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ROS Function in Redox Signaling and Oxidative Stress

Review

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Oxidative stress refers to elevated intracellular levels of reactive oxygen species (ROS) that cause damage to lipids, proteins and DNA. Oxidative stress has been linked to a myriad of pathologies. However, elevated ROS also act as signaling molecules in the maintenance of physiological functions — a process termed redox biology. In this review we discuss the two faces of ROS — redox biology and oxidative stress — and their contribution to both physiological and pathological conditions. Redox biology involves a small increase in ROS levels that activates signaling pathways to initiate biological processes, while oxidative stress denotes high levels of ROS that result in damage to DNA, protein or lipids. Thus, the response to ROS displays hormesis, given that the opposite effect is observed at low levels compared with that seen at high levels. Here, we argue that redox biology, rather than oxidative stress, underlies physiological and pathological conditions.

Introduction

Reactive oxygen species (ROS) are by-products of aerobic metabolism. ROS include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·), all of which have inherent chemical properties that confer reactivity to different biological targets. ROS are often associated with the principle of oxidative stress, which suggests that ROS induce pathology by damaging lipids, proteins, and DNA [1]. However, in the past two decades it has become apparent that ROS also serve as signaling molecules to regulate biological and physiological processes [2]. It appears that, early in evolution, nature selected for ROS as a signal transduction mechanism to allow for adaptation to changes in environmental nutrients and the oxidative environment [3]. Indeed in prokaryotes, there are well-described mechanisms whereby ROS directly activate transcription factors for adaptation to stress [4].

An understood mechanism of redox signaling involves H_2O_2-mediated oxidation of cysteine residues within proteins [5]. Cysteine residues exist as a thiolate anion ($Cys–S^-$) at physiological pH and are more susceptible to oxidation compared with the protonated cysteine thiol (Cys–SH) [6]. During redox signaling, H_2O_2 oxidizes the thiolate anion to the sulfenic form (Cys–SOH), causing allosteric changes within the protein that alter its function. The sulfenic form can be reduced to thiolate anions by the disulfide reductases thioredoxin (Trx) and glutaredoxin (Grx) to return the protein function to its original state [7]. Hence, first-degree oxidation of cysteine residues within proteins serves as a reversible signal transduction mechanism. It is estimated that thiolate oxidation in living cells occurs in the nanomolar range of H_2O_2, whereas higher levels of H_2O_2 further oxidize thiolate anions to sulfinic (SO_2H) or sulfonic (SO_3H) species. Unlike sulfenic modifications, sulfinic and sulfonic modifications can be irreversible and result in permanent protein damage (i.e. oxidative stress). Cells therefore have professional enzymes dedicated to prevent the build-up of intracellular H_2O_2 — primarily, peroxiredoxins and glutathione peroxidases (Figure 1).

H_2O_2 is generated from superoxide produced by mitochondria and NADPH oxidases [8,9]. Superoxide results from the one-electron reduction of molecular oxygen (O_2) and, within the cell, is rapidly converted by superoxide dismutases 1 and 2 (SOD1 and 2) into H_2O_2. SOD1 is primarily located in the cytosol and mitochondrial intermembrane space, whereas SOD2 localizes to the mitochondrial matrix. SODs prevent the accumulation of superoxide, which can damage and inactivate proteins containing iron–sulfur clusters [10]. Accumulation of superoxide is therefore more associated with oxidative stress than redox signaling. However, it is important to note that superoxide does not indiscriminately damage proteins: a specific set of proteins that are sensitive to inactivation by superoxide activate signaling pathways that either promote adaptation to elevated superoxide or initiate cell death [11]. This supports our current view of oxidative stress as a combination of cellular damage- and stress-responsive signaling. The third type of ROS is the extremely reactive hydroxyl radical, which indiscriminately oxidizes lipids, proteins, and DNA, resulting in damage or genomic instability [12]. Typically, hydroxyl radicals are generated from H_2O_2 in the presence of ferrous ions (i.e. the Fenton reaction). Therefore, cells have multiple mechanisms to maintain iron homeostasis to prevent the formation of toxic hydroxyl radicals. It is important to note that the changes in H_2O_2 required for signaling do not cause significant changes in intracellular ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH), or in the ratio of nicotinamide adenine dinucleotide phosphate (NADPH) to its oxidized form, NADP+ (glutathione is the most abundant antioxidant in the cell, while NAPDH is utilized to regenerate a myriad of antioxidants, including glutathione) [13]. In fact, large changes in these parameters are usually a sign of oxidative stress causing toxicity rather than signaling associated with redox biology [14].

Aside from the specificity and selectivity of ROS on their targets, the compartmentalization of ROS production within cells is an important determinant of whether damage or redox signaling occurs. In order for effective redox signaling to take place, the H_2O_2-dependent oxidation of a given protein is likely to occur close to the source of H_2O_2 production. For example, the protein targets of H_2O_2 generated from plasma membrane NADPH oxidases are also located at the plasma membrane. Mitochondria are known to move dynamically towards their targets, thus allowing mitochondrially generated H_2O_2 to activate signaling pathways [15]. Similarly, superoxide accumulation in the mitochondrial matrix has different outcomes from superoxide accumulation in the cytosol, in part due to a high content of iron–sulfur cluster proteins in the mitochondrial matrix. Indeed, SOD2 knockout mice have a dramatically severe pathological phenotype compared with that of SOD1 knockout mice. Accordingly, both the type of ROS and its local concentration

