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Biological Production, Detection, and Fate of Hydrogen Peroxide

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Abstract

Significance: Hydrogen peroxide (H_2O_2) is generated in numerous biological processes. It transmits cellular signals, contributes to oxidative folding of exported proteins, and, in excess, can be damaging to cells and tissues. Although a strong oxidant, high activation energy barriers make it unreactive with most biological molecules. Its main reactions are with transition metal centers, selenoproteins and selected thiol proteins, with glutathione peroxidases (GPxs) and peroxiredoxins (Prxs) being major targets. It reacts slowly with most thiol proteins, and how they become oxidized during redox signal transmission is not well understood.
Recent Advances: Kinetic analysis indicates that Prxs and GPxs are overwhelmingly favored as targets for H_2O_2 in cells. Studies with localized probes indicate that H_2O_2 can be produced in cellular microdomains and be consumed by highly reactive targets before it can diffuse to other parts of the cell. Inactivation of these targets alone will not confine it to its site of production. Kinetic data indicate that oxidation of regulatory thiol proteins by H_2O_2 requires a facilitated mechanism such as directed transfer from source to target or a relay mediated through a highly reactive sensor.
Critical Issues and Future Directions: Absolute rates of H_2O_2 production and steady-state concentrations in cells still need to be characterized. More information on cellular sites of production and action is required, and specific mechanisms of oxidation of regulatory proteins during redox signaling require further characterization. *Antioxid. Redox Signal.* 29, 541–551.

Keywords: hydrogen peroxide, thiol protein, peroxiredoxin, redox regulation

Biological Chemistry

Hydrogen peroxide (H_2O_2) is a major biological reactive oxygen species, produced as a by-product of respiration and an end-product of numerous metabolic reactions. It is a transmitter of cellular signals, and in excess can be damaging to cells and tissues. This special issue focuses on different techniques for the detection of H_2O_2 in living systems. The objective of this article is to discuss aspects of its production and fate that have implications for the detection and interpretation of the results. The biological chemistry of H_2O_2 has been covered in more detail elsewhere (120) and here, I will provide a brief overview. H_2O_2 is a strong two-electron oxidant. It is more oxidizing than hypochlorous acid or peroxynitrite, but in contrast to these highly reactive species, H_2O_2 has a high activation energy barrier that must be overcome to release its oxidizing power. It reacts poorly or not at all with most biological molecules. Although H_2O_2 is a weak one-electron oxidant, if it can be reduced, the hydroxyl radical is one of the strongest oxidants known and it reacts widely with most biological targets at near-diffusion controlled rates.

Biologically, direct reactions of H_2O_2 are restricted to transition metal centers and relatively few other targets. It reacts with cysteine and selenocysteine residues, although with minor but important exceptions (see below), these reactions are slow (Table 1). The reaction occurs with the thiolate anion, so at physiological pH, low pK_a thiols are more ionized and, therefore, more reactive. Methionine residues react with H_2O_2, but ~100 times more slowly than thiols. Pyruvate has similar reactivity to reduced glutathione (GSH) (Table 1) and at typical intracellular concentrations (or when present in culture media) would be competitive with most thiols. Another relevant reaction is with carbon dioxide.

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Table 1. Main Reactions of Hydrogen Peroxide with Biological Compounds

Species	*Rate constant* (M^{-1} s^{-1})	*Initial product*	*References*
GSH	0.9	Sulfenic acid (–SOH)	(125)
Low pK_a thiol proteins	20–200	–SOH	
Prxs and GPxs	10^5–10^8	–SOH or –SeOH	(27, 28, 58, 77, 79, 106)
Met residues	0.007	Sulfoxide	(85)
Pyruvate	2.2	Acetic acid/CO_2	(110)
Carbon dioxide	0.02 (K=0.4)	Peroxymonocarbonate (HCO_4^-)	(6, 105)
Heme peroxidases/catalase	$\sim 10^7$	Compound I	(22, 52)
Metalloproteins	~200–	Higher oxidation state	(44)
Low molecular weight transition metal complexes	$\sim 5–20 \times 10^4$	Hydroxyl radical or higher oxidation state of metal	(32, 98, 114, 117)
oxyR	$(\sim 10)^5$	–SOH	(54)

GHS, reduced glutathione; GPx, glutathione peroxidise; Prx, peroxiredoxin.

