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Comprehensive Invited Review

Hydrogen Peroxide: A Signaling Messenger

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ABSTRACT

Hydrogen peroxide (H_2O_2) is a well-documented component of living cells. It plays important roles in host defense and oxidative biosynthetic reactions. In addition there is growing evidence that at low levels, H_2O_2 also functions as a signaling agent, particularly in higher organisms. This review evaluates the evidence that H_2O_2 functions as a signaling agent in higher organisms in light of the known biology and biochemistry of H_2O_2. All aerobic organisms studied to date from prokaryotes to humans appear to tightly regulate their intracellular H_2O_2 concentrations at relatively similar levels. Multiple biochemical strategies for rapidly reacting with these low endogenous levels of H_2O_2 have been elucidated from the study of peroxidases and catalases. Well-defined biochemical pathways involved in the response to exogenous H_2O_2 have been described in both prokaryotes and yeast. In animals and plants, regulated enzymatic systems for generating H_2O_2 have been described. In addition oxidation-dependent steps in signal transduction pathways are being uncovered, and evidence is accumulating regarding the nature of the specific reactive oxygen species involved in each of these pathways. Application of physiologic levels of H_2O_2 to mammalian cells has been shown to stimulate biological responses and to activate specific biochemical pathways in these cells. *Antioxid. Redox Signal.* 8, 243–270.

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I. INTRODUCTION

THERE IS NOW little doubt that hydrogen peroxide (H_2O_2) plays important roles in many biological processes in most if not all living organisms, from relatively simple bacteria to complex multicellular plants and animals. It is clear that prokaryotes and yeast have evolved mechanisms to sense and respond to H_2O_2 in the environment, and that many living systems intentionally generate H_2O_2 for diverse purposes, including oxidative biosynthesis and host defense. In addition, there is an increasing body of literature indicating that this intentionally generated H_2O_2 may also function in signal transduction pathways in multicellular organisms, particularly vertebrates.

The concept that H_2O_2 may function as a signaling agent has arisen during the time of general acceptance of other small, relatively reactive compounds serving as signaling agents, most notably nitric oxide (•NO). There has also been a growing appreciation of the prominent role of oxidative stress in many pathologic states, and of the presence of multiple distinct reactive oxygen species (ROS) and reactive nitrogen species (RNS) in living systems. The study of H_2O_2 as a signaling agent has in fact been an area of intense research with well over 2000 publications pertaining to H_2O_2 listed each year on PubMed. However the precise nature by which H_2O_2 functions as a signaling agent in vertebrates has remained unclear. This has been due in part to the relative difficulty in utilizing specific techniques to study H_2O_2 generation and action, and thus the reliance on much less specific techniques. However, a picture is emerging of the specific roles of H_2O_2 in living systems, and how these processes are distinct from those of oxidative stress, and those involving other specific ROS or RNS.

This review will focus on the evidence generated thus far that H_2O_2 is able to function as a signaling agent as part of signal transduction pathways in higher organisms. This analysis will be conducted in the context of other biologic functions involving H_2O_2, particularly oxidative biosynthesis and the sensing of H_2O_2 in the environment in lower forms of life. This review will also highlight the distinctions between H_2O_2 signaling and oxidative stress. However, this review will not be an in depth analysis of oxidative stress, nor will there be an attempt here to catalogue all of the pathways and signaling components potentially modified under such conditions. Importantly, this review will also analyze the potential involvement of H_2O_2 versus other specific ROS or RNS in signal transduction pathways that have been implicated as having an oxidation-dependent step or component. This review will rely heavily on placing experimental data in context with our current knowledge concerning the physiologic levels of H_2O_2 in living cells.

II. THE STUDY OF HYDROGEN PEROXIDE BIOLOGY AND BIOCHEMISTRY

Clearly a major obstacle in studying the roles of H_2O_2 in biology has been the lack of widely available specific tools and methodologies. ROS in general are difficult to study due to their relative instability and small size. It is also important to take into account that there are numerous distinct types of ROS and RNS in biology and that there are important interactions between some of these species, such that experimentally perturbing one system has secondary effects on distinct reactive species. One of the most important of such interactions is the very rapid (almost diffusion limited) reaction of superoxide (O_2^-) with •NO to generate peroxynitrite (148). This reaction has enormous implications for the interpretation of experimental results. This is due to the fact that •NO itself is a signaling agent with its principal sensor being the soluble isoform of guanylate cyclase (25). Importantly, the cGMP formed in response to •NO has inhibitory effects on growth and proliferation in many cell types (165). Thus in an experimental set-up in which the production of O_2^- is altered, such as by the application of xanthine with xanthine oxidase, which can produce both O_2^- and H_2O_2 (97), or by altering the expression or activity of an NADPH oxidase component, it is often not clear whether the observed effects are due to alteration of the H_2O_2 levels or to alteration of •NO levels. For example, downregulation of an NADPH oxidase component could potentially result in inhibition of cell proliferation by either downregulating the level of H_2O_2 or by upregulating the level of •NO.

A second common difficulty involves the use of antioxidants. Many antioxidants such as *N*-acetylcysteine, are very nonspecific, and do not allow for the discrimination of a role by H_2O_2 versus other distinct ROS or RNS. Likewise the use of a peroxiredoxin, a thiolate-dependent thioredoxin peroxidase, also does not allow for definitive identification of H_2O_2 as the specific reactive species involved, as peroxiredoxins react quite well with distinct reactive species such as peroxynitrite and lipid peroxides (178, 231). Of the antioxidants typically added to cells, catalase is generally considered as being the most specific for H_2O_2, but even here problems exist. Cells in culture lack their physiologic •NO sink, namely the hemoglobin of red blood cells, and in such situations catalase is able to metabolize •NO (32, 34). The catalytic consumption of •NO by catalase requires the presence of H_2O_2. However both the mechanism of this reaction and the rate constants governing the reaction are unclear. A second potential confounding phenomenon is that the marked reduction of H_2O_2 by added catalase can lead to reduced steady-state levels of O_2^-, preventing peroxynitrite formation (137). Thus unfortunately, it cannot be claimed with certainty that an observed effect in a cell culture system subsequent to the addition or overexpression of catalase is solely due to the removal of H_2O_2.

