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Chemistry and biology of reactive oxygen species in signaling or stress responses

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Abstract

Reactive oxygen species (ROS) are a family of molecules that are continuously generated, transformed and consumed in all living organisms as a consequence of aerobic life. The traditional view of these reactive oxygen metabolites is one of oxidative stress and damage that leads to decline of tissue and organ systems in aging and disease. However, emerging data show that ROS produced in certain situations can also contribute to physiology and increased fitness. This Perspective provides a focused discussion on what factors lead ROS molecules to become signal and/or stress agents, highlighting how increasing knowledge of the underlying chemistry of ROS can lead to advances in understanding their disparate contributions to biology. An important facet of this emerging area at the chemistry-biology interface is the development of new tools to study these small molecules and their reactivity in complex biological systems.

Biomolecules can be modified by oxidation-reduction (redox) reactions in a temporal and sequence-specific manner to adjust their three-dimensional structure and function. Perhaps nowhere is this fact better illustrated than in the first enzyme crystal structure, which revealed that hen egg lysozyme forms four disulfide bonds through oxidation of eight cysteine side chains to stabilize its folded form[1]. Despite the rich history of redox chemistry and its broad consequences for health, aging and disease, researchers are now only beginning to scratch the surface in understanding of the multitude of redox-regulated chemistries that can occur in biological systems. In this context, ROS formed from electrontransfer reactions at oxygen are major molecular sources of redox equivalents at the cell and organism level. ROS are often the small molecules responsible for mediating redox modifications of various biomolecules and are prevalent in diseases ranging from cancer to neurodegenerative diseases to diabetes. The overproduction and/or mismanagement of ROS leads to the general phenomenon of oxidative stress that is implicated in aging and death[2,3]. However, a more sophisticated and nuanced view of ROS is emerging that goes beyond a simple story of stress and disease, as organisms have also evolved a growing number of increasingly well understood, diverse mechanisms to harness the reactivity of ROS for a wide variety of essential physiological processes[4–10].

This Perspective, in the context of the theme of sensors and switches, will highlight the signal and stress dichotomy of ROS chemistry and how it affects biological systems from a molecular, cellular and organismal level. In this spirit, the purpose of this Perspective is not

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to collate citations for a comprehensive review but to provide an introduction to how cells and organisms sense and use ROS as signal and/or stress agents through the exquisite control of chemistry. Although beyond the scope of this review, the related reactive nitrogen species (RNS) such as nitric oxide ($[NO]^{\bullet}$) are also important for human physiology[11,12] and can react with ROS to form oxidants such as peroxynitrite[13]. We begin by describing biologically relevant ROS molecules and where they are generated, move on to address what and how biomolecules are modified by ROS and how they are controlled at a cellular level and close by highlighting select examples of biological processes that are mediated by ROS and the chemical tools that are available for studying the functions of these small molecules in complex systems. The common theme of this Perspective is that the chemistry of ROS is the key feature in determining the downstream biological outcome.

Biologically relevant ROS

The term ROS remains useful for global descriptions of downstream phenotypes, but because ROS encompass a family of molecules, and not one discrete chemical entity, the molecular identity of each ROS is often of critical importance in determining both its chemical reactivity and the biological response(s) to those reactions. Superoxide ($[O_2]^{\bullet-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), lipid peroxides (ROOH), ozone (O_3) and hydroxyl radical ($[OH]^{\bullet}$) are some of the major ROS in living systems, and determining whether an individual ROS is present at sufficient concentrations to participate in productive chemistry in a given situation is crucial to elucidating its biology.

Where ROS are generated

Another key consideration for ROS chemistry and biology is the subcellular location where a particular metabolite is generated, as microenvironments can dictate what targets these ROS molecules will potentially encounter in a spatial and temporal manner. The classic examples of organelles with localized ROS generation for physiology include phagosomes within specialized cells of the immune system used for pathogen killing[14], and peroxisomes[15], which mediate catabolic oxidation reactions for energy metabolism. In addition to these canonical ROS sources, we highlight three other main locales for ROS production in cells under physiological conditions (mitochondria, the endoplasmic reticulum (ER) and cell membranes), noting that other organelles (such as the nucleus and Golgi) as well as ROS cross-talk between subcellular regions are also open fields for study.

Mitochondria and electron transfer

Mitochondria house the electron transport chain (ETC), which transfers electrons from NADH and succinate along a controlled redox path that ends in the four-electron reduction of O_2 to H_2O during respiratory ATP synthesis. However, either by accident or for a purpose, the flow of electrons through the ETC is an imperfect process, and occasionally oxygen molecules undergo one- or two-electron reduction reactions to form ROS, particularly H_2O_2 and $[O_2]^{\bullet-}$ (ref. 16). Mice with mitochondria-targeted overexpression of catalase, an enzyme that quickly and specifically destroys H_2O_2, live longer[17] and show protection against age-related decline in mitochondrial function and insulin resistance[18]. For this reason, it has been commonly assumed that mitochondrial leakage of ROS is an inevitable consequence of aerobic respiration, similar to pollution from an automobile, and that cells are constantly combating these aberrant ROS fluxes. However, newer data suggest that cells have evolved exquisite mechanisms to use mitochondrial ROS in a controlled fashion for physiological benefits. For example, ETC-generated H_2O_2 can regulate neuronal dopamine release through ATP-sensitive potassium channels[19,20]. In addition, the Src homology and collagen homolog protein (p66shc) is an adaptor protein for receptor protein