The product, peroxymonocarbonate, is ~200 times more reactive than H_2O_2 with thiols and methionine (6, 105). However, the reaction is an equilibrium with only ~1% of the H_2O_2 in a physiological bicarbonate buffer being present as peroxymonocarbonate, and the forward step is slow. Acceleration of the reaction by carbonic anhydrase (6) may enhance its physiological relevance.

Transition metal centers

Transition metals such as iron(II) and copper(I) can cleave the O–O bond of H_2O_2 to generate hydroxyl radicals or activated metal complexes (45). These species are more reactive and less discriminating than H_2O_2 itself and are initiators of free radical reactions (44, 117, 119, 124). Physiological transition metal centers include low-molecular-weight chelates and redox-active metalloproteins (Table 1). The reaction is commonly referred to as the Fenton reaction (although strictly, Fenton chemistry refers only to iron) and in its simplest form, it can be written (where iron is complexed to ligand L) as follows:

$$L - Fe^{2+} + H_2O_2 \rightarrow L - Fe^{3+} + OH^{\bullet} + OH^- \quad \text{(Reaction 1)}$$

The mechanism is more complicated, however, and depending on the nature of the complex, the product could be the hydroxyl radical or a higher oxidation state of the metal (such as ferryl $[FeO]^{2+}$). Physiological forms of non-protein iron have not been directly characterized (mainly because as soon as cells or tissue are disrupted, this is likely to change). However, intracellular conditions would favor iron(II), and based on binding constants with potential chelators, GSH has been proposed as the most likely ligand with a lesser contribution by citrate (41). Most iron(II) complexes undergo Reaction 1, although rates vary substantially. For ongoing Fenton chemistry, the iron(III) formed needs to be recycled, for example, by GSH or ascorbate. This mechanism is also complicated, as the preferred ligand changes after oxidation and reduction rates also vary (1, 41). An important variation is where the metal is bound to a biological molecule, in which case site-localized oxidation can occur (18, 96). Reactions involving low-molecular-weight complexes are likely to be more prevalent in lysosomes, where they are formed by degradation of metalloproteins and their reactivity is enhanced by the low pH (128). However, there are many metalloproteins at other sites in the cell that are potential targets for H_2O_2.

Heme peroxidases, for example, myeloperoxidase, eosinophil peroxidase, and lactoperoxidase, react rapidly with H_2O_2 (Table 1). The initial product is a two-electron oxidized species (referred to as Compound 1) that oxidizes a range of substrates, to generate free radicals or two-electron oxidants such as hypochlorous acid. These major peroxidases are largely restricted to specialist cells that are involved in host defense (124), so although important for dictating the reactivity of H_2O_2 in inflammatory conditions, they are unlikely to have a universal role in cellular H_2O_2 metabolism. However, other heme proteins, including hemoglobin, myoglobin, and cytochromes, have surrogate peroxidase activity. Although they are less reactive than dedicated peroxidases, for example, the rate constant for cytochrome *c* is 200 M^{-1} s^{-1} (48), they are feasible intracellular targets and regulators of H_2O_2 action.

Thiol proteins

Due to the interest in thiol proteins as redox regulators of cell function, they have received particular attention as targets for H_2O_2. The vast literature on thiols and redox regulation will not be discussed here but is well covered in other reviews (30, 59, 84, 92, 123). The initial product of the reaction between a thiol and H_2O_2 is the sulfenic acid (–SOH). Sulfenic acids are transient species that react with another thiol to form a disulfide, which may be internal within a protein or intermolecular, such as a mixed disulfide with GSH. Reactions of low-molecular-weight forms are fast, with a lower limit of 10^5 M^{-1} s^{-1} measured for the rate constant for CysSOH reacting with $CysS^-$ (73). In serum albumin, the sulfenic acid is protected from its surroundings, and rate constants with CysSH and GSH of 22 and 3 M^{-1} s^{-1} (pH 7.4) have been measured (16). The active site CysOH of peroxiredoxin (Prx)2 reacts with GSH with a rate constant of 400 M^{-1} s^{-1} (80). In some proteins, the sulfenic acid reacts reversibly with a neighboring amide group to form a sufenyl amide, which protects it from further oxidation (86). Sulfenic acids can be further oxidized by H_2O_2 (hyperoxidized) to give the sulfinic acid. These rates tend to be 1000-fold slower than for the equivalent thiol (75, 78), and sulfinic acids should, therefore, reflect high oxidant exposure.