An equally troubling problem concerns the detection of H_2O_2 generation for which highly sensitive and specific methodologies are not in general use. One methodology frequently erroneously considered as being specific for H_2O_2 involves the use of 2′,7′-dichlorofluorescin diacetate (DCF), which is cell permeable and when oxidized displays enhanced fluorescence. Unfortunately this compound does not react directly with H_2O_2 to any significant degree (147). DCF does react avidly with peroxynitrite and intracellular peroxidases that have been previously oxidized by either H_2O_2 or another reactive species such as peroxynitrite or lipid peroxides. Thus DCF does not yield much discriminating information con-

cerning the nature of the oxidant involved in a process under study, and certainly DCF can never be used to assess the location of H_2O_2 generation. A common method for quantitating H_2O_2 *in vitro* involves the utilization of a heme-dependent peroxidase, such as horseradish peroxidase, with monitoring of the oxidation of the reductant in the experimental system (44). Since many heme-dependent peroxidases also react avidly with alkyl-peroxides and peroxynitrite (43, 82, 87), when using this approach it is typically necessary to include catalase in a negative control to verify that H_2O_2 is actually the oxidant being measured by the peroxidase. The traditional method for assessing the intracellular level of H_2O_2 involves monitoring the endogenous steady-state level of catalase compound I, the reaction product of H_2O_2 and catalase (44, 155).

Clearly one of the most specific methods to study H_2O_2 versus other reactive species is to simply use H_2O_2 itself in the experimental system. When adding H_2O_2 to cells, it is important to realize that H_2O_2 can permeate through plasma membranes at reasonably rapid rates with permeability coefficients ranging from 0.01 to 0.7 cm/min (44, 134). These permeability coefficients result in rates of permeation that are comparable to the rates at which H_2O_2 is being metabolized inside the cell by peroxidases and catalases. Thus when a bolus of H_2O_2 is applied to the exterior of cultured cells, a rapid equilibrium will be established in which the intracellular concentration of H_2O_2 is approximately 7- to 10-fold less than the extracellular concentration (6, 134, 186). Another important consideration when adding H_2O_2 to cells is that H_2O_2, being a reactive species, can at high enough concentrations react with almost any cellular component. As we will see, the interaction of H_2O_2 with cellular proteins is governed by kinetics. Thus it is critically important to be cognizant of the physiologic levels of H_2O_2 and to ensure that the effects observed in the experimental system are not solely *in vitro* phenomena resulting from the presence of supraphysiologic levels of H_2O_2 present in the system.

III. THE PRESENCE OF HYDROGEN PEROXIDE IN LIVING SYSTEMS

A. *Assessments of the physiologic intracellular concentrations of hydrogen peroxide*

As discussed above, a key aspect of studying H_2O_2 in living systems involves ensuring that the concentration of H_2O_2 in the experimental system is physiologically relevant. Thus it is important to understand the precise nature of the physiologic range for H_2O_2 concentrations. For mammals, in early studies careful quantitative measure of the generation of H_2O_2 by rat liver subcellular fractions, perfused whole rat liver, and livers in anesthetized rats as determined by monitoring steady-sate catalase compound I levels, combined with assessment of total catalase activity in this tissue, allowed for the calculation of intracellular H_2O_2 concentrations ranging from a low of ~0.001 μ*M* to a high of ~0.1 μ*M* during peak H_2O_2 generation (44, 155). Similarly, mathematical modeling of the generation and catabolism of H_2O_2 in mitochondria utilizing experimentally determined rate constants for these processes resulted in a calculated steady-state level of mitochondrial H_2O_2 of 0.04 μ*M* (7). In accordance with these calculated levels, by careful modulation of H_2O_2 levels, it was demonstrated that Jurkat T-cells remain in a proliferative state as long as the intracellular H_2O_2 level is below 0.7 μ*M*, above which level apoptosis occurs (5). Suspensions of mammalian cells have been shown to generate steady-state extracellular levels of H_2O_2 ranging from 0.02 μ*M* to 2 μ*M* (26, 144). Given the H_2O_2-gradient across the plasma membrane discussed above, this would correspond to 0.002–0.2 μ*M* intracellularly, in excellent agreement with the above studies. Utilization of an amperometric microsensor in anesthetized rats revealed dopamine infusion or direct electrical stimulation of the brain resulted in a peak extracellular H_2O_2 concentration of 2–4 μ*M*, which would correspond to a peak intracellular concentration of 0.2–0.4 μ*M* (113). Thus in mammals the physiologic H_2O_2 concentration may reach as low as 0.001 μ*M*, and the maximal intracellular H_2O_2 concentration that can be generated for signaling purposes would be 0.5–0.7 μ*M*.

Interestingly, this physiologic concentration range appears not to be unique to mammals but remarkably consistent throughout diverse forms of life. In *Escherichia coli*, the intracellular H_2O_2 level was reported to be relatively constant at ~0.2 μ*M*, despite fluctuations in potentially H_2O_2–generating metabolic activities during cell growth (85). The transcription factor OxyR was attributed a key role in maintaining this intracellular level of H_2O_2 (see below). Although, based on experimentally determined rates of H_2O_2 generation and H_2O_2 catabolism, the physiologic steady-state level of H_2O_2 in *E. coli* in the absence of an external H_2O_2 source was calculated to be closer to 0.02 μ*M* (186). Similar to the studies with mammalian cells, a steady-state intracellular level of 0.5 μ*M* H_2O_2 was determined to be toxic to *E. coli* by stimulating DNA damage, indicating normal physiologic levels to be below this concentration (159). For plants, metabolic modeling of H_2O_2 levels based on the calculated concentrations of antioxidant enzymes and kinetic rate constants has been performed for chloroplasts, an organelle with a likely relatively oxidizing environment. This analysis revealed the steady-state concentration of H_2O_2 in chloroplasts to be ~0.37 μ*M* (168), remarkably similar to the peak physiologic levels of H_2O_2 determined for both animals and microbes.

Thus among all of these aerobic forms of life, the physiologic range for the intracellular H_2O_2 concentration is relatively uniform, and appears to vary from a low of ~0.001 μ*M* up to a maximum of ~0.5–0.7 μ*M*. Is it simply a coincidence that these physiologic ranges appear to be so similar across different forms of life? It is in fact likely that all of the cells analyzed to date are subject to similar toxic reactions of H_2O_2 with DNA, lipid membranes, and proteins. It would appear that these reactions do not pose a substantial threat to wild-type cells when the intracellular level of H_2O_2 is maintained below ~0.5–0.7 μ*M*, setting this level as the maximal intracellular level of H_2O_2 which a cell would likely routinely utilize for signaling.