tyrosine kinase signaling but also has a redox signaling and stress role by catalyzing electron transfer from cytochrome *c* to reduce O_2 to $[O_2]^{\bullet-}$ and H_2O_2 (ref. 21). In response to cellular stress, p66shc mediates ROS generation, usually signaling for apoptosis[22], but certain cell types use p66shc-generated ROS for growth signaling[23], suggesting that this redox protein can serve to generate ROS for either proapoptotic or proliferative processes.

Endoplasmic reticulum and oxidative protein folding

The primary source of ROS from the ER results from oxidative protein folding—secreted proteins often undergo disulfide bond formation as a post-translational modification as they fold in the ER lumen. The vast majority of protein disulfide formation reactions in the ER are initiated by the glycoprotein Ero1, which triggers a two-electron oxidation of the thioredoxin protein disulfide isomerase (PDI)[24]. In its oxidized disulfide form, PDI is then used to introduce disulfide bonds into protein targets through thiol-disulfide exchange. Ero1 uses O_2 as a two-electron acceptor to form one equivalent of H_2O_2 for each disulfide bond formation catalyzed, providing a strong flux of ROS in this cellular locale. In addition to the oxidative folding machinery, the ER can also house an isoform of NADPH oxidase (Nox4) that primarily generates H_2O_2 from O_2 by a two-electron reduction[25].

Cell membranes and NADPH oxidases

Another major source of physiological ROS, in the form of either $[O_2]^{\bullet-}$ or H_2O_2, are NADPH oxidases (Nox) and their dual oxidase relatives (Duox), which are localized to various cellular membranes[4,26,27]. Nox proteins are classically known as important ROS producers for the phagocytic killing of pathogens during the immune response. However, the discovery of a vast array of different Nox and Duox isoforms in virtually every cell type throughout the body suggests more general roles for these ROS-producing enzymes[28]. Indeed, beginning with the first report that receptor tyrosine kinase signaling activates Nox-derived ROS production at the plasma membrane[29], the list of receptor-ligand interactions connected to Nox-regulated redox signaling continues to expand at a rapid pace[6]. Because of the widespread yet differential expression of Nox and Duox isoforms across organelles, cell types and organisms, the use of H_2O_2 and $[O_2]^{\bullet-}$ signaling in this manner can potentially be placed in the same class as other ubiquitous small-molecule messengers such as calcium ions (Ca^{2+}) and $[NO]^{\bullet}$.

Chemical targets and reactions mediated by ROS

Once specific types of ROS are generated at a given time and place, they can mediate a diverse array of reversible or irreversible redox modifications on biomolecules ranging from proteins to lipids to DNA and RNA. In this context, the chemical reactivity of an individual ROS is generally dictated by whether it prefers one- or twoelectron oxidations. Additionally, as many highly reactive oxidants have short half-lives within the cellular milieu, ROS reactivity is also regulated by which biomolecule targets are within close proximity of the site of ROS generation (see next section). With these principles in mind, we highlight a selection of ROS targets classified by their chemical functionality, using protein-based examples (Fig. 1). We primarily focus on cysteine residues owing to the greater knowledge base about the pathology and physiology of the oxidation of this amino acid, and we then extend our discussion to other amino acid targets of ROS.

Cysteines

Oxidation of the thiol side chains of cysteine, usually by an initial two-electron oxidation mediated by H_2O_2 or HOCl, is the most commonly recognized and studied redox post-translational modification[7] of proteins. Such reactions generally exploit the electrophilic nature of these particular ROS, as nucleophilic attack by deprotonated thiols releases either

H_2O or Cl^- to form a sulfenic acid, which can then go on to form internal or mixed disulfides or other products. HOCl reacts rapidly with thiolates in a relatively nonspecific manner, as illustrated by the difference in reaction rate of glutathione with HOCl (3×10^7 M^{-1} s^{-1}) as compared to H_2O_2 (0.9 M^{-1} s^{-1})[14]. However, the thiolate reactivity of H_2O_2 can be tuned and substantially accelerated by the local structure and environment of the target residue. Indeed, this fact is highlighted by the large difference in reaction rate constants of H_2O_2 with redox-active peroxiredoxin 2 (Prx2; 2×10^7 M^{-1} s^{-1})[30] and protein tyrosine phosphatase, nonreceptor type 1B (PTP1B; 20 M^{-1} s^{-1})[31], two major targets of redox signaling in cells. Thus, H_2O_2 can serve as a ubiquitous yet selective second messenger that can oxidize and modulate a wide array of thiol or thiolate-containing targets with kinetic control[14]. Sulfenic acids are reactive intermediates that can be trapped by internal thiols to form an intramolecular disulfide, react with glutathione (glutathionylation) or other external thiols to form intermolecular disulfide species or cyclize into sulfenamide structures[32,33]. Moreover, sulfinic and sulfonic acids can be formed by subsequent two- and four-electron oxidations of sulfenic acid congeners, and the discovery of Sulfiredoxin proteins, which can convert sulfinic acids back to thiols, presages an additional layer of redox regulation at this higher oxidation state[34,35].