A select group of thiol proteins, most notably the Prxs and glutathione peroxidases (GPxs), react with H_2O_2 many orders of magnitude faster than other proteins and much faster than can be explained by the presence of an ionized thiol (Table 1) (27, 59, 123). Members of both families are widely distributed and, combined with GSH or thioredoxin recycling systems, they are the main contributors to cellular peroxide-metabolizing antioxidant activity (83, 104). The exceptional reactivity of the GPxs is, in large part, due to their reactive selenocysteine at the active site. For Prxs, ionization of the active site thiol is only one contributor to high reactivity, and they require additional activation of the H_2O_2 through hydrogen bonding in the transition state of the enzyme (35, 74). These proteins are highly reactive with other peroxides and peroxynitrite (107), all of which also contain an O–O bond that can be activated, but their reactivity with other oxidants or thiol reagents is unexceptional (79). Prxs are susceptible to hyperoxidation by excess H_2O_2, and it is noteworthy that their sulfenic acids undergo this reaction faster than do most protein thiol groups (43, 78). A likely explanation for this is that the structural features that activate the H_2O_2 for reaction with the thiol also increase its reactivity with the sulfenic acid. The bacterial transcriptional activator oxyR, which upregulates the expression of antioxidant defense proteins, also reacts rapidly with H_2O_2 (Table 1).

Non-reactions of H_2O_2

Just as relevant for understanding the biology of H_2O_2 is to appreciate what species do not react, or react too slowly to be important physiological targets. This is particularly pertinent when using compounds that are loosely classified as ''antioxidants.'' Many of these are good free radical scavengers but are essentially unreactive with H_2O_2. These include vitamin E and its analogues such as Trolox, polyphenols, and carotenoids. Where they do inhibit a reaction that involves H_2O_2, it is likely that they are scavenging a secondary radical in the reaction pathway. Another compound commonly used as an ''antioxidant,'' *N*-acetylcysteine, does react with H_2O_2, but for a thiol reaction, its high thiol pK_a (9.2) makes it particularly slow (125).

Sources of H_2O_2

It has been known for many years that H_2O_2 is produced in biological systems. Historically, it has been regarded as an unavoidable by-product of oxygen metabolism, with the exception of when it is produced in specialist cells such as the neutrophil where it acts as a peroxidase substrate and contributes to anti-microbial defense. Indeed, a small fraction of the oxygen consumed by mitochondria is converted to superoxide and then H_2O_2, and this can increase under stress conditions such as ischemia/reperfusion (72). H_2O_2 is also produced during autoxidation or redox cycling of a wide range of xenobiotics, and this is an important toxicity mechanism (119). Autoxidation of physiological compounds, particularly flavoproteins and heme proteins, is another significant source. It has been calculated that the rate of H_2O_2 production within *Escherichia coli* could be up to 20 μM/s, primarily as a consequence of autoxidation reactions (46, 91). The superoxide and H_2O_2 produced in these processes could be regarded as a trade-off against the advantages of oxygen metabolism that can usually be dealt with through antioxidant defenses. However, H_2O_2 (either directly or *via* dismutation of superoxide) is also formed as an intentional product of a wide range of enzymatic reactions that occur in diverse situations and diverse locations.

One common misconception is that superoxide dismutase (SOD), by increasing the rate of superoxide dismutation, increases the amount of H_2O_2 produced. This is not necessarily the case. Superoxide dismutates to oxygen and H_2O_2 (Reaction 2) regardless of the presence of SOD, and SOD will only change the stoichiometry of H_2O_2 production if it prevents the superoxide from carrying out a reaction other than dismutation. If this reaction involves superoxide acting as a reductant (*e.g.*, with cytochrome *c*, Reaction 3), then SOD would result in more H_2O_2 being produced. Alternatively, if superoxide were acting as an oxidant (as in Fe–S protein oxidation, Reaction 4), SOD would decrease H_2O_2 production. A mechanism whereby SOD can enhance H_2O_2 production is by displacing an unfavorable equilibrium that would usually result in little superoxide being generated (*e.g.*, Reaction 5) (122). Displacement of such an equilibrium between ubisemiquinone and superoxide occurs in mitochondria (29), providing a mechanism for MnSOD in the matrix and CuZnSOD in the intermembrane space to regulate the spatial release of H_2O_2.