B. *The interaction of hydrogen peroxide with peroxidases and catalases*

To aid in our discussion of H_2O_2 as a signaling agent, it is useful to consider the biochemistry of H_2O_2, and specifically

how, when present at low endogenous levels, H_2O_2 is able to react with proteins in general, and thus how H_2O_2 may be reacting with a sensor as part of a signaling pathway. Of course given high enough concentrations of H_2O_2 and long enough reaction times, nearly every protein will react in some fashion with H_2O_2. We will limit our consideration to physiologically relevant modes of interactions. Most of our understanding of the biochemical mechanisms by which H_2O_2 interacts with proteins under physiologic conditions has derived from the study of enzymes that degrade H_2O_2, particularly peroxidases and catalases. Peroxidases reduce H_2O_2 to water and subsequently oxidize a secondary reductant such as glutathione (glutathione peroxidases) or ascorbate (ascorbate peroxidases). Catalases, when oxidized, are able to utilize H_2O_2 itself as a reductant, oxidizing H_2O_2 to molecular oxygen. Peroxidases and catalases of course need to react rapidly with H_2O_2 in order to maintain H_2O_2 at the physiologic low intracellular levels that have been observed. Interestingly there are a relatively limited number of mechanisms by which peroxidases and catalases react with H_2O_2 (Fig. 1). Peroxidases and catalases combine the features of a reactive moiety cable of directly reacting with H_2O_2, with additional residues in the active-site pocket that typically interact with and lower the energy of the transition-state intermediate (TSI) facilitating the reaction. Both the reactive moiety and the accessory TSI-stabilizing residues are required for these enzymes' rapid rates of reaction with H_2O_2, which are characterized by second order rate constants of 10^5–10^7 $M^{-1}s^{-1}$ (191).

Certainly one theme of peroxidases and catalases is the use of transition metals at their active sites. The heme-containing peroxidases and catalases utilize an iron-protoporphyrin IX (heme) complex as the reactive moiety (152, 199). The heme with the iron in the +3 (ferric) oxidation state reacts with H_2O_2 to generate compound I, an oxyferryl (FeIV = O) porphyrin radical cation. Although in some of these enzymes the radical is most likely located on an adjacent tyrosine residue. The heme-dependent peroxidases and catalases typically react with H_2O_2 with second-order rate constants on the order of 10^7 $M^{-1}s^{-1}$ (64, 78, 83, 136). Less common strategies involving the use of transition metals for reacting with H_2O_2 are the utilization of vanadium by certain haloperoxidases, and the utilization of a di-manganese center in manganese-dependent catalases. The vanadium haloperoxidases from marine algae and terrestrial fungi utilize a bound vanadium complex to catalyze biosynthetic halogenating reactions (37, 58). The vanadium center is able to react with H_2O_2 with a

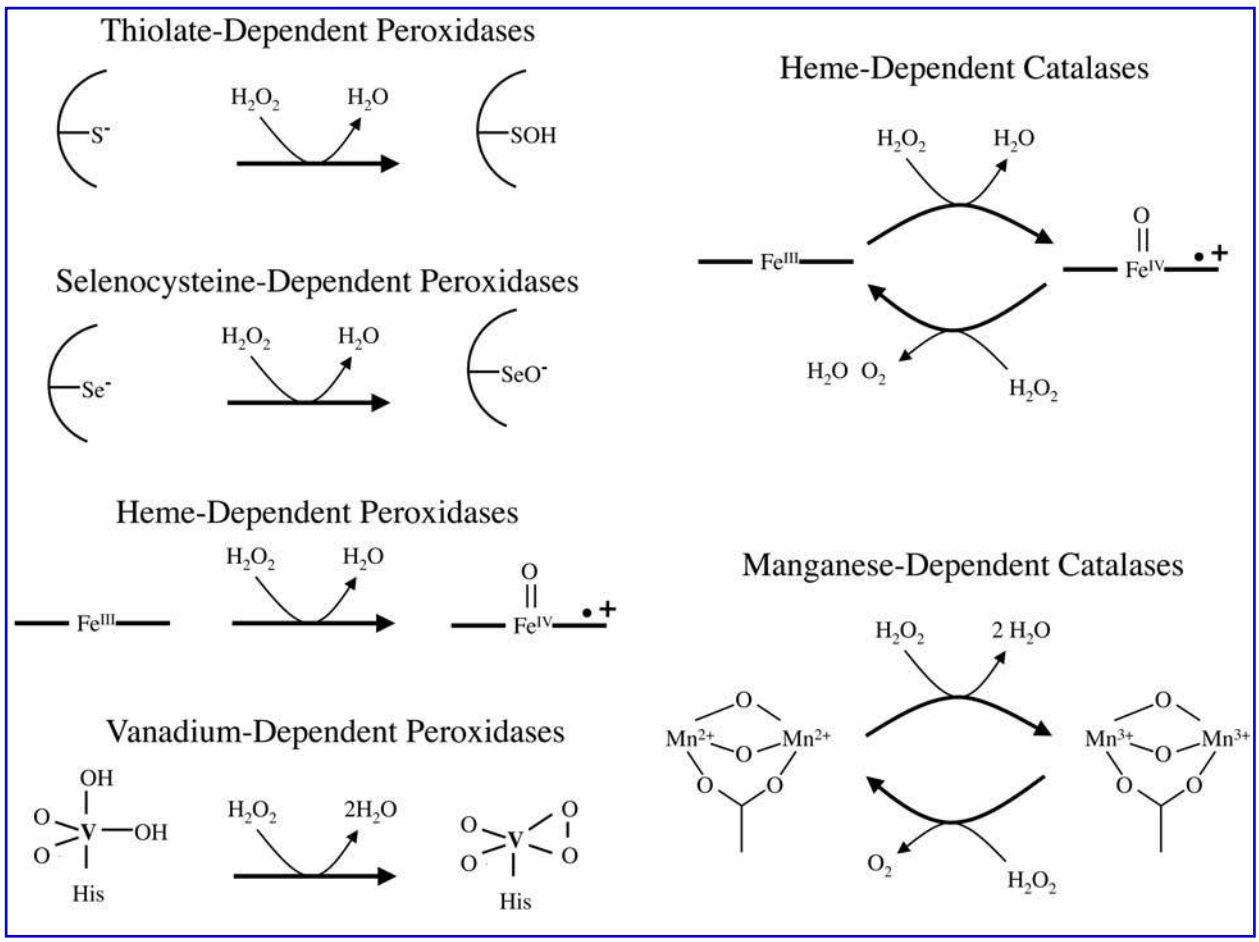

FIG. 1. The interaction of H_2O_2 with peroxidases and catalases. Depicted are the reactions by which H_2O_2 at physiologic levels interacts with cellular peroxidases and catalases. Typically these reactions occur with second order rate constants on the order of 10^5-10^7 $M^{-1}s^{-1}$.

second-order rate constant of ~10^5 $M^{-1}s^{-1}$ (177). A similarly less common active site is the utilization of a di-manganese center in manganese catalases from select prokaryotes including *Salmonella enterica* (232). In these enzymes, the reduced Mn^{+2}-Mn^{+2} site reduces H_2O_2 to water with oxidation of the Mn cluster to the Mn^{+3}-Mn^{+3} state. This oxidized enzyme is then itself able to oxidize H_2O_2 to molecular oxygen, completing the catalytic cycle.