The number of cellular targets of H_2O_2 that undergo reversible cysteine oxidation is rapidly growing and encompasses a range of different biological processes; we highlight a few examples here. Phosphatases such as PTEN[5,36] and PTP-1B[32] can be reversibly deactivated by H_2O_2 production, forming an intermolecular disulfide or sulfenamide, respectively, to enhance forward kinase signaling for various receptor-ligand interactions. Transcription factors such as Yap1 (ref. 37) in yeast and FoxO4 (ref. 38) in mammals can detect H_2O_2 and activate genes associated with redox regulation. The activity of matrix metalloproteinase-7 can be regulated *in vitro* by oxidation, in which the addition of HOCl results in activation of the proenzyme[39]. The nucleocytoplasmic shuttling of the histone deacetylase HDAC4 is controlled by a thioredoxin 1—dependent, H_2O_2-mediated disulfide formation to regulate cardiac hypertrophy[40].

Other amino acids

In addition to cysteine thiols, various other amino acids can be oxidized by ROS. The aberrant oxidation of lysine, arginine, proline and histidine residues, usually catalyzed by redox-cycling metal ions such as Fe^{2+} and Cu^{2+}, can result in the conversion of the side chain amines to carbonyls, potentially altering a protein's function, and the protein carbonyl content of an organism or tissue often serves as a marker of general oxidative stress[41]. HOCl, which is generated from the activity of myeloperoxidase, can react with tyrosine residues to form 3-chlorotyrosine, 5-chlorotyrosine and 3,5-chlorotyrosine[42], which are implicated in impaired protein function associated with high-density lipoprotein in atherosclerosis[43] as well as diminished airway function in children with cystic fibrosis[44]. However, organisms have evolved mechanisms to both sense and recycle oxidation of at least some amino acids other than cysteine, suggesting that these modifications may be physiological in nature. For example, methionine thioethers can be oxidized to the corresponding sulfoxides, and methionine sulfoxide reductase, an enzyme that is crucial for normal lifespan in mammals, can reverse this modification[45]. PerR is a transcription factor found in *Bacillus subtilis* that regulates redox defense genes and can detect low levels of H_2O_2 by metal-catalyzed oxidation of histidine[46]. Protein cofactors can also serve as redox sensors, as exemplified by the SoxR transcription factor, which senses $[O_2]^{\bullet-}$ by oxidation of an iron-sulfur cluster[47], as $[O_2]^{\bullet-}$ oxidizes iron-sulfur clusters at a rate that is almost diffusion limited as a result of high charge attraction[8].

How cells control ROS chemistry for signaling functions

Because of their transient and reactive nature, a major question for ROS chemistry in living systems is how do cells funnel these small molecules selectively toward physiological redox signaling over uncontrolled oxidative stress pathways? Here are a few recent findings that illustrate the principles of how ROS selectivity can be regulated in a spatial and temporal manner at the subcellular level to promote kinetically competent redox reactions; it is likely that multiple layers of control are working in conjunction to mediate physiological ROS signaling (Fig. 2).

Colocalization of ROS sources and targets

Many types of ROS will not migrate far from their source of production because of their inherent instability and reactivity, and because of the redox-buffering capacity of a cell. For example, the cellular half-life of $[OH]^{\bullet}$ is only about 10^{-9} s because of its reactivity (the reduction potential of the $[OH]^{\bullet}$, $H^{+}-H_2O$ couple is 2.31 V) compared to about 1 ms for H_2O_2 (ref. 8). This means that $[OH]^{\bullet}$ will react with or very near to the biomolecule that produced it, whereas H_2O_2 can diffuse away from its source. Moreover, as shown by the reactivity of H_2O_2 with various cysteine thiols, the wide range of observed reaction rates between ROS sources and targets affords another level of discrimination. As such, a primary layer of control for ROS signaling is the colocalization of sources and targets of ROS by generation of the small-molecule oxidant in proximity to a given substrate. This form of regulation directly influences the kinetics of a putative chemical signaling reaction by controlling the local concentrations of the participating molecular reactants. For example, Nox proteins that influence receptor tyrosine kinase signaling via H_2O_2 and $[O_2]^{\bullet-}$ are often colocalized with their putative physiological targets, such as phosphatases and kinases, at the plasma membrane. This also prevents oxidation of pathological targets such as nucleotides that are confined to other parts of the cell[48,49]. Indeed, recent data show that ROS generation is localized for signaling in various cell types[50]. Other examples of colocalization of ROS signaling sources and targets is ER localization of the H_2O_2 generator Nox4 and its phosphatase target PTP1B[51] and the localized generation of HOCl by myeloperoxidase in phagosomes for pathogen defense[14].

