment of many diseases. Interestingly, reversible PH secondary to vitamin C deficiency and clinical scurvy has been described [197, 237]. Furthermore, low levels of ascorbate have been observed in patients with high altitude PH [22], suggesting a potential beneficial role of vitamin C in PAH. Xiang et al. investigated the effect of vitamin C supplementation in broilers with pulmonary hypertension syndrome (PHS) induced by low temperatures [379]. Vitamin C supplementation reduced PHS incidence and attenuated the percentage of thick-walled peripheral lung vessels and associated muscularization of pulmonary arterioles [379]. Paradoxically, however, Walton et al. found that broilers with PHS secondary to low temperatures and fed with flax seed oil had higher incidence of PHS when vitamins C and E were

added to the diet [354]. On the other hand, Belaiba et al. showed that vitamin C inhibits the production of ROS and HIF-1α protein, as well as the increase of VEGF mRNA in PASMC stimulated with thrombin or $CoCl_2$ in vitro [31]. No clinical trials have explored the effects of vitamin C on PH in humans. One clinical trial found no benefit of vitamin C supplementation in the prevention of acute mountain sickness [23]. Currently, there are two ongoing clinical trials registered in the NIH that aim to determine the use of antioxidants, including vitamin C, as prophylaxis for acute mountain sickness (NCT01182792, NCT01571687).

Tocopherols

Vitamin E is the most important lipophilic antioxidant in the lung and plays a key role in scavenging hydroxyperoxyl radicals produced during lipid peroxidation [189, 341]. Severe oxidative stress leads to increased concentration of vitamin E in the lung [189]. Patients with IPAH appear to have decreased levels of α-tocopherol in the plasma and vitamin E levels have been shown to correlate with pulmonary function better than other antioxidants [278, 308]. These findings suggest that there is a mobilization of vitamin E from other tissues to reach adequate levels in the lung [189]. There is limited and variable evidence on the effect of vitamin E in models of PH. In a model of broilers with PHS induced by cool temperatures, high dietary vitamin E attenuated mitochondrial dysfunction [161], lowered PHS-induced mortality, and improved antioxidant capacity [44]. However, a subsequent study demonstrated no mortality benefit of vitamin E supplementation in broilers with PHS [45]. Additional studies found that α-tocopherol [182] and vitamin E failed to improve RVH in broilers with PHS, nor improved cardiopulmonary performance or NOS activity in isolated pulmonary arteries [216]. Further studies are needed to further elucidate the effects of vitamin E in PAH.

Carotenoids

The antioxidant activity of carotenoids is due to their multiple conjugated double bonds, which makes them susceptible to oxidative cleavage [314]. The antioxidant properties of vitamin A have been of great interest in the study of many diseases, including lung cancer [123]. The role of retinol in lung development, vasculogenesis, and angiogenesis has been well documented [304, 307]. In PH, it has been demonstrated that patients with IPAH have reduced levels of retinoic acid, and treatment of hPASMC with this vitamin suppressed 5-HT-induced cell growth in vitro [278]. In a rat hypoxia model, treatment with all-*trans* retinoic acid (ATRA) significantly reduced muscularization of peripheral PAs and medial wall thickness of small muscular arteries; however, it did not attenuate PH or RVH [392]. Similarly, in MCT-induced PH in rats, Swamidas et al. found that dietary retinol resulted in less vascular inflammation in the lung and RV, but did not improve RVH [333]. Conversely, Qin et al. found that ATRA treatment in rats

with MCT-induced PH lowered mPAP and inhibited collagen accumulation and MMP1 mRNA overexpression in the lungs [281]. No clinical trials have evaluated the benefits of carotenoids in human PAH.

Flavonoids

The antioxidant properties of flavonoids have been well documented in vitro [217]. They act through different mechanisms including chelation of metal ions, stimulation of antioxidant enzymes, and inhibition of enzymes that increase oxidative stress [80]. The benefits of flavonoids have been evaluated in a wide array of pathologies, including cardiovascular diseases, type II diabetes, neurodegenerative diseases, and cancer [217]. Many investigators have been interested in the effects that flavonoids may have on oxidative stress in PH. In rat models of MCT-induced PH, administration of flavonoids, such as quercetin and genistein, has been shown to decrease mPAP, RVSP, RVH, medial wall thickness, and neomuscularization of PAs, as well as inhibit hPASMC proliferation and progression to right heart failure [127, 150, 228]. In rats exposed to hypoxia, puerarin was shown to lower levels of ET-1 and type I collagen, enhance the activity of SOD, and improve pulmonary vascular remodeling [206]. Similarly, breviscapine was shown to decrease mPAP, RVH, and vascular remodeling as well as decrease fractalkine and Rho-kinase mRNA expression in a rat hypoxia model [63, 383]. In addition, genistein was shown to inhibit the mean change in tension caused by ET-1 in IPA of rats previously exposed to chronic hypoxia [367]. Finally, genistein has been shown to significantly attenuate PH, activate eNOS, restore endothelial function, and decrease vascular remodeling in broilers with PH [384]. No clinical trials have yet explored the effects of flavonoid administration in patients with PAH.

Resveratrol

Resveratrol is commonly found in foods such as grapes, plums, and peanuts, and has become a substance of interest because of its potential benefits in cardiovascular disease and cancer [86]. Resveratrol exerts its antioxidant effects possibly through scavenging superoxide radicals formed in the mitochondria, inhibiting lipid peroxidation, and competing with coenzyme Q to decrease the oxidative chain complex [86]. Other antioxidant mechanisms of resveratrol include upregulation of antioxidant enzymes, decrease in NOX levels, and regulation of GTP-cyclohydrolase 1, which increases BH_4 levels and reverses eNOS uncoupling [378]. In the rat MCT-induced PH model, resveratrol attenuates elevation in RVSP, RVH, and thickening of IPAs [79, 268, 269]. In addition, resveratrol normalizes alterations in BMP receptors and SMAD signaling molecules, upregulates NOX subunits, and attenuates expression of IL-6, IL-1β, TNF-α, PDGF-α, PDGF-β, MCP-1, iNOS, and ICAM-1 in vivo. Furthermore, resveratrol prevented proliferation of PASMC after PDGF

stimulation, and inhibited cytokine-induced NF-κβ activation in PASMC in vitro [79]. Finally, Chun et al. also showed that resveratrol reduced mPAP and monocyte chemoattractant protein-1 expression in rats with PH induced by infusion of autologous blood clot in the PA [68].

15.4.2.2 Gases

Nitric Oxide

Currently, inhaled NO is indicated for the treatment of term or near-term neonates with hypoxemic respiratory failure associated with PH, and is clinically used in acute vasoreactivity testing in the cardiac catheterization laboratory in patients with PAH [2, 29]. Inhaled NO has also been shown to be beneficial in patients that undergo surgery for CHDs or heart transplant [160]. There have been non-controlled observational clinical studies that show improved PVR and PAP and minimal adverse events in patients with PAH treated with long-term inhaled NO [29, 58, 164, 274, 275, 319]. However, there are still concerns about the potential risks of long-term inhaled NO therapy in PAH patients, including rebound PH upon sudden discontinuation, and toxicity due to production of NO_2 and methemoglobin [29, 160]. Further clinical trials are needed to determine the safety profile of inhaled NO in the treatment of PAH.

Most of the rationale behind the studies of inhaled NO in the treatment of PAH are based on the fact that NO is a selective pulmonary vasodilator, rather than the role it may play as antioxidant. However, recent studies have demonstrated that inhaled NO increases antioxidant defenses, decreases DNA damage, and improves lung inflammation in rabbits exposed to conventional mechanical ventilation [119, 299]. In addition, inhaled NO treatment in infants with hypoxemic respiratory failure reduced oxidative stress biomarkers, namely MDA and total glutathione [139]. The potential antioxidant mechanisms of NO are very complex, since this molecule is also involved in the production of RNS and nitrosative stress, as discussed in previous sections. Nevertheless, recent studies have shown that NO participates in scavenging of lipid peroxyl radicals, and some RNS such as $ONOO^-$ might even participate in cell signaling pathways that activate cellular antioxidants resulting in cytoprotective, rather than cytotoxic, effects [271].

Hydrogen Sulfide

The toxic effects of excessive hydrogen sulfide (H_2S) inhalation have been well documented and include pulmonary edema, bronchiolitis, reactive airways disease, pulmonary interstitial fibrosis, and death [64]. Its main mechanism of toxicity is due to inhibition of cytochrome oxidase and other cellular respiratory enzymes, which is dependent on concentration and duration of exposure [64]. However, H_2S is produced endogenously in the lung and studies have now shown potential benefits

of H_2S or H_2S donors in the treatment of chronic pulmonary diseases including COPD, asthma, and PH [64]. Antioxidant mechanisms of H_2S include increasing glutathione levels and activation of Nrf2 with subsequent upregulation of antioxidant response elements [277].

H_2S levels have been shown to be low in rats exposed to hypoxia [282], and in patients with acute exacerbations of COPD who have elevated PAP, compared to those with normal PASP [65]. Treatment of hypoxia-exposed rats with an H_2S donor, sodium hydrosulfide (NaHS), reduces mPAP and RVH [365], decreases vascular remodeling, and enhances total antioxidant capacity compared with controls [282]. Similarly, administration of NaHS to broilers exposed to hypoxia significantly reduced PH compared with untreated controls [385]. In addition, H_2S has been shown to relax rat aortic arteries and inhibit vascular SMC proliferation in vitro [102, 152, 282, 394]. Additionally, H_2S or injected NaHS has been shown to be protective in mouse lung injury models [121]. Investigations of H_2S still remain in a preclinical phase.

Carbon Monoxide

Carbon monoxide (CO) is very well known for its toxic effects both in chronic cigarette smoke exposure or acute intoxication [128]. The interest in the role of CO as a therapeutic gas is relatively recent and has been based on observations that, at low doses, CO may have cytoprotective properties involving inhibition of inflammatory and proliferative signals [128]. The anti-inflammatory effects of CO have been shown in many in vivo and in vitro studies [128, 244], but its antioxidant properties are less known. In fact, some studies have found that CO inhibits cytochrome c oxidase in the mitochondria, increasing accumulation of electrons within the electron transport chain resulting in increased generation of ROS in this organelle [402]. In contrast, other studies have shown that CO inhibits NOX, limiting ROS production [323].

Low dose CO has been shown to be protective in the FHR model, as well as in the hypoxia and MCT-induced PH rat models [403]. Daily treatment with 1 h of inhaled CO at 250 ppm protected FHRs from the development of spontaneous PH and prevented both hypoxia and MCT-induced increases in RVSP, RVH, and pulmonary vascular remodeling [403]. Although effects on ROS were not assessed, CO was found to attenuate PASMC proliferation, decrease apoptosis, and induce eNOS expression in PAEC [403]. In addition, CO has been shown to attenuate PVR elevation in hypoxemic sheep [256], and decrease vascular remodeling in iliac arteries in a porcine model of balloon angioplasty [285]. CO has also been shown to have protective effects in other lung diseases including bleomycin-induced fibrosis [399], lung transplantation [187, 188], and ventilator-induced lung injury [95, 149, 244]. Furthermore, treatment of ex-smoking COPD patients with CO inhalation decreased sputum eosinophils and improved responses to methacholine testing [30]. Further studies are needed to determine the efficacy of CO in patients with PAH, as well as to elucidate the role of CO in modulating oxidative stress in PAH.

15.4.2.3 Antioxidant Enzyme Mimetics

Substances that mimic the functions of antioxidant enzymes can also be used to counteract oxidative stress in the pulmonary vasculature. The antioxidant enzyme mimetics investigated have the same mechanism of action previously described for the enzymes that they emulate.

MnTE-2-PyP

MnSOD mimetics have high selectivity for mitochondria and decrease superoxide levels in the mitochondrial matrix, increasing the levels of diffusible H_2O_2 [98]. The SOD mimetic MnTE-2-PyP has been shown to be protective in a mouse model of hypoxia-induced PH [351]. Treatment of mice with MnTE-2-PyP attenuated hypoxia-induced increases in RVSP, RVH, and pulmonary vascular remodeling [351]. Furthermore, MnTE-2-PyP attenuated hypoxia-induced NALP3 inflammasome activation, caspase cleavage, and IL-1β and IL-18 production [351]. Other Mn porphyrin-based SOD mimetics have demonstrated similar efficacy in the MCT model and FHR [380].

Tempol

Tempol is also an SOD mimetic that has been studied in various animal models of PH. In rats exposed to chronic hypoxia, tempol normalized RVSP and reduced RVH [108], while combined treatment with tempol and tadalafil significantly prevented elevation in RVSP and RV dP/dt(max) and reduced oxidative stress in rats exposed to acute hypoxia [289]. In addition, tempol has been found to inhibit LY83583-mediated constriction of rat IPAs [185], reduce hypoxia-induced SMC proliferation and remodeling in rat PAs, as well as inhibit lung ROS production [184]. Furthermore, treatment with tempol attenuated PH in a sheep model [320], and prevented spontaneous development of PH in $ALK1^{+/-}$ mice [170]. Tempol has not yet been evaluated in any clinical trial.

Ebselen

There is minimal information on the use of the glutathione peroxidase mimetic ebselen in PH; however, recent studies suggest that ebselen may have protective effects in the pulmonary vasculature. Ebselen has been shown to attenuate hypoxia and peroxynitrite-induced proliferation of PASMC in vitro [3]. In addition, ebselen has been shown to decrease the sustained phase of hypoxic vasoconstriction of IPAs in rats [71]. More studies are needed to better understand the effects of ebselen on the pulmonary vasculature and determine whether ebselen has efficacy in animal models of PH.

15.4.3 Inhibitors of ROS Generation

15.4.3.1 Inhibitors of Oxidases

Inhibitors of oxidases include inhibitors of NOX, xanthine oxidase, and monoamine oxidase. These substances function by blocking the main enzymes that produce ROS in cells.

NADPH Oxidase Inhibitors

NOX inhibitors are perhaps the most studied of all the oxidase inhibitors tested in PH. Apocynin, an NADPH inhibitor, attenuates hypoxia-induced PH and vascular remodeling in lectin-like oxidized low-density lipoprotein receptor (LOX-1) transgenic mice that have enhanced ROS in response to hypoxia [264]. In addition, apocynin was shown to attenuate cold-induced PH and PA remodeling in rats [78], and restored pulmonary artery endothelial function and vascular responses in diabetic rats [214]. In lambs with PPHN induced by ductus arteriosus ligation, it has also been shown that apocynin significantly improves oxygenation, enhances PA relaxation and eNOS expression, and improves angiogenic activity of PAEC [339, 363]. Furthermore, in rat PASMC, apocynin reverses hypoxia-induced decreases in Kv current density [245], and suppresses U46619-induced inhibition of Kv currents [70].