Another approach used by peroxidases involves the incorporation of the amino acid selenocysteine into the protein. Approximately 25 selenocysteine-containing proteins are present in humans, including multiple glutathione peroxidases, thioredoxin reductase, multiple thyroid hormone deiodinases, and several for whom the biologic functions are presently not clear (109). This is in comparison to the presence of 24 selenocysteine containing proteins in mice, 3 in *Drosophila*, 1 in *C. elegans*, and none in yeast. Selenocysteine has an inherently lower pK_a than cysteine (5.2 *vs*. 8.4) stabilizing the H_2O_2-reactive nucleophilic selenolate (R-Se^-) moiety under physiologic conditions. Mammalian glutathione peroxidases display rates of reaction with H_2O_2 on the order of 10^7 $M^{-1}s^{-1}$ (81). The isolated selenolate moiety itself is only expected to react with H_2O_2 with a second order rate constant of 35 $M^{-1}s^{-1}$ (40). The high rates of reactivity of the glutathione peroxidases appear to be achieved by both the presence of a H_2O_2–reactive nucleophilic moiety and additional active-site residues that most likely interact with and stabilized the TSI.

A large class of peroxidases utilizes a deprotonated cysteine thiol or thiolate as the reactive moiety, an example being the ubiquitous peroxiredoxins, which often employ thrioredoxin as their reductant (178, 231). In these thiolate-dependent peroxidases, the active site Cys residue typically has a reduced pK_a in the range of 4.5–5.9, secondary to the advantageous positioning of positively charged residues, resulting in the deprotonated thiolate (R-S^-) moiety under physiologic conditions. This Cys-thiol deprotonation alone accounts for an enhancement in the rate of reaction with H_2O_2 by 10- to 100-fold over what would be achieved with a protonated Cys-thiol (22, 191, 226). The active site Cys-thiolate reacts directly with H_2O_2 yielding the sulfenic acid (R-SOH) (54). The sulfenic acid is itself unstable and susceptible to nucleophilic attack, typically from a second Cys-thiol, to generate a disulfide bond. What is impressive about the thiolate-dependent peroxidases is that their active sites are so finely constructed that they are able to achieve rates of reaction with H_2O_2 on the order of 10^5–10^6 $M^{-1}s^{-1}$ without the use of transition metals or unusual amino acids (18, 57, 149). However, other residues in addition to the nucleophilic thiolate are essential for these enzymes' rapid rates of reaction with H_2O_2. For example, in the thiolate-dependent NADH peroxidase, His-10 is believed to facilitate the reaction with H_2O_2 by stabilizing the TSI and also by binding the resulting Cys-sulfenic acid (237). Mutation of this residue to a Gln does not appear to alter the pK_a of the active-site Cys residue, but does diminish the rate of reaction with H_2O_2 by 150-fold (56). Thus the presence of a H_2O_2-reactive nucleophilic thiolate moiety in the setting of an active site finely tuned for a rapid reaction with H_2O_2, allows thiolate-dependent peroxidases to react quickly with low endogenous levels of H_2O_2.

Of course thiolate-dependent peroxidases are not the only enzymes that utilize nucleophilic thiolate moieties in their active sites. In fact humans possess 143 thiolate-dependent proteases, 103 thiolate-dependent phosphatases, as well as multiple thiolate-dependent thiol-*S*-transferases, thiol/disulfide isomerases, transglutaminases, and dehydrogenases (2, 124, 173). These nonperoxidase thiolate-dependent enzymes lack an active site designed to react quickly with low endogenous levels of H_2O_2, and demonstrate rate constants for the reaction with H_2O_2 on the order of 10–100 $M^{-1}s^{-1}$, 3–5 orders of magnitude lower than those exhibited by the thiolate-dependent peroxidases (9, 42, 70, 128, 129, 190, 191). Thus cellular proteins with reactive thiolate moieties in their active sites appear to be nicely divided into two distinct groups, those with a physiologic role in reacting with endogenous H_2O_2 and that do so with second order rate constants of 10^5–10^6 $M^{-1}s^{-1}$, and those enzymes, much more numerous in number, with other distinct functions in the cell that react with H_2O_2 with second order rate constants of 10–100 $M^{-1}s^{-1}$. If we assume a constant intracellular H_2O_2 concentration at the highest allowable (nontoxic) level of 0.7 μ*M* as discussed above, then for each thiolate-dependent peroxidase, 50% of the peroxidase in the cell will have reacted with this endogenous H_2O_2 in 1–10 sec. In contrast, under these conditions, it will require 2–30 hours for 50% of any one of these nonperoxidase thiolate-dependent enzymes to react with H_2O_2, a time scale so long as to be physiologically insignificant, as the reduction of these enzymes with glutathione and/or thioredoxin is likely to be much faster. Thus clearly one advantage of maintaining intracellular H_2O_2 levels below 0.7 μ*M* is preventing the inadvertent oxidative inhibition of the numerous thiolate-dependent enzymes in the cell.

C. Functions of hydrogen peroxide in biology

This analysis allows us to define some specific contexts within which H_2O_2 appears to function in cells. One of the earliest recognized roles of H_2O_2 in biology was in fact the generation of oxidative stress (61, 187, 238). Clearly upon exposure to increasing stress levels of H_2O_2, eukaryotic cells by virtue of their peroxidases will begin to oxidize their pools of free thiols. Due to the large amount of thioredoxin peroxidases in cells, initially the thioredoxin pool specifically is likely to become increasingly oxidized, with much less to no alteration to the reduced glutathione pool and the overall reduction potential of the cell (91). Such a state can be defined as mild oxidative stress. In this setting of stress levels of H_2O_2, the nonperoxidase thiolate-dependent enzymes will begin to become inhibited by oxidation of their active-site thiolate moieties. In addition under such conditions, many of the peroxiredoxins will also become inhibited by "over-oxidation" of the active site thiolate to the sulfinic acid (R-SO_2^-) (230, 234). This over-oxidation process is due to reaction of the sulfenic acid with a second equivalent of H_2O_2 with second order rate constants on the order of 10–100 $M^{-1}s^{-1}$, identical to the rate constants governing the rates of reaction of nonperoxidase active-site thiolates with H_2O_2 (191). With increasing oxidative stress, there will be significant oxidation of glutathione and alteration of the overall redox potential of the cell. This latter condition would be best

defined as a severe oxidative stress. Due to the harmful effects of oxidative stress, prokaryotes and yeast clearly need to sense and respond to excess H_2O_2 in their environment. In addition it is clear that many higher multicellular organisms intentionally generate stress levels of ROS, including H_2O_2, to aid in host defense against pathogens.