Modulation of local redox buffer capacity

Through various measurements and calculations, the intracellular concentration of H_2O_2 has been determined to fluctuate between the low-nanomolar to low-micromolar range[6]. These estimates, however, assume an even distribution of H_2O_2 throughout the cell. As previously explained, the sources of each ROS are localized to specific regions, suggesting that ROS fluxes may not be homogenous in concentration across a living cell and that the concentration of ROS near a source of generation can reach a high local concentration.

In addition to localizing a transient increase in ROS concentrations in proximity to a given target, another layer of physiological ROS control occurs through alterations in local redox buffering capacity. As described above, the millimolar concentrations of cellular glutathione provide a substantial redox buffer for many ROS such as HOCl, but they react too slowly with H_2O_2 to provide much buffering capacity. Peroxiredoxins, however, have remarkably fast reaction rates with H_2O_2 and provide a prime example of local redox control of H_2O_2 owing to two different mechanisms that can modulate peroxiredoxin activity.

Peroxiredoxin proteins typically cycle between reduced dithiol and oxidized disulfide forms mediated by glutathione and H_2O_2, respectively[52]. However, the Prx2 isoform is also susceptible to overoxidation by reaction of the sulfenic acid form of the protein with a second equivalent of H_2O_2, resulting in a transient catalytically inactive protein. In this way,

low levels of H_2O_2 are quickly and efficiently quenched by the redox-buffering capacity of Prx2. However, H_2O_2 generation at specific subcellular locales can cause a localized overoxidation and deactivation of Prx2, thereby allowing the redox signal to build up in a defined and controlled region as dictated by the source. This so-called `floodgate' model illustrates an elegant and powerful mechanism by which a cell can control localized fluxes of ROS for selective cellular chemistry[52,53].

A second example of localized redox buffer control has been identified recently, in which the Prx1 isoform, which is substantially less susceptible to overoxidation than its Prx2 congener, can be selectively deactivated by phosphorylation in cells stimulated with growth factors or in mice during cutaneous wound healing[54]. In this model, receptor activation is directly coupled to local changes in redox-buffering capacity through discrete kinase signaling cascades.

Membrane transport and sequestration

We recently discovered a third form of physiological ROS regulation that involves membrane ROS transport and sequestration, providing a physical barrier to off-site redox reactions. Building on previous work in plant and yeast models[55,56], we showed that the transport of H_2O_2 across mammalian cell membranes can be controlled by specific classes of aquaporins, integral membrane proteins originally identified as transporters of water and other small-molecule metabolites. Members of the aquaglyceroporin and unorthodox families of aquaporins, but not classical aquaporins, can enhance the permeability of mammalian cell membranes to H_2O_2, and models with both Nox and aquaporin use the latter to regulate transport of extracellularly generated H_2O_2 across the plasma membrane to mediate intracellular signaling cascades[57]. This work suggests that individual cell and tissue types can potentially be tuned for their susceptibility to H_2O_2-mediated cellular signaling or stress, depending on which aquaporins or related channels are displayed on their cell surfaces.

Physiological processes mediated by ROS signaling

Various redox-regulated physiological processes have been identified that cement ROS signaling as a diverse, important and widespread biological phenomenon. Owing to space limitations, the discussion here is limited to three recent examples in which ROS signaling contributes to physiology and shows the breadth of redox regulation at the cellular and organismal scale (Fig. 3).

Cell migration

Redox signaling can regulate cell migration at both the molecular and whole-organism levels. At a molecular level, cells respond to various stimuli by generation of Nox-derived H_2O_2, which can then modulate the local cytoskeleton organization and hence cell migration. This process is mediated by cofilin, an important regulator of cellular actin dynamics, through redox modulation of its opposing phosphatase Slingshot-1L. Slingshot-1L is activated by its release from a regulatory complex through H_2O_2-mediated oxidation, which in turn induces cofilin-mediated membrane ruffling and cell motility[58]. Specifically, colon cancer cells rely on c-Src tyrosine kinase–induced, Nox1-generated H_2O_2 to form functional invadopodia for normal cell migration[59]. At the whole-organism level, zebrafish have been shown to produce tissue-scale fluxes of H_2O_2 generated from the Nox isoform Duox upon tail lacerations[60]. The H_2O_2 signal traverses hundred of micrometers through the zebrafish epithelium to recruit leukocytes to the wounded area.

Circadian rhythm

Another exciting new area of physiological redox signaling is the circadian rhythm, with the recent discovery that the oxidation state of peroxiredoxin proteins can provide a way for cells to keep time without transcription or translation. Red blood cells, which lack a nucleus and most other organelles, including mitochondria, use 24-h redox cycles of peroxiredoxin proteins that persist for many days, under constant conditions that are entrainable and temperature compensated[61]. Similar findings were reported for *Ostreococcus tauri*, a green algae that suspends all transcription when kept in the dark but does not reset its clock upon reintroduction into light. *O. tauri* keeps track of time in the dark, without transcription, through similar peroxiredoxin redox cycling events[62]. Although the molecular basis and ROS responsible for these redox fluctuations are yet to be identified, the correlations between fluctuations in ATP and NADPH suggest a link between peroxiredoxin oxidation, central metabolism and circadian rhythm. This fascinating finding could potentially define a general and conserved regulatory mechanism for controlling circadian rhythms in various contexts.