Xanthine Oxidase Inhibitors

Allopurinol has been the mainstay of treatment for gout for many years and has recently become of great interest in the study of ischemic heart disease, chronic heart failure, and inflammatory diseases. In mice and rats exposed to hypoxia, allopurinol has been shown to decrease superoxide production, reduce PH, attenuate vascular remodeling, and alleviate the increased RVSP and RVH [26, 151, 167]. In addition, Shen et al. found that isolated rat lungs exposed to hypoxic challenges had attenuated HPV when treated with allopurinol ex vivo [315].

15.4.3.2 Iron Chelators

Iron normally exists in cells in the form of ferric ions (Fe^{3+}), which can react with superoxide releasing highly reactive hydroxyl radicals. These radicals can cause lipid peroxidation, DNA oxidation, and protein oxidation [374]. Based on this rationale, it has been suggested that iron chelation may have a potential benefit on oxidative stress in the lung, but most investigations have failed to support this hypothesis. Treatment of rats exposed to chronic hypoxia with desferroxamine prevented PH and vascular remodeling in vivo, and inhibited human PASMC growth in vitro [374].

However, human studies have demonstrated that healthy volunteers exposed to desferroxamine develop increased PVR [25], and hypoxia-induced pulmonary vasoconstriction is enhanced by desferroxamine in healthy volunteers [318]. In addition, recent studies have found decreased iron levels in patients with IPAH and iron supplementation is now being evaluated as a potential treatment in this group of patients [153, 294]. Further studies are needed to better understand the role of iron in PAH pathogenesis.

15.4.4 Nrf2 Activators

Nrf2 promotes gene expression of antioxidant response element (ARE)-regulated antioxidant enzymes in response to oxidative stress [165]. Nrf2 is held in the cytoplasm by an inhibitor, and activation of the PKC signaling by oxidative stress leads to activation and translocation of Nrf2 to the nucleus with subsequent activation of ARE-regulated genes [165]. Nrf activators act by eliciting this response and increasing the level of ARE-regulated antioxidant enzymes in cells. Protandim, an Nrf2 activator prevented the development of right ventricular failure and fibrosis in the Sugen hypoxia rat model of PH, although it did not prevent the angio-obliterative vascular remodeling [352]. In addition, Nrf2-knockout mice develop exaggerated RVH in response to hypoxia, and the Nrf2 activator olipraz attenuates RVH and vascular remodeling in wild type, but not Nrf2-deficient, mice exposed to hypoxia [107]. Future studies on the potential benefits of Nrf2 activators in the treatment of PAH are necessary.

15.4.5 Tetrahydrobiopterin

The role of tetrahydrobiopterin (BH_4) in oxidative stress and eNOS uncoupling has been reviewed in previous sections. Deficiency of this cofactor has been associated with development of PH and IPF in animal models [10, 183, 338]. Sapropterin dihydrochloride (pharmaceutical preparation of BH_4) has been used in the treatment of hyperphenylalanemia [295]. Interest in the possible benefits of BH_4 supplementation for the treatment of PH is now increasing. Administration of BH_4 to MCT-treated rats attenuated PH and vascular remodeling [120, 180], as well as decreased HPV and increased NO synthesis in isolated lung preparations [120, 190]. In addition, while BH_4 did not improve endothelial dysfunction of IPAs in a porcine model of PPHN [258], treatment of PAEC from PPHN lambs decreased apoptosis, improved angiogenesis, increased NO and eNOS dimer formation, and decreased superoxide production [338]. Furthermore, treatment with sapropterin dihydrochloride, in addition to sildenafil and/or endothelin receptor antagonists, in 18 patients with PAH or inoperable CTEPH was well tolerated and improved 6-min walk distance, although did not significantly alter NO synthesis or oxidative stress [295].

As BH_4 supplements have been proven to be safe in humans, they represent an interesting therapeutic alternative for the treatment of PAH, but further studies are needed to determine their true efficacy.

15.4.6 Mitochondria-Activating Drugs and Mitochondria-Targeting Antioxidants

The hyperproliferative and antiapoptotic phenotype of PASMC observed in PAH is associated with mitochondrial suppression, altered glucose metabolism, and decreased mROS production [98]. These mechanisms are described in detail in previous sections.

15.4.6.1 Mitochondria-Targeting Antioxidants

There has been recent interest in therapeutic strategies that specifically target mitochondria in order to restore their normal function. The fact that this organelle is negatively charged has led to the development of strategies that increase mitochondrial selectivity such as the use of a positively charged ion, namely triphenylphosphonium (TPP^+), to deliver vitamin antioxidants [98]. One of the agents that uses this cation as vehicle and has been studied in vascular diseases is MitoQ, a ubiquinone analogue of the mitochondrial electron transport chain [98]. Treatment of spontaneously hypertensive rats with MitoQ protected against the development of hypertension, improved endothelial function, and decreased cardiac hypertrophy [134]. In addition, the mitochondrial-targeted SOD mimetic mitoTEMPO decreased mitochondrial superoxide production, reduced cellular NOX activity, restored NO expression, improved endothelial-dependent relaxation, and attenuated hypertension in mice exposed to angiotensin II infusion [93].

15.4.6.2 Mitochondrial-Activating Therapies

DCA and trimetazidine (TMZ) stimulate mitochondria and regulate metabolic substrate entry into the TCA cycle [98]. DCA also inhibits PDK, which ultimately results in the inhibition of normoxic HIF-1α production and increases in pro-apoptotic factors, reducing abnormal cell proliferation [98]. Several studies in animal PH models have demonstrated that DCA stimulates glucose oxidation, reduces mPAP, and decreases medial wall thickening of PAs [40, 98, 136, 235, 239, 331]. An early-phase clinical trial of DCA in PAH is currently being completed [98] (NCT01083524). TMZ has also been shown to increase glucose oxidation, suppress fatty acid oxidation, restore perfusion to distal PAs, and reverse established PH in animal models [98, 331]. Finally, phenylbutyrate (PBA), a chemical chaperone which prevents

disruption of the ER-mitochondrial unit, has recently been shown to attenuate PH, vascular remodeling, and RVH in both hypoxia-induced PH in mice and in MCT-induced PH in rats [98].

15.5 Conclusions

This review highlights the important role that oxidative stress and aberrant NO signaling play in the pathogenesis of PAH and emphasizes the mechanisms of ROS-induced pulmonary vascular remodeling in PAH. Although significant progress has been made in understanding the pathogenesis of PAH, currently available therapies that target the imbalance of vasoactive mediators do not improve mortality in PAH patients. Emerging studies implicate oxidative stress as a key mechanism in the pathobiology of PAH and therapies targeting ROS generation have shown efficacy in animal models of PH. Growing evidence of the importance of oxidative stress in the pathogenesis of PAH has led to the identification of potential new therapeutic targets in PAH. New approaches to target oxidative stress include ROS scavengers, inhibitors of ROS generation, Nrf2 activators, mitochondria-activating drugs, and eNOS recoupling agents. Developing novel therapeutics to target oxidative stress in PAH is an active and exciting area of research. Although human data is currently limited, antioxidant therapeutics may hold promise in the future for treatment of PAH.

Conflict of Interest The authors report no conflict of interest.

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Chapter 16
Role of Oxidants and Antioxidants in Pediatric Respiratory Disorders

Meenu Singh and Anil Chauhan

The first known oxidative challenge of life occurs as early as birth, when lung cells are exposed to a sudden several fold increase in oxygen (O_2) concentration. A fully-developed lung armed with sufficient defense is therefore critical in ensuring that the newborn lung is resistant to high O_2 tensions. This prerequisite is clearly highlighted in the problems that can arise following a premature birth at approximately 32 weeks or earlier, when the structural and biochemical components of the human lung, vital for normal respiration, are not sufficiently developed. The extent of pulmonary immaturity in an infant born at this stage necessitates ventilation and the provision of supplementary oxygen that, in the presence of a severely-reduced antioxidant defense system, has the potential to increase the risk of toxicity to lung cells. Indeed, O_2-related lung injury of prematurely born neonates can in turn play a role in the progression to broncho-pulmonary dysplasia, which is a common cause of morbidity and mortality in preterm infants.

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Further, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. The genetic and pharmacological evidence that Nrf2-dependent GSH-induced signaling plays a key role in lung Type II cell proliferation and cellular protection against oxidant-induced death was demonstrated in freshly isolated primary cultures. In addition to quenching high levels of ROS, signaling

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induced by GSH is critically required for proper cell proliferation but not essential for maintaining differentiation [1]. A large number of oxidants are known to induce oxidative stress in the lungs.

16.1 Allergy, Asthma, and Oxidants

Sensitization to a normally harmless allergen results in the immune system being biased to a predominant T-helper type 2 response. Re-exposure to the same allergen leads to a robust secretion of allergy-related mediators that eventually trigger symptoms. Our understanding of these disorders has enabled the search for therapeutic approaches that can either modulate the sensitization process or impact upon allergic mediators, thus helping to manage allergic symptoms. Importantly, asthma is one of the common respiratory allergy in children. Atopic dermatitis and food-allergies are other important allergic problems. Asthma is a chronic inflammatory disorder characterized by airway obstruction and airway hyper responsiveness (AHR). There is influx and activation of cells such as eosinophils, macrophages, and neutrophils to the site of inflammation with generation of reactive oxygen and nitrogen species (ROS/RNS) [2]. These ROS/RNS cause endothelial barrier dysfunction, activate redox sensitive transcription factors, and enhance AHR [3]. The reactive species cause increased lipid peroxidation, mediating direct tissue damage.

Oxidative stress plays an important role in exacerbating the asthmatic condition, which is caused by over production of reactive oxidants and overwhelming of endogenous antioxidants [1]. In the airways, the lung maintains an endogenous defense system consisting of both enzymatic and non-enzymatic components to balance between normal physiologic function and damage which is accompanied by the redox environment in neutralization of free radicals. There are enzymes which help in quenching of free radicals/oxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxide (GTPx), hemeoxygenase, glutathione-S-transferase, glutaredoxin, and thioredoxin (TRX). The non-enzymatic components include vitamins A, C, uric acid, and glutathione [3, 4]. The most important scavenger and inhibitor of lipid peroxidation present in the lung lining is Alpha tocopherol (vitamin E). It has been amply demonstrated that there is reduction in BHR and inflammation, low frequency of allergen sensitization, and immunomodulation with tocopherol alone or in combination with vitamin C [5–7]. Alphalipoic acid, another free radical scavenger can recycle other antioxidants and also accelerate synthesis of reduced glutathione (GSH) [8].

The GSH redox buffer serves as one of the important defense systems of the lung. It is crucial in maintaining intracellular GSH/GSSG homeostasis. Any alteration in the lung redox potential, influences activation of proinflammatory transcription factors such as NF-κB and AP-1 [9]. The enzyme glutathione-S-transferase catalyzes conjugation of reduced glutathione with electrophilic reactive compounds resulting from oxidant-mediated lipid peroxidation, thus effectively detoxifying them. Earlier studies had shown that supplementation of antioxidants in combination is more efficient in

reducing oxidative stress while maintaining cellular redox homeostasis (high GSH/GSSG) [3, 7]. The earlier work with antioxidants mutated glutathione-S-transferase (mGST) and GSH has also demonstrated a synergistic effect in ameliorating oxidative stress and airway inflammation [10]. A decreased antioxidant capacity in plasma and bronchoalveolar lavage (BAL) fluid of patients with asthma provided oxidant–antioxidant imbalance. Bronchial epithelial cells isolated from patients with asthma not receiving corticosteroids, were found to possess less Cu, Zn-SOD activity than epithelial cells obtained from control subjects [11]. Whilst there is a loss of SOD activity in the lungs of individuals with atopic asthma within minutes of an acute asthmatic response to segmental antigen instillation [12]. Children chronically exposed to high levels of ozone (O_3), the principal oxidant pollutant in photochemical smog, are more vulnerable to respiratory illness and infections. Early life ozone exposure causes persistent nasal epithelial alterations in infant monkeys and provides a potential mechanism for the increased susceptibility to respiratory illness exhibited by children in polluted environments [13].

The IL-17 is important for ozone-induced bronchial hyper responsiveness but not for the induction of emphysema and inflammation. This dependent effect of AHR on IL-17 is likely to be a direct effect of IL-17 on airway smooth muscle. It was also reported that IL-17A was necessary for the development of AHR in an ovalbumin-induced asthma model [14].

According to the statistical analysis through Poisson regression, it was analyzed that the week number and prior day accumulation of atmospheric gasses, i.e., CO, SO_2, NO_2, NOx, $PM_{2.5}$, and O_3 led to significant effect on asthma exacerbations among students with asthma [16]. Monitoring of air pollutants over time could be a reliable new means for predicting asthma exacerbations among elementary school children. Such predictions could help parents and school nurses implement effective precautionary measures [15].

Exhaled breath condensate (EBC) 8-isoprostane concentrations are increased in asthma. EBC 8-isoprostane concentrations did not change following any inhalational challenge, as compared to baseline, in either asthmatics or controls. EBC 8-isoprostane concentrations do not acutely change following broncho-provocation in subjects with mild asthma [16]. Aluminum (Al) is a non-essential mineral which human beings are exposed to in day-to-day life. Abnormal aluminum distribution in our body may further precipitate oxidative stress and inflammation, alter Th1/Th2 lymphocyte balance, and therefore contribute to the development of asthma [17].

16.2 Role of Antioxidants in Asthma

Polyphenols are one such class of compounds that are found in foods and plant sources and have been investigated for their anti-allergic effect in different disease models and in human clinical trials. Their anti-inflammatory profile is known to impact on the recruitment of immune cells to the skin and in preventing the development of secondary infections following disruption of the skin barrier.

The interaction of polyphenols with proteins can modulate the process of allergic sensitization and their direct effect on allergic effector cells such as mast cells inhibit mediator release, resulting in the alleviation of symptoms. In addition, their endogenous anti-oxidant ability limits the extent of cellular injury from free radicals during the allergic insult. Overall, polyphenols hold promise as anti-allergy agents capable of influencing multiple biological pathways and immune cell functions in the allergic immune response and deserve further investigations [18]. There are several other antioxidants such as SOD, CAT, GTPx, TRX, Peroxiredoxin (PRX), and glutathione transferase (GST) which play a role in regulating oxidative stress in our lungs.