$$2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \qquad \text{(Reaction 2)}$$

$$2O_2^{\bullet -} + 2\text{cyt}C^{3+} \rightarrow 2O_2 + 2\text{cyt}C^{2+} \qquad \text{(Reaction 3)}$$

$$2O_2^{\bullet -} + 2\text{Pr} - [4\text{Fe} - 4\text{S}]^{2+} + 2H^+ \rightarrow 2\text{Pr} - [4\text{Fe} - 4\text{S}]^{3+} + 2H_2O_2 \qquad \text{(Reaction 4)}$$

$$\text{semiquinone} + O_2 \leftrightarrows \text{quinine} + O_2^{\bullet -} \qquad \text{(Reaction 5)}$$

NADPH oxidases

NADPH oxidases (NOXs) catalyze the oxidation of NADPH and the reduction of oxygen and have been widely studied in relation to redox signaling. Most generate superoxide, which then dismutates to give H_2O_2, and some produce H_2O_2 directly (53, 55). The seven mammalian family members (NOX1-5 and DUOX 1 and 2) have a wide tissue distribution. They are all membrane-associated multi-component complexes, and they have the key feature of transferring electrons across the membrane from cytoplasmic NADPH to oxygen on the other surface. NOX activation occurs with a wide range of stimuli and at various membrane locations, including the plasma membrane, caveolae, endosomes, the endoplasmic reticulum (ER) membrane, and the nucleus, with the site varying depending on the NOX isoform, the cell type, and the stimulus. Thus, NOX-derived H_2O_2 will be formed locally and the site of generation will influence its reactions with cellular targets and detection systems.

Peroxisomal enzymes

There are enzymatic sources of H_2O_2 in cells that are not usually linked to signaling pathways. These include peroxisomal enzymes such as acylCoA oxidase, polyamine oxidase,

and D-amino acid oxidase (14, 23, 88), which reduce oxygen to H_2O_2 during the oxidation of their substrate. The catalase in most cells is restricted to peroxisomes, where it has a high capacity to break down and restrict the release of the H_2O_2. Peroxisomal catalase has the potential to break down H_2O_2 from other parts of the cell (14) and may, therefore, influence H_2O_2 metabolism at other sites.

Oxidative protein folding in ER

H_2O_2 is produced in the lumen of the ER during oxidative folding of exported proteins. Flavoprotein oxidases (ERO1 and QSOX) catalyze the reduction of oxygen to H_2O_2, with concomitant oxidation of Cys residues to disulfides (26, 34, 99). Although cellular production by this mechanism has not been directly measured, with one H_2O_2 produced per disulfide, large amounts could theoretically be produced (109). Thus, in a plasma cell making several million antibody molecules per minute (60), disulfide production by this mechanism would correspond to overall intracellular H_2O_2 production of tens of μM/min. The peroxide-metabolizing enzymes, Prx4 (100) and the GPxs, GPx7, and GPx8 are localized in the ER. GPx8 has been shown to trap H_2O_2 as it emerges from ERO1 and relays the oxidizing equivalents on to GSH (81). Further exchange reactions of glutathione disulfide enable the oxidizing potential of oxygen for disulfide bond formation to be maximized (5). Likewise, the H_2O_2 can be used for protein disulfide formation *via* Prx4, the oxidized form of which is recycled by several of the ER protein disulfide isomerases that promote protein folding (101). Presumably due to the effectiveness of these enzymes, there appears to be limited transfer of oxidative stress from the ER to other parts of the cell (36).

H_2O_2 Transport and Diffusion

There are multiple situations where cells generate or are exposed to H_2O_2. Depending on the site of H_2O_2 production and the specific targets it encounters, the responses of the cells can be quite diverse. One consideration is compartmentalization, and whether a membrane can constrain or direct the reactions of H_2O_2. Membranes do retard diffusion (3, 10), but they are not impermeable. Therefore, a high concentration of a reactive substrate needs to be present in the same compartment (such as catalase in peroxisomes) to restrict H_2O_2 diffusion through the membrane. H_2O_2 transport is facilitated by aquaporins, and, in particular, AQP3 and AQP8 have been shown to regulate cellular uptake (4, 9, 10, 65, 102). Thus, the diffusion of H_2O_2 from a cell compartment should be slower if aquaporins are not present in the membrane, and selective membrane localization could direct transport and promote specificity in cell signaling.