However there are also clearly situations in which H_2O_2 is functioning in biology in a role that is distinct from oxidative stress. The most extensively characterized phenomenon in this regard is that of H_2O_2-dependent oxidative biosynthesis (Fig. 2). One such example is the formation of Tyr–Try protein cross-links in the fertilization envelope of sea urchin eggs after fertilization. Such cross-links are believed to help prevent subsequent entry of additional sperm and to protect the embryo. These Tyr–Tyr cross-links are formed oxidatively by the heme-dependent ovoperoxidase, utilizing H_2O_2 generated by the dual (NADPH) oxidase Udx1 (151, 227). Plant cells in particular perform numerous oxidative biosynthetic processes in building and maintaining their cell walls, including the formation of diferulic linkages and the cross-linking of hydroxyproline-rich proteins named extensions, possibly through isodityrosine cross-links (160). In both of these cases, the cross-links appear to form as a result of the activity of peroxidases. In mammals, a well-characterized example of H_2O_2-mediated oxidative biosynthesis involves the formation of mono- and di-iodotyrosine residues during the biosynthesis of thyroid hormone, which is catalyzed by heme-dependent thyroid peroxidases (72, 132). In this system, H_2O_2 appears to be generated directly from the human dual (NADPH) oxidases DUOX1 and DUOX2 (3).

In consideration of these roles of H_2O_2 in biology, we can now define the characteristics of a system in which H_2O_2 could be considered to function as a signaling agent. This would be a system in which H_2O_2 was generated in a regulated fashion. However, this system would be distinct from oxidative stress in that the low level (< 0.7 μM intracellular) of H_2O_2 generated would not cause an alteration of the overall reduction potential of the cytosol, deplete the reduced thioredoxin pool, or nonspecifically inhibit the vast number of thiolate-dependent enzymes in the cell (Fig. 3). However, in such a system there would exist a limited number of sensors with the ability to react with these low endogenous levels of H_2O_2 in a manner probably not unlike that of the peroxidases and catalases. The oxidation of the sensor would alter its biologic function, either activating or inhibiting its activity. We can further envision potentially two distinct types of sensors, direct and indirect sensors (Fig. 4). Direct sensors would react directly with H_2O_2, with relatively rapid kinetics ($\geq 10^5\ M^{-1}s^{-1}$), similar to that of peroxidases and catalases. In contrast, indirect sensors would not react directly with H_2O_2, but rather with a peroxidase oxidized by H_2O_2. Such an indirect sensor would need to be preferentially oxidized by a particular peroxidase in the setting of much more numerous cellular reductants. For either type of sensor, to be a true signaling pathway the sensor would need to remain oxidized for some distinct period of time in the setting of cellular reducing agents, such as thioredoxin and glu-

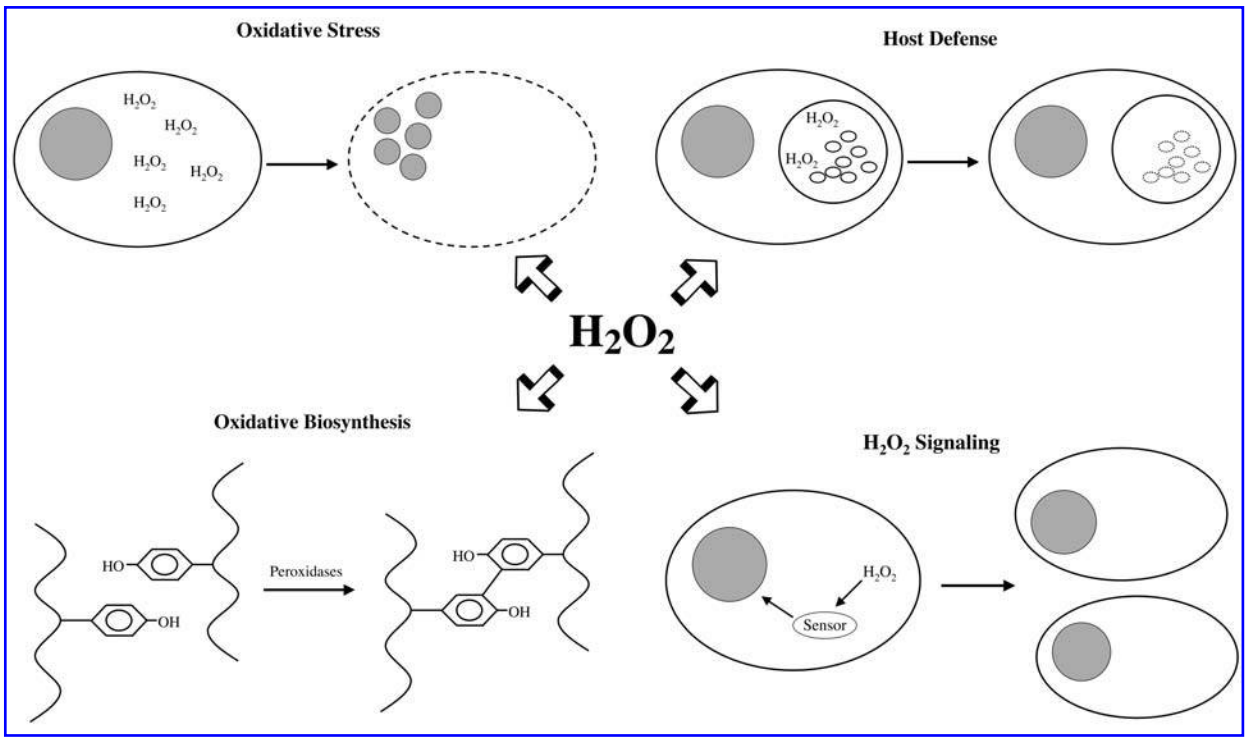

FIG. 2. Functions of H_2O_2 in biology. Potential roles for H_2O_2 in biological systems include the killing of cells by oxidative stress (*upper left*), host defense such as aiding in the killing of ingested microorganisms (*upper right*), oxidative biosynthesis such as Tyr cross-linking mediated by peroxidases (*lower left*), and signaling such as in mediating mitogenic signaling pathways (*lower right*).