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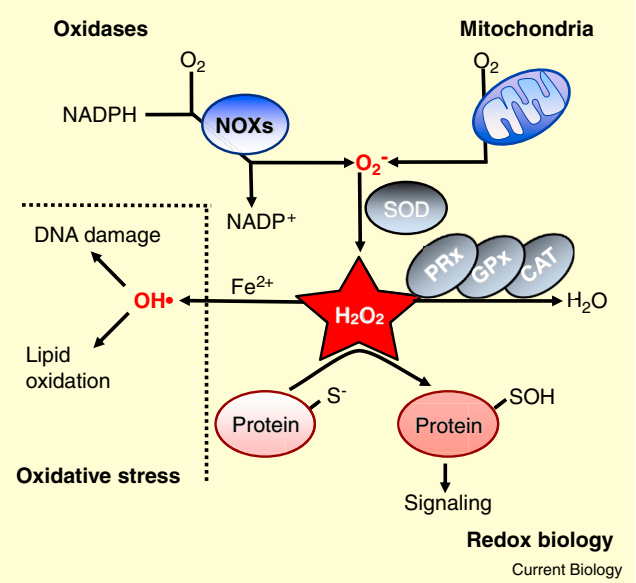

Figure 1. Basics of ROS.

Intracellular superoxide (O_2^-) is primarily produced by the oxidation of NADPH by NAPH oxidase enzymes (NOXs) or by electron leak from aerobic respiration in mitochondria. Superoxide is rapidly converted into hydrogen peroxide (H_2O_2) by compartment-specific superoxide dismutases (SODs). H_2O_2 is capable of oxidizing cysteine residues on proteins to initiate redox biology. Alternatively, H_2O_2 may be converted to H_2O by cellular antioxidant proteins, such as peroxiredoxins (PRx), glutathione peroxidase (GPx), and catalase (CAT). When H_2O_2 levels increase uncontrollably, hydroxyl radicals (OH·) form via reactions with metal cations (Fe^{2+}) and irreversibly damage cellular macromolecules.

collectively determine whether redox signaling or oxidative-stress-induced damage occurs.

In this review, we will discuss these two faces of ROS — redox biology and oxidative stress. We will focus on both physiological and pathological conditions using the examples of normal and cancer cell proliferation; beneficial and pathological inflammation; and the normal and accelerated aging process.

ROS and Regulation of Normal and Cancer Cell Proliferation

Metazoans use growth factors to coordinate mitogenic, survival, and nutrient uptake signals for cell growth and proliferation [16]. Growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), activate the intrinsic tyrosine kinase activity of their receptor tyrosine kinases (RTKs), leading to the autophosphorylation of specific tyrosine residues on the cytoplasmic tails of the receptor [17]. This recruits multiple proteins to the receptor, resulting in activation of several key signal transduction pathways — notably, phosphatidylinositol 3-kinase (PI3K)–AKT signaling and the RAS-MEK-ERK MAP kinase cascade — to promote cell proliferation, nutrient uptake, and cell survival [18]. However, RTKs and PI3K are negatively regulated by protein tyrosine phosphatases (PTPs) and the phosphatase (and tumor suppressor) PTEN, respectively, resulting in dampening of mitogenic signaling [19,20]. Thus, sustaining signal transduction pathways requires the inactivation of these phosphatases.

Initial experiments demonstrated that PDGF and EGF can rapidly and transiently increase ROS generation through NADPH oxidases and that these ROS were required for growth-factor-induced receptor tyrosine phosphorylation [21]. Subsequently, it was demonstrated that H_2O_2 produced in response to EGF led to the oxidation of the catalytic cysteine of protein tyrosine phosphatase 1B (PTP1B) to a sulfenic moiety, causing inactivation of this phosphatase [22]. PTP1B dephosphorylates tyrosine residues of the EGF receptor (EGFR) [23]. Thus, its inactivation by H_2O_2 results in increased tyrosine phosphorylation of EGFR and transmission of downstream growth signaling. The oxidized PTP1B is reactivated by thioredoxin, illustrating the reversibility of the redox signal [24]. Intracellular MAP kinase signaling following PDGF stimulation is also reinforced through oxidation and inactivation of the PDGFR-associated phosphatase SHP-2 [25]. In fact, H_2O_2 can reversibly oxidize a number of purified PTP family members *in vitro*, resulting in their inactivation [26]. Purified human PTEN is inactivated by H_2O_2 through oxidation and disulfide bond formation between Cys121 and Cys71, a modification reversed by thioredoxin activity [27]. Increased levels of oxidized PTEN can be measured in cells shortly after stimulation with various growth factors involved in PI3K activation [28]. Normally, the abundance of peroxiredoxins quickly decreases H_2O_2 upon growth factor stimulation [29,30]. However, recent data indicate that a local pool of peroxiredoxin 1 (PRX1) associated with cell membranes is phosphorylated and inactivated upon growth factor stimulation, allowing accumulation of local H_2O_2 and inhibition of phosphatase activity [31]. Since this PRX1 inactivation is localized to membranes it allows intracellular PRX1 pools to remain active, thus preventing peroxide build up in the cells. These data support a model where growth factor activation must be accompanied by a localized burst in ROS production at the plasma membrane. ROS then inactivates the action of phosphatases, reinforcing proliferative signaling pathways. Recent studies indicate that, in addition to NADPH oxidases, mitochondrial ROS can also inactivate phosphatases through oxidation [32].

