Oxygen radicals and signaling

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Recent evidence suggests that reactive oxygen species, such as superoxide anions and hydrogen peroxide, function as intracellular second messengers. This review will discuss the progress in understanding the intracellular pathways leading from ligand stimulation to the generation of oxidants, as well as some of the increasing number of cellular processes that appear to be subject to redox regulation.

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Abbreviations

ERK	extracellular-signal-regulated kinase
JNK	c-Jun amino-terminal kinase
MAPK	mitogen-activated protein kinase
PDGF	platelet-derived growth factor
PKC	protein kinase C
ROS	reactive oxygen species

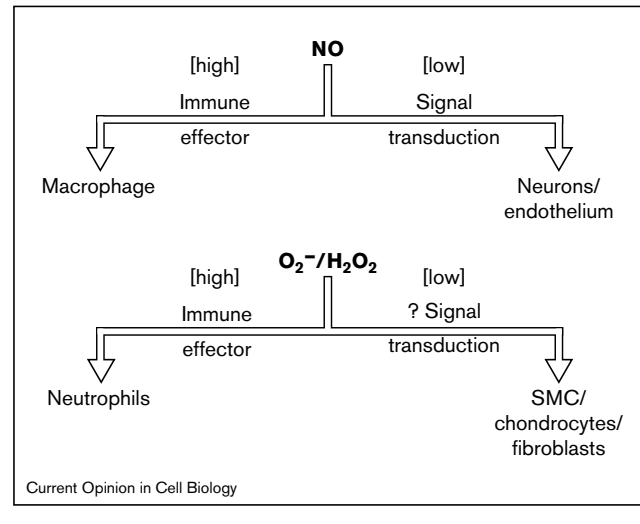
Introduction

Few biological entities have as bad a reputation as reactive oxygen species. For many years, these small diffusible molecules have been thought of as the unwanted and toxic by-products of living in an aerobic environment. Although the cell had clearly evolved multiple defenses for their elimination, their relentless production coupled with their damaging nature has led to the widely held belief that these molecules serve only a harmful function. The purpose of this review is to re-evaluate this prejudice and to provide a summary of some recent evidence suggesting that the production of reactive oxygen species (ROS) is tightly regulated and serves a physiological function.

The notion that molecules such as superoxide anion (O_2^-) and hydrogen peroxide could function in signal transduction in mammalian cells is not without precedent. Indeed, a wealth of information suggests that these molecules function in this fashion in both bacteria and plants. In bacteria, redox regulation of transcription occurs, with a different set of genes stimulated by H_2O_2 and O_2^- [1,2]. Similarly in the plant pathogen response, there appears to be a clear role for H_2O_2 as a signaling molecule [3].

In mammalian cells, the physiological role for O_2^- and H_2O_2 is less well characterized than that of another reactive oxygen species, namely nitric oxide. Analysis of the role of NO suggests it functions in two discrete fashions. Production of nitric oxide by macrophages and other immune-effector cells results in the high level production of NO, consistent with its role in host defense. In contrast, the nitric oxide synthase found in endothelial cells or neurons generates two to three orders of magnitude less NO when activated. Produced at this level, NO is widely believed to function in signal transduction. This dichotomy between immune function and signal transduction is likely to be preserved for other reactive oxygen species. As seen in Figure 1, O_2^- and H_2O_2 are produced in large amounts by cells of the immune system. In contrast, other cell types, including vascular smooth muscle cells, chondrocytes and fibroblasts appear to produce significantly lower amounts of these molecules. Emerging evidence suggests that this mini 'oxidative burst' appears to have an important role in signal transduction.

Figure 1



A potential analogy between nitric oxide and other reactive oxygen species. In both cases, high levels are produced by immune effector cells while lower amounts are used by other cell types for signal transduction.

ROS as second messengers in signal transduction

Studies over the past 10 years have demonstrated that ligand stimulation of non-phagocytic cells results in an increase in intracellular reactive oxygen species. This phenomenon has been observed in a wide variety of cell types and is stimulated by a diverse collection of ligands, including cytokines [4–6] as well as peptide growth factors acting through tyrosine kinase [6–8,9•] and G-protein-coupled receptors [10].