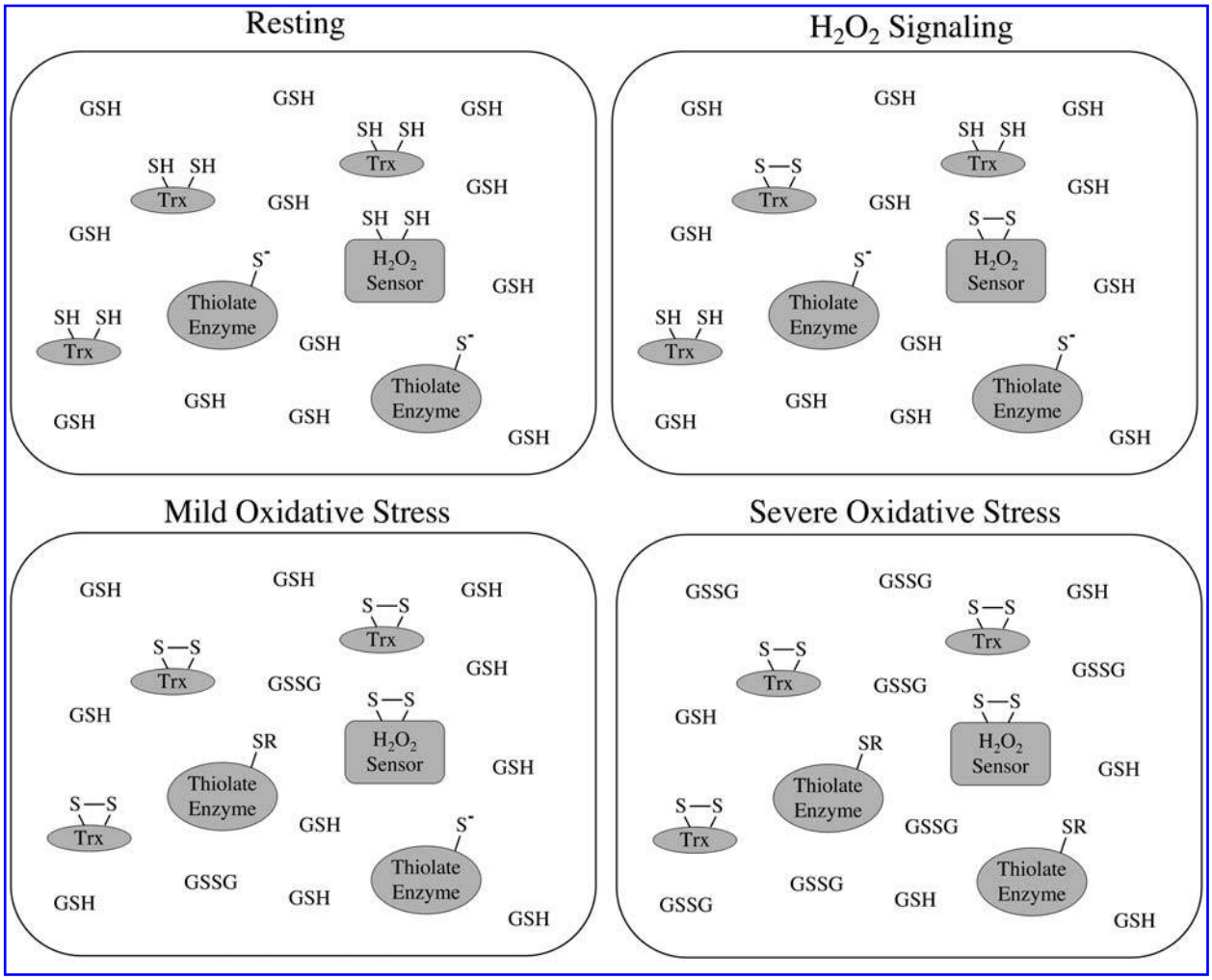

FIG. 3. Discriminating H_2O_2 signaling from oxidative stress. For a eukaryotic cell in the resting state (*upper left*) a hypothetical H_2O_2 sensor and most of the cellular thioredoxin (Trx) and glutathione (GSH) would be in the reduced state, as would the cellular nonperoxidase thiolate-dependent enzymes. During H_2O_2 signaling, the intracellular peroxide level would rise to no more than 0.5–0.7 μ*M*, oxidizing and activating the H_2O_2 sensor (*upper right*). Under such conditions a limited amount of the cellular Trx pool would become oxidized, but there would be no significant oxidation of GSH or most nonperoxidase thiolate-dependent enzymes. With increasing levels (> 1 μ*M*) of intracellular H_2O_2 the reduced Trx pool is depleted, and there is increasing oxidation of GSH and oxidative inactivation of general cellular thiolate-dependent enzymes, at first with only minimal alteration of the overall reduced GSH pool (*lower left*). With more severe oxidative stress a substantial fraction of GSH is oxidized (*lower right*).

tathione. Thus the rate of reduction of the sensor must be slow enough to allow for the oxidized sensor to carry out its function before being reductively deactivated. Also such a system should be distinct from those of oxidative biosynthesis, in which the H_2O_2 is functioning primarily as a biosynthetic substrate. Based on this conceptualization of a signaling agent, we will now discuss the current data implicating H_2O_2 as playing such a role in biology.

IV. SENSING HYDROGEN PEROXIDE IN PROKARYOTES

It is essential for prokaryotes to sense alterations in their environment and respond appropriately. Thus multiple pathways have been described by which prokaryotes can sense and respond to oxidative stress (67, 94, 171, 195, 242). Among these oxidative stress response pathways, it is becoming clear that there are sensors that sense and respond specifically to H_2O_2. The best characterized H_2O_2-sensor in prokaryotes is the transcription factor OxyR. OxyR was initially characterized in *Escherichia coli* and *Salmonella typhimurium* as a gene product mediating the upregulation of oxidative stress defense genes in response to exogenous H_2O_2 (52). OxyR homologues have been identified in numerous bacterial species including Gram-negative bacteria, Gram-positive bacteria, and mycobacteria. OxyR has also been reported to be upregulated during oxidative metabolism in *E. coli* in order to maintain the intracellular level of H_2O_2 close to 0.2 μ*M*, despite enhanced endogenous H_2O_2 production (85, 86). OxyR is a positive regulator of several oxidative

