# Chapter 2
# Reactive Oxygen and Nitrogen Species: General Considerations

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## 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).

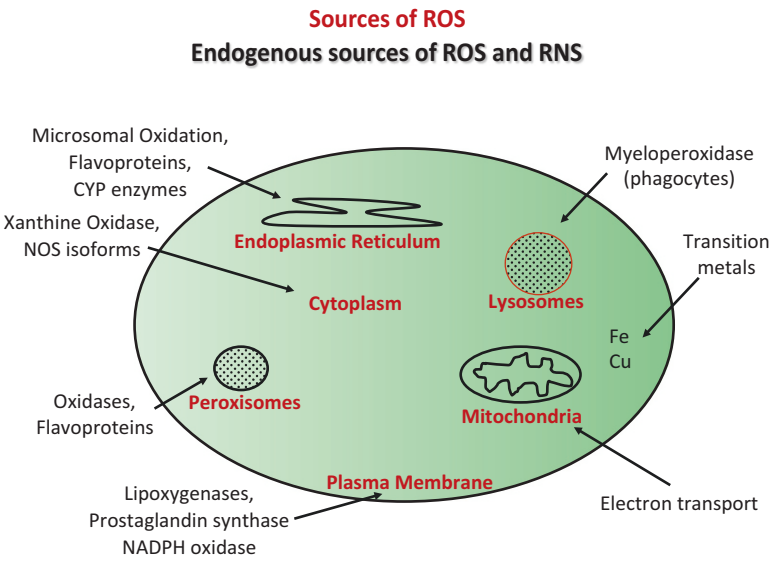


**Fig. 2.1** Various endogenous sources of ROS and RNS in the cell

## 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).

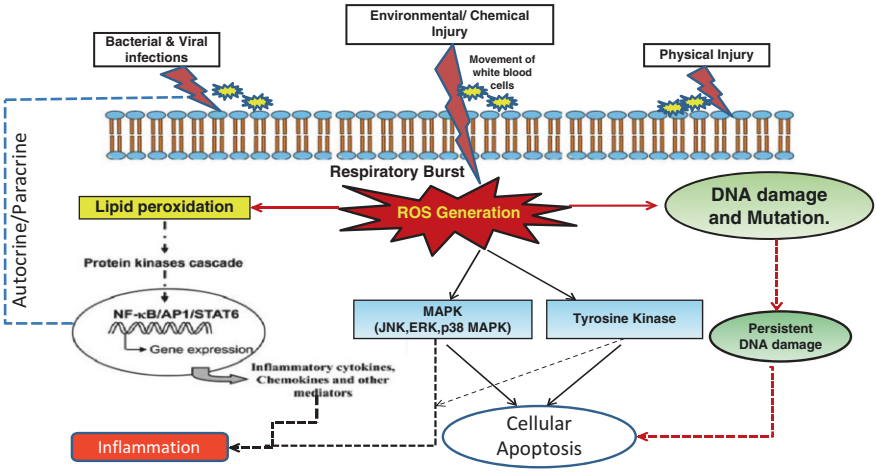


**Fig. 2.2** Role of ROS and RNS in tissue damage. Inflammation begins with a reaction to an irritant or infection that is characterized by movement of fluid and white blood cells into extravascular tissue. This is followed by cell proliferation and involves tissue repair and regeneration. Generation 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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