The importance of this rise in ROS following ligand activation has been appreciated only more recently.

Analysis in vascular smooth muscle cells demonstrated that stimulation by platelet-derived growth factor (PDGF) results in a rapid increase in ROS which peaks within minutes of ligand stimulation and then returns to baseline [8]. This time course is similar to the time course of growth-factor-stimulated tyrosine phosphorylation. The link between ligand-stimulated H_2O_2 production and phosphorylation was further strengthened by the observation that exogenous H_2O_2 mimics growth-factor-induced tyrosine phosphorylation. In addition, increasing the level of the peroxide-scavenging enzyme catalase blunted the increase in H_2O_2 and inhibited the ability of PDGF to stimulate tyrosine phosphorylation. Similar results were obtained in A431 cells stimulated with epidermal growth factor (EGF) [9•], again suggesting that ligand-stimulated ROS generation may have a general role in mediating tyrosine phosphorylation.

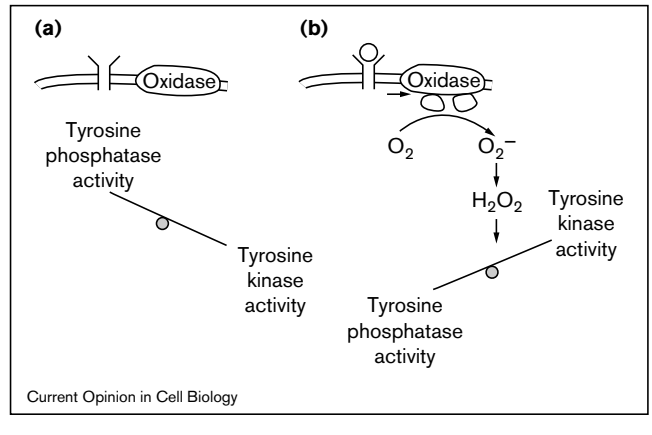
Although these results suggest that ROS may function as a second messenger system in the context of ligand stimulation, other evidence suggest that oxidative stress may also activate unique pathways. The transcription factor NF-κB is stimulated by a host of ligands leading eventually to the serine phosphorylation and subsequent proteosomal degradation of the IκB inhibitory subunit. ROS are important in ligand-stimulated NF-κB activation since most, if not all, such activation can be blocked by antioxidant treatment [11]. Nonetheless, direct oxidative stress such as hypoxic reoxygenation also activates NF-κB, but appears to do so through a unique proteolysis independent pathway involving tyrosine phosphorylation [12•]. Similarly, addition of H_2O_2 appears to activate various protein kinase C (PKC) family members [13•]. However, the activation of PKC by oxidants appears to be independent of lipid cofactors and thereby differs from the classical ligand-stimulated pathway.

Targets of ROS

The downstream targets of ROS have remained largely unexplored. Extracellular administration of non-lethal concentrations of H_2O_2 has been demonstrated to activate mitogen-activated protein kinase (MAPK) as well as the c-Jun amino-terminal kinase (JNK) [8,14,15]. In addition to the effects of exogenous ROS, the ability of ligands to activate MAPK has been shown in certain examples to be inhibited by treating cells with chemical or enzymatic antioxidants [8,16]. This has also been demonstrated for JNK activation, where it has been noted in several cell types that activation of the kinase is inhibited by pretreatment with the antioxidant N-acetylcysteine [17•,18]. Since this antioxidant affects the level of intracellular glutathione, it is consistent with other results suggesting that some, but not all, ligands that activate JNK are exquisitively sensitive to the intracellular glutathione status of the cell [19].

Although the activity of the extracellular-signal-regulated kinase (ERKs) are redox sensitive, they are probably not direct targets of ROS. Although not proven, one such direct target may be tyrosine phosphatase. All such molecules have in the active site a cysteine residue which is essential for biological activity [20] and which can be regulated in a redox-dependent manner [21]. This observation may provide a mechanistic explanation linking H_2O_2 generation and tyrosine phosphorylation. As demonstrated in Figure 2a, under basal conditions when ROS levels are low, tyrosine phosphatase activity would predominate since the specific activity of tyrosine phosphatase is several orders of magnitude greater than the corresponding activity of tyrosine kinases. Ligand stimulation would result in an increase in ROS which would function to transiently inactivate the activity of tyrosine phosphatase (Figure 2b). When ROS levels fell, phosphatase activity could be restored presumably by cellular reducing enzymes. Under such a scenario, growth-factor-stimulated ROS production would temporarily permit a burst of kinase activity through the transient inactivation of phosphatases. Recent evidence suggests that such a mechanism may also be common to other non-classical activators of receptor tyrosine kinase activity such as radiation and alkylating agents [22•].

Figure 2



A model for how ROS may regulate tyrosine phosphorylation. **(a)** Under basal conditions, ROS levels are low and the specific activity of tyrosine phosphatase exceeds that of the corresponding kinases. **(b)** Following ligand stimulation, ROS levels increase, perhaps as in the case of the neutrophil, through the recruitment of cytosolic proteins to a membrane bound oxidase. Increase ROS levels react with the redox-sensitive cysteine residues of tyrosine phosphatases leading to their transient inactivation. This leads to a burst of unopposed kinase activity until ROS levels fall and phosphatase activity can be restored through reduction of the oxidized cysteine residue.

The role of small GTP-binding proteins

The intracellular pathway in non-phagocytic cells leading from ligand activation to ROS generation appears to share some similarity to the better characterized neutrophil system. In phagocytic cells, the small GTP-binding protein Rac2 appears to have an important role in oxidase function [23]. Similarly, a requirement for small GTPases,

including Ras and Rac1, has been recently shown for ROS generation following stimulation by cytokines and growth factors [24•]. This study also demonstrated that expression of constitutively active mutants of *ras* or *rac1* lead to an increase in levels of ROS. Genetic evidence suggests that Rac1 acts downstream of Ras since expression of a dominant-negative *rac1* mutant inhibits Ras-stimulated ROS production [24•].

Surprisingly, although the small GTP-binding Ras and Rac1 may contribute to ROS production, they may themselves also be an important target of redox modulation. Recent experiments have demonstrated that a cysteine residue at position 118 of Ras can be regulated in a redox-dependent fashion [25•]. Redox modification of the residue appears to effect nucleotide exchange, and conversion of cysteine 118 to serine results in an inhibition of certain aspects of Ras-dependent signaling [25•].

The source of ROS

The enzymatic source or sources of ligand-stimulated ROS have remained elusive. A variety of cellular enzymes, including cyclooxygenases and lipoxygenases, are potential ligand-activated superoxide-generating systems. Perhaps the most intriguing possibility is that by analogy with the neutrophil system, an NADPH oxidase will be the primary source of ROS involved in signaling. Such a hypothesis is supported by the observation that a ligand-activated enzyme with NADPH/NADH oxidase activity appears to be present in a variety of non-phagocytic cells including smooth muscle cells [10], chondrocytes [6], and kidney epithelium [18]. Treatment with diphenyleneiodonium, a pharmacological inhibitor of the flavoprotein component of the neutrophil NADPH oxidase, appears to significantly effect ROS production in non-phagocytic cells stimulated by either ligands [6,10] or expression of activated small GTPases [24•]. In addition, components of the NADPH oxidase appear to be present in other cell types [26]. Inhibiting the expression of one of these ubiquitously expressed components, $p22^{phox}$, was recently shown to inhibit the ability of angiotensin II to stimulate superoxide production in vascular smooth muscle cells [27•]. Cells deficient in $p22^{phox}$ no longer hypertrophied in response to angiotensin II, again suggesting a physiological downstream role for ROS.

ROS in growth and death

The observation that the small GTP-binding proteins Ras and Rac regulate ROS production in non-phagocytic cells may be important in understanding the role of these proteins in growth control and transformation. Previous experiments have demonstrated that treatment of some cells with oxidant stress stimulated cell division and the expression of growth-related gene products [28,29]. Recent experiments with Ras-transformed NIH 3T3 cells demonstrated an increase in superoxide production compared to non-transformed or Raf-transformed 3T3 cells [30••]. The increase in O_2^- was inhibited by dominant-negative Rac1 expression. Treatment with an antioxidant inhibited S-phase progression in serum-starved Ras-transformed cells but not in Raf-transformed cells. These effects were independent of MAPK or JNK activation suggesting that oxidants may mediate a novel Ras-dependent pathway important for cell-cycle progression and transformation. Recent evidence further suggests that this redox-dependent, small GTPase-regulated pathway may have distinct and as yet uncharacterized downstream targets. In particular, a mutant of Rac, defective for superoxide production but still capable of activating both JNK and cytoskeletal reorganization appears to be unable to stimulate cell proliferation (D Bar-Sagi, personal communication).

Although ROS may mediate growth regulatory pathways, there is emerging, although sometimes conflicting, evidence that also suggests a role for ROS in apoptotic pathways [31]. As apoptosis is triggered by multiple agents and proceeds through multiple pathways it is likely that ROS may participate in some, but not all, aspects of programmed cell death. In this regard, it has been recently observed that stimulation with Fas ligand resulted in apoptosis through the generation of O_2^- [32]. This process was inhibited by expression of a dominant-negative *ras* gene, again suggesting a role for small GTPases in the control of the redox state of the cell. In addition, two recent studies have suggested that ROS may mediate p53-dependent apoptosis [33•,34••]. Both studies employed adenovirus-mediated gene transfer of wild-type p53. Overexpression of p53 resulted in a significant increase in ROS levels, while treatment of cells with antioxidants inhibited p53-mediated apoptosis. In addition, analysis of gene products induced via p53-dependent transcriptional activation included a number of proteins that function to regulate the intracellular redox state [34••].

Redox regulation of transcription

Reactive oxygen species may directly regulate the activity of transcription factors. As discussed previously, perhaps the most widely studied example is the activation of NF-κB. Activation of this factor, which can result from stimulation by diverse agents, appears to proceed through a common pathway involving ROS generation [11,35]. Using cells that overexpress either superoxide dismutase or catalase, H_2O_2 and not O_2^- has been demonstrated to be the relevant ROS [36]. Such specificity is reminiscent of the SoxRS and OxyR system of bacteria. Consistent with previous studies, the ligand-stimulated pathway leading to H_2O_2 production and subsequent redox activation of NF-κB has recently been shown to involve the Rho family of small GTP-binding proteins [37,38].

Another transcription factor whose activity appears to be regulated in a redox-dependent fashion is the hypoxia inducible factor, Hif-1 [39]. Studies over the few several years have demonstrated that the level of Hif-1 protein

increases under hypoxic conditions. Interestingly, treatment of cells with H_2O_2 before hypoxia inhibits the subsequent increase in Hif-1 levels [40,41]. Recent studies have shown that one reason for the difference in Hif-1 levels between hypoxic and normoxic conditions is that, in normoxic conditions, Hif-1 is subjected to degradation through the ubiquitin pathway [42]. One explanation may be that ambient levels of H_2O_2, which are higher under normoxic conditions than during hypoxia, may regulate ubiquitin activity. Such speculation is supported by some recent observations that ubiquitin conjugation activity could increase almost 10-fold in activity following a H_2O_2 challenge [43]. Alternatively, as has been suggested, ROS may alter Hif-1, perhaps through phosphorylation, and thereby alter its degradation rate [42].