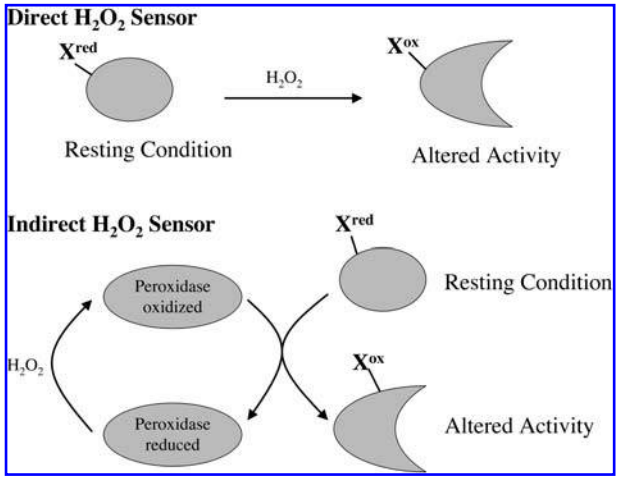

FIG. 4. Direct and indirect H_2O_2 sensors. Depicted are two general mechanisms by which H_2O_2 sensors could function. A direct sensor (*upper panel*) would be directly oxidized by H_2O_2 with rapid kinetics, and this oxidation would alter the activity of the sensor. An indirect sensor (*lower panel*) would not react directly with H_2O_2, but instead would be oxidized by a peroxidase that had previously been oxidized by H_2O_2.

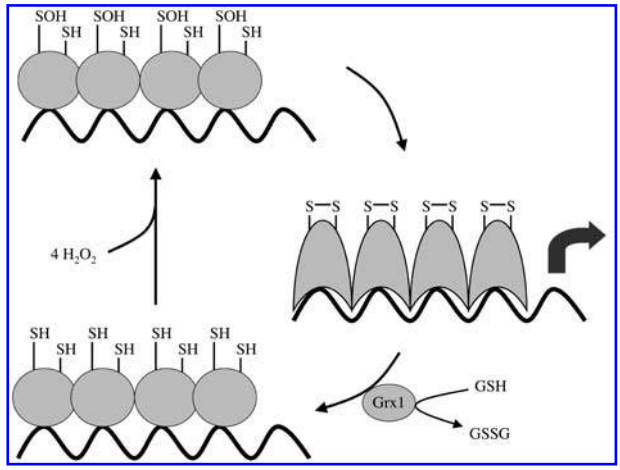

FIG. 5. The activation of OxyR by H_2O_2. The prokaryotic H_2O_2-sensor OxyR binds to DNA as a tetramer. Each monomer reacts rapidly with H_2O_2 to generate a sulfenic acid at Cys199. Sulfenic acid formation stimulates a conformational change and disulfide bond formation between Cys199 and Cys208. The conformational change results in a structure that stimulates transcription of OxyR-dependent genes. The activated transcription factor is deactivated through reduction by glutaredoxin-1 (Grx1) and glutathione (GSH).

stress defense genes including the hydroperoxidase (katG), an alkyl hydroperoxide reductase (*ahpCF*), glutathione reductase (gorA), and glutaredoxin (*grxA*), while also functioning as a negative regulator of its own gene (52, 195).

Sequence analysis and DNA-binding studies revealed OxyR to be a 34 kDa transcription factor belonging to the LysR family of bacterial regulators (53), and DNA footprinting analyses indicated that the protein binds to DNA as a tetramer (208). The protein contains an N-terminal helix-loop-helix DNA binding domain, and a larger C-terminal domain (residues 80–305) now realized to be a regulatory domain. It was determined that only an oxidized form of OxyR was active in stimulating transcription of the oxidative-stress inducible genes, suggesting that OxyR is oxidized either directly or indirectly in response to H_2O_2, resulting in the activation of the transcription factor (196). Oxidation, in fact substantially alters the contact sites between the protein and the DNA, suggesting that oxidation is accompanied by a substantial conformational change of the protein (Fig. 5) (208).

The reaction of H_2O_2 with OxyR involves the initial oxidation of Cys199 with subsequent formation of a disulfide bond between Cys199 and Cys 208 (12, 121, 203, 241). The redox potential of this disulfide bond has been determined to be –185 mv, about 90 mv higher than *E. coli* cytosol (-280 mv), indicating that the protein will be predominantly in the reduced form under resting conditions (241). Bolus addition of H_2O_2 to *E. coli* leads to a transient oxidation and activation of the protein at 10 min, with reversion to the reduced form at 60 min. Reduction and deactivation are catalyzed by glutaredoxin 1, the gene for which is in fact positively regulated by oxidized OxyR. This transient oxidation occurs even with the addition of low levels of H_2O_2 because the rate of reaction of OxyR with H_2O_2 is relatively fast, with a second order rate constant of 10^5 $M^{-1}s^{-1}$ (12, 121). This rate constant is comparable to the rate constants for the association of H_2O_2 with peroxidases (191) and allows for an oxidation rate that is significantly faster than the rate of reduction of OxyR in the bacterial cells. These relatively rapid kinetics result in the complete oxidation of OxyR in 30 sec with addition of just 5 μ*M* H_2O_2 to cells, or the addition of 0.1 μ*M* H_2O_2 to *in vitro* protein preparations. Thus OxyR is able to sense physiologic fluctuations of intracellular H_2O_2 levels.

It is expected that the primary reactive Cys199 in OxyR, like many other reactive cysteines in biology, will have a reduced p*Ka*, and thus be present predominantly in the thiolate form. In the crystal structure of the regulatory domain, in the reduced form, Cys199 is expected to be relatively close to Arg266, which could facilitate the lowering of the p*Ka* of Cys199 (50). Cys199 appears to react directly with H_2O_2 to generate a sulfenic acid (R-SOH) intermediate (121). Interestingly, in the reduced form of the protein Cys208 is separated from Cys199 by approximately 17 Å (50). Thus prior to disulfide bond formation, a substantial conformation change occurs. It has been proposed that this conformational change may be triggered in part by the small hydrophobic pocket surrounding Cys199, which may not accommodate the larger more polar sulfenic acid side chain created upon reaction with H_2O_2 (50).

Oxidative modulation of a protein's function has now been demonstrated for numerous proteins implicated in cellular responses to oxidative stress. However what is distinct concerning OxyR, is the kinetic data indicating that this protein is able to function as a specific and sensitive sensor for H_2O_2, analogous to the sensitivities and specificities of mammalian soluble guanylate cyclase for •NO and of the *Rhodospirillum rubrum* transcription factor CooA for carbon monoxide (25, 179). The specificity of OxyR for H_2O_2 has been highlighted

by the relatively minor contribution of OxyR in the overall response of *E. coli* to nitrosative stress from •NO and nitrosothiols (145). Thus in prokaryotes, OxyR not only senses oxidative stress, but it is a specific sensor of low endogenous levels of H_2O_2. Thus, our understanding of OxyR offers us insight into how a vertebrate H_2O_2-sensor could function.