Cancer cells 'hijack' normal cell machinery by constitutively activating growth factor pathways to sustain cellular growth and proliferation [33]. This allows cancer cells to take up abundant nutrients, survive stress, and continuously proliferate. Consequently, the 'hyper-metabolism' of cancer cells causes abundant generation of ROS from mitochondria and the endoplasmic reticulum, as well as by the action of NADPH oxidases [34]. Initial observations over two decades ago demonstrated that cancer cells generate higher levels of ROS than their non-transformed counterparts [35]. It was assumed that these elevated ROS levels caused genomic instability and thereby promoted tumorigenesis [36]. However, chromosomal instability is likely attributable to loss of p53 and other mechanisms that promote aneuploidy. Cancer cells driven by the *MYC* oncogene demonstrate no detectable increase in chromosomal instability and drive tumorigenesis through a ROS-dependent increase in signaling pathways [37]. Furthermore, treatment with the antioxidant N-acetyl-cysteine (NAC) or with inhibitors of NADPH oxidase prevents mitogenic signaling pathways in oncogenic *Kras*-driven mouse fibroblasts [38]. Human cancer cells driven by oncogenic *KRAS* require mitochondrial ROS for proliferation [39]. Mitochondrial mutations resulting in TCA cycle or electron transport chain dysfunction generate ROS to activate tumorigenic signaling pathways, including those involving PI3K and MAP kinase signaling [40–42]. Another

important target of ROS is the transcription factor NF-κB, which is known to control the survival of tumor cells [43]. NF-κB was of one of the earliest transcription factors discovered to be responsive to ROS [44].

In cancer cells, the high rate of ROS production is counterbalanced by an equally high rate of antioxidant activity in order to maintain redox balance [45]. If cancer cells do not control their ROS levels then they are susceptible to oxidative-stress-induced cell death [46,47]. Steady-state ROS levels in cancer cells are determined by both the rate of ROS production and also the rate of ROS scavenging. Thus, at steady state, cancer cells can display either an increase or a decrease in ROS compared with normal cells. Additionally, the signaling pathways that are responsive to H_2O_2 are localized close to the sources of ROS generation, allowing activation of these pathways despite the high overall antioxidant activity in cancer cells that protects against oxidative-stress-induced cell death.

The major mechanism by which cancer cells increase their antioxidant proteins is through activating the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) [48]. Normally NRF2 interacts with Kelch-like ECH-associated protein 1 (KEAP1) and is thereby targeted for proteasomal degradation. Elevated ROS oxidizes redox-sensitive cysteine residues on KEAP1, resulting in dissociation of KEAP1 from NRF2. Subsequently, NRF2 translocates to the nucleus, heterodimerizes with the small MAF protein and binds to antioxidant-responsive elements (AREs) within the regulatory regions of multiple antioxidant genes. Aside from elevated ROS, signaling pathways involving ERK MAP kinase and PI3K can also activate NRF2. Furthermore, certain tumor cells display mutations of KEAP1, leading to constitutive activation of NRF2 [49]. The loss of NRF2 in cancer cells increases oxidative stress, resulting in diminished tumorigenesis [50]. It is important to note that loss of NRF2 reduces multiple antioxidant defense systems, thus making multiple types of ROS (i.e. superoxide, peroxide and hydroxyl radicals) increase at a threshold that invokes damage to cancer cells. However, the loss of a specific antioxidant defense system might not elevate ROS levels above the threshold that causes damage. In this scenario, the elevated ROS levels hyperactivate signaling pathways to promote tumorigenesis, as observed following the loss of PRX1 [51,52].

If increased ROS levels are essential to promote and reinforce proliferative signals, one might predict that tumor suppressors could serve as antioxidants, reducing cellular ROS to levels that do not support proliferation. The highly mutated tumor suppressor p53 controls the expression of a variety of antioxidant genes [53]. Tumor formation in p53-deficient mouse models can be suppressed through dietary supplementation with NAC, suggesting that a primary tumor-suppressive function of p53 in certain cancers is to decrease ROS [54]. Furthermore, a recent study suggested that the major tumor-suppressive function of p53 might be the regulation of antioxidant and metabolism genes rather than apoptosis and cell-cycle arrest [55]. The induction of expression of the p53 target TIGAR is one mechanism by which p53 regulates metabolism to control antioxidant function [56]. TIGAR functions as a fructose-2,6-bisphosphatase, lowering the levels of fructose-2,6-bisphosphate, a positive regulator of phosphofructokinase-1 [57]. This results in a decrease in glycolytic flux and shunting of glucose carbons into the pentose phosphate pathway to produce NADPH, which is required to maintain many antioxidant systems. Other tumor suppressor genes, such as the FOXO transcription factors, also repress tumorigenesis by inducing the expression of antioxidants [58].

While ROS play a key role in maintaining mitogenic signals to drive cancer cell proliferation, they are also integral in adapting to the metabolic stress that occurs when highly proliferative tumors outstrip their blood supply [59]. The resulting tissue hypoxia stabilizes the family of transcription factors termed hypoxia-inducible factors (HIFs) [60]. HIFs are heterodimeric species, consisting of an oxygen-sensitive subunit, HIFα, and a constitutively stable subunit, HIFβ. HIFα is hydroxylated at proline residues by prolyl hydroxylases (PHDs) and these hydroxylated proline residues are then recognized by the E3 ubiquitin ligase von Hippel-Landau protein (pVHL), which targets HIFα to the proteasome [61]. Under hypoxic conditions, HIFα is not hydroxylated by PHDs, thereby preventing pVHL from targeting HIFα to the proteasome. Subsequently, HIFα translocates to the nucleus and dimerizes with HIFβ, regulating metabolic adaptation to hypoxia and the expression of pro-angiogenic genes such as vascular endothelial growth factor (VEGF) [62]. Hypoxia increases ROS production, leading to HIFα stabilization through the inhibition of PHDs [63,64]. ROS-mediated induction of HIFs can promote tumorigenesis of certain cancer cells [37,65,66]. Furthermore, the tumor suppressor activity of the sirtuin protein SIRT3 involves the upregulation of antioxidant defenses to prevent HIF activation [67,68].

In summary, we support a model in which tumorigenic cells generate high levels of ROS to activate proximal signaling pathways that promote proliferation, survival and metabolic adaptation (i.e. redox biology). At the same time, cancer cells maintain a high level of antioxidant activity to prevent build-up of ROS to levels that could induce cell death i.e. oxidative stress (Figure 2). This presents a conundrum in how to approach ROS therapy in cancer: should treatments focus on lowering ROS levels to prevent signaling or on increasing ROS to selectively kill cancer cells? A systematic review of randomized control studies with the antioxidants β-carotene, vitamin A, and vitamin C concluded no significant benefit, and possibly a detrimental effect, of these agents in cancer prevention [69]. However, it is possible that more targeted antioxidants that specifically become enriched in cancer cells or prevent localized ROS production from mitochondria and NADPH oxidases may provide a clinical benefit. While randomized control human trials with pro-oxidant cancer therapy have not yet been completed, there is accumulating evidence that raising ROS levels through small molecules can selectively induce cancer cell death by disabling antioxidants [70–72]. There are two caveats to this approach, First, if the ROS levels are not sufficiently raised within the cancer cell then the therapy would simply further activate NF-κB, PI3K, HIFs and MAP kinases to promote tumorigenesis. Second, agents should disable only the antioxidants used by cancer cells and not those also used by normal cells. Since the role of ROS and sensitivity to both oxidants and antioxidants likely differs between cancer types, continuing to test both oxidants and antioxidants *in vivo* will hopefully yield new agents to add to existing chemotherapy regimens.

ROS and Regulation of Inflammation

The innate and adaptive immune systems are critical for pathogen-specific defense and immunological memory.

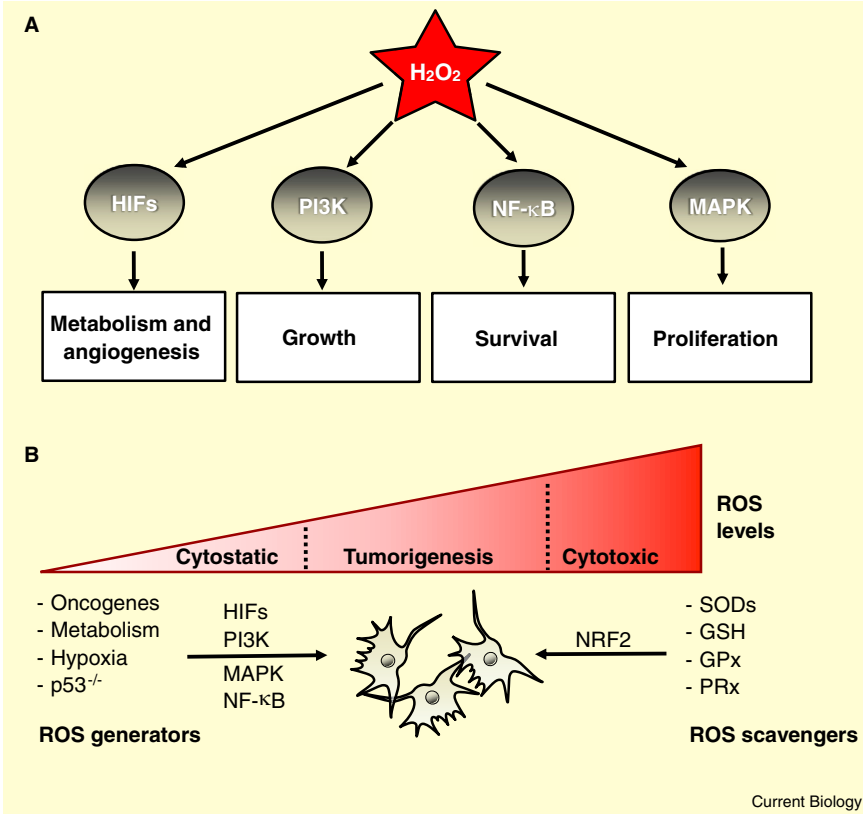

Figure 2. ROS regulation of normal and cancer cell proliferation.

(A) H_2O_2 is required for activation of a number of cellular pathways involved in cellular growth, survival and proliferation and in metabolism and angiogenesis. (B) Cancer cells generate higher levels of ROS that are essential for tumorigenesis. Genetic alterations leading to activation of oncogenes (PI3K, MAP kinase, HIFs, NF-κB) and loss of tumor suppressors (p53) coordinate an elevated redox state. ROS is also generated by increased oxidative metabolism and hypoxia in rapidly expanding tumors. In addition, cancer cells express elevated levels of cellular antioxidants (SODs, GSH, GPx, and PRx), in part through NRF2, to protect against oxidative-stress-induced cell death.

Furthermore, the immune system is crucial for tissue repair. However, if the immune system either fails to be properly activated or is persistently activated it can contribute to multiple diseases, including autoimmunity and cardiovascular disease, and can accelerate the normal aging process. In the past two decades, substantial evidence has revealed that ROS are essential second messengers in innate and adaptive immune cells [73,74]. Yet increased levels of ROS within immune cells can result in hyperactivation of inflammatory responses, resulting in tissue damage and pathology [75].