A largely unresolved conundrum related to NOX function is why H_2O_2 is generated on the non-cytoplasmic surface of the membrane when its targets are presumed to be in the cytoplasmic compartment. Transport *via* an aquaporin may, in some cases, provide a clue. Several investigators have observed that knockdown of one of the aquaporins inhibits both uptake of H_2O_2 into the cells and NOX-dependent growth factor or chemokine signaling (8, 38–40, 65). Although this mechanism would facilitate entry of H_2O_2, NOX activation is often not replicated by the addition of exogenous H_2O_2 and it is not obvious as to how an aquaporin would distinguish between the two sources. However, specificity could be achieved if an interaction between the NOX and aquaporin were able to channel H_2O_2 into the cell at the site of generation. At present, this is largely speculative, although evidence of NOX2 interacting with AQP3 has been reported (40).

Regardless of membrane barriers, the fate of H_2O_2 will depend on how far it diffuses before reacting. In cells, it appears that this distance is short. This has been nicely demonstrated by Belousov and colleagues (11, 67–69) by using the H_2O_2-specific genetically encoded probe, Hyper. They targeted fluorescently distinguishable Hyper variants to different cellular sites, including cytoplasm, ER surface, and cytoskeleton, and determined how far H_2O_2 can travel from a site of generation. With both physiological stimulation, and with targeted expression of D-amino acid oxidase, which produces H_2O_2 when supplied with D-alanine (61), their results showed H_2O_2 reacting very close to the site where it was generated. As the distance travelled before being consumed is inversely related to how rapidly it reacts with available targets, these observations imply that there are highly reactive targets in its path. To date, studies that have used this approach are limited, but it has considerable potential for exploring determinants of diffusion distances and establishing how they vary in different cell types and compartments.

Targets for H_2O_2

There are very few biological molecules that react fast enough with H_2O_2 to prevent it from diffusing from a localized site in a cell. Diffusion distance is inversely related to the concentrations of species that can react with H_2O_2 and how fast they react (defined by their rate constant). On this basis, it can be calculated as to how far H_2O_2 should diffuse in the presence of known cell constituents (118). Using this information for GSH and low pK_a thiol proteins at typical cell concentrations, it is clear that neither would prevent H_2O_2 from traveling beyond the dimensions of the cell. The only compounds known to have sufficient reactivity (if we ignore catalase because of its confinement in peroxisomes and heme peroxidases because they are present only in specialist cells) are reduced forms of the Prxs and the Gpxs. They consume H_2O_2 about a million times more rapidly than other thiol proteins (Table 1), and at cellular concentrations, would permit only limited diffusion. The Prxs are generally more effective because they are more abundant.

If Prxs and GPxs consume H_2O_2 rapidly and restrict diffusion, what is the likelihood of other targets being oxidized? This can be modeled by using a competitive kinetic approach based on concentration and rate constant data. For example, if we consider representative thiol and selenoproteins, any one of the Prxs and GPxs, provided it remained active, would outcompete other targets for more than 99% of the H_2O_2 (Table 2). The remainder would preferentially react with GSH over thiol proteins involved in redox signaling, because of their much lower abundance (21, 118). The strong preference for Prxs and GPxs is in line with the recognized activity as protective enzymes against deleterious reactions of H_2O_2. It is also evident experimentally in *E. coli* and *Saccharomyces cerevisiae*, which become highly sensitive to H_2O_2 when their main Prx (AhpC and Tsa1, respectively) is knocked out (46, 70, 90).

Redox signaling mechanisms

H_2O_2 does not act solely as a damaging species that needs to be removed. It is also a physiological regulator of

Table 2. Kinetic Hierarchy for Representative Cellular Targets of Hydrogen Peroxide

Protein	*Rate constant* (M^{-1} s^{-1})	*Estimated cell concentration* (μM)	*Percent of H_2O_2 consumption*	*[H_2O_2] for response time of 5 min* (μM)
Peroxiredoxin 2	2×10^7	20	~78	0.2
Glutathione peroxidase	6×10^7	2	~21	0.1
GSH	0.9	2000	~1	3000
KEAP1	140	1	~0.07	17
Cdc25B	160	<0.1	~0.01	14
PTP1B	20	<0.1	~0.001	120

Simulation of competition between a Prx (Prx2), GPx (GPx 1), KEAP1, GSH, and two protein tyrosine phosphatases (PTP1B and Cdc25B). Kinetic modeling was performed by using rate constants and estimated cell concentrations and by assuming homogeneous distribution. Response time refers to half the substrate being oxidized. See Marinho *et al.* and Winterbourn (59, 118), where more details of the modeling procedure and the parameters used are given.