V. RESPONSES TO HYDROGEN PEROXIDE IN YEAST

Analogous to prokaryotes, yeast have been extensively studied in terms of their responses to oxidative stress, particularly resulting from the application of stress levels of H_2O_2. Correspondingly multiple oxidative stress responsive pathways have been identified (156, 204, 207). In the budding yeast *Saccharomyces cerevisiae*, the transcription factor Yap1 has been implicated as playing a primary role in the response to H_2O_2, and a homologous protein Pap1 has been implicated in playing a similar role in the fission yeast *Schizosaccharomyces pombe*. Yap1 and Pap1 belong to the AP-1 family of transcription factors with homology to mammalian c-jun (143, 206, 210). Yap1 was recognized as conferring resistance to multiple oxidative stress inducing agents including mendione, methyl viologen, diamide, and hydroperoxides. The genes regulated by Yap1 include multiple oxidative stress resistance genes such as peroxidases, superoxide dismutases, thioredoxin, thioredoxin reductase, and glutathione reductase. Yap1 and Pap1 are regulated predominantly by a cytoplasmic to nuclear translocation induced by oxidative stress (111, 209). Application of H_2O_2 prevents the interaction of Yap1 with the Crm1 nuclear export receptor, leading to the accumulation of Yap1 in the nucleus (112, 233).

It has been demonstrated that the application of stress levels of H_2O_2 to intact yeast cells stimulates the formation a disulfide bond in Yap1 (65). The C-terminal of Yap1 contains a nuclear export signal with a C-terminal cysteine rich domain termed c-CRD. Oxidation of one or more of cysteine residues in the c-CRD is sufficient to prevent nuclear export (110). In addition, an N-terminal cysteine rich domain (n-CRD) is required for activation by H_2O_2 (55, 223). A H_2O_2-stimulated disulfide bond in Yap1 was determined to form between Cys303 located in the n-CRD and Cys598 in the c-CRD in cells treated with stress levels of H_2O_2 (65). An additional disulfide bond was identified between Cys310 of the n-CRD and Cys629 of the c-CRD in purified Yap1 oxidized by exposure to air *in vitro* (228, 229). All four of these Cys residues are highly conserved among Yap1 homologues. The Cys303-Cys598 disulfide bond formed in response to H_2O_2 is in contrast to the effects of relatively nonspecific thiol modifying agents such as diamide or *N*-ethylmaleimide, which cause the nuclear localization of Yap1 independent of the n-CRD, *via* modification of Cys residues located completely within the c-CRD (15, 55, 223). The solution structure of the regulatory portion of Yap1 in the reduced and oxidized forms has confirmed that disulfide bond formation blocks access to the nuclear export sequence (229). Yap1 appears to be reduced in cells principally by thioredoxin, and to be relatively insensitive to glutaredoxin and the glutathione reduction system (41, 102). Deficiency of thioredoxin results in constitutive activation of Yap1.

Overall, there is some resemblance of the Yap1 system to that of OxyR, with H_2O_2 stimulating the formation of a specific disulfide bond that results in the functional activation of the protein. However in marked contrast to OxyR in which complete oxidation *in vivo* is achieved by the addition of just 5 μ*M* H_2O_2 to cells, Yap1 is not oxidized *in vivo* by the application of even 25–50 μ*M* H_2O_2. Oxidation of Yap1 in yeast cells requires at least 100 μ*M* H_2O_2, and is maximal with 800 μ*M* H_2O_2 (65), indicating that there are significant differences between the OxyR and Yap1 systems. In addition to the relatively low sensitivity of Yap1 to H_2O_2 in cells, purified Yap1 is not oxidized to an appreciable extent in 10 min with 100 μ*M* H_2O_2 *in vitro* (66), indicating that Yap1 is not a direct target of H_2O_2.

The oxidation of Yap1 in *S. cerevisiae* by 400 μ*M* H_2O_2 also results in the transient formation of a minor form of higher molecular mass (66). Utilization of an overexpressed Cys303Ala mutant of Yap1 enhanced the formation of this minor species, and enabled the identification of this species as a mixed disulfide between Cys598 of Yap1 and the active site cysteine, Cys36, of the phospholipid hydroperoxidase Gpx3 (66). Gene deletion of Gpx3 inhibited formation of the disulfide bond in Yap1 in response to 400 μ*M* H_2O_2. In addition, nuclear accumulation of Yap1 in response to 400 μ*M* H_2O_2 was substantially but not completely reduced in the absence of Gpx3. Gpx3 was originally identified by its homology to the family of phospholipid hydroperoxide glutathione peroxidases (PHGPx), and Gpx3 has been demonstrated to mediate resistance to linolenic acid induced lipid peroxide stress in *S. cerevisiae* (13, 14). This classic function of Gpx3 is apparently not dependent on the presence of Yap1, as Yap1 deficient cells show no enhanced sensitivity to linolenic acid. In contrast to mammalian glutathione peroxidases, *S. cerevisiae* Gpx3 contains an active site Cys (Cys36) rather than a selenocysteine. However, Gpx3 is also capable of functioning as a thioredoxin peroxidase, catalyzing the reduction of H_2O_2 with the reducing equivalents from thioredoxin. This latter function appears to require disulfide bond formation between Cys36 and Cys82 (66). Although the phospholipid hydroperoxidase activity of Gpx3 appears to be adequately supported by glutathione, presumably with glutathione directly reducing the sulfenic acid formed upon oxidation of Gpx3 by phospholipid hydroperoxides (13).

There are two lines of evidence indicating that the thioredoxin-dependent H_2O_2 peroxidase activity of Gpx3 is not essential for the activation of Yap1. First, Cys82Ser mutants of Gpx3, which appear to lack thioredoxin peroxidase activity, fully reverse the enhanced H_2O_2 sensitivity of Gpx3 deficient cells (66). Also, a chimeric form of Gpx3 containing an oligomerization insert found in multimeric mammalian cytosolic glutathione peroxidases (cGpx3), was demonstrated to maintain high peroxidase activity to t-butylperoxide, but as expected showed much lower activity toward the larger phosphatidylcholine hydroperoxide. The typical monomeric structure of PHGPx enzymes is believed to result in a more open substrate binding site, allowing for the reduction of larger substrates. Not surprisingly, cGpx3 could not compensate for Gpx3 in activating Yap1 in response

to H_2O_2 (14). An additional protein, