

Reactive Oxygen Species: Drivers of Physiological and Pathological Processes

This article was published in the following Dove Press journal:
Journal of Inflammation Research

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Abstract: Since the Great Oxidation Event, about 2.4 billion years ago, the Earth is immersed in an oxidizing atmosphere. Thus, it has been proposed that excess oxygen, originally a waste product of photosynthetic cyanobacteria, induced oxidative stress and the production of reactive oxygen species (ROS), which have since acted as fundamental drivers of biologic evolution and eukaryogenesis. Indeed, throughout an organism's lifespan, ROS affect directly (as mutagens) or indirectly (as messengers and regulators) all structural and functional components of cells, and many aspects of cell biology. Whether left unchecked by protective antioxidant systems, excess ROS not only cause genomic mutations but also induce irreversible oxidative modification of proteins (protein oxidation and peroxidation), lipids and glycans (advanced lipoxidation and glycation end products), impairing their function and promoting disease or cell death. Conversely, low-level local ROS play an important role both as redox-signaling molecules in a wide spectrum of pathways involved in the maintenance of cellular homeostasis (MAPK/ERK, PTK/PTP, PI3K-AKT-mTOR), and regulating key transcription factors (NFκB/IκB, Nrf2/KEAP1, AP-1, p53, HIF-1). Consequently, ROS can shape a variety of cellular functions, including proliferation, differentiation, migration and apoptosis. In this review, we will give a brief overview of the relevance of ROS in both physiological and pathological processes, particularly inflammation and aging. In-depth knowledge of the molecular mechanisms of ROS actuation and their influence under steady-state and stressful conditions will pave the way for the development of novel therapeutic interventions. This will mitigate the harmful outcomes of ROS in the onset and progression of a variety of chronic inflammatory and age-related diseases.

Keywords: reactive oxygen species, oxidative stress, inflammation

Introduction

The detection of free radicals (chemical entities possessing highly reactive unpaired electrons) in biological systems¹ gave rise to the free radical theory,² which was supported by the discovery of superoxide dismutase (SOD)³ and by pioneering concepts such as oxidative stress and antioxidants.⁴ All these findings have placed aerobic metabolism and reactive oxygen species (ROS) as the most accepted cause of aging and numerous inflammatory diseases. ROS comprise both free radical and non-free radical oxygen intermediates (peroxides), like superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{\cdot}), and singlet oxygen (1O_2).⁵ These molecules are generated by plasma membrane proteins, such as the growing family of NADPH oxidases,⁶ by lipid metabolism within the peroxisomes,⁷ and by the activity of various cytosolic enzymes such as cyclooxygenases.⁸ Although all these sources contribute to the overall oxidative burden, the vast majority of cellular

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ROS (approximately 90%) come from mitochondria due to oxidative phosphorylation.⁹

Oxidative stress, defined by an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of oxidation-reduction (redox) reactions and/or molecular damage¹⁰ introduces the redox concept, that in living cells operates in fundamental processes, collectively termed “redox signaling” and “redox control”.¹¹ Thus, the original free radical theory has been substituted by the “redox hypothesis”, which postulates that oxidative stress occurs by disruption of thiol redox circuits. This is fundamental in cell signaling and physiological regulation without macromolecular damage. Indeed, thiols in proteins can function as transducing elements linking chemistry (ROS) and biologic structure.^{12,13}

Conversely, elevated levels of ROS may lead to more extensive and irreparable cell damage through oxidation of DNA, RNA, carbohydrates, proteins and lipids, resulting ultimately in cell death through apoptosis or necrosis.¹⁴ There are a high variety and range of pro-oxidant and antioxidant enzymes and compounds, prompting a classification of sub-forms of oxidative stress: nutritional, dietary, postprandial, radiation-induced and physiological. At the molecular level, oxidative stress can be photooxidative, nitrosative or reductive. Finally, different scales can be considered ranging from physiological oxidative stress to excessive and toxic oxidative burden.¹⁰

In pathological situations and exposure to stressful environmental insults, ROS are produced in excessive amounts.¹⁵ These pathological effects are usually mediated by ion channel opening, lipid peroxidation, protein modifications, and DNA oxidation. The pro-inflammatory molecules generated in stressful situations through ROS cause inflammation, which plays a major role in aging and the development of a variety of diseases.¹⁶ These include vascular disorders (coronary heart disease, hypertension, metabolic syndrome, atherosclerosis),¹⁷ autoimmune diseases (rheumatoid arthritis, inflammatory bowel disease),¹⁸ neurodegenerative diseases (Alzheimer's, Parkinson's, age-related macular degeneration),¹⁹ and respiratory diseases (asthma, chronic obstructive pulmonary disease, acute lung injury, cystic fibrosis).²⁰

This review will focus on the role of ROS at the physiological and physiopathological levels. Particularly, we will emphasize their relevance in the immune-inflammatory processes, highlighting the most important molecules/pathways involved. Finally, we will stress the

role of ROS in senescence and aging, and the status of the underlying antioxidant therapies.

ROS Production and Compartmentalization

Up to 1–2% of consumed oxygen by a cell can turn into oxygen radicals, which can lead to the production of ROS. The main source of ROS in vivo is through aerobic breathing. Nevertheless, ROS are also produced by peroxisomal β -oxidation of fatty acids, microsomal cytochrome P450, metabolism of xenobiotic compounds, stimulation of phagocytosis by pathogens or lipopolysaccharides, arginine metabolism, and tissue-specific cellular enzymes.^{21,22} Intracellular ROS generation and their local redox status are important for understanding cell pathophysiology. Some subcellular compartments are more oxidizing (such as endoplasmic reticulum (ER), lysosomes, or peroxisomes) whereas others are more reducing (mitochondria, nuclei). Thus, ROS levels may fluctuate between subcellular compartments and may lead to beneficial effects or pathology.²³

Mitochondria are considered the most redox-active compartment in the cell, accounting for more than 90% of oxygen utilization.⁹ Although most of the oxygen undergoes complete reduction to water at the level of cytochrome oxidase, partial reduction accompanied by ROS generation can occur as well.²⁴ The most common ROS is $O_2^{\cdot-}$, which is converted by mitochondrial SOD into H_2O_2 , which in turn transmute into powerful oxidants like OH^{\cdot} radicals. This occurs primarily through a Fenton reaction, that can oxidize a high number of important biomolecules. SODs, metalloenzymes found in all kingdoms of life, can be classified into four groups according to the metal cofactor present in their active site: copper-zinc-SOD (Cu-ZnSOD), iron SOD (FeSOD), manganese SOD (MnSOD) and nickel SOD (NiSOD).²⁵ From these, three isoforms are ubiquitously expressed in humans, all other mammals and most chordates: cytoplasmatic Cu-ZnSOD or SOD1, mitochondrial MnSOD or SOD2, and extracellular Cu-ZnSOD (EC-SOD) or SOD3.^{26,27} SOD1 is the most abundant and is constitutively expressed, although its overall expression in the lung is lower compared to several other organ systems. Its functional importance is most likely related to the regulation of cytosolic $O_2^{\cdot-}$ levels.²⁸ SOD2 is inducible by oxidative stress, hyperoxia, environmental pollutants and by inflammatory cytokines.²⁹ SOD3 expression is cell- and tissue-specific,

being highest in lung. Because SOD3 represents the only $O_2^{\cdot-}$ scavenging enzyme within the extracellular environment, it is likely of major importance in protection against exogenous and environmental stress.³⁰

The ER tubular network has a unique oxidizing environment, and under stress conditions, redox-signaling mediators play key roles in ROS generation and dictate the fate of protein folding and secretion.³¹ The microsomal cytochrome P450-dependent monooxygenase system, responsible for the oxidative metabolism of xenobiotics, is one of the major producers of ROS in the liver cell.³²

The peroxisomes contain peroxisomal oxidases and xanthine oxidase, that generate ROS and nitric oxide (NO).⁷ But catalase is the major oxidoreductase responsible for H_2O_2 metabolism. Under physiological conditions, H_2O_2 diffusion is prevented through its rapid conversion to O_2 . Interestingly, recycling endosomes, late endosomes, and lysosomes are as oxidizing as the ER.³³ The lysosomal electron transport chain, which promotes proton translocation to maintain an optimal pH for the acidic hydrolases, generates OH^{\cdot} radicals.³⁴

The main source of ROS in the plasma membrane corresponds to $O_2^{\cdot-}$ production by the membrane-bound NADPH oxidases, which help to kill bacterial intruders.³⁵ The NADPH oxidase proteins constitute the only enzyme family with the sole function of producing ROS.³⁶

Regarding the exogenous sources of free radicals, ROS, oxidative stress and environment hold a complex relationship which impact on human health and disease.³⁷ Environmental oxidative stress derives from different artificial and natural sources such as cigarette smoke, traffic exhaust emissions, solar radiation, nutrition, radiotherapy, cosmetic devices or industrial pollution, and is induced by different mechanisms.³⁸ Some of the influences of the environment on organic life can promote or generate oxidative stress. In general, the action of radiation includes the induction of ROS, namely $O_2^{\cdot-}$, H_2O_2 , and OH^{\cdot} .³⁹

Functions and Mechanism of Action of ROS at the Physiological Level

At physiological level, besides their role destructing pathogens in the immune defense against external insults⁴⁰ or the synthesis of cellular structures like protein complexes,⁴¹ ROS function as redox messengers (second messengers) (Figure 1). Cells can generate ROS constitutively and exogenously, and use them for intracellular signaling and for

stimulating redox-sensitive signaling pathways to modify the cellular content of the cytoprotective regulatory proteins.^{42,43} Thus, ROS control pro-inflammatory signaling, pro-fibrotic signaling, cell proliferation, apoptosis and a range of other biological processes without triggering a requirement for macromolecular damage.¹¹ In fact, the ROS-mediated redox messenger activity is thought to be larger than the ROS-mediated macromolecular damage activity.⁴⁴

The redox state in the cytosol is normally achieved by the “redox-buffering” capacity of intracellular thiols, such as glutathione (GSH) and thioredoxin (Trx). These are the main redox nodes controlling physiologically relevant processes. Cysteine (Cys)/cystine (CySS) inclusion as a redox control node apart from GSH and Trx1 significantly expands the redox range. These three central couples of proteins are maintained at stable and non-equilibrium values in different organelles. Their function entails controlling the redox state of oxidizable thiols (“sulfur switches”) in proteins.^{45,46} The high ratios of reduced to oxidized forms are maintained by the activity of GSH reductase and Trx reductase. These sulfur switches are used for the maintenance of protein structure, protein trafficking, and for the regulation of enzyme, cell signaling, receptor, transcription factor and transporter activities.⁴⁷

Most redox signaling research has focused, on the one hand, on H_2O_2 and other ROS as oxidants for signaling proteins. On the other hand, it has centered on oxidation of sulfur-containing side chains of cysteine and methionine by ROS, which can result in higher oxidation states of sulfur. While ROS such as OH^{\cdot} may cause irreversible damage with low specificity to macromolecules, the main targets of a mild oxidant such as H_2O_2 are the thiol groups of protein cysteine residues.⁴⁸ In fact, $O_2^{\cdot-}$, OH^{\cdot} , and 1O_2 are not second messengers because they have no specificity in their interaction with effectors in signaling pathways. H_2O_2 , instead, has an interesting chemistry that provides specificity for the oxidation of thiols and enables its function as a second messenger.⁴⁹ Accordingly, signaling enzymes and proteins containing cysteine residues have been proposed as potential targets for ROS.⁵⁰ The sulfur from the cysteine in these proteins can be reversibly or irreversibly oxidized to sulfenic acid (-SOH), sulfinic acid (-SO₂H), disulfide bond (-SSR) or sulfonic acid (-SO₃H)⁵¹, and these were initially thought to be the markers of oxidative damage. Formation of sulfinic and sulfonic acid is irreversible and, therefore, they are not involved in the

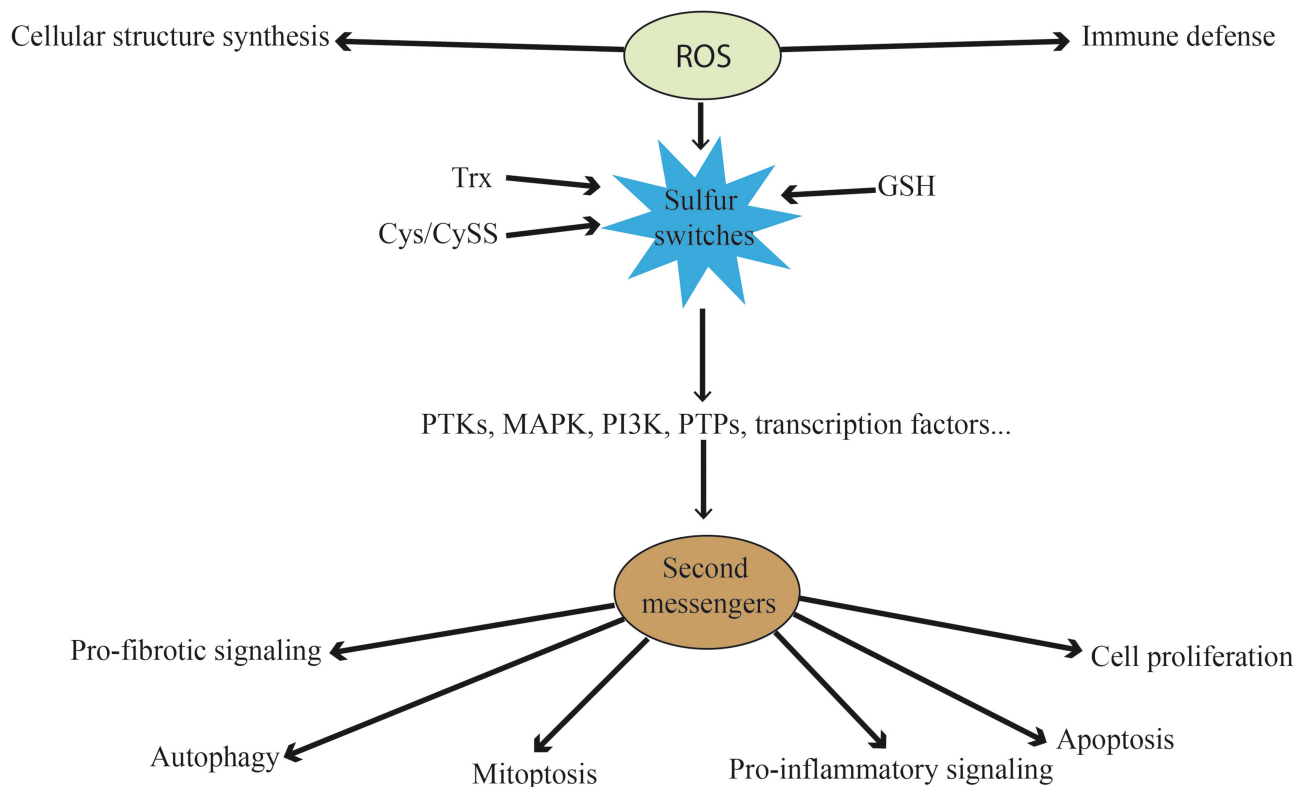


Figure 1 Functions of ROS at physiological level. Besides their direct functions in immune defense and cellular structure, ROS can act as second messengers. They oxidize the three main sulfur switches and indirectly influence pro-fibrotic signaling, autophagy, mitoptosis, pro-inflammatory signaling, apoptosis and cell proliferation. These processes are mediated by kinases, phosphatases and transcription factors, among others.

Abbreviations: ROS, reactive oxygen species; Trx, thioredoxin; Cys/CySS, cysteine/cystine; GSH, glutathione; PTK, protein tyrosine kinases; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PTP, protein-tyrosine phosphatases.

signaling reaction. Conversely, disulfide bonds and protein sulfenic acids can easily be reduced by systems such as Trx and peroxiredoxin (Prx) and are considered mediators of redox signaling.⁵²

Consequently, ROS formed in the mitochondria and the cytosol influence the redox state of cysteine residues in proteins. This constitutes a regulatory mechanism determining protein conformation and function. ROS are also critical for the establishment of protein–protein and protein–DNA interactions involving many aspects of the signal transduction pathway.

As a result of exposure to different stimuli, intracellular ROS increase and can lead to oxidation of cysteine residues in cytoplasmic proteins such as kinases and phosphatases, ultimately affecting signal transduction processes.⁵³ Overall, H₂O₂ can induce the phosphorylation of tyrosine residues from numerous cell proteins depending on the regulation of the redox status. This regulation is determined by the content of thiol compounds in the cell, primarily by the content of GSH. Through this mechanism, several proteins, notably kinases, are activated (eg,

protein-tyrosine kinases (PTKs)). They promote the effects of growth factors, cytokines, and hormones⁵⁴ or induce downstream signaling pathways involving protein kinases of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) cascades.⁴²

Conversely, the inhibition of phosphatases by oxidants is mediated through oxidation of the reactive cysteine residue in the active center and inhibition of its catalytic activity (eg, protein tyrosine phosphatases (PTPs)⁵⁵ capable of dephosphorylating PTKs). Thus, phosphatases are directly oxidized by ROS resulting in their inhibition and ultimately in the sustained activation of signaling pathways. This plays a key role in cell proliferation and survival in response to growth factor, hormone, and cytokine stimulation.

Furthermore, ROS can directly modulate the activation of key signaling molecules such as transcription factors. They act as master switch systems in higher organisms, significantly determining the gene expression profile and cellular response to oxidative stress, as detailed in the next sections.

Other functions of ROS include their role in mitoptosis⁵⁶ and autophagy.⁵⁷ Moreover, there is a cross-talk between ROS and Ca^{2+} .⁴² Multiple evidences show that intracellular Ca^{2+} modulates ROS generation and clearance processes and thereby shift the redox state from oxidized to reduced, and vice versa.^{58,59}

ROS in Inflammation

Imbalanced ROS are key players in the inflammatory process.⁶⁰ Oxidative stress-mediated signaling mechanisms are involved in inflammation and tissue injury. We acknowledge that many factors including activator protein 1 (AP-1), Redox factor-1 (Ref-1), p53 or hypoxia-inducible factor 1 (HIF-1) have been described to hold key effector roles in redox signaling. Nevertheless, in the following sections, we will focus on the involvement of ROS in a few fundamental aspects of the immune-inflammatory response. First, we will remark on the molecular mechanisms and relevance of ROS generation in inflammatory phagocytes. Indeed, NADPH oxidase-derived ROS has been associated with pathogen killing. Moreover, mitochondria-derived ROS hold also a relevant role in activating the inflammasomes, cytosolic multiprotein oligomers of the innate immune system driving the secretion of mature cytokines and pyroptosis, a pro-inflammatory form of programmed cell death. Furthermore, ROS range of actions encompasses the direct activation of the redox sensor nuclear factor erythroid 2-related factor (Nfr2). This ubiquitous transcription factor activates and modulates antioxidant, drug metabolism, anti-inflammatory detoxification and radical scavenging functions. Also, worth mentioning is its interplay with nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), another omnipresent redox-regulated transcription factor that controls cytokine production and cell survival. In fact, ROS overproduction impacts key pathways leading the inflammatory response such as the tumor necrosis factor receptor-alpha (TNF- α)-tumor necrosis factor receptor-1 (TNFR1) pathway, which balances cell survival, apoptosis and necroptosis through regulation of NF- κ B and, in turn, controls ROS signaling in immune cells. Finally, we will highlight the consequences of oxidative stress induced by polymorphonuclear cells (PMNs), stimulating the opening of inter-endothelial junctions and facilitating the migration of inflammatory cells across the endothelial barrier.

NADPH Oxidase-Derived ROS in Inflammatory Phagocytic Cells

NADPH oxidases (NOX) were first identified in phagocytes, acknowledging their role inducing respiratory or oxidative burst and bacterial killing.⁶¹ The oxidative burst is the first line of defense against environmental pathogens as a part of the innate immune system. It involves neutrophils and macrophages,⁶² and is characterized by the rapid production of large amounts of intracellular ROS and the activation of proteases that degrade phagocytosed microbes.⁶³ Non-mitochondrial ROS production during the oxidative burst results from the tightly regulated activation of NOX proteins. NOX1, NOX2 and NOX4, the major NOX isoforms expressed in the vascular system, are strongly implicated in inflammation-induced vascular injury.⁶⁴ Concomitantly, phagocytes such as granulocytes, neutrophils, monocytes and macrophages produce ROS using NOX2.⁶⁵

The generation of ROS begins with the rapid uptake of oxygen, activation of NOX and the production of the superoxide free radical anion, $\text{O}_2^{\cdot-}$. $\text{O}_2^{\cdot-}$ is then rapidly converted to H_2O_2 by SOD. These free radicals can destroy microorganisms or other foreign matter. In addition, $\text{O}_2^{\cdot-}$ and H_2O_2 react to provide OH^{\cdot} .⁶⁶ Neutrophil cytoplasmic granules contain $\text{O}_2^{\cdot-}$ molecules generated by NOX2 and the enzyme myeloperoxidase (MPO), which react with the ubiquitous ion chloride generating hypochlorous acid, a potent oxidant and antimicrobial agent.⁶⁷ Following phagocytosis, antigen presentation allows a more specific and targeted response against the pathogen. ROS play a role in this specific response triggering the amplification of intracellular signal transduction cascades in T lymphocytes.⁶⁸ Moreover, ROS regulate macrophages adjusting its intracellular redox state. This has direct consequences in the release of prostaglandins and of pro-inflammatory cytokines such as several interleukins (IL-6, IL-12), necessary to control the ratio of type 1 to type 2 helper T cells.⁶⁹

Microbes with common pathogen-associated molecular patterns (PAMPs) are recognized by specific pattern recognition receptors, complement components or growth factors, triggering NOX2 complex activation.⁷⁰ Analogously, exposure of a phagocyte to pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α), gamma-interferon (IFN- γ), and/or IL-1 β induces NOX2 complex formation. This significantly increases the ROS levels within the cell.⁷¹

The NOX2 complex is composed of five subunits: NOX2 (gp91phox) and the four phagocytic oxidases: p67phox, p47phox, p40phox and p22phox. To assemble the NOX complex under pathogen attack, the cytosolic p67phox, p47phox and p40phox subunits converge and recruit a small GTPase (Ras-related C3 botulinum toxin substrate 1 (RAC1) in monocytes or RAC2 in neutrophils), that colocalize with p22phox and NOX2 to the membrane.⁷² The NOX2 complex converts extracellular O₂ into O₂⁻, which is converted to H₂O₂ by SOD3 to generate ROS. These species penetrate the phagocyte membrane and act intracellularly to promote pathogen phagocytosis.⁶⁵

TNF-NOX2 signaling is believed to account for chronic inflammation and its associated tissue damage. ROS regulation is very important because it engages in a positive feedback loop in which the production of ROS is induced by TNF and, in turn, ROS trigger TNF expression through p38, NF-κB and c-Jun N-terminal kinases (JNK).⁷³

Riboflavin kinase (RFK) is important for ROS generation because it interacts with TNFR1 when coupled to the NOX2 complex, activated by pathogen stimulation.⁷⁴ Consequently, ROS are produced through a pathway involving Vav1, Rac2 and proline-rich tyrosine kinase-2 (Pyk2), which enhances the adherence of macrophages and neutrophils to endothelial cells.

NOX-generated ROS in macrophages induce the translocation of microtubule-associated protein 1 light chain 3 beta (MAP1A/MAP1BLC3B) to the phagosomal membrane, fusing lysosomes and clearing phagosomes in LC3-associated phagocytosis (LAP).⁷⁵ TLR4 signaling promotes the recruitment of mitochondria to phagosomes to enhance ROS production and kill bacteria.⁷⁶ This increases ROS production which, in turn, activates NF-κB in T cells.

Mitochondria-Derived ROS in Inflammation

Mitochondrial ROS (MtROS), a byproduct of mitochondrial respiration and metabolic enzyme activity, participate in key molecular functions and signal transduction pathways ensuing in inflammatory cells: 1) cytokine release induced by LPS, 2) NF-κB activation via inositol 1,4,5-trisphosphate receptor (IP3R), 3) Ca²⁺ signaling induced by thrombin, and 4) AP-1 activation induced by lysophosphatidylcholine (LPC), which triggers endothelial activation.⁷⁷

Another important role of MtROS is the regulation of the inflammasomes.⁷⁸ These high molecular weight

complexes activate inflammatory caspases (caspase-1 and 11) and cytokines (IL-1β and IL-18) in macrophages.⁷⁹ At least four different prototypes of inflammasomes (Nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain-containing, NLRP, also known as NALP) have been recognized: NALP1, NALP3 (also known as NLRP3, the best-characterized form), NLR Family CARD domain containing 4 (NLRC4 or ice protease-activating factor, IPAF), and absent in melanoma 2 (AIM2).⁸⁰ MtROS are involved in macrophage activation during inflammation and become primary regulators of NLRP3 inflammasome activation. Thus, in macrophages stimulated by LPS, MtROS regulate IL-1β transcription (inflammasome priming) and the maturation and secretion of IL-1β (inflammasome activation).⁸¹ On the other hand, thioredoxin-interacting protein (TXNIP), a negative regulator of Trx and a proapoptotic factor regulated by ROS, mediates the activity of MtROS and NOX4. TXNIP also influences inflammasome activation induced by glucose.⁸² Furthermore, an increased level of ROS in chronic inflammatory states inhibits mitophagy (the removal of malfunctioning mitochondria through autophagy)⁸³ and fosters inflammasome activation.⁸⁴

Master Switches and Pathways Regulated by ROS in Inflammation: NF-κB, Nrf2 and TNF-α -TNFR1 as Paradigms

Overall, ROS can activate NF-κB, a major redox-regulated transcription factor, in response to inflammatory agonists (Figure 2).⁸⁵ The translocation of NF-κB to the nucleus takes place in response to H₂O₂ through nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor-alpha (IκBα), tyrosine phosphorylation (Tyr42), phosphorylation of the serine/threonine PEST domain plus subsequent degradation via calpain and p65 phosphorylation.⁸⁶ In the nucleus, NF-κB initiates the transcription of pro-inflammatory molecules, including cytokines (IL-1, IL-6, TNF-α), cyclooxygenase-2 (COX-2), vascular adhesion molecules and inducible nitric oxide synthase (iNOS). ROS-induced NF-κB is inhibited by SOD2 overexpression,⁸⁷ and the NOX family of proteins influence, and are influenced by, NF-κB activity.⁸⁸ Specific inflammatory agonists use ROS as part of their signaling cascades. For example, LPS induces the activation of NF-κB through NOX4.⁸⁹

Nrf2 is a redox-sensitive transcription factor that regulates redox homeostasis, holding anti-oxidant, anti-inflammatory and detoxification responses.⁹⁰ Nrf2 resides