H_2O_2, hydrogen peroxide.

metabolic activities and signaling pathways that depend on the redox state of their components. There are numerous documented examples of thiol proteins and other regulatory enzymes becoming oxidized during receptor activation or mild oxidative stress (37, 82, 103, 108). How this occurs is something of conundrum when we consider their low reactivity in comparison to the professional thiol peroxidases. One possibility is that the peroxidases are simply overwhelmed at high or sustained H_2O_2 exposure, allowing sufficient to escape and undergo less favorable reactions. However, though feasible, this seems more applicable to oxidative injury rather than a regulatory process.

Alternatively, reversible or irreversible inhibition could limit the ability of Prxs and GPxs to compete for H_2O_2. These enzymes function within redoxin pathways. GPxs and 1-Cys Prxs are recycled by GSH and glutathione reductase/NADPH, and the eukaryotic 2-Cys Prxs are most efficiently reduced by thioredoxin and thioredoxin reductase/NADPH, or thioredoxin-related proteins such as protein disulfide isomerases (50, 83). If the reductive step becomes rate limiting, then the Prx or GPx will accumulate in its disulfide form and become less effective. The peroxidase activity of 2-Cys Prxs can also be decreased by phosphorylation and by irreversible inactivation through hyperoxidation (83, 126). However, whether these mechanisms alone would be sufficient to enable oxidation of other thiol proteins at the levels of H_2O_2 exposure expected during signaling is debatable. With Prxs/GPxs inactivated, H_2O_2 could diffuse throughout the cell, and GSH should still be a more favored target.

Similar conclusions can be reached by considering what steady-state concentration of H_2O_2 would be required to oxidize different targets. This is determined by the rates of formation and elimination (2, 59). Thus, if production increases, or removal is inhibited through inactivation of a Prx, then the H_2O_2 concentration will increase. Absolute intracellular concentrations of H_2O_2 have proved notoriously difficult to measure directly, and there is still a need to address this problem with current methodologies. The closest estimate for non-stress conditions is probably that obtained by Sies and coworkers in the 1970s (76, 93) by monitoring the spectral characteristics of catalase under controlled conditions. With the proviso that catalase should largely reflect peroxisomal conditions, they estimated rates of H_2O_2 generation for isolated perfused liver and calculated steady-state concentrations in the 10 n*M* range. Imlay (46), using turnover rates for the major H_2O_2-metabolizing enzyme, AhpC and knowledge that the H_2O_2-responsive transcription factor oxyR switches on at concentrations above 200 n*M* (33), estimated *E. coli* concentrations of around 50 n*M*. These concentrations would rise if Prxs were inactivated. However, to oxidize half of a thiol protein such as protein tyrosine phosphatase, PTP1B would require a sustained H_2O_2 concentration of 100 μM for at least 5 min (2, 59) (Table 2). Not only is it doubtful that such a concentration could be achieved, but also this mechanism seems unsuited for a rapid signaling response.

Thus, kinetic analyses based on the reactivity of H_2O_2 under homogeneous conditions do not provide a convincing explanation for how less favored cellular targets become oxidized. However, the cell with its numerous membranous structures and organelles is not homogeneous, and H_2O_2 production and targets will not necessarily be evenly distributed. As discussed earlier, confining H_2O_2 to react within a compartment or close to its site of generation requires a highly reactive target to be present, so this alone would not ensure oxidation of a less reactive target. Selective oxidation could be achieved if co-localization of the generator and target allowed facilitated oxidation without H_2O_2 being released in a freely diffusible form (Fig. 1). Such protein–protein interactions play an important role in many metabolic pathways and although the evidence is limited, there are indications that they could operate in a redox context. For example, a complex with NOX4 was found to facilitate oxidation of protein phosphatase 1 (87) and interactions between NOX4 and PTP1B (17), and between AQP3, NOX2, and the EGF receptor (40) have been observed.