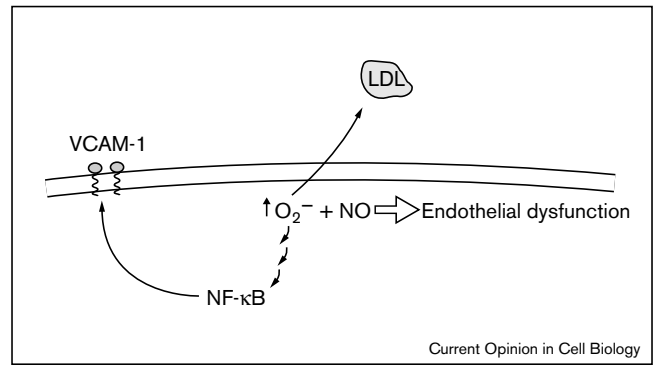
Finally, the binding of certain transcription factors to DNA appears to be regulated in a redox-dependent fashion through the oxidation–reduction of critical cysteine residues in the DNA-binding domain [44]. This area was considerably aided by the isolation of Ref-1 [45], a nuclear protein which appears to facilitate DNA binding by specifically reducing cysteine residues in the DNA-binding domain. Ref-1 activity is in turn regulated by the antioxidant protein thioredoxin [42,46]. Agents that induce oxidative stress, such as phorbol ester, appear to cause the translocation of thioredoxin into the nucleus where it directly associates with Ref-1 [47•]. Such a pathway may provide an explanation for how activity is restored for transcription factors which are activated by oxidative stress but require a reduced state to bind DNA.

ROS as mediators of disease

A variety of human diseases have been linked to an overproduction of ROS. The growing realization that oxidants may function in signaling pathways may, in turn, cause a re-evaluation of the pathways and sources of oxidant production linked to human disease. As an example, as shown in Figure 3, both human and animal data suggests that prior to the development of atherosclerotic plaque, the vessel wall appears to produce an increase in superoxide anions [48]. The increase in O_2^- has been postulated to contribute to atherogenesis by a variety of mechanisms including the inactivation of NO, the oxidation of low-density lipoprotein and the stimulation of NF-κB. In addition, a variety of clinical and epidemiological evidence supports a protective role for antioxidants in cardiovascular disease [49].

Could the pathways described above be activated in the pre-atherosclerotic vessel? Does hypertension or hypercholesterolemia activate Ras or Rac1 proteins, and is the observed increase in ROS simply a marker of a continual activation of these small GTPases? Although clearly speculative, it is interesting to note that one characteristic of atherosclerotic vessels is an increase in turbulent flow. Recent evidence using an *in vitro* model of this condition, namely cells exposed to an increase in sheer stress, demonstrate that endothelial cells appear to respond to an increase in flow through the activation of small GTP-binding proteins [50,51] and the subsequent induction of a host of gene products which appear to regulate the redox state of the cell [52,53]. In addition, *in vivo* infusion of agents linked to hypertension, such as angiotensin II, result in the increased expression of $p22^{phox}$ and augmented NADPH oxidase activity in the vessel wall, as well as a stimulation of O_2^- production [54,55•]. As such, the further delineation of these pathways may allow for the development of specific oxidase inhibitors that would presumably be significantly more specific and effective than the current class of scavenging antioxidants. Such inhibitors may have applications not only in atherogenesis, but in a host of human diseases that have been linked to ROS and which include reperfusion injury, Alzheimer's and aging.

Figure 3



Putative role of superoxide anions in the development of atherosclerosis. Increased O_2^- can interact with, and thereby inactivate, locally produced NO, a clinical syndrome known as endothelial dysfunction. Similarly, it can lead to the oxidation of low-density lipoprotein (LDL) cholesterol. Finally, the activation of NF-κB which is regulated by ROS and which in turn regulates the expression of various adhesion molecules (e.g. VCAM-1), has also been linked to atherogenesis.

Conclusions

This review has dealt with the hypothesis that reactive oxygen species such as O_2^- and H_2O_2 act as intracellular signaling molecules. The recent discovery of a role for key cellular proteins such as Ras and p53 in controlling oxidant levels suggest that the redox state is actively regulated. Indeed, the alteration of protein function by oxidants may be in many ways analogous to phosphorylation except that protein modification no longer occurs on specific serine or tyrosine residues but instead on redox-sensitive amino acids such as cysteine and histidine. Delineation of the pathways regulated by oxidants may have fundamental implication in the control of diverse cellular events including transcription, growth and death. Significant work needs to be done in identifying direct targets of ROS and the relevant sources of oxidant production. Such work will hopefully provide some understanding of how specificity can be obtained using ROS as signal transducers. In

addition to providing fresh insight into a host of biological questions, these studies might provide a new direction and therapeutic approach to a wide assortment of human diseases, in which ROS are implicated.

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