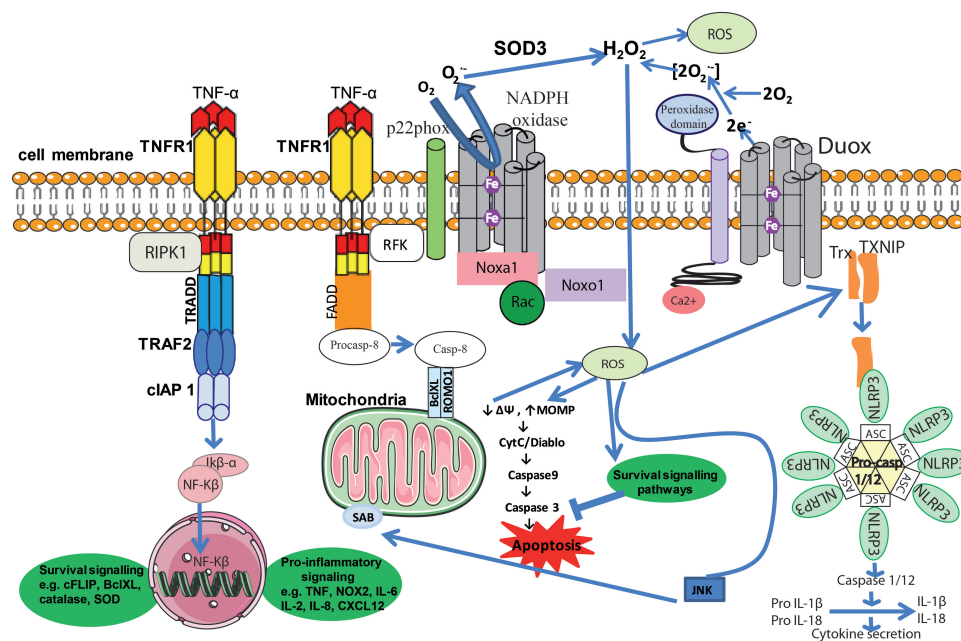


Figure 2 Inflammation and ROS. Elevated ROS production as a result of inflammatory signaling can mediate canonical NF- κ B activation and downstream inflammatory gene induction, proteasome activity, antioxidant gene transcription, inflammasome activation, and cytokine secretion. The intricate balance between cell death and cell survival is largely modulated by intracellular ROS generation. In general, high intracellular ROS generation causes cell death by activation of cell death pathways (mitochondrial-dependent and -independent), whereas low levels of ROS act as signaling molecules that facilitate cell survival. Death receptors such as TNFR1 cause cell death by inducing high intracellular ROS production. The binding of TNF- α to TNFR1 recruits proteins such as TRADD and TRAF2 to activate TNFR1 signaling. The associated TRADD further recruits FADD and pro-caspase-8, and this whole signaling complex known as DISC is endocytosed, resulting in caspase-8 activation. Similar to TNFR1, the extent of caspase-8 activation determines whether a cell will follow a mitochondrial-dependent pathway or an independent pathway. Low caspase-8 activation follows a mitochondrial-dependent amplification loop which causes Cyt-C release. The released Cyt-C then subsequently binds Apaf-1 and caspase-9 and activates effector caspases such as caspase-3, which causes cell death. In the event of significant caspase-8 activation, it directly activates caspase-3 independent of mitochondria. High intracellular ROS induce sustained JNK activation and causes mitochondrial Cyt-C-dependent cell death. TNFR1 signaling also activates the NF- κ B transcription factor by recruiting TRAF-2 and RIPK1 proteins. NF- κ B activation is involved in the transcription of anti-apoptotic signaling proteins such as cIAP-2 and Bcl-xL. On the other hand, TNF can activate the NOX1 (neutrophilic) or NOX2 (epithelial) complex. The first step of NOX1/2 activation is the interaction of the cytosolic domain of TNFR1 with RFK and p22phox. The activated NOX complex converts extracellular O_2 into $O_2^{\bullet-}$, which extracellular SOD3 then converts into extracellular H_2O_2 . The generated H_2O_2 passes freely through the plasma membrane and acts as a major source of intracellular ROS. Furthermore, TNF-induced formation of complex II leads to interactions between activated caspase-8, ROMO1 and Bcl-XL in the outer mitochondrial membrane, which reduce mitochondrial membrane potential, triggering MOMP and the production of MtROS. Conversely, if the levels of caspase-8 are high, TNF can activate directly caspase-3 independent of mitochondria, leading to apoptosis. Additionally, Ca^{2+} -activated DUOX produces even more H_2O_2 , which joins the cycle. Another important role of MtROS is the regulation of the inflammasome. MtROS are involved in immune cell activation during inflammation and are primary regulators of NLRP3 inflammasome activation. These high-molecular-weight complexes activate inflammatory caspases (caspase-1 and -11) and cytokines (IL-1 β and IL-18). Also illustrated are ROS activating NF- κ B directly or indirectly by inhibiting IKK phosphatases, and JNK stimulating mitochondrial ROS production through SAB. **Abbreviations:** TNF- α , tumor necrosis factor- α ; TNFR1, tumor necrosis factor receptor-1; RIPK1, receptor-interacting serine/threonine-protein kinase 1; TRADD, tumor necrosis factor receptor type 1-associated DEATH domain protein; TRAF2, TNF receptor-associated factor 2; cIAP1, cellular inhibitor of apoptosis proteins; I κ B- α , nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor- α ; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; cFLIP, cellular FLICE inhibitory protein; BclXL, B-cell lymphoma extra-large; SOD, superoxide dismutase; NOX2, NADPH oxidase 2; IL-, interleukin; CXCL, chemokine (C-X-C motif) ligand; FADD, Fas-associated death domain; Casp, Caspase; ROMO1, ROS modulator 1; SAB, SH3 homology associated BTK binding protein; e $^-$, electron; H_2O_2 , hydrogen peroxide; O_2 , oxygen; $O_2^{\bullet-}$, superoxide radicals; ROS, reactive oxygen species; DUOX, dual oxidase; Trx, thioredoxin; TXNIP, thioredoxin-interacting protein; NLRP3, NLR family pyrin domain containing 3; ASC, Apoptosis-associated speck-like protein containing a CARD; JNK, c-Jun N-terminal kinases; $\Delta\Psi$, mitochondrial membrane potential; MOMP, mitochondrial outer membrane permeability; Cyt-C, cytochrome C; DIABLO, direct inhibitor of apoptosis-binding protein with low pI; Ca^{2+} , calcium ion; Rac, Ras-related C3 botulinum toxin substrate; Noxo1, NADPH oxidase organizer 1; Noxa1, NADPH oxidase activator 1; Fe, iron; RFK, riboflavin kinase.

in the cytoplasm at a low basal level, but when induced by oxidative stress translocates to the nucleus⁹¹ where it binds to the antioxidant responsive element (ARE) sequence, necessary for ROS detoxification. Nrf2 is composed of seven functional domains (Neh1-7) that regulate its transcriptional activity and stability.⁹² The Neh1 is responsible for the translocation of Kelch-like ECH-associated protein 1 (KEAP1) into the nucleus.⁹³ The activity of the Nrf2-ARE signaling pathway is controlled by sequestration and degradation of Nrf2 into the cytoplasm by KEAP1.⁹⁴

Indeed, Neh2 is a negative regulatory domain that promotes Nrf2 degradation.⁹⁵ Neh3 modulates the transcriptional activation of ARE genes,⁹⁶ and Neh4 and 5 facilitate Nrf2 transcription.⁹⁷ Neh6 and 7 control Nrf2 degradation.⁹⁸

There is a crosstalk between Nrf2 and NF- κ B signaling pathways under stress or pathophysiological conditions.^{62,64,99} Studies using animal models¹⁰⁰ or different cell types, such as microglial cells and monocytes,^{101,102} suggest that Nrf2 up-regulation decreases pro-inflammatory and immune responses

that are regulated by NF- κ B. Thus, although not completely well understood, Nrf2 and NF- κ B influence each other to control anti-oxidant and inflammatory responses.¹⁰³ It has also been shown that Nrf2 overexpression inhibits RAC1-dependent activation of NF- κ B.¹⁰⁴ Interestingly, novel therapeutic approaches to prevent or reduce brain injury are being focused to restore Nrf2 activity while preventing NF- κ B activation. Thus, several phytochemicals such as curcumin, sulforaphane and metformin have shown a dual effect as Nrf2 activators and NF- κ B inhibitors.⁶³

TNF- α is a pro-inflammatory cytokine with important functions in mammalian immunity and cellular homeostasis.¹⁰⁵ This master multi-faceted regulator plays an important role in the control of cell survival, apoptosis, necroptosis and intercellular communication through two main pathways: 1) induction of apoptosis through ROS/JNK, and 2) reduction of the apoptotic signal through NF- κ B activation and triggering antioxidant gene expression.^{106,107} Dysregulation of these processes elicits inflammatory diseases and cancer. TNF can present in soluble (sTNF) or membrane-bound (mTNF) form. Its receptor TNFR1, is ubiquitously expressed on almost all cell types and can be activated by both TNF forms.¹⁰⁸ TNFR1 signaling under resting conditions is inhibited by association with a 60-kD cytoplasmic protein known as silencer of death domains (SODD). When TNF binds to TNFR1 the corresponding complex, TNF-TNFR1, recruits the adaptor protein tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD) and the receptor-interacting serine/threonine-protein kinase 1 (RIPK1),¹⁰⁹ initiating the assembly of membrane-bound TNFR complex I. That is, binding of TNF receptor-associated factor 2 (TRAF2) to cellular inhibitor of apoptosis proteins (cIAP1 and cIAP2).^{110,111} cIAP1 and cIAP2 facilitate the recruitment of the linear polyubiquitin chain assembly complex (LUBAC) to complex I^{106,112} and prevent inflammation¹¹³ and cell death. This is achieved through the recruitment of TGF β -activated kinase 1 (TAK1) and TAK1 binding protein 2/3 (TAB2/3), phosphorylation of I κ B α and activation of NF- κ B.¹¹⁴

RIPK1 acts as a molecular switch between cell survival and cell death, depending on its state of ubiquitination. When RIPK1 in complex I is deubiquitinated by cylindromatosis (CYLD) and TNFR1 is activated by TNF- α , RIPK1 dissociates from the membrane-bound TNFR1 signaling core. Then, it assembles in the cytosol with TRADD and Fas-associated death domain (FADD), and

the collective death-inducing signaling complex (DISC) is internalized in an endosome. The release of endosomal DISC to the cytosol further recruits initiator procaspase-8, resulting in its activation through complex IIa. This leads to apoptosis depending on the presence of cellular FLICE inhibitory protein (cFLIP_L).¹¹⁵ Complex IIb/necrosome is formed when dissociated and non-ubiquitinated RIPK1 assembles with the same molecular components of complex IIa, except TRADD, with further addition of RIPK3.¹⁰⁶

ROS are important regulators of TNF-TNFR signaling, leading to cell survival or death. The generally accepted hypothesis is that TNF-induced ROS suppress NF- κ B activation decreasing survival signaling and increasing cell death.¹¹⁶ Nevertheless, several evidences indicate that mitochondrial ROS can promote TNF-mediated NF- κ B activation.¹¹⁷ Thus, ROS can positively control NF- κ B signaling inhibiting the phosphatases involved in I κ B α phosphorylation, triggering its degradation and allowing NF- κ B activation.¹¹⁸

TNF-induced ROS signaling initiates with the activation of NOX1/2 (NOX2 concerning phagocytic cells and NOX1 non-phagocytic cells) through the interaction of TNFR1 with RFK and p22phox, which generates H₂O₂ via extracellular SOD3. This H₂O₂ traverses the extracellular membrane and is the main source of intracellular ROS. Moreover, TNF-induced formation of complex II (which contains procaspase-8) leads to interactions between BclXL and ROS modulator 1 (ROMO1) in the outer mitochondria membrane. This reduces the mitochondrial membrane potential ($\Delta\Psi$), which produces more MtROS through mitochondrial outer membrane permeability (MOMP). Thus, MtROS can contribute to apoptosis releasing mitochondria-derived pro-apoptotic factors such as cytochrome C (Cyt-C).^{119,120} High levels of ROS activate the JNK pathway by inhibiting MAPK phosphatases, while JNK stimulates MtROS production through SH3 homology associated BTK binding protein (SAB), an outer mitochondrial membrane protein.¹²¹ Increased MOMP can be induced by excessive Ca²⁺ entry and increased oxidative stress, resulting in mitochondrial membrane depolarization. Cyt-C then binds to apoptosis activation factor-1 (Apaf-1) and recruits initiator procaspase-9, which undergoes auto-activation. This whole complex is collectively known as “apoptosome”.¹²² Furthermore, caspase-9 activates effector caspases such as caspase-3. Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with

low pI (Smac/DIABLO) also facilitates the activation of effector caspases by removing the blockage by an inhibitor of apoptosis proteins (IAPs).¹²³ Apoptosis-inducing factor (AIF), on activation, induces DNA condensation and cleavage.¹²⁴ EndoG-mediated DNA fragmentation is the final event in the apoptosis process. The mitochondrial release of Cyt-C is considered the major determinant of cell death, and Smac/DIABLO, which activates caspase-9 and -3, executes apoptosis.¹¹⁵

Indeed, TNF-induced ROS production is important in the crosstalk between the NF- κ B-induced cell survival pathway and the JNK-induced cell death pathway.^{125,126} SAB activates JNK and thus increases MtROS generation, sustaining JNK activation in a self-amplifying loop. Moreover, the quantity of activated caspase-8 will determine whether a cell follows a mitochondrial-dependent (low levels) or -independent (high levels) pathway. Furthermore, dual oxidase (DUOX), the major NOX isoform expressed preferentially in the lungs¹²⁷ is activated by Ca^{2+} , the concentration of which is enhanced by ROS, and further increases H_2O_2 production, fostering the self-amplifying cycle.

Oxidative Stress Regulates the Opening of Endothelial Junctions and Immune Cell Migration Across the Endothelial Barrier

Cell adhesion molecules (CAMs) are expressed on activated endothelial cells and leukocytes. They mediate the migration of inflammatory cells through the endothelium of postcapillary venules. Neutrophils, the first cells to reach the inflamed scene, cross the endothelial barrier mainly through endothelial junctions.¹²⁸ The extravasation process in response to inflammatory stimuli is regulated by oxidative stress induced by the own extravasating leukocytes.^{129,130} This can occur directly through CAMs activation: intracellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin^{131–133} (Figure 3), including integrins that bind to ICAM-1.¹³⁴ Alternatively, neutrophil extravasation may involve indirect activation through a transcription-dependent mechanism involving redox-sensitive transcription factors (ie, NF- κ B and AP-1). Thus, oxidative stress induced by leukocytes at the site of inflammation plays a crucial role disturbing endothelial junction assembly. Both adherens junctions (AJs) and tight junctions (TJs) initiate their disassembly, leading to gap formation between cells.¹³⁵

ROS-mediated regulation of intracellular free Ca^{2+} concentration is also a major mechanism of increased vascular permeability, the hallmark of inflammation. An increase in intracellular Ca^{2+} was associated with enhanced H_2O_2 generation.¹³⁶ Moreover, the major Ca^{2+} driveway in endothelial cells is through store-operated Ca^{2+} channels (SOC channels: TRPC1 and TRPC4), receptor-operated Ca^{2+} channels (ROC channels: TRPC3, TRPC6 and TRPC7), and transient receptor potential melastatin channels (TRPM), presumably regulated by ROS.¹³⁷