Another concept for redox signaling that is gaining recognition is that it involves a relay mediated by initial oxidation of a highly reactive sensor. It has become evident that high reacting proteins such as the Prxs and GPxs function not solely to remove H_2O_2 but can also act as sensors and transmit oxidizing equivalents to less reactive thiol proteins (30, 84, 97, 123). This mechanism has been well characterized in yeast, where a Prx or Prx-like GPx acts as a sensor for the oxidative activation of the transcription factors Yap1 and Pap1 (24, 112), and more recently in mammalian cells for the transcription factor STAT3 (94) and for ASK1 (47). The most likely mechanism involves a mixed-disulfide intermediate between the sensor and the target (Fig. 1), but other mechanisms are possible. Protein–protein interactions should

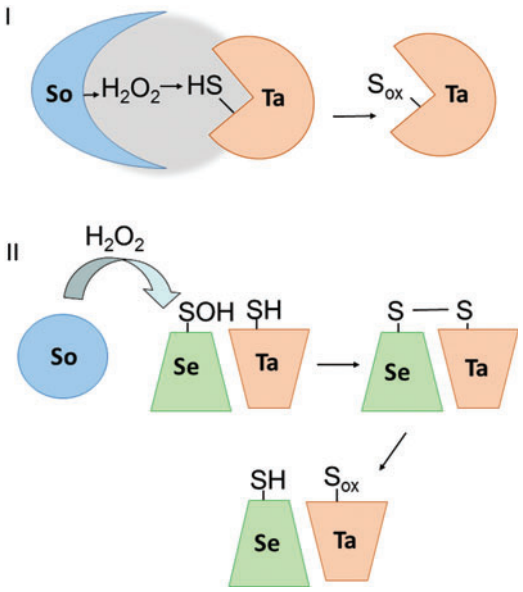

FIG. 1. Possible mechanisms for transmission of a redox signal initiated by H_2O_2 involving (I), site-directed oxidation of a target protein (Ta) without release of freely diffusible H_2O_2; and (II), oxidation *via* a relay from a highly reactive sensor protein (Se). So, H_2O_2 source; *gray shading*, restricted H_2O_2 zone. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars H_2O_2, hydrogen peroxide. To see this illustration in color, the reader is

facilitate oxidation *via* a sensor, as well as add selectivity, and indeed interaction with a scaffold protein is required for the Orp/Yap1 relay to occur (7, 111).

In summary, kinetic data predict that highly reactive thiol and selenoproteins would usually consume the majority of cellular H_2O_2. This agrees well with the antioxidant function of these proteins under oxidative stress but highlights the need for other factors to explain how redox regulation involving less reactive thiol proteins occurs. Site localization alone may not be sufficient. It is unlikely that a single mechanism covers all, but evidence is emerging for cases where directed transfer from source to target or facilitated oxidation *via* a sensor enables is involved.

Detection in Cells

An essential requirement for understanding the biology of H_2O_2 is to be able to detect it in living cells and tissue. Several methodological approaches are available, as described in detail in this special issue or elsewhere (11, 12, 49, 63, 121). These are invaluable tools for addressing fundamental questions related to the signaling and toxicological actions of H_2O_2. However, results can be misinterpreted and it is important to appreciate what information the different approaches can and cannot deliver. The main methodologies can be broadly classified on the basis of whether they use (i) small molecules that are oxidized indirectly by radical mechanisms, (ii) small molecules that are oxidized by non-radical mechanisms, and (iii) genetically encoded green fluorescent protein (GFP)-based probes that react directly with H_2O_2. This article focuses on the mechanisms underlying the methodologies and how this determines what aspect of H_2O_2 that they detect.

First, several caveats must be considered. As with many fluorescence methods, there is the potential for the probe itself to generate H_2O_2, for example, through light-induced oxidation (15, 127) or reaction with pyridine nucleotides (31, 113). As discussed later, specificity is an issue with some approaches. Also, the reaction of H_2O_2 with the probe occurs in competition with its reactions with other cell constituents. Even the highly reactive GFP-based probes will not scavenge all the H_2O_2 in the cell and the response reflects the balance between the detection reaction and other competing reactions. This is important for interpreting a change in probe response, as it may not necessarily indicate a change in H_2O_2 production but rather altered efficiency of removal by competing reactions. For example, it could represent a decrease or upregulation of Prx activity. Thus, none of the live cell methodologies are currently able to give absolute numbers for the amount of H_2O_2 being produced in cells or tissue, or its steady-state concentration. Their value lies in detecting whether H_2O_2 is being produced, and monitoring relative responses in different experimental situations.

