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.