Other enzymes that contribute to the opening of endothelial junctions include: 1) myosin light chain (MLC), which is activated by ROS and contributes to actin reorganization in endothelial cells,¹³⁸ 2) Cell division control protein 42 (Cdc42), a member of the Rho GTPase family involved in ethanol-induced NADPH oxidase activation and ROS generation, rearranging actin filaments in endothelial cells;¹³⁹ 3) protein kinase C (PKC) isoforms, activated by H_2O_2 , targets for multiple tyrosine phosphorylation events by various tyrosine kinases, including the Src family kinases;¹⁴⁰ and 4) toll-like receptors (TLRs), a subset of the pathogen-associated pattern recognition receptors family expressed in a wide range of immune cells the most relevant of which is TLR4, receptor for LPS signaling.¹⁴¹

ROS and Aging

The multifactorial and inexorable phenomenon of aging deteriorates human homeostasis at multiple levels, reducing gradually the ability to resist stress, damage, and illness.¹⁴² Aging mechanisms have been classified into two main categories: 1) genetically programmed by developmental processes (cell senescence, neuroendocrine alterations, immunological alterations), and 2) caused by random damage, that is, accumulation of somatic mutations and oxidative stress. Here we will focus on the “free radical theory of aging” and its evolved “mitochondrial free radical theory of aging”, highlighting the ROS ability to induce cell senescence in aging processes. Nevertheless, ROS generation might not be the initial trigger of the aging process. In fact, several studies present an alternative point of view to Harman’s hypothesis,² whose free radical theory remains inconclusive. These reports suggest that: 1) oxidative damage is just one type of damage, 2) biological imperfectness drives the aging process, and 3) cumulative damage defines lifespan.^{143,144} Accordingly,

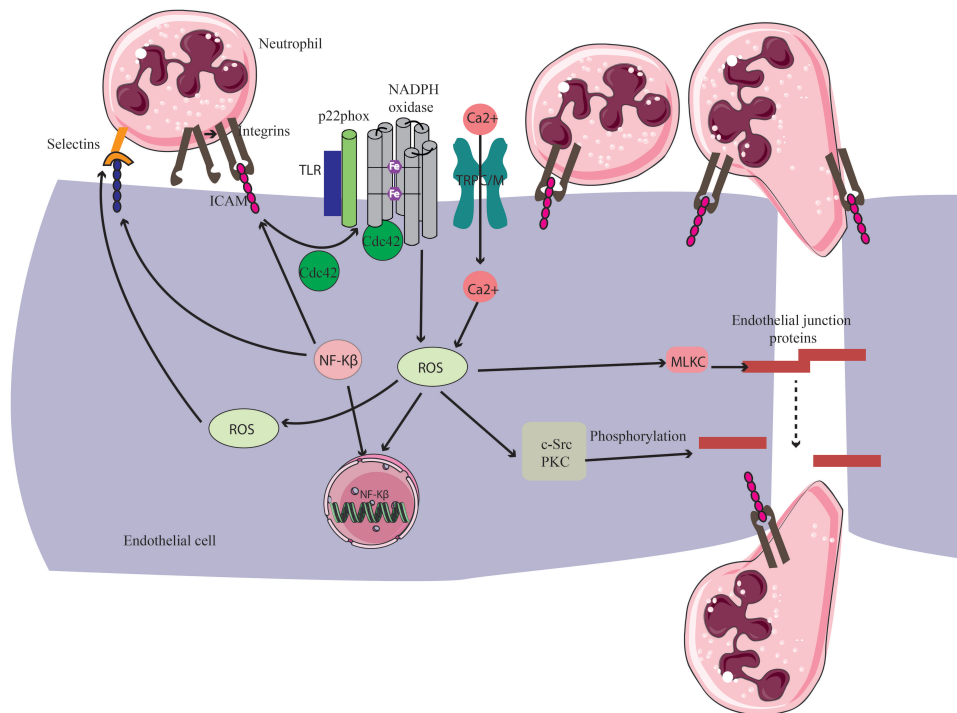


Figure 3 Role of ROS in neutrophil migration through endothelial cells. Integrins bind to ICAM-1 expressed in activated endothelial cells and arrest neutrophils on the endothelial surface. This leads to the activation of Cdc42 and Rac, which induce intracellular ROS generation through NOX. Increased ROS are involved in activation of different kinases, including c-Src and PKC, and thus induce phosphorylation and destabilization of endothelial junction proteins such as adherens junction. Moreover, ROS enhance the expression of P-selectin on the endothelium. Furthermore, ROS are known to activate NF- κ B, inducing the expression of cell adhesion molecules such as ICAM-1, VCAM-1 and E-selectin which, in turn, enhance neutrophil binding on the endothelium. Acute exposure to pro-inflammatory factors triggers NOX-mediated ROS, which results in an increase of $[Ca^{2+}]$ by TRPC/TRPM ion channels. Increased $[Ca^{2+}]$ activates calmodulin and several downstream kinases including myosin light chain kinase (MLCK), which contribute to the disassembly of endothelial junction proteins.

Abbreviations: NOX, NADPH oxidase; TRPC/M, transient receptor potential canonical/transient receptor potential melastatin channels; Ca^{2+} , calcium ion; Cdc42, cell division control protein 42; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; ROS, reactive oxygen species; TLR, toll-like receptor; ICAM, intracellular cell adhesion molecule; c-Src, proto-oncogene tyrosine-protein kinase Src; PKC, protein kinase C.

antioxidant strategies against aging will be briefly considered.

The Free Radical Theory of Aging

One of the preferential causal theories of aging is the “free radical damage theory”, firstly proposed by Denham Harman in the 1950s to explain aging focusing on ROS generation. Free radicals cause oxidative damage to the macromolecular components of the cell, affecting homeostasis (cell death, inflammation) and shaping the organism’s pathophysiological network. Indeed, multiple studies have reported age-related oxidative modifications to a large variety of proteins. These include structural proteins, enzymes, and proteins important in signal transduction pathways.^{145–149} Furthermore, telomerase shortening is affected by oxidative damage.^{150,151} As stated previously, ROS such as $OH\cdot$, $O_2\cdot^-$, and H_2O_2 are extremely reactive and are the major cause of damage to proteins, lipids, and DNA. Oxidative damage to these biomolecules seems to depend on H_2O_2

and a reduced transition metal.¹⁵² Therefore, molecules that contain transition metals, such as aconitase, are likely to undergo oxidative damage.¹⁵³

Any perturbation of the redox balance during aging can activate the redox-sensitive transcription factors and generate numerous pro-inflammatory mediators. In addition to the apoptotic and senescence pathways, redox modification of transcription factors can regulate cellular proliferation, differentiation, senescence, death, and aging. For instance, NF- κ B altered signaling has been causally linked to aging and diverse pathological conditions.¹⁵⁴ Macrophages, central effectors of the innate immune system, are strongly involved in inflammation and generate reactive species during age-associated diseases.¹⁵⁵ However, there is still a lack of evidence to conclusively determine that inflammation is one of the causal factors influencing biological aging and longevity in mammalian species.

Mitochondria play a significant role in the free radical theory of aging.¹⁵⁶ According to the endosymbiotic theory¹⁵⁷,

aging started 1 billion years ago with mitochondria. Because mitochondria are mostly responsible for the production of ROS, in the 1970s the free radical theory of aging was extended into the “mitochondrial free radical theory of aging”.¹⁵⁸ It states that aging is produced by the toxicity of ROS through a malicious cycle in which ROS injury to mitochondrial elements precedes the generation of more ROS. The mitochondrial activity declines with age because of replication errors of mitochondrial DNA (mtDNA) and toxic adducts on mitochondrial proteins.¹⁵⁹ These adducts are harmful to the function of proteins, being promoted by high levels of acetyl-CoA and succinyl-CoA, which acylate the ϵ -amino groups of lysines. Sirtuins like SIRT3, SIRT4 and SIRT5, which are enriched in mitochondria and possess deacetylase activity, catalyze the removal of the adducts.¹⁶⁰ This decline in mitochondrial function includes a decreased activity of the respiratory chain and ATP synthase, Krebs cycle fluxes, oxidative alterations of cardiolipin, disrupted regulation, and defective mtDNA regulation and activity.¹⁶¹ This results in an increase in the production of $O_2^{\bullet-}$ and H_2O_2 in mitochondria, causing oxidative damage to mtDNA (more sensitive than nuclear DNA) and mitochondrial membrane lipids.¹⁶² Consequently, changes in mitochondrial membrane permeability ensue, which culminates in the release of apoptogenic factors mediating cell apoptosis through the previously described pathway.¹⁶³

The cellular senescence process, which influences somatic, tumor and stem cells¹⁶⁴ is considered an aging hallmark. It drives the cell through longevity, by hampering tumorigenesis and cell death, and is involved in many age-related diseases.

The DNA damage caused by ROS as mutating agents induce and maintain the cell senescence process.¹⁶⁵ Moreover, attention has also been focused on the ROS involvement as signaling molecules in cell senescence induction without DNA damage, through Ras, p53, p21 or p16.¹⁶⁶ Furthermore, ROS contribution at the epigenetic level seems to modulate cell senescence.¹⁶⁷ Hence, senescent cells have been identified as a novel potential therapeutic target in aging and age-related diseases.¹⁶⁸

Therapeutic Strategies Against Aging

Studies in different model organisms indicate that inhibition of oxidative stress contributes to an increase in the life span. For example, administration of Vitamin E was previously shown to extend the life span of different animals.^{169,170} Nevertheless, despite a vast amount of publications on ROS and the implications of oxidative stress

and free radical damage in almost all pathological conditions, no pharmaceutical drugs are yet available or prescribed for antioxidant therapies in the clinical practice. Similarly, the commercial use of many natural antioxidants, mainly in the form of dietary supplements is a fact. Nevertheless, the results of a number of clinical trials suggest that, as yet, there is no sound clinical evidence that such antioxidants can represent effective treatments in any clinical condition.^{171,172} Notable exceptions are GSH (administered either orally or by inhalation),^{173,174} although questioned,¹⁷⁵ and perhaps β -carotene at high doses.^{176,177} Indeed, antioxidants have met with limited success because only a small fraction reaches mitochondria.¹⁷⁸ To circumvent this limitation, antioxidants are being conjugated with lipophilic cations so that they can accumulate in mitochondria.¹⁷⁹ Alternatively, human clinical trials regarding dietary supplementation have reported to reduce ROS injury related to aging.¹⁸⁰ Thus, vitamin D supplementation can reduce the risk of hip and other fractures in housebound elderly.¹⁸¹ Moreover, functional foods and nutraceuticals possessing antioxidant activity, such as supplemented blueberry extract, might slow the aging process.¹⁸²

Human aging could also be influenced by the concentration and activity of enzymes involved in the antioxidant defense system. Several reports suggest that the levels of SOD, catalase, and glutathione peroxidase from a variety of tissues (liver, brain, kidney, heart, etc.) are reduced in senescent animals.¹⁸³ In this respect, SOD has demonstrated great physiological relevance and therapeutic potential in various pathological conditions such as cancer, inflammatory diseases, cystic fibrosis, ischemia, aging, rheumatoid arthritis, neurodegenerative diseases and diabetes.¹⁸⁴ Accordingly, SOD has proved effective in preclinical studies.¹⁸⁵ SOD is also considered an anti-aging enzyme. In animal models such as *Drosophila*, a reduction in SOD activity accelerated loss of olfactory behavior on aging.¹⁸⁶ Furthermore, SOD mimetics, synthetic compounds that have an equivalent function as SOD, attenuate aging.¹⁸⁷ On the other hand, energy restriction diet, moderate reduction of nutrient availability without malnutrition, significantly increased the expression of SOD1 and SOD2 and extended lifespan by 16% in fruit flies.¹⁸²

Although contrasting reports and inconclusive studies have emerged, as stated previously, it can be postulated that the increase in oxidative stress and damage to cellular constituents associated with aging could be due to

a decline in antioxidant defense systems. For many years, coenzyme Q 10 (CoQ₁₀) has been considered a key factor in the progression of aging-associated complications.¹⁸⁸ However, although several studies have supported the safety and the potential of CoQ₁₀ reducing oxidative stress biomarkers, adequate large-scale clinical trials are needed to confirm the beneficial effects of CoQ₁₀ supplementation.

Concluding Remarks

A double-edged sword of ROS in health and pathology is being increasingly appreciated, although the underlying mechanisms are still incompletely understood. In physiological processes environmental and metabolic ROS levels are tightly regulated and play an essential role as signaling molecules, contributing to cell and tissue homeostasis. Conversely, excess of ROS generation and/or impaired antioxidant activity under acute and chronic oxidative stress associate with metabolic “reprogramming”, inflammation, tissue damage/dysfunction and toxicity, leading to senescence or death. Emerging “omics” technologies together with single-cell analyses and novel imaging tools will allow specific, spatiotemporal, comprehensive and quantitative dissection of the whole spectrum of pathways and processes induced by ROS at whole tissue or organism level. In this scenario, further advances in personalized redox medicine may combine dietary/nutritional and pharmacological strategies (anti-inflammatory drugs, activators of mitophagy, uncouplers, hTERT activators, antioxidants). Whether successful, these approaches might enable to tip the balance equilibrating the redox state of the tissues towards homeostasis, while maintaining the ability of immune cells to clear infections (eg, pathogen killing through the respiratory burst of neutrophils). This will surely become highly beneficial in a variety of age-related pathologies and ultimately in the health of the elderly population.

Acknowledgments

We thank CERCA Programme/Generalitat de Catalunya for institutional support. J.C. is supported by a grant from the “Associació Catalana de Fibrosi Quística”. J.M.A. has received support from the Ministerio de Economía y Competitividad (Madrid, Spain) (grants FIS-ISCIPII13/01490 and PI16/00377, co-funded by FEDER funds/European Regional Development Fund (ERDF)-a way to build Europe-), and from the Generalitat de Catalunya (grant 2014S GR 541). J.M.A. is sponsored by the

“Researchers Consolidation Program” from the SNS-Dpt. Salut Generalitat de Catalunya (Exp. CES06/012).

Disclosure

The authors report no conflicts of interest in this work.

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