Consider first the specific features of the radical-based probes, which include the widely used dichlorofluorescein, dihydrorhodamine, and luminol methods. They can (with appropriate controls) give an indication of whether a cell shifts to a more oxidizing state but, as discussed in more detail elsewhere (25, 49, 115, 121), they are not good detectors for attributing a reaction to H_2O_2. Most importantly, they do not react directly with H_2O_2, but require a transition metal catalyst. This could be a low-molecular-weight chelate, a peroxidase or cytochrome, or another metalloprotein center. Also, the intermediate probe radical can react with oxygen to generate H_2O_2 or be quenched by radical scavengers. Therefore, a change in availability of the metal catalyst, oxygen tension, or the radical scavenging capacity of the cell can affect the response without any change in the generation or metabolism of H_2O_2. These probes are also oxidized by peroxynitrite, hypochlorous acid, and other oxidants; thus, taking all these points together, interpretation must be limited.

One example where a radical-generating probe can be used with more confidence is for quantifying how much H_2O_2 is released by cells. Amplex red is most commonly used, and horseradish peroxidase is added to scavenge all the released H_2O_2 and to catalyze oxidation to fluorescent resorufin (66). Extracellular radical scavengers [and superoxide (51)] will suppress the signal by reacting with the detector radical but with appropriate controls, reliable data can be obtained. Azide is usually added to inhibit catalase, but as other intracellular enzymes also consume H_2O_2, this does not necessarily represent the total amount of H_2O_2 produced by the cells.

Small-molecule probes that react directly with H_2O_2 are predominantly boronate compounds, as first developed by the Chang laboratory (56, 64). The reaction releases a masked fluorescent center and an advantage is that the complications of a radical mechanism are avoided. A limitation is that the probes react very slowly and are inefficient at trapping H_2O_2. As an illustration, oxidation of 10 μM probe by 100 μM H_2O_2 typically takes more than an hour (19, 64). This can, to some

extent, be overcome by high fluorescence intensity, and because the probe reaction consumes very little H_2O_2, it provides a bioorthogonal approach that causes little perturbation to the system (56). This is good for monitoring stable steady-state concentrations (20), but low reactivity limits their ability to respond to short-term fluxes of H_2O_2. Also, the signal is highly dependent on the cellular concentration of probe and this needs to be accounted for, for example, by using a ratiometric version (19, 95). The possibility that oxidation could be due to peroxynitrite or hypochlorous acid (129) also needs to be considered.

The development of genetically encoded GFP derivatives has been a major advance in cellular H_2O_2 detection. These have been engineered to contain a dithiol switch that changes the fluorescence of the protein when oxidized (11, 12, 42, 89). High reactivity and good specificity is achieved by coupling to an H_2O_2-sensitive thiol protein [oxyR, Orp1, and, recently, the yeast Prx, TSA2 (71)] that transfers oxidizing equivalents intramolecularly to the GFP. When expressed in cells, they have the advantage of being ratiometric, although the pH dependence of Hyper means that it should be used in conjunction with its redox-inactive counterpart (42, 116). A key difference from the small-molecule probes, and an important point when interpreting results, is that oxidation is reversed by cellular reductants, including the thioredoxin and GSH/glutaredoxin systems. The response, therefore, reflects a balance between the rates of probe oxidation and reduction rather than the rate of H_2O_2 production. roGFP itself is also reduced by GSH (62) and can provide a useful control.

By incorporating appropriate targeting sequences, the probes can be directed to different cell compartments, including mitochondria, ER, nucleus, and cytoplasm (11, 13, 57, 69). Studies with targeted probes have shown that in unstressed cells only the ER maintains an oxidizing environment. The use of a targeted probes on its own will not distinguish whether a change in response is due to H_2O_2 generated in the same compartment or to H_2O_2 diffusing into it. However, this can be addressed by expressing probes with distinguishable fluorescence at different sites in the cell, and establishing whether the response is evenly distributed or localized. Recent studies have demonstrated the power of this methodology for probing the microenvironment in the cell, for example, by using the PTP1B targeting sequence to locate Hyper to the cytoplasmic side of the ER. Using this approach, Belousov and coworkers detected H_2O_2 only near the cytoplasmic surface of the ER after platelet-derived growth factor stimulation, and localized to endosomes after activation with epidermal growth factor (67, 69). This methodology has considerable potential for elucidating sites where H_2O_2 is produced and reacts, and further application should help answer questions surrounding redox activity in many cell signaling and oxidative stress conditions.

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Abbreviations Used

ER = endoplasmic reticulum
GFP = green fluorescent protein
GPx = glutathione peroxidase
GSH = reduced glutathione
H_2O_2 = hydrogen peroxide
NOX = NADPH oxidase
Prx = peroxiredoxin
SOD = superoxide dismutase