The innate immune system responds to microorganism-derived pathogen-associated molecular patterns (PAMPs) and endogenous cell-derived damage-associated molecular patterns (DAMPs) of tissue injury [76]. PAMPs and DAMPs bind to specific receptors, including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) and promote the secretion of cytokines that are essential for fighting pathogens or for repairing tissue damage (Figure 3A) [77–79]. Initial studies implicating ROS in innate immunity demonstrated that the TLR ligand lipopolysaccharide (LPS) activates inflammatory cytokines by stimulating the generation of ROS by NADPH oxidase and mitochondria [80,81]. More recent studies have shown that mitochondrial ROS are essential for pathways initiated by other TLRs, including TLR1, TLR2, and TLR4, and for optimal bactericidal activity of macrophages [82]. RLRs also signal through mitochondrial ROS [83], which might not be surprising since the outer mitochondrial membrane serves as a platform for formation of the RLR signaling complex [84]. The NLR NLRP3, a component of the inflammasome, also requires NADPH and mitochondrial ROS for activation [85–89]. Interestingly, patients with tumor necrosis factor receptor-associated periodic syndrome (TRAPS) have heightened responsiveness to LPS, due to increased mitochondrial ROS production, which promotes inflammation, again suggesting that redox biology, and not oxidative stress, is regulating inflammatory diseases [90].

Adaptive immunity involves the expansion of pathogen-specific T cells and B cells via rapid proliferative responses. Initial evidence for redox signaling in this process stemmed from the observations that treatment of primary T cells with pharmacological antioxidants inhibited proliferation and production of the cytokine interleukin-2 (IL-2) following T-cell receptor stimulation *in vitro* [91]. Antioxidants also diminished the expansion of T cells *in vivo* [92]. The major initial source of ROS required for T-cell activation is mitochondria [93,94]. Pharmacological or genetic disruption of mitochondrial ROS generation can diminish T-cell activation *in vitro* and *in vivo*. However, NADPH oxidase can be invoked in response to mitochondrial ROS to further sustain ROS levels to maintain T-cell activation [95]. ROS generated by NADPH oxidase and mitochondria have also been implicated in B-cell activation and proliferation upon stimulation of the B-cell receptor [96,97]. Thus, both T- and B-cell receptor signaling requires the generation of ROS to mount the proper adaptive immune response.

What happens when ROS levels are elevated during immune responses? The simple answer is that this depends on the degree to which ROS levels are elevated beyond what is expected during a normal immune response. Under certain conditions, a slight elevation could be beneficial or detrimental [98]. For example, mice lacking uncoupling protein 2 (UCP2) have higher levels of mitochondrial ROS and increased immunity to bacterial pathogens [99], suggesting that a low elevated level of ROS in the immune system might enhance normal immune function. Indeed, mice heterozygous for Mclk1 (a mitochondrial hydroxylase necessary for ubiquinone synthesis) have increased mitochondrial ROS with elevated normal innate and adaptive immune responses and fight pathogens without incurring tissue damage [100]. By contrast, high levels of ROS generation due to loss of NRF2 lead to elevated levels of pro-inflammatory cytokines [101]. NRF2-deficient mice have exacerbated inflammatory

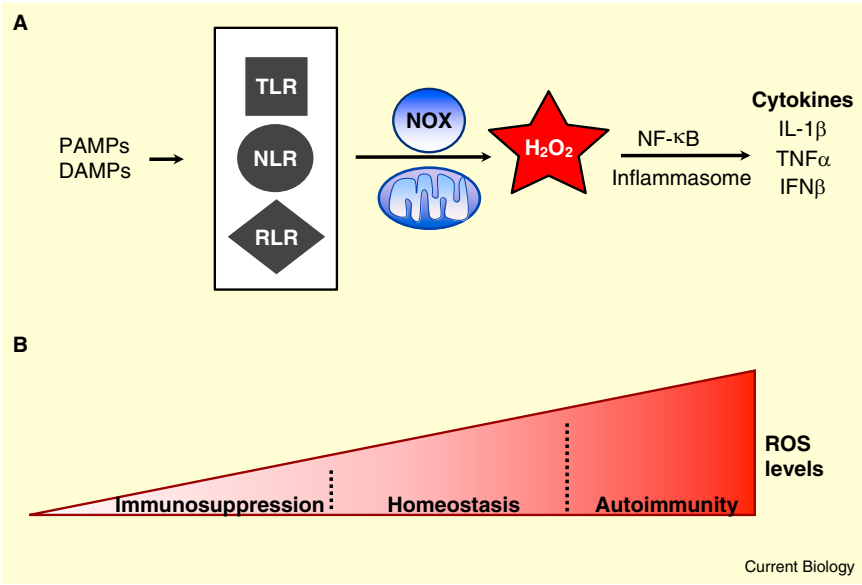

Figure 3. ROS regulation of inflammation.

(A) Activation of the innate immune system requires ROS signaling. Common patterns associated with pathogens or cell damage (PAMPs or DAMPs) activate surveillance receptors (TLR, NLR, RLR), which increase ROS through NAPDH oxidase (NOX) enzymes and mitochondria. ROS is required for the release of pro-inflammatory cytokines (IL-1β, TNFα, IFNβ) to effect an appropriate immune response. (B) Low levels of ROS maintain a healthy immune system. Decreasing ROS levels inhibits activation of proper immune responses, leading to immunosuppression. Elevated ROS levels contribute to autoimmunity through increasing the release of pro-inflammatory cytokines and proliferation of specific subsets of adaptive immune cells.

responses to pathogens, resulting in worsened pneumonia and sepsis [102]. Antioxidants improve the survival of NRF2-deficient mice in these models of sepsis. Antigen-specific adaptive immunity induced by an experimental model of asthma is also intensified by NRF2 deficiency [103]. Thus, slight elevations in ROS levels may enhance immune system function, whereas high levels of ROS could promote a pathological response (Figure 3B).

The finding that ROS function in normal innate and adaptive immunity presents a challenge regarding when antioxidants could be utilized as an immunomodulatory therapy. Clearly antioxidants should not be administered in healthy individuals that have a robust antoxidant defense and a healthy immune system, since ROS are intimately tied to optimal pathogen clearance. However, when the immune system becomes dysregulated, as observed in autoimmune disease, antioxidants could be helpful in ameliorating the heightened immune response. As with ROS therapy in cancer, there are obstacles that need to be considered when using antioxidants for immunomodulation. For example, the dosage of antioxidants should not be so high as to interfere with normal immune responses. Furthermore, the timing of antioxidant treatment is crucial during the progression of an inflammatory disease. This is certainly the case in critically ill patients in the intensive care unit. These patients often display signs of elevated ROS and heightened inflammatory responses that result in multi-organ failure and mortality. Even in the cases of an acute infection, it is possible that a pro-inflammatory cytokine storm is primarily responsible for admission to the intensive care unit. Yet, multiple clinical trials have consistently showed no efficacy or even an increase in mortality in intensive care unit patients with critical illness that have been treated with antioxidants [104]. The reasons for this failure are not fully understood but we speculate that the antioxidants might interfere with the normal responses to pathogens in certain immunosuppressed populations. It is possible that these immunosuppressed patients might even benefit from pro-oxidant therapy to boost their immune system.

Going forward, it will be important to characterize how different inflammatory cells respond to changes in ROS levels. There is growing appreciation that different T-cell and macrophage subsets can have pro- or anti-inflammatory activity, but it is not fully understood whether these different subsets have differential responses to ROS. Along these lines, it might be beneficial in the amelioration of immune system pathologies to increase a particular subset of T cells or macrophages by either increasing or decreasing ROS levels.

ROS and Regulation of Aging

The ability to regenerate tissues as well as prevent damage to existing tissues are two key determinants of aging. One of the original theories of aging formulated over 50 years ago is Denham Harman's free radical theory of aging, which proposed that ROS contribute to aging through their reactivity towards cellular macromolecules, particularly in the mitochondria [105]. Damaged mitochondria, through inefficient oxidative phosphorylation, produce escalating amounts of ROS, inevitably impairing cellular function [106]. However, interventions in reducing ROS levels have had mixed results and it is not clear whether ROS-induced damage is the underlying cause of aging [107]. On the contrary, recent evidence suggests that ROS signaling is required for the maintenance of tissues and that increasing ROS can activate cellular stress pathways to dampen tissue degeneration and promote healthy aging [108].

Longevity studies in multiple model organisms have not consistently demonstrated that antioxidants prevent aging. Early studies in *Drosophila* suggested that increasing SOD and catalase activity in the cytosol extend longevity [109], although other investigators could not duplicate these experiments [110]. Furthermore, careful measurements of ROS in *Drosophila* have not found any correlation between ROS levels and longevity. In mice, overexpression of cytosolic SOD together with catalase or mitochondrial SOD also does not increase longevity [111]. By contrast, the overexpression of mitochondrial matrix catalase (CAT^{mm}), but not cytosolic or nuclear catalase, in mice does extend longevity [112]. The conventional interpretation is that CAT^{mm} detoxifies matrix-generated H_2O_2 to water, preventing H_2O_2-induced oxidative damage to mitochondria. An alternative explanation is that detoxification of matrix-generated H_2O_2 prevents leakage of H_2O_2 into the cytosol, thereby interfering with normal ROS signaling pathways

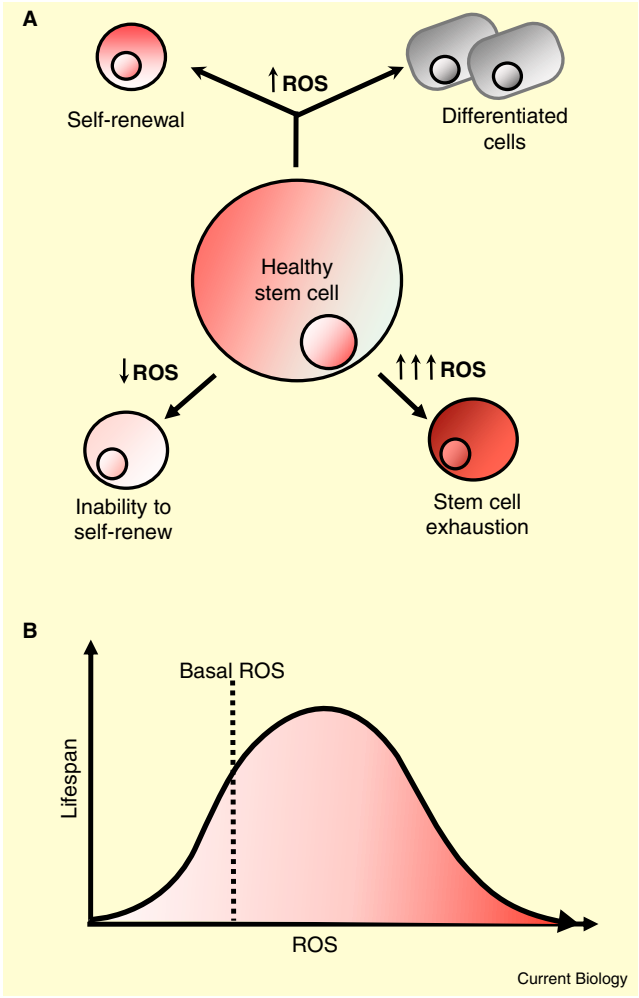

Figure 4. ROS regulation of aging.

(A) Moderate ROS levels are required for proper stem-cell differentiation and renewal through activation of signaling pathways. While decreased ROS levels impair stem-cell function, ROS levels that are too high lead to stem-cell exhaustion and premature aging through activation of signaling pathways. (B) Increased ROS levels are not always detrimental to lifespan. Activation of cellular responses due to slight increases in ROS can drive signaling pathways that counter the normal aging process. However, high ROS levels can hyperactivate signaling pathways that promote inflammation, cancer and cell death, leading to an accelerated aging phenotype.

that prevent pathologies such as cancer, a major cause of death in laboratory mice. Since mitochondria are a major source of ROS in the cell, the mitochondrial genome is often thought to be particularly susceptible to oxidative damage, yet deletion of mitochondrial matrix SOD in mice increases mitochondrial DNA damage and increases cancer incidence, but does not accelerate aging [113,114]. Interestingly, loss of SOD enzymes in *C. elegans* can even extend lifespan [115].

An observation that further questions the free radical theory of aging is that elevation of ROS through signaling mechanisms can increase longevity from yeast to mice [116]. In yeast, inhibition of target of rapamycin (TOR) or caloric restriction extends chronological lifespan by increasing mitochondrial ROS [117,118]. In *C. elegans*, glucose restriction, mutation of mitochondrial electron transport components, and diminished insulin-like growth factor (IGF) signaling all extend lifespan by increasing mitochondrial ROS [119–121]. Paraquat, a direct generator of mitochondrial ROS, is sufficient to increase lifespan in *C. elegans* [122]. Sirtuin-dependent extension of lifespan in *C. elegans* has also been shown to be dependent on an increase in ROS production. This result was unexpected as the contribution of sirtuins to lifespan regulation was previously thought to be primarily mediated by deacetylation of proteins, including histones [123]. There is also increasing evidence for a conserved mitochondrial longevity pathway in mammals. Mice heterozygous for MCLK1, which is required for proper electron transport, have increased mitochondrial ROS [124]. However, these mice have less oxidative damage to cytosolic proteins and are long-lived, supporting a model whereby elevated ROS levels are paradoxically protective through the induction of stress response pathways [125]. A common model of human aging in cell culture is replicative senescence. Initial support for the free radical theory came from the observation that hypoxia increased the replicative lifespan of human diploid fibroblasts [126]. The original interpretation was that hypoxia decreased ROS, resulting in reduced accumulation of oxidative damage and leading to an increase in replicative lifespan. However, later studies have demonstrated a paradoxical increase in mitochondrial ROS during hypoxia, resulting in activation of HIFs and increased replicative lifespan of human fibroblasts [127]. The long-lived mitochondrial mutants in *C. elegans* also depend on ROS-dependent activation of HIF for increased lifespan [119]. Beyond HIF, there are likely to be multiple signaling pathways that ROS activates to increase lifespan.

Aging is accelerated when the tissues that are damaged are not repaired [128]. The maintenance of adult tissue and organ systems requires removal of damaged cells and replenishment from undifferentiated stem cell populations. Stem cells have to both self-renew to maintain the stem cell pool and also differentiate to generate specialized tissue. The best-studied example is the hematopoietic stem cell, which differentiates to provide myeloid and lymphoid progenitors throughout lifespan. An emerging model in hematopoietic stem cells is that generation of low levels of ROS by NADPH oxidases or mitochondria is required to activate proliferative pathways, serving as a 'go' signal to support stem-cell proliferation. By contrast, high levels of ROS impair stem-cell function by activating signaling pathways that limit self-renewal, but do not necessarily cause cellular damage (Figure 4A). For example, hematopoietic stem cells from mice that lack the gene encoding the DNA-damage checkpoint kinase ATM have higher ROS levels that activate p38 MAP kinase and the cell-cycle inhibitor p16INK4a, resulting in a reduction of the repopulating capacity of these stem cells and exhaustion of the stem-cell population [129]. Interestingly, the rise in p16INK4a expression increases with age and has been directly shown to limit stem-cell renewal and function [130]. The antioxidant NAC rescues defects resulting from the loss of ATM [131]. Other defects caused by loss of ATM include development of thymic lymphoma and innate and adaptive immune dysregulation [132]. These defects are alleviated by the expression of mitochondrial catalase, suggesting that the normal function of ATM might to be to control ROS levels [132]. Indeed, ROS oxidize a specific cysteine residue on ATM to generate disulfide-linked activated ATM dimers that promote antioxidant responses

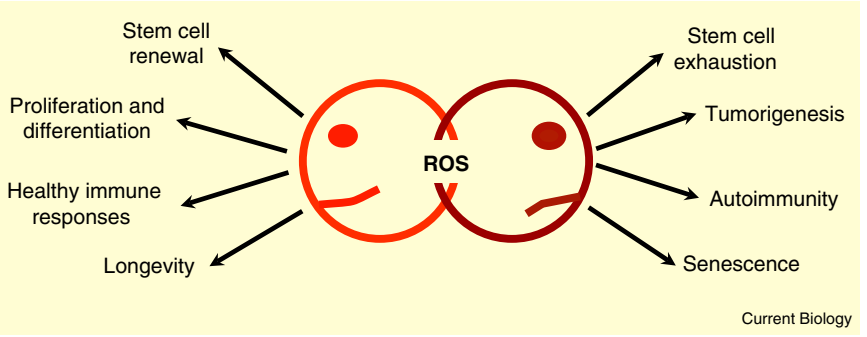

Figure 5. Janus of ROS: a therapeutic conundrum.

Redox biology encompasses both the physiological and pathological roles of ROS. Determining whether to use pro-oxidant therapy to promote physiological ROS responses or antioxidant therapy to prevent ROS pathologies remains the central question in redox biology.

by regulating NADPH production from the pentose phosphate pathway [133,134]. ATM also maintains a low level of ROS to sustain stem-cell function in part through the pro-apoptotic Bcl-2 family protein BID [135].

Consistent with the ATM observation in hematopoietic stem cells, loss of FOXO transcription factors, the Polycomb group protein Bmi1, or tuberous sclerosis 1 (Tsc1) triggered an increase in ROS levels in these cells, limiting their repopulating capacity [136–138]. Aside from hematopoietic stem cells, neural stem cells are also sensitive to an increase in ROS. The loss of PRDM16, a transcription factor that regulates brown fat but is also highly expressed in hematopoietic stem cells and neural stem cells, triggers defects in the function of both types of stem cell [139]. However, NAC only rescued these defects in neural stem cells, not in hematopoietic stem cells, suggesting that redox biology is dependent on cellular context. Thus, ROS levels have to be maintained within a range that allows for stem cells to function properly, and this concentration range may differ between tissues.

ROS are also essential for stem-cell differentiation. Mouse hematopoietic stem cells deficient in both AKT1 and AKT2 have reduced levels of ROS, leading to impaired differentiation [140]. Similarly, in *Drosophila* hematopoietic progenitors, increasing ROS triggers differentiation, whereas decreasing ROS impairs differentiation [141]. Furthermore, human bone marrow mesenchymal stem cells also require ROS for differentiation into adipocytes, and mitochondrial ROS generated from complex I can trigger muscle differentiation [142,143]. Within the skin epidermis, undifferentiated cells along the basement membrane undergo a regulated transformation into mature apical epidermal cells. Lowering mitochondrial ROS impairs this differentiation process, which can surprisingly be restored by supplementation with exogenous H_2O_2 [144]. Similarly, lowering ROS levels decreases the regenerative capacity of neural stem cells and spermatogonial stem cells [145,146]. However, aberrant elevation of ROS levels impairs cardiac myocyte differentiation [147]. This raises two questions: what levels of ROS are required for stem-cell renewal compared with stem-cell differentiation? And, what levels of ROS inhibit stem-cell renewal compared with stem-cell differentiation? We speculate that quiescent stem cells are present at low levels of ROS and a slight increase in ROS provides the signal for self-renewal and cellular differentiation; ROS levels above those required for self-renewal or differentiation impair these two critical stem-cell functions.

The idea that stem-cell and tissue impairment are not a consequence of oxidative-stress-induced damage is further supported by experiments in mice harboring mitochondrial mutations. Mice engineered to accumulate mitochondrial DNA mutations due to defective DNA polymerase proofreading prematurely age and display impaired neural stem cell and hematopoietic stem cell function beginning *in utero* [148]. Remarkably, administration of NAC rescued the dysfunction in both of these stem-cell types, suggesting that the genetic mutations caused by the error-prone polymerase were dispensable for self-renewal. Other mutations of mitochondrial DNA, such as A1555G, result in maternally inherited phenotypes by increasing ROS levels [149]. However, these ROS cause their pathologies through activation of signaling pathways and apoptosis, rather than oxidative damage. Consequently, the stem cell, tissue degeneration, and aging communities have undergone a revolution in the past two decades from viewing ROS simply as toxins that cause cellular damage to seeing them as molecules that regulate cellular signaling pathways to invoke beneficial or detrimental effects (Figure 4B).

Conclusions

Studies over the past two decades in various organisms, tissues and cell types have led to a shift in our understanding of ROS: we no longer view them just as molecules that invoke damage (i.e. oxidative stress) but now also appreciate their role in regulating signaling pathways that impinge on normal physiological and biological responses (i.e. redox biology). The levels and compartmentalization of H_2O_2 dictate redox biology, while high levels of superoxide or hydroxyl radicals invoke oxidative stress. Redox signaling is required for numerous cellular processes, as indicated by the role of ROS in proper cellular differentiation, tissue regeneration, and prevention of aging. However, we propose that redox signaling, and not oxidative stress, is also crucial in regulating signaling pathways that control various disease states, including tumorigenesis, autoimmunity, and loss of tissue regeneration with age (Figure 5). This conceptual shift makes it difficult to interfere with redox biology by administering antioxidants, which would affect redox biology of both normal and abnormal responses. To date, physical exercise is one strategy that increases ROS, resulting in activation of beneficial pathways that diminish cancer, diabetes, and ageing [150]. But, since most of us find it difficult to spend time at the gym, it will be important to identify and distinguish the molecular effectors of redox biology that maintain normal biological and physiological responses from those that promote human pathologies. Such studies would allow for the development of selective therapies that would alleviate disease without interfering with healthy tissue.

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