16.3 Pediatric Infections

Pathogenic organisms can be considered as pro-oxidant agents because they produce cell death and tissue damage. In addition, organisms can be eliminated by a specific cell-defense mechanism, which utilizes in part, reactive oxygen radicals formed by oxidative stress responses. This necessarily is a defense process that however results in cell damage, thereby leading to the development of inflammation, a characteristic oxidative stress situation. This fact shows the duality of oxidative stress in infections and inflammation: oxygen-free radicals protect against microorganism attack, and can produce tissue damage during this protection to trigger inflammation. Iron, a transition metal which participates in generating oxygen-free radicals, also displays this duality in infection. Different pathologies, such as sickle cell anemia/malaria and acquired immunodeficiency syndrome (AIDS), may display this duality in part. In addition, it should be noted that oxidative damage observed in infectious diseases is mostly due to the inflammatory response than to the oxidative potential of the pathogenic agent. The last point is exemplified in cases of respiratory distress and in glomerulonephritis [19].

Various biochemical events taking place during pulmonary inflammation were examined in the BAL fluids from patients with acute respiratory distress syndrome (ARDS) and in experimental animal models. In patients with ARDS, active neutrophil elastase was found in the BAL fluids. In these fluids, there occurred an inactivation of the major elastase inhibitor alpha 1-protease inhibitor (alpha 1-PI). This was caused by the oxidation of a methionine residue at the active site of the alpha 1-PI, and offered indirect evidence of oxidation occurring in the inflamed pulmonary tissues. Studies with experimental animals have been initiated to gain understanding of the relative roles of proteases, oxidants, arachidonate metabolites, complement, contact system components, and other mediators in the pathogenesis of pulmonary inflammation. Intrabronchial instillation of glucose oxidase/glucose to produce oxidants or formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbolmyristate acetate (PMA) as leukocytic stimuli induced severe acute pulmonary injury in New Zealand white rabbits and rhesus monkeys. The injury was accompanied by leukocytic protease

(acid cathepsins) release in the rabbit lungs and oxidant formation, which could be inhibited by neutrophil depletion. Oxidant formation was demonstrated by the inactivation of catalase by 3-amino-1,2,4-triazole in the presence of H_2O_2, a drop in intracellular glutathione levels, and in the rhesus monkey by inactivation of alpha 1-PI [20].

Neutrophil infiltration into the lungs and oxidative injury is associated with bronchopulmonary dysplasia. However, the pathological importance of neutrophil oxidants is still not clear. Nosocomial pneumonia is also implicated, but the evidence is limited, in part because of the difficulty in distinguishing genuine infection from bacterial colonization. Good biomarkers of neutrophil oxidant activity and lung infection are needed. It has been previously tested that glutathione sulfonamide, a product of glutathione oxidation by myeloperoxidase-derived hypochlorous acid (HOCl) and a potential new neutrophil oxidant biomarker, is detectable in endotracheal aspirates from ventilated preterm infants. As infectious organisms stimulate neutrophils to generate HOCl, it was determined that levels of HOCl-specific biomarkers were increased in samples that were bacterial culture-positive. Glutathione sulfonamide was detected in 66 of 87 endotracheal aspirate samples. Levels correlated with myeloperoxidase activity and another HOCl-specific marker, chlorotyrosine. Median levels of glutathione sulfonamide (fourfold) and other biomarkers (twofold) were significantly higher in culture-positive aspirates.

Staphylococcus epidermidis, a frequent colonizer, was associated with glutathione sulfonamide levels, no different from those in negative samples. Glutathione sulfonamide showed good sensitivity and specificity for detecting bacterial growth and had a promise for detecting lung infection [21]. Secreted hypothiocyanous acid (HOSCN) kills pathogens but paradoxically is tolerated by many mammalian cells. Mammalian thioredoxin reductase (H-TrxR) reduces HOSCN while bacterial L-TrxR is inhibited by it corresponding to differential cytotoxicity. Mammalian H-TrxR confers resistance against HOSCN enabling its use as a selective biocide. Findings directly link mammalian H-TrxR to innate immunity and inflammatory lung disease [22].

16.4 Tuberculosis

Tuberculosis (TB) is associated with oxidative stress and the induction of host anti-oxidants to counteract this response. Heme oxygenase-1 (HO-1) is a critical promoter of cytoprotection in diverse disease models including mycobacterial infection. Systemic levels of HO-1 were dramatically increased in individuals with active pulmonary and extra-pulmonary tuberculosis, particularly in children with bilateral lung lesions and elevated bacillary loads in sputum [23]. HO-1 levels effectively discriminated active from latent tuberculosis with higher predictive values than either C-reactive protein or serum amyloid protein. Moreover, there was a marked reduction in HO-1 levels in active TB cases following anti-tuberculous therapy but not in those who failed treatment [23]. These findings establish HO-1 levels

as a potentially useful parameter for distinguishing active from latent or treated pulmonary tuberculosis that is superior in this respect to the measurement of other acute inflammatory proteins [23].

16.5 Cystic Fibrosis

Cystic fibrosis (CF) is a fatal autosomal recessive condition caused by a defect of the trans-membrane conductance regulator gene that has a key role in cell homeostasis. A dysfunctional cystic fibrosis transmembrane conductance regulator impairs the efflux of cell anions such as chloride and bicarbonate, and also that of other solutes such as reduced glutathione. This defect produces an increased viscosity of secretions together with other metabolic defects of epithelia that ultimately promote the obstruction and fibrosis of organs. It is largely accepted that neutrophils migrating inside the bronchial lumina of CF patients release large amounts of ROS, including the superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl-free radical (OH), mainly by the activation of the NADPH oxidase (NOX). To such exaggerated ROS production, both the continuous interaction of neutrophils with bacteria and bacterial degradation products and the inability to engulf bacteria in bio-films contribute, leading to a condition of "frustrated phagocytosis." Neutrophils are therefore recognized as a major source of ROS in the airway surface liquid (ASL) of young children with CF [24, 25].

Oxidative stress and inflammation in cystic fibrosis can affect surfactant biophysical activity, thus leading to early alterations of lung function in patients with CF [26]. Oxidative damage of surfactant may involve both lipid and protein components. Alteration of lipid components can in turn generate toxic lipid species with cytotoxic activity towards nearby epithelial cells [27]. Altered protein components have been shown in cystic fibrosis [28]. Notably, surfactant protein D, which is an important innate host defense molecule, becomes unable to agglutinate bacteria when it is modified by oxidation, which facilitates pathogen colonization in the lung [29].

The fat-soluble vitamin supplementation is of utmost importance in daily practice together with energy intake requirements and pancreatic enzyme replacement therapy [30]. Among these, vitamin E, β-carotene, and ω-3FA have been observed to alleviate selected biochemical signs of oxidative stress as measured, for instance, with well-established laboratory indices of lipid peroxidation, and in some studies these effects were preliminarily associated with positive clinical outcomes. The randomized-controlled clinical trials on antioxidant supplements (including ω-3 FA) so far carried out in CF, have failed to conclusively demonstrate their significant beneficial effects on respiratory symptoms, and on the consequent impact that these have on the quality of life of these patients [30, 31]. There is lack of evidence to support the use of these supplements in CF. Well-timed (early) interventions with appropriate antioxidant formulations/protocols need to be proposed for the next generation of trials, and the development of novel CF-tailored antioxidant and anti-inflammatory agents should be promoted.

Conflict of Interest There has been no conflicts of interest to disclose.

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Chapter 17
Oxidative Stress and Respiratory Diseases: The Critical Role of Nrf2

Thomas E. Sussan and Shyam Biswal

17.1 Nrf2 is a Key Regulator of Oxidative Stress

The rise of oxygen in Earth's atmosphere that began approximately 2.5 billion years ago was vital for eukaryotic and metazoan development. However, this oxidative environment presented challenges to early life forms, necessitating the development of a cellular system to detoxify oxidative stress. The primary function of the *nuclear factor* (*erythroid-derived 2*)*-like 2* (Nrf2) pathway is to regulate baseline antioxidant capacity and to sense changes in the oxidative environment and initiate a cellular response to this environmental stress. Nrf2 is conserved across many diverse species, and an ortholog has even been detected in yeast (YAP1).

The Nrf2 protein is a basic leucine zipper transcription factor that is characterized by its conserved structural domain referred to as the cap'n'collar (CNC) domain, which was first discovered as a regulator of mandibular development in *Drosophila* [1]. In mammals, the family of CNC-containing transcription factors consists of NF-E2 [2], Nrf1 [3], Nrf2 [4, 5], Nrf3 [6], and the more distantly related Bach1 [7] and Bach2 [8]. These CNC transcription factors function as heterodimers, binding to accessory proteins such as Mafs, to activate gene expression [9]. Early studies revealed that Nrf2 bound the antioxidant response element (ARE), which is a cis-element in the promoters of many anti-oxidative genes that is critical to their inducible activation. Over-expression of Nrf2 in in vitro models increases ARE-dependent transcriptional induction [10], while Nrf2-deficient mice do not exhibit inducible expression of ARE-containing genes [9]. Recent chromatin immune-precipitation-sequencing analysis identified more than 650 direct inducible targets of Nrf2 [11]. Among the Nrf2-dependent genes are the phase II detoxification genes and many other genes that regulate redox states.

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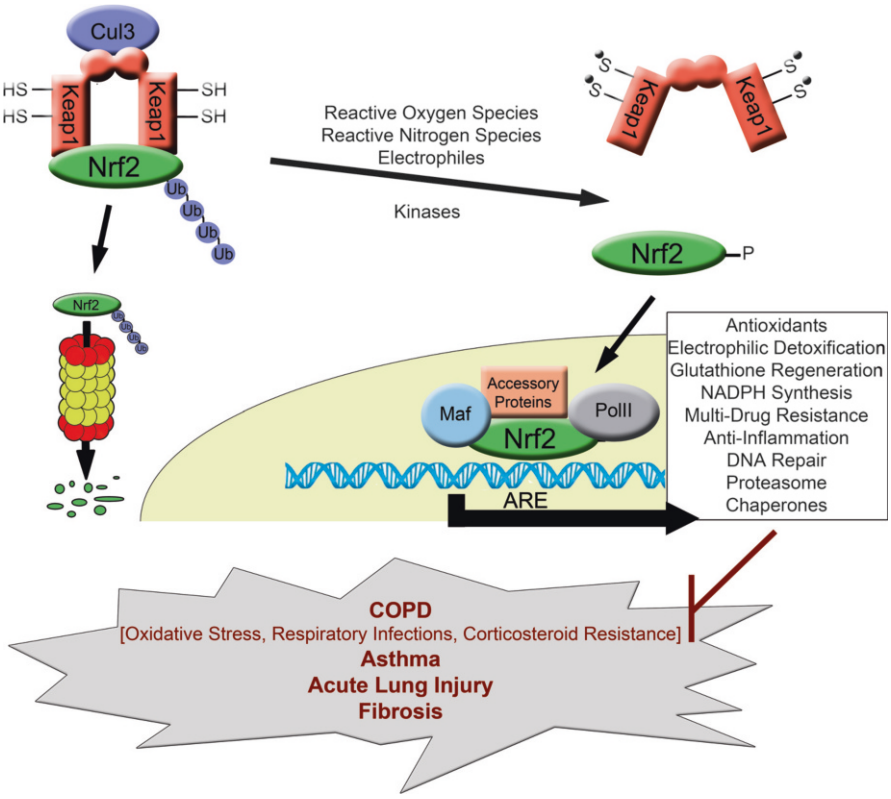

Fig. 17.1 Under basal conditions, Nrf2 is bound to Keap1 in the cytoplasm, resulting in Cul3-mediated ubiquitination and proteasomal degradation of Nrf2. Oxidative/nitrosative stress causes Nrf2 to release from Keap1, where it is free to translocate to the nucleus, associate with accessory proteins, and coordinate the transcriptional activation of numerous antioxidative pathways. The Nrf2 pathway attenuates oxidative stress and inflammation, resulting in reduced susceptibility to a variety of pulmonary diseases

Under non-stressed conditions, Nrf2 persists at low levels in the cytoplasm where it is bound to its inhibitor Keap1. Keap1 is a cysteine-rich protein that binds both the actin cytoskeleton [12] and the Cul3-based E3 ubiquitin ligase [13, 14]. Keap1 serves to anchor Nrf2 in the cytoplasm and also to signal its ubiquitination and subsequent proteosomal degradation, resulting in low baseline expression of the Nrf2-dependent cytoprotective genes. However, the disulfide bonds in Keap1 are highly sensitive to oxidative stress, and exposure to a wide variety of electrophiles/oxidants triggers a conformational change in Keap1, caused by modification of thiol residues, releasing Nrf2 [15–18]. Other post-translational modifications also facilitate this dissociation, including phosphorylation of Nrf2 and S-nitrosylation of Keap1 [19–21]. Upon dissociation from Keap1, Nrf2 translocates to the nucleus, heterodimerizes with Maf proteins, binds the ARE, and activates the coordinate expression of hundreds of genes. The net result is an adaptive cytoprotective response that detoxifies oxidative and environmental stressors (Fig. 17.1).

17.2 Nrf2-Regulated Genes

Nrf2 has been shown to directly or indirectly alter the expression of approximately 15,000 genes [11], and the number of directly inducible genes has been estimated at 654. Of these 654 genes, 224 are regulated under both basal and inducible conditions [11]. Nrf2 is a pleiotropic regulator of numerous pathways, although the inducible targets of Nrf2 are primarily categorized as antioxidative genes. The Nrf2-dependent antioxidative response utilizes multiple pathways, such as (a) providing direct antioxidants [22, 23], (b) encoding enzymes that directly inactivate oxidants [24], (c) increasing levels of glutathione and thioredoxin synthesis and regeneration [25, 26], (d) stimulating NADPH synthesis [27, 28], (e) enhancing toxin export via the multidrug response transporters [28], (f) inhibiting cytokine-mediated inflammation [29], (g) enhancing recognition, repair, and removal of damaged proteins [30], and (h) increasing chaperones and regulating post-translational modifications [11]. Additionally, Nrf2 may regulate the expression of multiple microRNAs [31], although the functional significance of this is currently unknown. Thus, Nrf2 is a prolific and ubiquitous regulator of multiple pathways that counteract oxidative stress.

17.3 Role of Nrf2 in Disease

Unlike some CNC family members, Nrf2 has not been shown to regulate developmental processes. Most studies reveal only minor phenotypes in $Nrf2^{-/-}$ mice under non-stressed conditions. Nrf2 activity declines with aging, resulting in enhanced age-related oxidative stress [32]. However, one analysis of Nrf2-deficient mice revealed that they exhibited shortened life-spans, glomerulonephritis, multi-organ inflammatory lesions, edema, and neurological symptoms [33]. $Nrf2^{-/-}$ mice exhibit exacerbated phenotypes in a wide variety of disease models and in response to a large number of toxicant exposures, and these exacerbated phenotypes are often typified by elevated oxidative stress, inflammation, and fibrosis.

17.4 Role of Nrf2 in Pulmonary Diseases

Lungs are constantly exposed to numerous oxidants, from both endogenous sources and environmental exposures. For example, a single puff of cigarette smoke contains an estimated 10^{15}–10^{17} oxidant molecules [34]. Other sources of oxidants, including outdoor and indoor air pollution and ionizing radiation, place a tremendous oxidative burden on the lungs that results in oxidized macromolecules (nucleotides, lipids, proteins) that have altered or impaired functions, leading to cell damage or death. Thus, the Nrf2-dependent antioxidant defense pathways are vital for removing this stress and modifying disease susceptibility.

17.5 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD), which is the third-leading cause of death in the world, is characterized as a progressive decline in lung function that is driven by aberrant non-resolving inflammation, oxidative stress, and protease/antiprotease imbalance. COPD consists of both emphysema and chronic bronchitis, and is predominantly associated with exposure to environmental stressors, such as cigarette smoke, outdoor air pollution, household air pollution, and occupational exposures. Experimental models of emphysema have revealed that $Nrf2^{-/-}$ mice develop enhanced airspace enlargement, alveolar destruction, apoptosis, inflammation, oxidative stress, and protease/anti-protease imbalance after either chronic cigarette smoke exposure [35–37] or elastase treatment [38]. Not only has Nrf2-deficiency been shown to alter susceptibility to emphysema in mice, but some individual Nrf2-dependent genes have also been shown to play a role in the pathogenesis of emphysema. For example, mice deficient in the Nrf2-dependent genes NAD(P)H: quinone oxidoreductase 1 (Nqo1) [39] and thioredoxin-1 [40, 41] show enhanced susceptibility to emphysema. The role of Nrf2 in patients with COPD was confirmed, as lung biopsies and alveolar macrophages from COPD patients exhibited declining Nrf2 activity that correlated with disease severity [42–44].

Emphysema, which is specifically characterized by remodeling and destruction of the alveoli and small airways, can be considered a disease in which the homeostasis between cell death and proliferation becomes unbalanced. Oxidative stress (and in turn Nrf2) have been shown to play a role in the regulation of both cell death and proliferation. In cigarette smoke-exposed mice, Nrf2-deficiency results in decreased proteosomal activity and an increase in misfolded proteins, leading to endoplasmic reticulum stress and apoptosis [45]. Concomitantly, oxidative stress can trigger cell-cycle arrest, and indeed lung cells from $Nrf2^{-/-}$ mice exposed to cigarette smoke exhibit reduced mitochondrial responses compared to cigarette smoke-exposed wild-type mice [46, 47].

Studies showing that Nrf2-deficient mice have enhanced susceptibility to emphysema are complemented by studies showing that genetic or pharmacologic activation of Nrf2 can attenuate pathological damage caused by cigarette smoke. Mice containing a tissue-specific deletion of Keap1 in Clara cells exhibit constitutive Nrf2 activity in the airway epithelium, and these mice show decreased oxidative stress and inflammation after exposure to cigarette smoke [48]. Additionally, pharmacologic activation of Nrf2 via a synthetic triterpenoid (CDDO-Im) significantly attenuates oxidative stress, airspace enlargement, and alveolar destruction in wild-type, but not $Nrf2^{-/-}$ mice [49]. Clinical studies using Nrf2-activating drugs are currently being pursued in Phase II trials, and hold substantial promise as therapeutics for delaying or preventing disease progression.

In parallel with these studies on the role of Nrf2 in COPD, numerous studies have been conducted to assess the ability of antioxidants to attenuate COPD. Studies, on the role of the antioxidant *N*-acetylcysteine, which is a precursor to

glutathione, in cigarette smoke-induced emphysema in mice, are conflicting [50, 51]. Likewise, the role of *N*-acetylcysteine as a therapy for COPD is also controversial, although several studies indicate that *N*-acetylcysteine decreases exacerbation rates, reduces hospital admissions, and improves lung function [52–56]. Nrf2 activates numerous antioxidative pathways, including pathways that enhance glutathione synthesis and regeneration, and thus, Nrf2 activation may provide greater benefits than enhancing individual antioxidant pathways.

17.6 COPD Exacerbations

COPD is complicated by frequent and recurrent acute exacerbations, which are described as sudden episodes of worsening respiratory symptoms. These symptoms include dyspnea, cough, and sputum production that are often followed by subsequent clinical deterioration [57, 58]. The frequency of these exacerbations correlates strongly with decline in lung function [59–61], and approximately 10 % of COPD patients with frequent and severe exacerbations account for 70 % of the total COPD-related health care cost [62]. Thus, therapeutic targets that mitigate COPD exacerbations could have substantial benefit to patient outcomes. These COPD exacerbations are primarily attributed to infectious agents, such as bacteria and viruses. During exacerbations, patients with COPD exhibit significant increases in airway inflammatory cells (neutrophils, macrophages, and eosinophils), cytokines (TNF-α, IL-8), proteases (neutrophil elastase), and oxidative stress (H_2O_2 and 8-isoprostane), compared to patients with stable COPD [63–65].

Multiple studies indicate that Nrf2 reduces susceptibility to bacterial and viral infections [66, 67], and Nrf2 also attenuates inflammation and oxidative stress in cigarette smoke-induced bacterial [68] and viral [69] exacerbations in mice. Host phagocytic cells release oxidants as part of their anti-microbial defense, but failure to detoxify this oxidative stress can result in damage to the lung parenchymal cells. Despite increased oxidative stress and inflammatory cells in the lungs of COPD patients, bacteria and viruses have higher rates of proliferation and colonization than in lungs of non-COPD patients, which can be attributed to a failure of the host innate immune defense. Alveolar macrophages from COPD patients have defective bacterial phagocytosis, compared to either blood macrophages from the same COPD patients or alveolar macrophages from non-COPD patients [70]. A recent study using both cigarette smoke-exposed mice and alveolar macrophages from COPD patients demonstrated that activation of Nrf2 by sulforaphane restored defective bacterial phagocytic function by inducing the expression of macrophage scavenger receptors [68]. Thus, Nrf2 reduces exacerbation severity both through direct detoxification of oxidative stress and enhancement of innate immune defense, which suggests that Nrf2 may be an important therapeutic target for improving COPD-related morbidity and mortality.

17.7 Corticosteroid Resistance in COPD

Current therapies for COPD patients consist of anti-inflammatory corticosteroids and bronchodilators. However, even high-dose corticosteroid therapy produces only mild symptomatic relief, and may increase the risk of developing pneumonia. Steroids are effective anti-inflammatory therapies for many diseases, including asthma, but COPD patients are more refractory to corticosteroid therapy, due to a decline in the activity of histone deacetylase 2 (HDAC2) [71]. Nrf2-deificent mice contain reduced HDAC2 levels in the lungs and increased steroid-resistant inflammation after exposure to cigarette smoke [72]. Declining HDAC2 activity also results in deacetylation and destabilization of Nrf2 [73]. Activation of Nrf2 by sulforaphane improves corticosteroid responsiveness in alveolar macrophages from COPD patients, via regulation of S-nitrosylation of cysteine residues on HDAC2 [74]. This indicates that activation of Nrf2 may attenuate nitrosative modifications of HDAC2 to restore HDAC2 activity, and a combination of a corticosteroid and Nrf2 activator may be a valuable therapy for resolving inflammation in COPD patients.

17.8 Asthma

Allergic asthma is characterized by reversible bronchial constriction, eosinophilic inflammation, Th2 cytokine secretion, and mucus hypersecretion in response to a normally harmless inhaled allergen. Oxidative stress plays an important role in the regulation of the immune response; depletion of the antioxidant glutathione within antigen-presenting cells skews the immune response toward a Th2-mediated response [75]. Using a model in which mice are sensitized and subsequently challenged with ovalbumin, Nrf2-deficient mice exhibit heightened airway resistance, Th2-mediated inflammation, mucus hyper-secretion, and reduced antioxidant status compared to wild-type controls [76]. Additionally, airway macrophages from asthmatic patients exposed in vivo to allergens show decreased Nrf2 activity, which can be restored by consumption of vitamin E [77]. Dendritic cells, which are responsible for the priming of CD4+ T cells during sensitization, play an important role in eliciting T-cell responses after allergen challenge. In response to an allergen, dendritic cells from Nrf2-deficient mice contain elevated oxidative stress, increased surface expression of activation markers, cytokine secretion, and enhanced ability to prime T cells, compared to wild-type dendritic cells [78, 79]. The role of Nrf2 in dendritic cells of asthmatic mice or humans has not been directly assessed, but the potential role of Nrf2 in allergen sensitization provides interesting possibilities for the management of asthma.

A few studies have addressed the potential for Nrf2 activators to be used as therapeutics in asthmatics. Small molecules that activate Nrf2, such as sulforaphane [80] and artesunate [81] have been shown to attenuate features of allergic asthma in mice when given prior to challenges, although neither study demonstrated conclusively

that these drugs were working through an Nrf2-dependent mechanism. Further studies are needed to determine whether Nrf2 activators could be used in a therapeutic model to reduce asthmatic symptoms. In airway smooth muscle cells (ASMCs) from asthmatic and non-asthmatic patients, activation of Nrf2 via sulforaphane activated antioxidant responses and reduced proliferation in a TGF-β dependent manner [82]. On the other hand, ASMCs from severe asthmatics exhibit reduced Nrf2 activity compared to ASMCs from patients with no or non-severe asthma [82].

Collectively, the current evidence suggests that Nrf2 modifies sensitivity to asthma in both animal models and samples from asthmatic patients. Acute asthmatic responses are typically well controlled by corticosteroids, so it is not clear that Nrf2 activators would be used as a rescue therapy. However, the notion that Nrf2 may potentially decrease sensitization and development of asthma is intriguing. Additionally, 5–10 % of asthmatics are not well controlled by corticosteroids. These patients typically demonstrate an elevated Th17-mediated inflammation. Thus, there would be tremendous value in novel therapeutics that reduce this Th17 response. To date, Nrf2 activators have not been shown to regulate Th17 responses in the lungs; however, the Nrf2 activator CDDO-trifluoroethyl amide significantly suppressed multiple sclerosis in a mouse model that is driven by Th17-induced inflammation [83].

17.9 Acute Lung Injury

Acute lung injury (ALI) is caused by inhalation exposure to a variety of different agents, including infectious pathogens and toxic chemicals, and can develop as a consequence of respiratory therapy (hyperoxia and mechanical ventilation). ALI, and its more severe form of acute respiratory distress syndrome (ARDS), leads to diffused pulmonary inflammation, edema, and respiratory failure. Patients with ALI and ARDS exhibit high rates of morbidity and mortality, accounting for approximately 150,000 annual deaths in the US [84, 85]. Oxidative stress is an important contributor to ALI, regardless of the root cause. Numerous in vivo and in vitro studies indicate that Nrf2 attenuates toxicity after exposure to a wide variety of toxicants, and Nrf2 also mitigates susceptibility to a wide range of pulmonary viral and bacterial pathogens.

Mechanical ventilation is vital for critically ill patients and premature newborns; however, mechanical ventilation initiates ALI due to hyperoxia and cyclic stretch, which generate oxidative stress and inflammation. Hyperoxia frequently results in respiratory symptoms and permanent functional abnormalities, including bronchopulmonary dysplasia in infants. In response to hyperoxia exposure in either neonatal or adult mice, Nrf2-deficiency enhances mortality, edema, inflammation, and cell death, compared to wild-type controls [86–89]. Meanwhile, activation of Nrf2 via CDDO-imidazole attenuates hyperoxia-induced inflammation, edema, and apoptosis in adult mice [90].

In addition to the direct effects of hyperoxia on epithelial and endothelial cell injury, hyperoxia-induced ALI also leads to enhanced susceptibility to bacterial or viral

infections during the recovery phase. Using an animal model of hyperoxia exposure followed by infection with *Pseudomonas aeruginosa*, Nrf2-deficient mice exhibit enhanced mortality, bacterial pulmonary burden, edema, and inflammation, compared to wild-type controls [91]. Even in the absence of hyperoxia, cyclic stretch caused by mechanical ventilation can induce oxidative stress and inflammation. Nrf2-deficient mice demonstrate elevated oxidative stress and inflammation in ventilator-induced lung injury, which is suppressed by antioxidant supplementation [92]. Thus mechanical ventilation causes injury via both exposure to high concentrations of oxygen as well as cyclic stretch, and Nrf2 mediates the harmful effects of both. There is also an association between traumatic brain injury (TBI) and subsequent ALI. In a mouse model of TBI-induced ALI, Nrf2-deficient mice contain elevated pulmonary capillary permeability, edema, apoptosis, and inflammation, compared to wild-type animals [93]. Thus, Nrf2 regulates susceptibility to ALI in response to multiple stressors.

17.10 Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a progressive fatal disease of unknown origin. While the pathogenesis of IPF is unknown, oxidative stress may play an important role, as evidenced by the elevation of reactive oxygen species in airway cells of IPF patients and decreased glutathione in airways and sputum. A role for Nrf2 has been demonstrated in a mouse model of bleomycin-induced pulmonary fibrosis. Intratracheal delivery of bleomycin to Nrf2-deficient mice results in elevated fibrosis, inflammation, weight loss, and mortality, compared to wild-type controls [94, 95]. Nrf2-deficient mice also exhibit elevated Th2 cytokines (IL-4 and IL-13) and an increased number of Th2 cells [95], supporting the notion that the lung Th1/Th2 balance is an important underlying mediator of disease susceptibility. In further support of this notion, Nrf2 selectively regulates Eotaxin-1, a key chemokine for eosinophil recruitment, in normal human lung fibroblasts [96], suggesting that Nrf2 reduces allergic inflammation via signaling in fibroblasts. Fibroblasts isolated from IPF patients exhibit reduced Nrf2 expression, compared to control fibroblasts [97]. Activation of Nrf2 in IPF fibroblasts via either sulforaphane treatment of siRNA-mediated inhibition of Keap1 resulted in reduced oxidative stress and promoted myofibroblastic dedifferentiation. Thus, activation of Nrf2 may suppress pathogenesis of this fatal disease.

17.11 Polymorphisms

Genetic variation accounts for the observable differences among individuals, and these variants are also determinants of disease susceptibility. The most common genetic variants are single nucleotide polymorphisms (SNPs), which are substitutions of individual base-pairs. The human genome contains approximately ten million SNPs, although the majority of these SNPs have no functional significance. In a recent study of COPD patients, four functional polymorphisms were identified in the promoter of Nrf2, and those haplotypes with a high expression of Nrf2 were

associated with decreased severe COPD, compared to those patients with low Nrf2-expressing haplotypes [98]. A second analysis of SNPs in COPD patients demonstrated that SNPs in Nrf2 and Keap1 were associated lower and higher lung function, respectively [99]. However, no polymorphisms in Nrf2, Nrf2-regulating genes, or Nrf2-dependent genes have been identified that significantly correlate with the rate of lung function decline [100]. It is not clear whether these polymorphisms result in altered levels of antioxidants in these patients. A recent study demonstrated that polymorphisms in Nrf2, SOD2, and GSTP1 marginally influence COPD- and asthma-related hospital admission rates after exposure to elevated air pollution [101]. Air pollution is often acutely linked to hospital admission rates, and this link between Nrf2 and air pollution-induced hospital admissions suggest that oxidative stress underlies this link. Additionally, a functional polymorphism in Nrf2 is strongly associated with increased risk of ALI after trauma [102], further demonstrating a link between Nrf2 expression and pulmonary disease.

17.12 Conclusions

The role of oxidative stress in numerous pulmonary disorders is clear, and susceptibility to these diseases is strongly influenced by the ability of the host to detoxify oxidative stress. Nrf2 is a ubiquitous and pleiotropic transcription factor that regulates hundreds of genes that provide anti-oxidative and cyto-protective functions in the cell. As such, Nrf2 serves protective roles in a variety of pulmonary and extrapulmonary diseases through its induction of hundreds of ARE-containing genes, resulting in a coordinated response to environmental stressors. Experiments using animal models and human in vitro or ex vivo cells demonstrate that Nrf2 can protect from COPD, asthma, ALI, and IPF. These studies are supported by analysis of polymorphisms in patient samples, demonstrating that alterations to the Nrf2 pathway correlate with pathogenic responses. Nrf2 activators present viable therapeutic options for these and other diseases, and these are currently being explored in clinical trials. The Federal Drug Administration (FDA) recently approved the first Nrf2-activating drug, Tecfidera, as a therapy for multiple sclerosis, and it is likely that similar drugs will be developed for other diseases.

Conflict of Interest Shyam Biswal and the Johns Hopkins University hold intellectual property on the development of Nrf2-based therapeutics for COPD. Cureveda LLC has licensed this intellectual property. Thomas Sussan has no conflict.

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Chapter 18
Development of Novel Antioxidants

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18.1 Introduction

Oxidative stress is a pernicious component of all inflammatory pathways, which are triggered by environmental insults and infections. Oxidative stress has demonstrated associations with almost all pathophysiologies of diseases, which are chronic and acute inflammatory in nature. Unfortunately, research in the development of new antioxidants has been rather low paced, primarily because the pathophysiological implication of oxidative stress in a disease has always been subjected to the paucity of knowledge. This can well be cited as in spite of clear realization of oxidative stress as key component of inflammatory disease *N*-acetyl cysteine (NAC) is the only antioxidant molecule available to the treating physicians globally. Further, the antioxidant pathways are also very complex, which make it difficult to identify single pathway pharmacology.

However, now, resources are being focused in developing molecules, which have potential to reduce oxidative stress. There are some possibilities that these molecules may have potential to incarcerate inflammation of asthma, acute respiratory distress syndrome (ARDS), COPD, interstitial lung disease (ILD), tuberculosis, and pneumonia, which may change the course of the disease. Further, new mechanisms of oxidative stress pathways and their counteracting mechanisms are being deciphered, to an extent that genetic manipulation through transcription factors modulation is being considered as future of antioxidant therapies [1]. However, the most ubiquitously prevalent antioxidant pathways such as glutathione–thioredoxin pathways and superoxide dismutase (SOD)–catalase (CAT) synergistic mechanisms have been studied extensively. Therefore, current development of most antioxidants is in context to modulations of these pathways [2–4]. Also, genetic research in lung inflammation

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and oxidative stress has been primarily based on transcription factors such as nuclear factor (NF)-κB. Therefore, molecules, which modulate transcription factor NF-κB, are of the prime interest in development as potential antioxidant [1]. This chapter will primary deal with the newer antioxidant molecules, which are in the developmental phase for treating chronic inflammatory respiratory diseases (Table 18.1).

18.2 Glutathione Pathway-Based Antioxidants

Glutathione is one of the most ubiquitously prevalent antioxidants in the cellular systems, which formulates one of the prime protective mechanisms against the oxidative stress in various disease processes. In lungs, glutathione may also assist in maintaining the integrity of airway and alveolar epithelial cells, which are prone to damage due to adverse environmental conditions [2]. In chronic inflammatory lung diseases there is a persistent depletion of glutathione in the respiratory system and systemic circulation. Therefore, glutathione is one of the primary targets in the development of novel antioxidants.

Glutathione is synthesized in the body from the amino acids L-cysteine, L-GLUTAMIC acid, and glycine. Cysteine is the rate-limiting factor in cellular glutathione synthesis. Further, in the cells glutathione is synthesized in two-step mechanisms chronologically involving enzymes glutamate cysteine ligase (GCL) and glutathione synthetase. There is a possibility of modulating glutathione metabolism at every step, which can be potential targets for antioxidant therapies. One of the most intriguing examples of this is NAC, which provides cysteine residues in acetylated form for glutathione synthesis. NAC itself works as an antioxidant, provides direct source of cysteine, and also converts intracellular cystine to cysteine. NAC has been widely used in the management of ILDs and COPD. Unfortunately, chronic utility of NAC has been questioned in COPD management primarily due to its poor bioavailability in oral form [5]. Also, its acidic nature prevents its use in inhaled form [6]. However, the idea of providing cysteine for glutathione formation is so compelling that research has been directed towards formulation of new compounds, which may enhance the cysteine delivery to the cells.

18.2.1 Nacystelyn

Nacystelyn (NAL) is lysine salt of NAC which is neutral in nature. Therefore, it can be safely delivered locally to the lungs through inhaled route [1, 7]. In vitro studies have shown that NAL has potential to enhance intracellular GSH levels twice as effectively as NAC [8] and also modulate oxidant-mediated inflammation mechanisms [7, 9, 10]. A study has also shown that NAL, at concentrations obtainable in vivo by inhalation, can reduce neutrophil response, and production of cytotoxic hydroxyl and hypohalite radical; 50 % inhibition in production of these radicals was

Table 18.1 List of antioxidants under development for chronic lung diseases

Type of antioxidant	Stage of studies in lung diseases and other organ system diseases
Glutathione pathway	
a. Nacystelyn (NAL)	a. Animal and in vitro
b. Procysteine	b. Human in ARDS
c. *N*-isobutyrylcysteine (NIC)	c. Human in COPD exacerbation (similar to placebo)
d. Erdosteine	d. Human in COPD
e. *N*-acetyl cysteine proline cysteine amide (CB3)	e. Animal and in vitro studies
f. Glutamate cysteine ligase (GCL)	f. Animal and in vitro studies
Peroxidase mimetics	
a. Ebselen	a. Human studies in cardiovascular and nervous system diseases
b. BXT-51072	b. Phase 1 and preclinical trials in COPD patients
c. Diselinide and delluride compounds	c. Animal and in vitro studies
d. Cyclodextrin compounds of diselinide and delluride	d. Experimental stage only
Thioredoxin pathway	
a. Recombinant thioredoxin	a. Animal and in vitro studies
b. p38 mitogen-activated protein kinase	b. Animal and in vitro studies
c. Nuclear factor-κB	c. Animal and in vitro studies
d. Phosphatidylinositol 3-kinase (PI3-K)	d. Animal and in vitro studies
Superoxide dismutase mimetic	
a. MnTE-2-PyP^{5+}	a. Animal and in vitro studies
b. Pentaazamacrocyclic ligand-based mimetic	b. Animal and in vitro studies
Superoxide dismutase mimetic + catalase	
a. AEOL-10150	a. Animal and in vitro studies
b. Mn-TBAP	b. Animal and in vitro studies
c. AEOL-11027	c. Animal and in vitro studies
d. AEOL-10113	d. Animal and in vitro studies
e. Salens	e. Animal and in vitro studies
Anti-NF-κB-based antioxidants	
a. Pyrrolidine dithiocarbamate	a. Animal and in vitro studies for respiratory; current human applications in HIV and heavy metal poisoning
b. BAY 11-7085	b. Animal and in vitro studies
Spin traps	
a. STANZ	a. Animal and in vitro studies
b. NXY-059	b. Animal and in vitro studies
Neu-164 and Neu-107	Animal and in vitro studies
Tanshinone IIA and cryptotanshinone	Animal and in vitro studies
Nano-particles	Animal and in vitro studies

achieved at concentration five times lesser than that of NAC and three times lesser than that of captopril [11]. Further, a drug deposition study with inhaled NAL has shown that its deposition in the peripheral airways is unaffected by the presence of mucus in cystic fibrosis patients [12]. A study on cystic fibrosis patients illustrated that inhaled form of NAL is well tolerated in wide range of doses (4–16 mg) [13]. This indicates that NAL could graduate to become potential futuristic antioxidant therapy in the management of obstructive airway diseases involving both large and small airways and ILDs, which are primarily neutrophilic-mediated lung ailments.

18.2.2 Procysteine (L-2-Oxothiazolidone-4-carboxylate/OTC)

This is another cysteine donating compound with a better bioavailability than NAC [1]. Procysteine can effectively replete glutathione in oxidative stress conditions. Animal model studies have shown that procysteine has potential to improve survivals in pneumonia conditions [14]. Procysteine also improves efferocytosis and glutathione availability in the airway macrophages in smoking murine models [15]. A defective macrophage efferocytosis is one of the key components of COPD pathophysiology. Animal model studies of allergic asthma have also shown that procysteine has an ability to reduce expression of IL-4, IL-5, IL-13, and IL-18 [15], which are key inflammatory mediators that orchestrate allergic inflammation and airway-remodelling and induce broncho-hyper-responsiveness (Figs. 18.1 and 18.2). This antioxidant can also modulate expression of NF-κB, which is a key transcription factor for the expression of inflammatory genes. Therefore, it is likely that treatment with procysteine could have significant therapeutic benefits in chronic and acute inflammatory diseases of lungs. Bernard and colleagues [16] had shown that administration of 63 mg/kg procysteine can significantly reduce the duration of acute lung injury and significantly improves cardiac index in 17 patients with ARDS. However, its therapeutic potential in the management of chronic inflammatory disease of the lungs is lacking.

18.2.3 N-Acetyl Cysteine Proline Cysteine Amide (CB3)

There is a compelling evidence to show that converting the carboxyl group of NAC to an amide increases hydrophobicity of the compound, which enhances its membrane permeability and hence ultimately can remarkably restore intracellular glutathione [17–21]. Evidences from animal studies have shown that amide-NAC can attenuate airway inflammation and hyper-responsiveness by regulating activation of NF-κB and hypoxia-inducible factors (HIFs)-1α, and reduces oxidative stress in allergic airway disease [22]. NAC proline cysteine amide (CB3) is a novel amide-NAC, which is relatively more potent than other NAC-amide derivates in reducing inflammation in allergic diseases [21]. Animal model studies have shown that CB3 is more potent than NAC in reducing reactive oxygen species (ROS) [23]. It has been demonstrated to

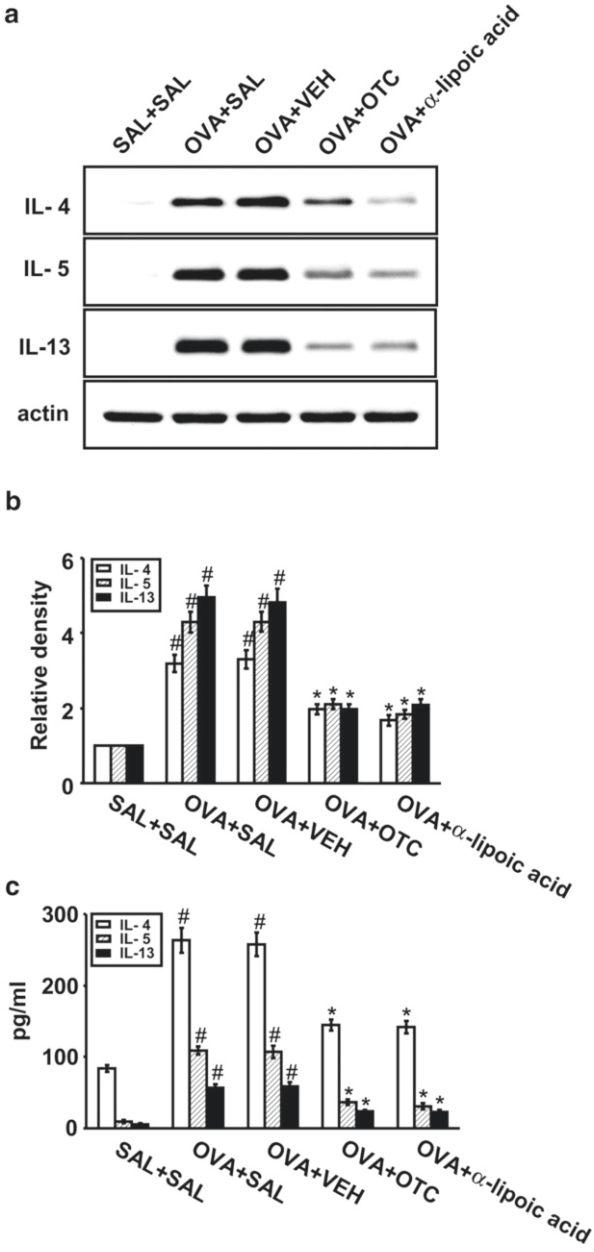

Fig. 18.1 Effect of OTC or α-lipoic acid on IL-4, IL-5, and IL-13 protein levels in lung tissues and in BAL fluids of ovalbumin-sensitized and -challenged mice. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), ovalbumin-inhaled mice administered saline (OVA + SAL), ovalbumin-inhaled mice administered drug vehicle (OVA + VEH), ovalbumin-inhaled mice administered OTC (OVA + OTC), and ovalbumin-inhaled mice administered α-lipoic acid (OVA + α-lipoic acid). (**a**) Western blotting of IL-4, IL-5, and IL-13 in lung tissues. (**b**) Densitometric analyses are presented as the relative ratio of each molecule to actin. The relative ratio of each molecule in the lung tissues of SAL + SAL is arbitrarily presented as 1. (**c**) Enzyme immunoassay of IL-4, IL-5, and IL-13 in BAL fluids. *Bars* represent mean ± SEM from eight mice per group. #, $p < 0.05$ versus SAL + SAL; *, $p < 0.05$ versus OVA + SAL (Reproduced with permission from [15])

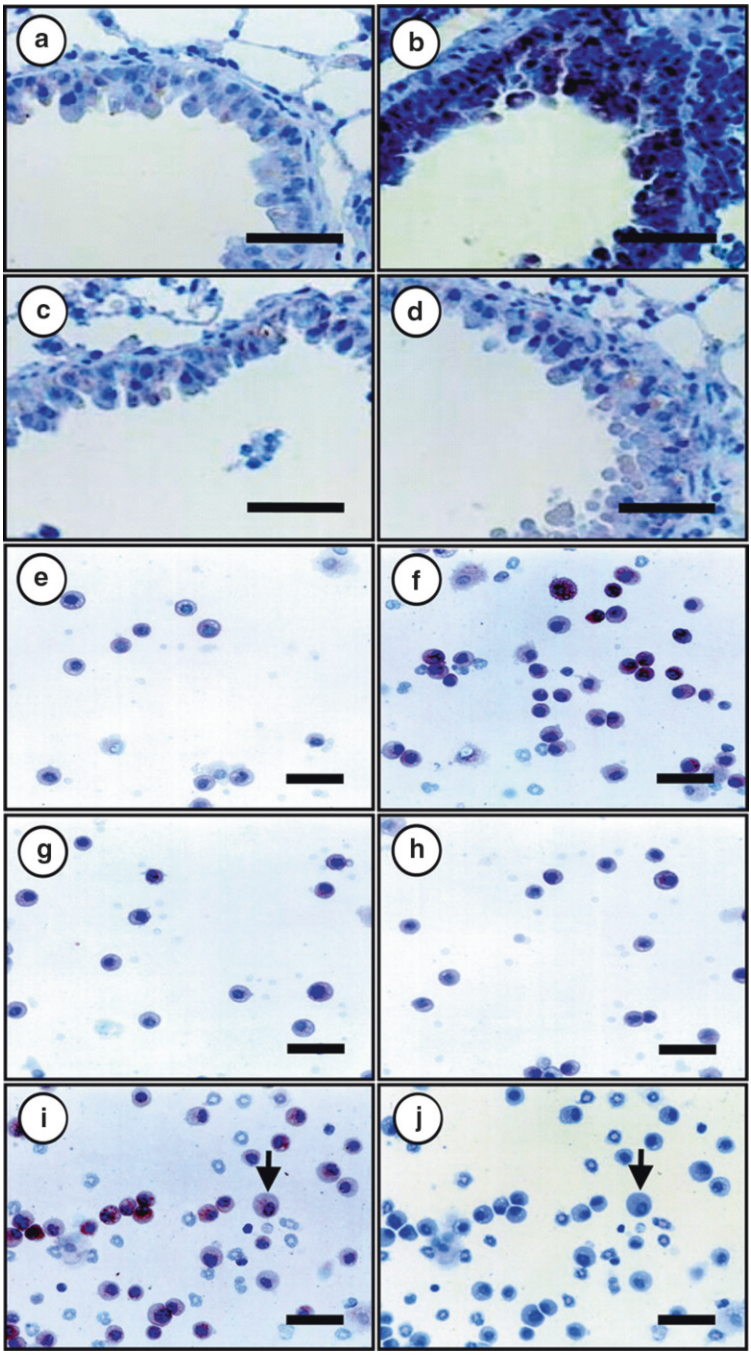

Fig. 18.2 Localization of immunoreactive IL-18 in lung tissues and in BAL fluids of ovalbumin-sensitized and -challenged mice. Sampling was performed 72 h after the last challenge in lung tissues from sensitized mice challenged with saline (**a**), from sensitized mice challenged with ovalbumin (**b**), from ovalbumin-inhaled mice administered OTC (**c**), and from ovalbumin-inhaled mice administered α-lipoic acid (**d**). Sampling was also performed in BAL fluids from sensitized mice challenged with

have reducing effects on airway inflammation and decrease airway hyper-responsiveness. CB3 has been shown to prevent translocation of NF-κB into the nucleus and prevent the expression of IL-4, IL-5, and IL-13, which are typical to allergic diseases [23]. However, here it is also important to mention that NF-κB is a universal inflammatory mediator of transcription factors, which may promote CB3 to have wider applicability in other inflammatory diseases as well. Many inflammatory diseases such as asthma and COPD have been associated with suppression and dysfunction of regulatory immune system. It has been shown that CB3 has an ability to increase IL-10 which is the key immune regulatory cytokine [23–25]. Hence, it can be assumed that CB3 could emerge as potent antioxidant in inflammatory diseases of the lungs; however, currently there is no published human data with this molecule.

18.2.4 Glutamate Cysteine Ligase (GCL)

This is a cardinal enzyme implicated in rate-limiting step of glutathione synthesis. This enzyme consists of a catalytic subunit (GCLC) and a modifier subunit (GCLM). Studies have shown that GCLM has antioxidant potential and its depletion can generate oxidative stress [26, 27]. Further studies have shown that smokers have decreased expression of GCL-light chain unit [24]. A substitution in the promoter region of GCLM is known to reduce glutathione levels, and has been associated with a threefold increased risk of COPD in populations [28]. Similarly, substitution in GCL's catalytic subunit (GCLC) has also shown to significantly enhance the risk of COPD [OR 1.83, 95 % CI 1.00–3.36] [29]. Both GCLC and GCLM expressions have been shown to increase in the inflammatory cells of COPD patients compared to smokers without COPD [27, 30–32]. Therefore, molecules, which have potential to manipulate the expression of GCL, are being developed as probable innovative antioxidants in future for various inflammatory diseases of the lungs.

18.3 Peroxidase Mimetic

Glutathione peroxidase and thioredoxin peroxidase are one of the most abundant peroxidase systems found in the cytosol of almost all mammalian cells [33]. Their primary function is to scavenge peroxides such as H_2O_2 and lipid peroxides, and

Fig. 18.2 (continued) saline (**e**), sensitized mice challenged with ovalbumin (**f**, **i**, and **j**), ovalbumin-inhaled mice administered OTC (**g**), and from ovalbumin-inhaled mice administered α-lipoic acid (**h**). **a**–**i**, representative light microscopy showing IL-18-positive cells in the BAL fluids; the *brown* color indicates IL-18-positive cells. (**j**) To examine the cell differentials in BAL cells prepared from the control mice, the slides used for the detection of IL-18 (**i**) were destained with 70 % ethyl alcohol. The smears of BAL cells were stained with Diff-Quik solution and were viewed under a light microscope. The *arrow* indicates a macrophage. Bars, 50 μm (Reproduced with permission from [15])

neutralize peroxides to less toxic compounds and water. Over-expression of these peroxidases has been shown to be protective against oxidative stress in cultured cells and animal models [3]. Hence, it is likely that molecules, which will mimic these peroxidases, could emerge as potential antioxidant in treatment of various chronic inflammatory diseases. Most of these compounds contain selenium or tellurium. They can scavenge O_2^-, H_2O_2, $ONOO^-$, and a variety of lipid peroxides.

18.3.1 Ebselen

One of the most promising and primary peroxidase mimetics which is extensively being researched is ebselen [2-phenyl-1,2-benzisoselenazol-3(2*H*)] [3]. Ebselen is not only a weak glutathione peroxidase mimetic but also possesses thioredoxin peroxidase properties [34]. Ebselen is known to mediate propagation of decomposition of ROS, hypochlorous acid, and oxygen and nitrous radicals and inhibit lipoxygenase, NADPH oxidase, and nitric oxide synthetase [3, 35–37]. Unlike other peroxidase mimetics, ebselen is well tolerated in humans. One hundred and fifty milligrams of twice-a-day ebselen has been shown to improve outcomes in stroke, ischemic damage of brain, and neurodegenerative diseases in human patients [38–40]. However, in context to chronic inflammatory disease of the lungs, benefit of ebselen has been largely limited to animal models and in vitro studies. Newer analogue of ebselen, BXT-51072 (Oxis, USA), has been developed with better activity and potency in cell systems and is currently in Phase 1 and preclinical trial for COPD treatment [1, 41].

Peroxidase mimetics such as diselinide and delluride compounds and peptide compounds such as selenosubstilisin have been shown to possess higher glutathione peroxidase-like activity than ebselen [42]. It has been observed that these compounds release free selenium and are electrophilic in nature, hence, possessing cytotoxic, genotoxic, and mutagenic potential that is a hindrance in their therapeutic application [43, 44]. However, cyclodextrin derivatives of these compounds with relatively less cytotoxic effects are being developed [45, 46]. Currently, there is a paucity of data, even at the in vitro level, to implicate their effects on living cells.

18.4 Thioredoxin Pathway-Based Antioxidants

Thioredoxin system is a ubiquitous thiol oxidoreductase system that regulates cellular reduction/oxidation (redox) status, which is induced in response to stress conditions [47]. Recent studies have shown that changes in thioredoxin status may contribute to the pathogenesis of COPD, asthma, and lung injury [44, 48]. Thioredoxin is known to protect lungs from ischemia/reperfusion injury, influenza infection, bleomycin-induced injury, and/or lethal inflammation caused by IL-2 and IL-18 [44, 49]. Therefore, potential role of recombinant human thioredoxin-1 as

treatment for lung inflammatory diseases cannot be undermined. The therapeutic data with recombinant thioredoxin is largely limited to animal studies; however, its clinical application is being evaluated for lung injury, ARDS, and COPD. New evidence with ambroxol, a mucolytic agent that has been used in clinical practice for decades, indicates that the molecule has antioxidant effects partly mediated by thioredoxin system at physiological concentration [50]. Further, thioredoxin pathway signalling molecules, such as autophagic proteins, p38 mitogen-activated protein kinase, NF-κB, and phosphatidylinositol 3-kinase, are also being considered as important antioxidants in near future [44]. However, the major limitation in this context is that each of these molecules has diverse signalling functions in cellular physiology; therefore, their application in treatments could interfere with other signalling pathways, which may be essential for cell survival.

18.5 Superoxide Dismutase and Catalase Pathway-Based Antioxidants

18.5.1 Superoxide Dismutase Mimetics

Free oxygen radicals undergo detoxification process in two-stage processes. First, free radicals are dismuted to oxygen and H_2O_2. This is catalyzed by an enzyme superoxide dismutase (SOD). Second, H_2O_2 formed by dismutation is further neutralized into water and oxygen with another enzyme called catalase (CAT). Further SOD is also known to scavenge reactive nitrogen and carbon radicals. Both SOD and CAT are metalloproteins. Inflammatory diseases have high exogenous and endogenous ROS production and human cells have a cutoff ability to generate SOD and CAT to counteract the oxidative stress. This becomes more prominent if the inflammatory stimulus is perpetual. Therefore, pertinent oxidant–antioxidant imbalance is cardinal to most of the chronic inflammatory diseases [3]. Replenishing SOD and CAT exogenously is an attractive antioxidant strategy.

SOD and CAT primarily function by enhancing redox-reaction to neutralize highly charged oxides, nitrogen, and carbon oxides. Naturally derived SOD and CAT usually do not reach the intracellular compartments where they are most required [51]. Studies have shown that active metals such as manganese and iron with rich coordination chemistries have potential to mimic SOD and CAT functionality of enhancing redox reactions [49, 52, 53]. Intriguingly nature has also provided cells with such compounds, particularly porphyrins, such as haem molecules, which form an integral component of various haem-proteins, and provide natural defense against oxidative stress in various cellular systems. Therefore, compounds with manganese and iron have emerged as natural choice for development of SOD and CAT mimetics. However, current data suggests that Mn-SOD mimetics are safer in cellular redox reactions. These SOD mimetics have more intracellular reachability.

MnTE-2-PyP^{5+} is one of the novel Mn-SOD mimetics that has been shown to have potential therapeutic benefits in stroke [54, 55], cardiac diseases [56, 57], malignancies [58], radiation injuries [59], lung diseases [60], and osteoarthritis [61] in the animal model studies. Another manganese compound, pentaazamacrocyclic ligand-based mimetic, is under process of development and has unique property of being relatively specific to O_2^- scavengers. In this compound, manganese atom (Mn) is held by five coordination points in the macrocyclic structure and is available only for one-electron transfers [62]. This compound can therefore function specifically as SOD.

18.5.2 Superoxide Dismutase Plus Catalase Mimetics

It is still not clear which is a better antioxidant target; is it SOD or CAT? Logically, targeting both looks superior to targeting single enzyme. Therefore, another group of mimetics of metalloporphyrin series constituting meso-substituted synthetic porphyrin, and iron or manganese as metallic redox element that is coordinated by four axial ligands, possessing either CAT-like activity or both CAT and SOD activity, are extensively being probed [3, 50, 51]. They are widely propagated as AEOL series compounds. AEOL-10113 and -10150 possess high SOD- and CAT-like activity [63], while AEOL-11207 and Mn-TBAP possess primarily CAT activity with low SOD activity [64]. Animal studies have shown that treatments with Mn-TBAP can protect against silica-induced, bleomycin-induced [65], and paraquat-induced [66–68] fibrosis. AEOL-10150 has been shown to attenuate inflammation by protecting epithelium from cigarette smoke-induced precancerous lesions and attenuate hemorrhage-induced acute lung injury [69]. AEOL-10113 has been shown to attenuate lung inflammation and bronchial hyper-reactivity in rats [70]. Therefore, it can be assumed that these drugs could emerge as important therapeutics in diseases such as ILDs, fibrotic lung diseases, occupational lung diseases, asthma, COPD, and ARDS.

Aromatic-substituted ethylenediamine metal complexes of manganese also known as salens are also being extensively researched. Animal studies have shown protective effects of salens against lung irradiation injury and ARDS [3]. However, currently its stability in biological matrix is questionable.

18.6 Anti-NF-κB-Based Antioxidants

18.6.1 Pyrrolidine Dithiocarbamate (PDTC)

Dithiocarbamates possess thiol structure and therefore function as antioxidants either by eliminating free radicals by thiol group or by stopping Fenton reaction. However, now it has been realized that dithiocarbamates also possess direct

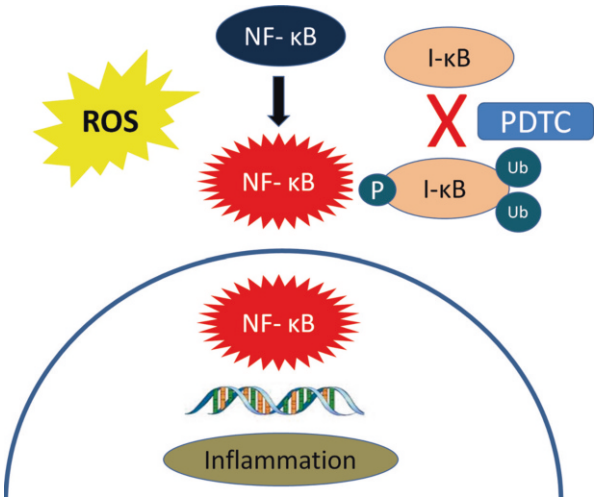

Fig. 18.3 Schematic diagram of the mechanism of action of pyrrolidine dithiocarbamate (PDTC)

anti-NF-κB properties. Oxidative stress is known to induce inflammatory gene expression through activation of a pleitropic transcription factor called NF-κB. Activation of NF-κB is the prime and the most sought inflammatory and oxidative stress pathway in pathogenesis of various chronic inflammatory diseases. Therefore, concept of using dithiocarbamates in inflammatory diseases provides an interesting antioxidant therapy in management of various diseases.

Pyrrolidine dithiocarbamate (PDTC) is a pyrrolidine derivative of dithiocarbamate, which is primarily being used in treatments of heavy metal toxicity and HIV disease in humans. Addition of pyrrolidine into dithiocarbamate enhances the compound's entry into the cell and provides prolonged stability in physiological environments. Intriguingly, PDTC does not have direct anti-NF-κB properties, but indirectly reduces its activity by inhibition of IκBα degradation, which is prime inactivator of NF-κB in inflammatory models [71] (Fig. 18.3). Its usage in metal poisoning and HIV suggests that this antioxidant may not be toxic for chronic use in humans.

PDTC has never been used in chronic inflammatory lung diseases. However, data from animal model and in vitro studies have shown that treatment with PDTC can significantly reduce expression of molecules and mediators, such as HIF-1α, haem oxygenase-1, VEGF, TNF-α, cycloxygenase-2, intercellular adhesion molecules (ICAM-1), vascular endothelial cell adhesion molecules (VCAM-1), and cytokine-induced neutrophil chemoattractant (CINC), which are known to have primary role in inflammatory disease pathophysiologies [72]. Further, PDTC is also known to inhibit activity of myeloperoxidase, which is a viscous neutrophilic enzyme. Animal model studies have shown that PDTC possesses protective effects in ARDS. The current understanding of PDTC indicates that it could be of significant therapeutic application in neutrophil-mediated lung diseases. However, substantial clinical data are needed before recommendation in chronic inflammatory disease of the lungs.

18.6.2 BAY 11-7085

BAY 11-7085 [(*E*)-3-(4-*t*-butylphenylsulfonyl)-2-propenenitrile] is a molecule, which inhibits NF-κB activation, although its primary role is induction of apoptosis. Studies have shown that BAY 11-7085 can reduce inflammation in ovalbumin-induced hyper-responsive mice (23) and also has potential to minimize hyperoxia-induced lung damage by inhibiting NF-κB signalling system [73]. The therapeutic application of this molecule has been studied for diseases such as endometriosis [74], malignancies [75], and cardiac morphogenesis [76] and even lung diseases such as non-small cell lung cancer and animal models of asthma [77, 78]. However, more experiments are needed to establish its efficacy as antioxidant in lung diseases.

18.7 Other Antioxidant

18.7.1 Spin Traps

Spin traps are nitrogen oxide molecules which trap the free reactive oxidant species in nitrone and nitro-oxide regions. The usual parent compound of the nitrone spin trap family is α-phenyl-*N*-*tert*-butyl nitrone (PBN), which forms a more stable nitroxide with ROS and hence removes harmful free radicals from circulation. Besides their direct free radical scavenging capabilities, nitrone spin traps also inhibit cyclooxygenase-2 (COX-2) and nitric oxide synthase (NOS). They may also decompose into nitric oxide, which itself activates many cellular mechanisms.

The problem with spin traps is their very short half-life and capability of generating hydroxyl radicals on decay, which has a damaging potential on the living tissues. Animal studies have shown that high doses of spin traps can cause impaired respiration, abnormal blood chemistry, seizures, and tissue damage [79, 80]. Now newer spin traps are being developed, such as stilbazulenyl nitrone (STANZ) and disodium-[(*tert*-butylimino)methyl]benzene-1,3-disulfonate-*N*-oxide (NXY-059), which have longer half-life and less toxicity [8]. The pharmacokinetic profile of NXY-059 from Phase IIa studies indicates that the drug is well tolerated and safe in stroke patients [8, 70]. STANZ exhibits highest antioxidant potential compared to all other nitrone spin traps. The spin traps have shown promising role in various neurodegenerative diseases such as ischemic brain damage, Alzheimer's disease, and Parkinson's disease [81]. However their role in chronic lung disease has not been explored yet.

18.7.2 Neu-164 and Neu-107

Two novel polycyclic molecules Neu-164 and Neu-107, containing benzene ring structures, have been developed, which exhibit pertinent antioxidant properties. In accordance with the little scientific evidence available with animal models and in

vitro experiments, these molecules demonstrate a potential towards reducing IL-6 and macrophage inflammatory protein-2 and also inhibit inflammatory enzymes such as myeloperoxidase and 5-lipooxygenase. These antioxidants have been shown to have salutary role in cigarette-induced cell damage of the lung cells in animal model experiments [82]. However, their application in chronic inflammatory disease of lungs is still at nascent stage plus there is lack of their toxicity data.

18.7.3 Tanshinone IIA and Cryptotanshinone

In spite of path-breaking advancements in designing synthetic compounds to treat diseases, ancient herbal therapy has not been abolished. Tanshinone IIA (TIIA) and cryptotanshinone (CT) are enantiomers found in the roots of *Salvia miltiorrhiza* and are the key ingredients in Chinese traditional medicine. In the last couple of years, many experiments have shown their multiple beneficial effects in many diseases. Their protective roles in kidney diseases [83, 84], hepatic diseases [85], and cardiac diseases [86] have been well established. These molecules have been found to ameliorate acute pancreatitis and neuronal diseases.

In vitro studies have shown that TIIA and CT are effective to minimize intracellular ROS and alleviate antioxidant enzymes activity. These therapies have shown protective effects from hypoxia-induced cell damage, which is largely induced by reducing intracellular NO production and mitochondrial superoxides. However, data on respiratory cells of these molecules are relatively very few. This herbal therapy can be beneficial to reduce oxidative stress-induced lung tissue damage.

18.8 Nano-particles

Nano-particles are chemically synthesized organic polymers or inorganic elements in various forms within a size of 1–100 nm of two or more dimensions, and are now primarily being envisaged as drug delivery mediums for future therapies. Liposomes, dendrimers, and polymetric micelles are organic nano-materials, while gold, silver, cerium, and carbon nano-materials belong to the inorganic class of nano-carriers. Nano-materials are generally used as vehicles for drugs and nuclear materials, although some nano-materials are themselves biologically active and can act directly with different biological molecules. Metal-based nano-particles such as gold, silver, and cerium possess some antioxidant properties as well. Oxides of cerium have been shown to possess SOD mimetic effects [87] and have potential to inhibit NF-κB activity by inhibiting translocation of its p65 subunit into the nucleus. Cerium oxides also reduce the expression of inducible nitric oxide synthase (iNOS) [88].

Another intriguing applicability of nano-particles is the antioxidant delivery to the target sites. This enables easy accessibility to intracellular compartments, which are otherwise not easily reached. The gold and polyamidoamine (PAMAM) dendrimers-based nano-particles delivery systems for natural antioxidants, such as

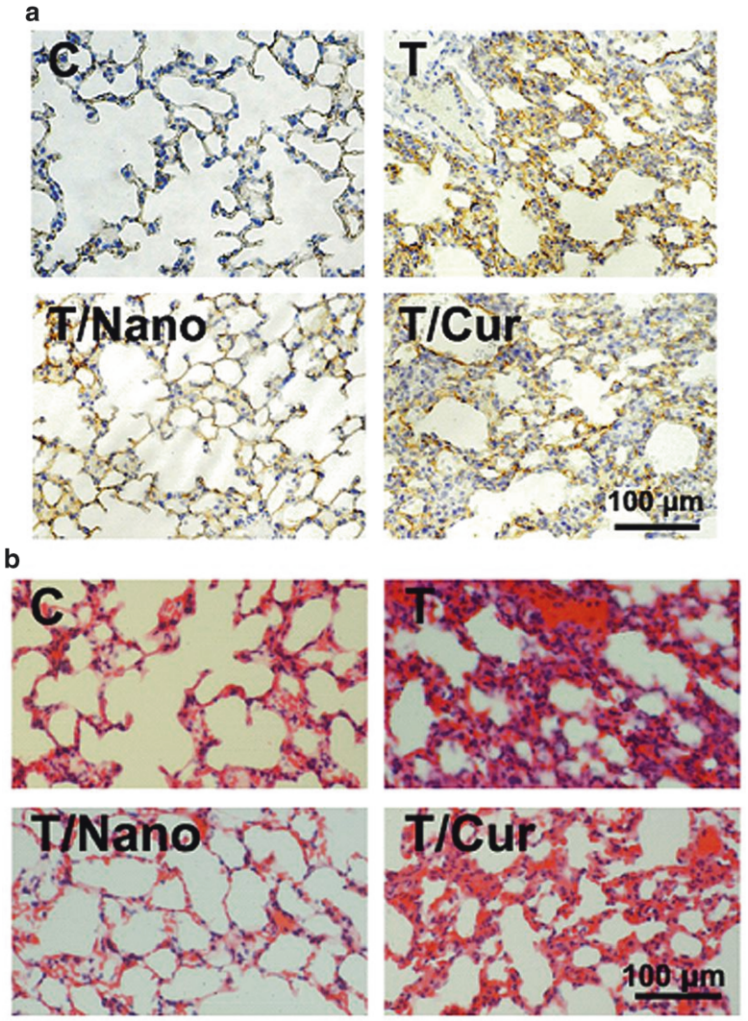

Fig. 18.4 CURN prevents TNF-α-induced acute lung inflammation in vivo. The sections were stained with ICAM-1 (**a**) and hematoxylin–eosin (**b**). Reproduced from: Yen F-L, Tsai M-H, Yang C-M, Liang C-J, Lin C-C, Chiang Y-C, Lee H-C, Ko H-H, Lee C-W. Curcumin nanoparticles ameliorate ICAM-1 expression in TNF-α-treated lung epithelial cells through p47^{phox} and MAPKs/AP-1 pathways (Reproduced from: [89])

flavonoids, curcumin, genistein, and resveratrol, are being developed for better drug deliveries. Experimental evidences suggest that the nano-particles-based flavonoids (e.g., quercetin and catechin) scavenge molecular oxygen more effectively than the free flavonoids [89]. Today we do not know whether nano-delivery or nano-particle therapies can have any potential role in chronic anti-inflammatory diseases of the lungs. However, currently this is the future of antioxidant research (Fig. 18.4).

18.9 Conclusion

With the current evidences from animal model studies and in vitro experiments, it is now possible to modulate oxidative stress at different levels of glutathione metabolism and peroxide detoxification mechanisms. Even manipulation of anti-inflammatory genes with transcription factor seems as promising development in the pharmacological development. However, the development of new antioxidants is still at very early stages.

Conflict of Interest The authors declare that they have no potential conflicts of interest.

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Chapter 19
Ayurvedic and Other Antioxidant Mimics

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Antioxidants are substances that inhibit oxidation of other substances generally by removing potentially damaging oxidizing agents in a living organism, thereby decreasing free radical-induced damage. Their popularity can be gauged from the fact that, as of May 2013, Google search with the word "antioxidant" gave close to 40 million hits whereas a PubMed query with the same term raised more than 385,000 hits. Most of the lay press and other forms of media are full of advertisements about antioxidants' putative (but mostly unproven) benefits coupled with lack of toxicity, in spite of the fact that most of the recent scientific evidence about antioxidants has been unfavorable. In this chapter, we will objectively review the current status and future implications of some of the Ayurvedic and other herbal antioxidants.

Normal human diet contains a large number of components that serve as antioxidants, the important ones being vitamins and trace elements. There is also no doubt that these nutrients are responsible for keeping us in good health. While there are no randomized controlled trials (RCTs) to prove, most nutritionists agree that diet plays an important role in health maintenance, and most of us consume these dietary antioxidants [1, 2]. There is sufficient evidence from observational studies to show that higher intake of fruits and vegetables is correlated with reduced risk of several diseases [3, 4].

Since dietary intake of antioxidants in fruits and vegetables has been shown to be a useful strategy to lower a person's risk of certain diseases, supplementation with antioxidants as medications was expected to be taken up by the pharmaceutical

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industry. For the past several years, markets are flooded with such antioxidant formulations and as per some estimates [5] products with antioxidant claims which include foods, beverages, supplements, and cosmetics had annual retail sales of about US$ 65 billion, showing an annual growth of a little less than 10 % with projections to reach US$ 86 billion by 2016.

These enormous volumes were attained without good quality evidence showing unequivocally the benefit of antioxidant supplements. On the contrary evidence from RCTs was far from encouraging and, in fact, showed harm. One of the first RCTs to show that antioxidants could increase the risk of cancer was the ATBC (alpha tocopherol beta carotene) Trial [6]. Subsequently, other trials included by us in acute pancreatitis showed similar results [7]. A recent Cochrane review which included 78 RCTs having a total of close to 300,000 participants showed that antioxidants significantly increased mortality (11.7 % versus 10.2 %; relative risk [RR] 1.03, 95 % confidence interval [CI] 1.01–1.05) in a fixed-effect model although the effect was small. In a random-effects model, the increase in mortality was not significant (RR 1.02, 95 % CI 0.98–1.05).

Importantly, if only trials with a low risk of bias were included (56 trials), the effect on mortality was greater (12.9 % versus 10.6 %; RR 1.04, 95 % CI 1.01–1.07). Among these trials, effect on mortality was greatest with beta-carotene and vitamin E. Mortality with beta-carotene (26 trials) was 13.8 % versus 11.1 % (RR 1.05, 95 % CI 1.01–1.09) whereas with vitamin E (46 trials), mortality was 12.0 % versus 10.3 % (RR 1.03, 95 % CI 1.00–1.05). Effect on mortality was not significant for vitamin A (14.0 % versus 13.6 %; RR 1.07, 95 % CI 0.9 7–1.18), vitamin C (9.9 % versus 9.3 %; RR 1.02, 95 % CI 0.98–1.07), and selenium (6.7 % versus 6.4 %; RR 0.97, 95 % CI 0.91–1.03).

The authors of this meta-analysis concluded that there was no evidence in favor of the use of antioxidant supplements for either primary or secondary prevention; it was recommended that antioxidant supplements should be considered as drugs, thereby emphasizing the need for systematic and thorough evaluation prior to marketing as is done for new drugs.

On the other hand, diet also contains a large number of antioxidants, most of which come from plant sources, and are called phyto-antioxidants. Beneficial effects of fruits and vegetables in promoting health are well established although it is not clear what is the exact contribution of antioxidant effect to these benefits.

Several studies have shown that herbal preparations are used very commonly by patients across a diverse range of disorders [8, 9]. Many of the herbal compounds are consumed in excess of what a normal diet would provide, presumably for their antioxidant and other benefits. The chemical compounds that provide herbal antioxidant protection include, but are not limited to, polyphenols, flavonoids, catechins, lignans, and others. Indian Systems of Medicine and Chinese Traditional Medicine describe various properties of these plant-based compounds and have been extensively reviewed [10–13].

With this background, we will look at some of the natural and herbal/Ayurvedic antioxidants and analyze the evidence available for each of these. Typically, many of these preparations are used as normal components of Indian diets, whereas others are predominantly used for their medicinal properties.

19.1 *Ocimum sanctum* Linn. (Lamiaceae) (Holy Basil; *Tulsi*)

Several compounds with potent antioxidant activity (at 10-μM concentrations) were isolated from fresh leaves and stem extracts of *O. sanctum* [14]. Out of the seven compounds isolated (cirsilineol, cirsimaritin, isothymusin, isothymonin, apigenin, rosmarinic acid, and eugenol), five (cirsilineol, isothymusin, isothymonin, rosmarinic acid, and eugenol) showed good to excellent antioxidant activity. Another compound, ursolic acid, isolated from *O. sanctum* protected against lipid peroxidation in liver microsomes in vitro [15].

Two other flavonoids, orientin and vicenin, isolated from the leaves of *O. sanctum*, were shown to increase survival time in lethally irradiated mice [16]. When animals were pre-treated with either of the flavonoids, a significant decrease in chromosome aberration following gamma irradiation was seen, with vicenin providing the best protection. In another study, *O. sanctum* demonstrated protective effects against copper sulfate toxicity (mediated by free radicals) in rats [17].

Oral treatment with very high doses of *O. sanctum* leaf extract for 15 days was shown to result in significantly elevated activities of enzymes (cytochrome P-450, cytochrome b5, aryl hydrocarbon hydroxylase, and glutathione-S-transferase) involved in the detoxification of carcinogens and mutagens in mice [18]. *O. sanctum* extract was also found to elevate hepatic and extrahepatic levels of glutathione, which is well known to be an important part of the body's protective mechanism against free radicals.

Several animal studies have shown anticancer activity; in one such study, chemopreventive activity for *O. sanctum* seed oil, which contains fatty acids including linolenic acid, has been shown in 20-methylcholanthrene-induced fibrosarcoma tumors in the thigh region of Swiss albino mice [19]. Antioxidant activity was deemed to be partly responsible for the chemopreventive effect.

19.2 *Curcuma longa* Linn. (Zingiberaceae) (Turmeric; *Haldi*)

Used for multiple indications like anticancer, antiseptic, anti-inflammatory, and other similar actions, curcumin has also been shown to possess antioxidant actions. Neuroprotection, partly attributable to antioxidant effect shown in alcohol-fed rats, has been described [20]. It is believed that curcumin may be more potent than alpha-tocopherol in its antioxidant effect [21]. It contains several known antioxidant curcuminoids like demethoxycurcumin, bis-demethoxycurcumin, 5′-methoxycurcumin, and dihydrocurcumin, as well as others like phenylheptanoids, monoterpenes, sesquiterpenes, diphenylalkanoids, phenyl propene derivatives of cinnamic acid, and terpenoids [22, 23].

19.3 Mineral Pitch (Shilajit)

Antioxidant and antiarthritic activities of shilajit were evaluated in an in vitro study [24]. It was shown that aqueous extract of shilajit exhibited free radical scavenging activity in a dose-dependent manner with IC50 value of 11.9 μg/mL, which was similar to that of standard ascorbic acid. This activity was attributed to the high phenolic content of shilajit.

In another study, it was shown that shilajit provided almost complete protection against hydroxyl radical injury and efficiently trapped nitric oxide free radicals [25]. These effects were concentration dependent. In an in vitro *study*, it was demonstrated that shilajit decreased the oxidation of reduced glutathione in rat liver homogenate [26].

19.4 *Withania somnifera* Dunal (Solanaceae) (Winter Cherry or *Aswagandha*)

Considerable in vitro and animal research shows that withanolides (withaferin A) and sitoindosides, the active compounds in *W. somnifera*, possess antioxidant properties in terms of increasing the activity and/or levels of free radical scavenging enzymes and glutathione peroxidase. It is likely that there are more constituents in *W. somnifera* that might also contribute to its significant antioxidant properties. Moreover, in some studies several compounds have been used together and it is difficult to attribute all the effects to *W. somnifera*; for instance in one study Transina, a polyherbal formulation comprising *W. somnifera* and other herbs like *Tinospora cordifolia*, *Eclipta alba*, *Ocimum sanctum*, *Picrorrhiza kurroa*, and shilajit, was used for superoxide dismutase activity in hyperglycemic rats [27].

19.5 *Terminalia arjuna* Roxb. W. & A. (Combretaceae)

Used traditionally as a cardiotonic, various studies have shown that bark extract of *T. arjuna* also possesses antioxidant effects. In alloxan-induced model of diabetes mellitus in rats, 500 mg/kg *T. arjuna* was shown to produce significant reduction in lipid peroxidation and increase in superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase and glucose-6-phosphate dehydrogenase, reduced glutathione, vitamin A, vitamin C, vitamin E, total sulfhydryl groups (TSH), and nonprotein sulfhydryl groups (NPSH) in liver and kidney tissues [28].

The effect on antioxidant status by administration of ethanolic extract of *T. arjuna* bark was evaluated in *N*-nitrosodiethylamine-induced liver cancer in male Wistar albino rats in a study; it showed that peroxide levels were reduced and the antioxidant enzyme levels were increased [29].

The compounds responsible for this action include polyphenols (~44 %), flavon-3-ols (catechin, gallocatechin, and epigallocatechin), and phenolic acids (gallic acid, ellagic acid, and its derivatives) [30].

19.6 Polyherbal Preparation (Triphala)

Another polyherbal formulation comprising aqueous extract of the fruits of *Emblica officinalis* Linn., *Terminalia chebula* Retz., and *Terminalia belerica* (Gaertn.) Roxb. was shown to inhibit gamma-radiation-induced lipid peroxidation and strand break formation in plasmid DNA (pBR322) in rat liver microsomes [31]. Individual components as well as the mixture were effective, the activity being possible due to the presence of phenolic compounds which were present in concentrations ranging from 33 to 44 %. Triphala also prevented superoxide-induced hemolysis of red blood cells and lipid peroxidation induced by Fe^{3+}/ADP/ascorbate system in rat liver mitochondria with tannins being the major phenolic compounds responsible for activity [32].

19.7 Gold Ash (*Swarnabhasma*)

Swarnabhasma is an old formulation used for a large number of indications. Prepared according to Ayurveda, it consists of realger (As(2)S(2)), lead oxide (Pb(3)O(4)), pure gold (Au), and latex of *Calotropis gigantea*; after purification and calcination, it does not contain any organic compound but has several other elements like Fe, Al, Cu, Zn, Co, Mg, Ca, As, and Pb [33]. The amount of heavy metals in "bhasma" is measured by atomic absorption spectrometry. In an animal model of cerebral ischemia, *Swarnabhasma* was shown to significantly improve antioxidant levels as measured by enzymatic parameters like peroxidase, reduced glutathione, catalase, glutathione reductase, glutathione-S-transferase, and others although effect on infarct size was not mentioned [34].

19.8 *Giloysatva* (*Tinospora cordifolia*) (Willd.) Miers ex. Hook.f. & Thomas. (Menispermaceae) and *Curculigo orchioides* Gaertn. (Liliaceae)

Giloysatva is a traditional Ayurvedic formulation. The antioxidant effects of *giloysatva* and hydro-alcoholic extract of *C. orchioides* (*Kali musali*) were evaluated in vitro [35]. Potent free radical scavenging activity was demonstrated which was presumably due to the presence of various flavonoids, alkaloids, and saponins.

19.9 *Terminalia belerica* (Roxb. Combertaceae) (Belleric Myrobalan or Vibhitaka)

In an animal model of alloxan-induced hyperglycemia, dried 75 % methanolic extract of fruits of *T. belerica* improved the antioxidant defense mechanism besides improving the blood glucose levels [36].

19.10 *Cissampelos pareira* Linn. (Menispermaceae) (Patha)

C. pareira extract was shown for the first time to scavenge oxygen free radicals in an in vitro model as well as in an animal model (benzo(a)pyrene-induced gastric toxicity) of tissue injury in doses ranging from 50 to 400 μg/kg [37].

19.11 *Bacopa monniera* Linn. (Scrophulariaceae) (Indian Pennywort or *Nirbrahmi*)

Traditionally used in Ayurvedic medicine as a memory enhancer, antiepileptic agent, and sedative, it was also shown to possess significant antioxidant activity, particularly with the alcoholic extract although this was about 50 % of that seen with vitamin E [38].

19.12 *Convulvulus plauricaulis* Linn. (Convolvulaceae) (Bindweed; *Shankhpushpi*)

Popularly known as a brain tonic, *Convolvulus pleuricaulis* has also been used as an antianxiety and antiepileptic preparation. Various studies have shown its antioxidant effects in animal models of learning and memory impairment [39, 40]. It contains several neuroactive alkaloids such as shankhpushpine, and convolamine, as well as other substances including hextriacontane, scopoletin, *beta*-sitosterol, ceryl alcohol, 20-oxodotriacontanol, tetratriacontanoic acid and 29-oxodotriacontanol, flavonoid (kaempferol), and phytosteroids such as phytosterol and *beta*-sitosterol [41–43].

19.13 *Aloe vera* Linn. (Aloaceae)

Alcoholic extract of *A. vera* leaf gel was demonstrated to possess antioxidant activity in a rat model of diabetes besides decreasing blood glucose and glycosylated hemoglobin and also led to improvement in hemoglobin [44]. Other studies have

also shown that it affords some protection against free radical-induced oxidative stress, which could be a combination of a direct antioxidant action and indirect action mediated by stimulation of endogenous antioxidant systems [45].

19.14 *Asparagus racemosus* Willd. (Liliaceae) (Common Asparagus; *Shatavari*)

Used traditionally for various actions like an antacid and as a tonic, its antioxidant actions have also been evaluated. Some studies have shown that it has nootropic effects which are partly due to its free radical scavenging action [46–48]. It contains a large number of compounds that can contribute to this action—vitamins A, B1, B2, C, E, folic acid, steroidal saponins, Mg, P, Ca, Fe, essential oils, asparagine, arginine, tyrosine, flavonoids (kaempferol, quercetin, and rutin), resin, and tannin [49].

19.15 *Piper nigrum* Linn. (Piperaceae, Black Pepper) and Piperine

In a rat model of metabolic syndrome induced by high fat diet, *P. nigrum* was shown to have beneficial effects on tissue lipid peroxidation and increased antioxidant levels [50].

19.16 *Celastrus paniculatus* Willd. (Intellect Tree; *Malkangani*)

Known to have a wide range of beneficial actions in conditions including asthma, leprosy, inflammation, dysmenorrhoea, and pruritus and possess laxative, expectorant, appetite-stimulant, aphrodisiac, anti-inflammatory, and diuretic properties, it has also been shown to be an antioxidant [51].

19.17 *Acorus calamus* Linn. (Sweet Flag, *Vacha*)

Having known sedative effects, this plant was demonstrated to have free radical scavenging actions in animal models of neurological disorders [52, 53]. It is postulated that its sedative effects are due to α- and β-asarone [54] but whether they are responsible for the antioxidant effect remains unknown.

19.18 Major Challenges

As can be seen from a brief description given above about the various effects of many of the plants, it is clear that antioxidant actions have been reported as secondary actions on the evaluation of other primary activity(ies) of the plant. While many of these studies are published in Medline-indexed journals, other publications in non-indexed and lay journals are also common. The quality of the published work in this field has always never been strong. Even though the results may be quite consistent, these are often viewed with some skepticism by the mainstream medicine experts. It is beyond the scope of this chapter to delve into the details of the reasons of whether this skepticism is justified or not, but it will be appropriate to mention that one finds significant arguments both for and against this skepticism. Also, we fear that the evaluation of antioxidant effects in most cases is the result of what can be termed as "convenience research" since facilities for estimating the various markers of oxidative stress are available in the researchers' laboratories, at relatively low costs. As a case in point, we have evaluated the role of various herbal formulations and extracts in animal models of metabolic syndrome and pancreatitis without looking at the oxidant/antioxidant status of those compounds [55–57].

Another related issue is lack of availability of good evidence about use of these antioxidants in humans. Limitations of antioxidants as promoters of good health have been challenged as pointed out earlier. Therefore, antioxidants are not recommended routinely to patients as drugs. On the other hand, antioxidants in foods are known to promote health and most guidelines in diverse groups of diseases recommend their intake. There is an extreme dearth of evidence whether herbal antioxidants will provide any benefit in humans.

The third challenge for those working in this field is related to the issue of bioavailability. Bioavailability refers to the rate and extent to which a chemical compound (drug, active component of a plant) is absorbed and becomes available for systemic use. Many of these plant-based products have poor oral bioavailability. Lack of good oral bioavailability can render a compound ineffective in an animal model or during human use. This has been very well described in case of curcumin [58]. Several methods of improving the bioavailability of curcumin have been evaluated. One traditional way of improving bioavailability of various compounds is by using black pepper or its combination with other herbs, for example, in *trikatu* on which there are data from our institution [59] as well. Unfortunately, such data on bioavailability of most of the other herbal compounds do not exist and, to the best of our knowledge, systematic and thorough attempts for doing the same are also lacking, although individual researchers are working in isolation.

We have not listed all the plants having antioxidant actions; it is likely that many more plants will show such effects due to the presence of several chemicals such as polyphenols and others. We have tried to include only those for which there is sufficient evidence.

19.19 Conclusion

Certain foods like fruits and vegetables, which are rich in antioxidants, have several health benefits. Whether the antioxidant effect contributes to any specific benefits has not been conclusively proven. Antioxidant effects of various vitamins and related compounds have not withstood the scrutiny of well-conducted clinical trials for their routine use. Research on the antioxidant effects of Ayurvedic compounds has focused on evaluating primary activity in a disease model along with measurement of various markers of oxidative stress and antioxidant enzymes. Most of these studies have failed to show whether the antioxidant actions have contributed towards the primary activity of the herb. There are several future challenges for researchers in this field to address the various issues.

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