Stem cell proliferation and neurogenesis

Finally, recent studies have established that physiological H_2O_2 signaling is essential for stem cell proliferation, as illustrated in neural stem cell models, and can also influence subsequent neurogenesis. Using a newly developed H_2O_2-responsive fluorophore, Peroxyfluor-6 (PF6), in combination with *in vitro* biochemistry and cellular assays and *in vivo* knockout mice studies, we recently discovered that adult neural hippocampal progenitors use Nox2-derived H_2O_2 to regulate growth signaling and maintain normal stem cell population sizes and levels of neurogenesis[63]. Concurrent with our work, another report documented that a population of neural stem cells located in the subventricular zone also use Nox2-derived ROS to modulate stem and progenitor pools and neurogenesis[64]. Taken together, these two studies show that brain-derived ROS are not solely detrimental to the fitness of a living organism and can in fact provide tangible benefits. These results also provide a molecular theory for the cognitive deficits observed in mice and humans lacking Nox2, and they suggest a link between H_2O_2, brain health and memory formation. Moreover, these findings provide one physiological mechanism to explain why nonspecific administering of antioxidants is generally a poor therapeutic.

chemical tools for studying ROS biology

The broad physiological and pathological consequences of ROS biology and the chemical complexities associated with these reactive small molecules provide a need for new and better methods to monitor the origins and fates of ROS, particularly those that can be used in intact living specimens and give real-time information. We present a subset of the most current chemical tools for studying ROS biology (Fig. 4), highlighting new opportunities for innovation.

ROS detection

As emphasized in the previous sections, each type of ROS molecule will have its own distinct reactivity in terms of selectivity and kinetics in a given biological context, so a primary need is to devise methods that allow detection of specific ROS metabolites in living cells and organisms. Traditional probes such as dichlorodihydrofluorescein remain useful as global ROS indicators, but because the chemistry for C-H oxidation in this dye and related fluorophores is not selective, one must be cautious about overinterpreting data and attributing biological effects to a single ROS[65]. To address this concern, a growing number of small-molecule and protein-based detectors have been introduced for monitoring various ROS, including $[O_2]^{\bullet -}$, H_2O_2, HOCl, $^1O_2$ and O_3, as well as global redox changes and

related reactive nitrogen species such as $[NO]^{\bullet}$ and peroxynitrite ($ONOO^{-}$)[66–79]. Protein-based ROS detectors take advantage of redox-active domains, often transcription factors or antioxidant defense proteins, tethered to fluorescent proteins, which generally rely on cysteine oxidations to modulate a fluorescent response. Two notable examples include HyPer[73], a H_2O_2-specific protein sensor that uses the bacterial transcription factor OxyR, as well as circularly permuted yellow fluorescent protein (cpYFP), which was fortuitously discovered to respond to reaction with $[O_2]^{\bullet-}$ (ref. 77). Small-molecule probes generally attempt to selectively detect a single ROS by targeting a unique reactivity of that particular ROS. For example, [NO] probes have taken advantage of the $[NO]^{\bullet}$-mediated conversion of diamines into triazoles[68] as well as the redox reaction between $[NO]^{\bullet}$ and Cu(II)[69].

Our laboratory has developed reaction-based approaches to selective H_2O_2 detection that exploit the H_2O_2-mediated conversion of aryl boronates to phenols or oxidative decarboxylation of α-ketoacids and incorporated these organic switches into fluorescent, bioluminescent and magnetic resonance imaging (MRI) modalities[80–87]. Recently, we have used the simple and versatile boronate switch to create H_2O_2-selective fluorescent turn-on probes with color modularity for dual imaging of multiple ROS simultaneously during the phagocytic respiratory burst[85], targeting groups for imaging of mitochondrial-localized ROS production in disease states[83] and methyl ester or acetoxymethyl ester functionalities as cytosolic trapping groups for sensitive detection of H_2O_2 during growth factor signaling in colon cancer and neural stem cells[57,63] (Fig. 4a). In addition, nanoparticle- and luciferase-based systems have proven useful for *in vivo* imaging of H_2O_2 (refs. 88,89). More recently, a quantitative mass spectrometry approach using a mitochondrial-targeted boronate was developed that allows the measurement of mitochondrial H_2O_2 levels in various tissues *in vivo* to address issues in aging and longevity[10] (Fig. 4b).

As the identity of the ROS generated in a given system is crucial to dictating the chemistry and, hence, downstream biology, extreme care must be taken to clearly delineate which molecules are involved in initiating these processes. Although many creative and useful specific ROS detection systems have been developed, specifically those for $[O_2]^{\bullet-}$, H_2O_2, HOCl and $[NO]^{\bullet}$, improved technologies are still in great demand. In particular, the discovery of new chemical reactions that can specifically detect other ROS, the creation of probes with faster reaction rates and the creation of reversible probes that can detect transient ROS fluxes would all help to decipher the complex redox processes that take place in biological systems.

Controlled ROS production

Because the timing and location of ROS chemistry is tightly regulated in living systems, exogenous addition of reactive molecules to whole-cell cultures or animal models cannot, in many cases, accurately mimic true physiological situations. As such, another potentially powerful set of tools to dissect ROS biology includes reagents that can locally produce a particular ROS on demand and in a controlled fashion. One technology for chemically controlled H_2O_2 generation uses D-amino acid oxidase, an enzyme that produces H_2O_2 upon reaction with the substrate *N*-acetyl-D-alanine[90]. In this way, local H_2O_2 generation from a genetically encodable system can be initiated using a chemical trigger, and this method has been applied to astrocyte H_2O_2 production. General oxidative stress photosensitizers that target nuclei and mitochondria were created using organelle-specific peptide delivery systems[91]. In parallel, our laboratory has developed a caged small-molecule H_2O_2 generator that rapidly produces H_2O_2 on demand upon cleavage of a photolabile protecting group[92]. We used this new reagent to produce H_2O_2 in living cells by light activation and trigger downstream redox regulation of cofilin, leading to actin polymerization and cell migration.

ROS target identification

A final key area in ROS chemical biology is to develop new approaches to measure and detect ROS-mediated modifications in living systems. Classically, oxidative stress conditions are globally assessed by measuring oxidized DNA or RNA (for example, 8-oxoG) and/or oxidized proteins (for example, protein carbonyl content). Additionally, hyperoxidized protein cysteines can be detected by western blot analysis with an antibody that detects sulfinic and sulfonic acids in proteins. However, technologies for detecting the transient and reversible oxidations associated with physiological events, particularly those that can be used in live-cell or even live-animal settings, are more informative to ROS signaling. For example, proteomics approaches that interrogate entire populations offer unbiased assessments of redox-active proteins. In this context, isotope-coded affi nity tags have been used to distinguish oxidant-sensitive cysteines in complex protein mixtures by using reactivity differences between free and oxidized thiols[93,94], and computational approaches comparing homologous proteins to identify sporadic incorporation of selenocysteine at cysteine active sites have been used to predict putative redox-active cysteine residues[95]. In addition, recent advances in activity-based protein profiling allow the quantification of nucleophilic, reactive cysteine residues that are more likely to be oxidized by ROS such as H_2O_2 (ref. 96). In terms of specific chemical modifications, elegant tools that exploit the selectivity of dimedone for sulfenic acid–modified cysteines coupled to fluorophores, affinity tags or both can be used to mark and identify proteins that have undergone this particular redox modification[97–99], and an antibody has been produced against a dimedone-modified cysteine residue for analysis by western blotting or pull-down assays[100] (Fig. 4c). This area is a particularly fruitful one as chemical biologists become more sophisticated in their thinking of redox chemistry and discrete reactions in the context of more complex systems.

Summary and prospects

The chemistry of a given ROS, which is influenced by its identity, concentration and local environment, is the key determinant of its downstream biological responses. As such, the study of ROS is an inherently well-suited area for the intellectual and practical approaches of chemical biology, as the invention of new chemical technologies that enable the assignment of sources, identities, concentrations and targets of ROS in complex living systems will greatly aid in elucidating the basic principles that control redox biology at the molecular, cellular and organismal levels.

Proteomics and other unbiased screening approaches should allow the identification of many new redox-sensing biomolecules and, when coupled with ROS-specific detection methods, allow chemical biologists to uncover many new processes mediated by ROS signaling and stress. Indeed, recent discoveries of ROS signaling in chemotaxis, stem cell proliferation, neurogenesis and circadian rhythm presage that redox chemistry can regulate a diverse array of biological processes. However, the molecular basis for much of this regulation is still largely unexplored. For example, it is clear that cell migration is regulated by specific redox modifications of proteins directly involved in cytoskeletal rearrangements and that external ROS cues can also signal cells to move toward an ROS source over relatively large distances. However, many of the links between the biochemical and whole-organism responses are still missing, and this will be a rich area for exploration. Studying various modes of ROS production, sensing and signaling at both the cellular and organismal levels is crucial for providing a coherent picture of how ROS are used by biological systems and offers an exciting set of challenges for understanding the contributions of these small molecules to health, aging and disease.

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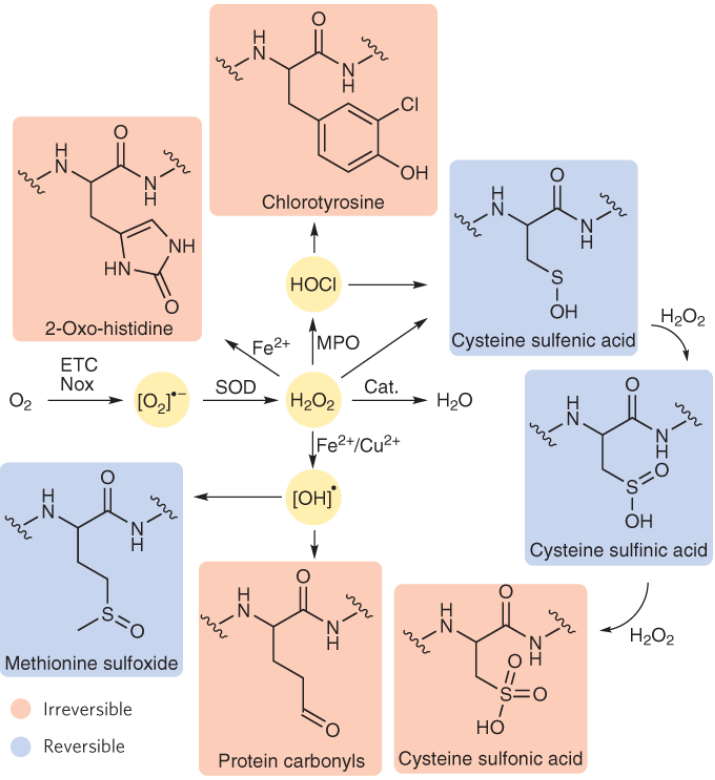

Figure 1. Reactions of primary ROS with functional groups on proteins

A one-electron reduction of molecular oxygen, either from the electron transport chain (ETC) or through the action of NADPH oxidases (Nox), yields superoxide ($[O_2]^{\bullet-}$). Superoxide is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) or by dismutation in aqueous solution. H_2O_2 can react with various functional groups; for example, this ROS can oxidize cysteine residues to form sulfenic acids or histidine residues to form 2-oxo-histidines. Sulfenic acids can then go on to form disulfide bonds or be further oxidized to sulfinic and then sulfonic acids by a second and third equivalent of H_2O_2, respectively. H_2O_2 can also be converted to hydroxy radical ($[OH]^{\bullet}$) by catalysis with redox-cycling metals such as Fe^{2+} and Cu^{2+}, which can then oxidize functional groups such as methionine residues to form methionine sulfoxides or other amino acids such as lysine, arginine, proline and histidine to form protein carbonyls. The enzyme myeloperoxidase (MPO) can convert H_2O_2 to the highly reactive hypochlorous acid (HOCl), which can oxidize cysteine residues to form sulfenic acids or tyrosine residues to form chlorotyrosine. Oxidized products in blue are those with known pathways to reverse the redox modification, whereas those products highlighted in red are thought to be irreversibly oxidized.

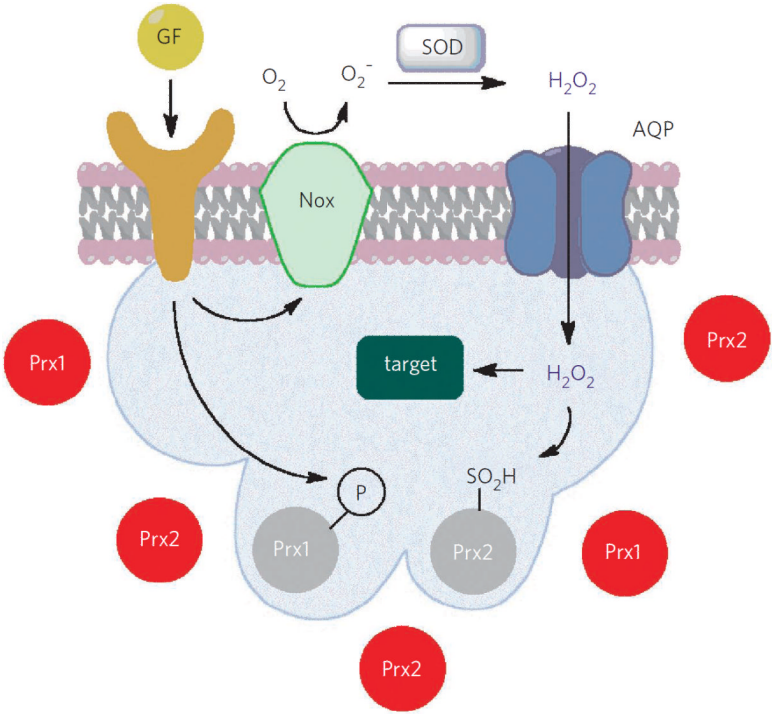

Figure 2. Potential layers of regulation for membrane-localized H_2O_2 signaling

Receptor activation, often by growth factors (GF) or other ligands, leads to superoxide ($[O_2]^{\bullet -}$) generation at the cellular membrane by Nox proteins, with subsequent production of H_2O_2 by dismutation or action of SOD. H_2O_2 can then pass through specific aquaporins (AQP) to reach the intracellular cytosol. Concomitantly, receptor activation also leads to localized Prx1 phosphorylation and deactivation, decreasing the redox-buffering capacity near the cell membrane. Localized rises in intracellular H_2O_2 levels can cause further deactivation of Prx2 by overoxidation. These various points of regulation can work together to lead to transient rises in H_2O_2 concentrations and the subsequent oxidation of local redox targets.

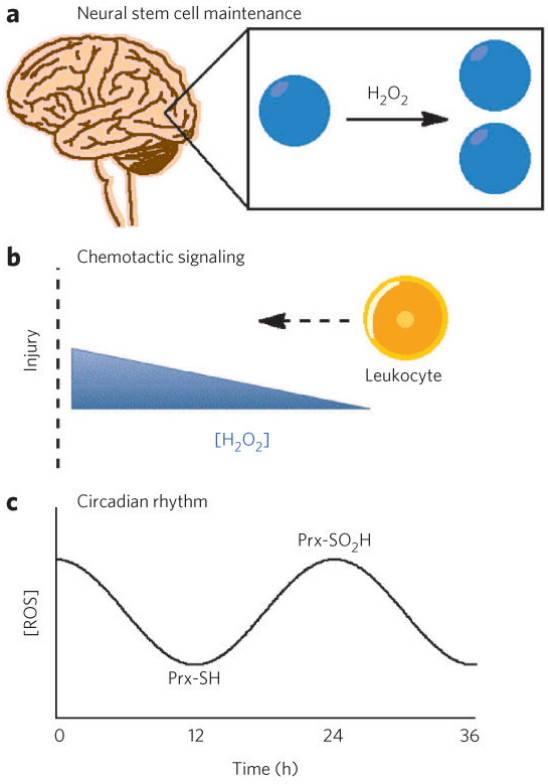

Figure 3. ROS signaling in physiology

(**a**) ROS have recently been discovered as second-messenger signaling agents used to control growth and maintenance of neural stem cells located in both the subgranular zone of the hippocampus as well as the subventricular zone of the lateral ventricles. (**b**) ROS have also been discovered as signaling agents at both the biochemical and whole-organism level to trigger chemotaxis and recruitment of leukocytes to damaged tissue. (**c**) Finally, the oxidation state of peroxiredoxins (Prx) have been shown to be modulated between reduced (Prx-SH) and oxidized (Prx-SO_2H) forms to regulate circadian rhythms in the absence of transcription or translation.

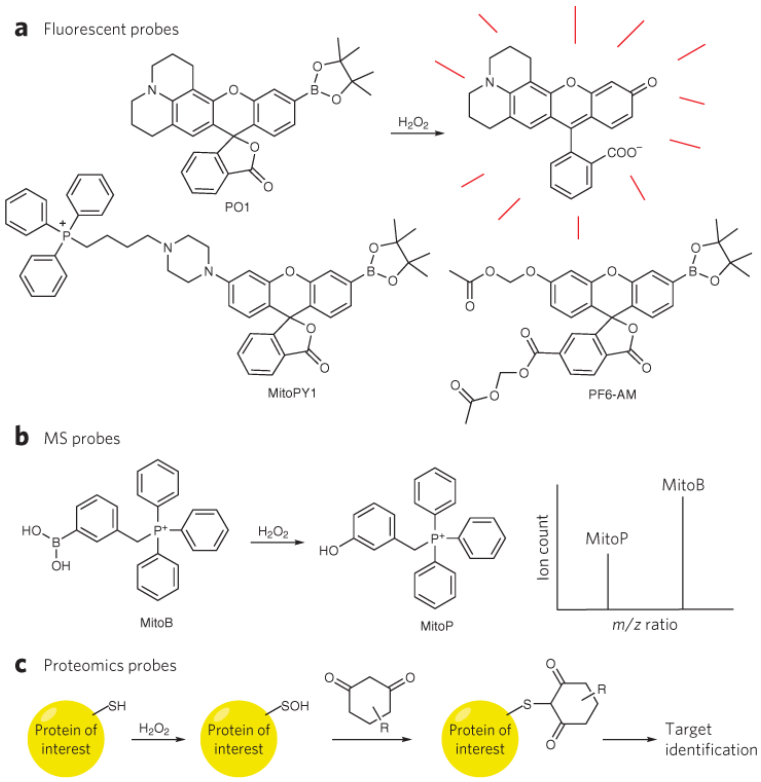

Figure 4. Chemical tools to study redox biology

(**a**) The conversion of boronates to phenols by H_2O_2 has been used to create a suite of novel fluorescent probes with various properties, such as red-shifted emission (Peroxy Orange 1, PO1), mitochondrial localization (Mitochondria Peroxy Yellow 1, MitoPY1) and enhanced sensitivity through cytosolic trapping groups (Peroxyfluor-6 acetoxymethyl ester, PF6-AM). (**b**) A mitochondrial-targeted MS probe, which similarly uses the conversion of a boronic acid to a phenol, allows ratiometric detection and quantification of H_2O_2 *in vivo* by analysis of the ion count ratios between the protected and deprotected form of the probe, which can be distinguished by differences in mass to charge (*m*/*z*) ratios. (**c**) Dimedone-based reactivity probes can trap oxidized cysteine residues from a sulfenic acid and when coupled to purification or labeling groups, allow the identification of the redox-modified target.