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ROS-dependent signal transduction

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Abstract

Reactive oxygen species (ROS) are no longer viewed as just a toxic by-product of mitochondrial respiration, but are now appreciated for their role in regulating a myriad of cellular signaling pathways. H₂O₂, a type of ROS, is a signaling molecule that confers target specificity through thiol oxidation. Although redox-dependent signaling has been implicated in numerous cellular processes, the mechanism by which the ROS signal is transmitted to its target protein in the face of highly reactive and abundant antioxidants is not fully understood. In this review of redox-signaling biology, we discuss the possible mechanisms for H₂O₂-dependent signal transduction.

Introduction

Reactive oxygen species (ROS), by-products of cellular respiration, protein folding, and end products of a number of metabolic reactions, include the superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and the hydroxyl radical (OH•). ROS are intracellular chemical species that are formed upon partial reduction of oxygen (O₂). As their name suggests, ROS are more chemically reactive than O₂; thus, historically, ROS were thought to function exclusively as cellular damaging agents, indiscriminately reacting towards lipids, proteins, and DNA [1]. However, over the past two decades, there is growing appreciation for the role of ROS as mediators of intracellular signaling to regulate numerous physiological and biological responses (i.e. redox biology) [2,3]. Examples of these cellular processes include growth factor signaling, hypoxic signal transduction, autophagy, immune responses, and stem cell proliferation and differentiation [4]. Thus, it appears that ROS are not simply an unwanted product of an imperfect system, but have instead been selected by nature for their specificity in signaling. This dual function of ROS seems counterintuitive given the evolution of a robust cellular antioxidant defense system. Therefore, a critical question in

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ROS-dependent signaling remains, how do ROS find their specific molecular target in the presence of highly reactive and abundant antioxidants? In this review, we will discuss (1) the sources and regulation of ROS signal in the cell and (2) the possible mechanisms for how the ROS signal achieves transmission specificity.

Tight regulation of ROS within cells

Specificity in ROS signaling takes advantage of the distinct biological properties of each oxidant species, which include their chemical reactivity, stability, and lipid diffusion capabilities. O_2^- is generated by the one-electron reduction of O_2 through cytosolic NADPH oxidases (NOXs) and in mitochondrial electron transport chain (ETC) complexes I, II, and III (Figure 1) [5,6]. Cytosolic O_2^- is rapidly converted to H_2O_2 by the enzymatic activity of superoxide dismutase 1 (SOD1). Furthermore, O_2^- generated by the mitochondrial ETC is released into the matrix where it is quickly dismutated to H_2O_2 by superoxide dismutase 2 (SOD2) [7]. Complex III-generated O_2^- is also released into the intermembrane space where it can traverse through voltage-dependent anion channels into the cytosol and be converted into H_2O_2 by SOD1 [8]. In addition, H_2O_2 is produced as a by-product of protein oxidation in the endoplasmic reticulum (ER), as an end product in numerous peroxisomal oxidation pathways such as in the β -oxidation of very long-chain fatty acids, and by a wide range of enzymes including cytochrome P450 [9]. It is important to note that the specific targets of ROS are proximal in location to these oxidant-generating systems.

The stability and membrane diffusibility of H_2O_2 , which has selective reactivity towards cysteine residues, provides an advantage with regard to signaling capacity. Indeed, the best characterized mechanism by which H_2O_2 act as signaling molecules is through the oxidation of critical cysteine residues within redox-sensitive proteins [10]. Susceptible cysteine residues have a low pK_a and exist as a thiolate anion (S^-) at physiological pH, making them more reactive than the protonated cysteine thiol group (SH) and providing a level of selectivity and specificity [11]. H_2O_2 oxidation of the thiolate anion to the sulfenic form (SO^-) impinges on cellular signaling by altering protein conformation and activity. In the presence of high concentrations of H_2O_2 , SO^- is further oxidized to form sulfinic (SO_2^-) and sulfonic (SO_3^-) acids (i.e. hyperoxidation), where SO_3^- generally represents an irreversible oxidative modification. To prevent irreversible cysteine oxidation, the SO^- intermediate is commonly incorporated into a disulfide (S-S) or sulfenic-amide (S-N) bond. These modifications are reversible by the actions of glutaredoxin (GRX) and thioredoxin (TRX), which restore protein function; the oxidized protein is returned to its reduced state (Figure 2).

A third type of ROS, the extremely reactive hydroxyl radical ($OH^\bullet$), is produced when H_2O_2 reacts with metal cations (Fe^{2+} or Cu^+) via the Fenton reaction. Given its very short half-life, strong oxidizing potential, and lipid insolubility, hydroxyl radicals cause irreversible oxidative damage to virtually any cellular macromolecules within the vicinity of their production. Since uncontrolled levels of H_2O_2 lead to $OH^\bullet$ formation, and the concentration of H_2O_2 associated with signaling is estimated to be in the low nanomolar range, tight regulation of ROS is critical for their role in redox-dependent signaling. To prevent the buildup of H_2O_2 and the toxicity of $OH^\bullet$, robust antioxidant systems exist to spatially and

temporally regulate intracellular ROS levels. H_2O_2 is converted to water (H_2O) by the enzymatic activity of several antioxidants including glutathione peroxidases (GPXs), peroxiredoxins (PRXs), and catalase. While GPXs and PRXs are present in a variety of cell compartments (e.g. the cytosol, mitochondria, and ER), catalase is confined to the peroxisomes of most cells. The high abundance of PRXs makes them ideal candidates for scavenging signaling-associated H_2O_2 . The PRX family of proteins (PRX1-6) contains an active site redox-sensitive cysteine, which is oxidized by H_2O_2 and subsequently reduced by thioredoxin (TRX) to complete the catalytic cycle [12]. Using NADPH, the oxidized TRXs are reduced by thioredoxin reductase (TR). A significant feature of PRXs is their ability to undergo reversible hyperoxidation by a second H_2O_2 molecule to form sulfinic acid (SO_2^-), a modification that transiently inactivates the protein. The hyperoxidized form of PRX is reduced back to the SO^- intermediate by sulfiredoxin (SRX) through an ATP-dependent process [13].

In contrast to PRXs, GPXs have higher rate constants but are less abundant; therefore, they are likely to function only during oxidative stress when the intracellular levels of H_2O_2 are elevated [14]. The GPX family of proteins (GPX1-8) catalyzes the reduction of H_2O_2 to H_2O by oxidizing reduced glutathione (GSH) to glutathione disulfide (GSSG). GSH reductase reduces GSSG back to GSH using NADPH as an electron donor. Thus, NADPH plays a critical role in both the generation (NOXs) and detoxification (PRXs and GPXs) of ROS. It is noteworthy to highlight that despite an abundance of antioxidants, residual intracellular ROS remain to participate in signal transduction.

H_2O_2 -dependent signal transduction specificity

The findings that H_2O_2 can oxidize critical cysteine thiol groups of phosphatases including PTEN, PTP1B, and MAPK opened up the possibility that H_2O_2 serve as signaling molecules [15–20]. However, the mechanism(s) by which H_2O_2 transmits its signal to oxidize these targets is not fully understood. How does H_2O_2 find its target protein given the high reactivity and abundance of antioxidants (i.e. kinetic competition)? How is the signal still transmitted?

Redox relay

It has been suggested that perhaps the professional scavenger enzymes themselves, such as PRX or GPX, participate in sensing and transducing H_2O_2 [21]. In a mechanism termed redox relay, scavenging enzymes receive the initial oxidation by H_2O_2 and subsequently transfer the oxidation to the intended target protein (Figure 3A). This mechanism is well characterized in *Saccharomyces cerevisiae* where the oxidant receptor peroxidase-1 (Orp1) enzyme, a GPX-like antioxidant, reacts with H_2O_2 in order to mediate the oxidation of the transcription factor Yap1 [22]. Briefly, H_2O_2 oxidation of Orp1 leads to two consecutive thiol-disulfide exchange reactions with Yap1. Thus, two intramolecular disulfide bonds are formed within Yap1 at critical cysteine residues [23]. These modifications alter Yap1 protein conformation, promoting Yap1 nuclear import and the initiation of a transcriptional response involving the upregulation of TRX [24]. Importantly, oxidation of Yap1 by Orp1 requires the Yap1-Ybp1 interaction [25]. It is thought that the Ybp1 protein acts as a scaffold to bring Yap1 in close proximity to Orp1 such that Orp1 can oxidize Yap1 before

Orp1 is reduced by TRX, completing the typical peroxidase catalytic cycle. Thus, this mechanism in yeast regulates ROS homeostasis. Furthermore, selectivity for signaling may be provided by the protein-protein interactions, which facilitate the thiol-disulfide exchange. Interestingly, in response to H_2O_2 , human glutathione peroxidase 4 (GPX4) but not peroxiredoxin 6 (PRX6) participated in a redox relay in mammalian cells with the redox-sensitive green fluorescent protein (roGFP2) [26]. This observation suggests that at least some human peroxidases are capable of functioning as oxidant acceptors, providing support for the possible existence of a redox relay mechanism *in vivo* for H_2O_2 -dependent signal transduction. Indeed, the redox relay mechanism has been observed in human cells whereby elevated H_2O_2 oxidation of the apoptosis signaling kinase (ASK1) protein and subsequent phosphorylation of its substrate p38 MAPK is dependent on the formation of a PRX1-ASK1 disulfide intermediate [27].

Floodgate model

A second mechanism to promote target protein oxidation by H_2O_2 may involve the inactivation of scavenging enzymes, thereby flooding the area with H_2O_2 and allowing for H_2O_2 -mediated oxidization of a specific thiol within the target protein (i.e. floodgate model) (Figure 3B) [28]. A unique feature of eukaryotic PRXs is their ability to be inactivated following reversible hyperoxidation to sulfinic acid (SO_2^-). Since PRX hyperoxidation has been selected for by nature, the floodgate model for H_2O_2 -mediated redox-dependent signaling is appealing. Indeed, H_2O_2 produced by cytochrome P450 enzymes during the synthesis of corticosterone in mice negatively regulates steroidogenesis through the hyperoxidation of PRX3 in the mitochondria [29]. In addition, it is possible that the scavenging enzymes are transiently inactivated by post-translational modifications (e.g. phosphorylation) allowing for H_2O_2 accumulation and cell signaling. An example of this mechanism, which involves inactivation of membrane-associated PRX1 and growth factor stimulated NOX-induced H_2O_2 , has recently been described in mammalian cells [30]. Specifically, growth factor stimulation and receptor activation induces the localized production of H_2O_2 by NOX (i.e. compartmentalization) while simultaneously inducing Src-mediated PRX1 phosphorylation and inactivation. This allows for the co-localization of elevated H_2O_2 with numerous signaling components without risking oxidative damage to the rest of the cell.

Other mechanisms

Although to date the redox relay and floodgate model seem most feasible, there are a few additional mechanisms to consider. Instead of a redox relay mechanism whereby scavenging enzymes accept H_2O_2 oxidation and transfer the oxidation to the target protein, a mechanism may exist which includes an intermediate step involving the oxidation of TRX or GSH (Figure 3C) [31]. A recent study highlighted the importance of TRX1 interaction with AMPK as an essential regulatory step for AMPK activation during ischemia [32]. Furthermore, it is conceivable that a signaling pathway could be initiated by the dissociation and resultant activation of a target protein following H_2O_2 oxidation of a scavenger enzyme (Figure 3D). Indeed, this mechanism has been observed with the ASK1 protein and TRX. H_2O_2 -induced oxidation of TRX changes the protein conformation, thereby disrupting the ASK1-TRX interaction and activating ASK1 kinase activity [33,34].

Conclusions

With the shift in our understanding of ROS, from toxic chemical species to critical signaling molecules, it is important to elucidate the molecular mechanisms of ROS signaling. Given the high reactivity and abundance of antioxidants, a mechanism of facilitated oxidation, like those mentioned here, is likely to mediate ROS-dependent signaling. The identification of specific targets of ROS that are essential for biological responses will ultimately fortify ROS as signaling molecules and potentially provide new therapeutic avenues for a myriad of diseases linked with excessive ROS.

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Figure 1. Endogenous sources of ROS signal

Intracellular ROS is primarily produced by NADPH oxidase enzymes (NOXs), the mitochondria, the endoplasmic reticulum, and the peroxisome. Cytosolic superoxide (O₂⁻) is rapidly converted into hydrogen peroxide (H₂O₂) by superoxide dismutase 1 (SOD1). H₂O₂ can either act as a signaling molecule by oxidizing critical thiols within proteins to regulate numerous biological processes, including metabolic adaptation, differentiation, and proliferation or be detoxified to water (H₂O) by the scavenging enzymes peroxiredoxin (PRX), glutathione peroxidase (GPX), and catalase (CAT). In addition, H₂O₂ can react with metal cations (Fe²⁺ or Cu⁺) to generate the hydroxyl radical (OH•), which causes irreversible oxidative damage to lipids, proteins, and DNA.

Figure 2. H₂O₂-mediated cysteine oxidation of redox-sensitive proteins

Critical cysteine thiol groups of target proteins exist as a thiolate anion (S⁻) and are readily oxidized by hydrogen peroxide (H₂O₂) to yield sulfenic acid (SO⁻), a reversible modification which alters protein activity. When H₂O₂ levels are high, SO⁻ can be hyperoxidized to generate sulfinic (SO₂⁻) and sulfonic (SO₃⁻) acids (orange box). While SO₃⁻ generally represents an irreversible oxidative modification, SO₂⁻ can be converted back to the SO⁻ intermediate by the enzymatic activity of sulfiredoxin (SRX). To protect the target protein from irreversible oxidation, the SO⁻ intermediate commonly forms reversible disulfide (S-S) or sulfenic-amide (S-N) bonds (green boxes). SO⁻ can either form a disulfide bond by reacting with an intra- or intermolecular cysteine or with glutathione (S-SG) or form a sulfenic-amide bond by reacting with the backbone amide nitrogen atom. The enzymatic activity of glutaredoxin (GRX) and thioredoxin (TRX) restore protein function by returning the oxidized protein back to its reduced state.

Figure 3. Possible mechanisms for H₂O₂-dependent signal transduction

(A) The redox relay mechanism uses a scavenging enzyme such as glutathione peroxidase (GPX) or peroxiredoxin (PRX) to transduce the H₂O₂ signal and oxidize the target protein.

(B) With the floodgate model, H₂O₂ inactivates the scavenger, perhaps through hyperoxidation to sulfinic (SO₂⁻) acid or through a post-translational modification (PTM), to allow for H₂O₂-mediated oxidation of the target protein. (C) The scavenging enzymes accept H₂O₂ oxidation and transfer the oxidation to an intermediate redox protein such as

thioredoxin (TRX), which subsequently oxidizes the target protein. **(D)** Dissociation of the target protein from the oxidized scavenging enzyme results in target protein activation.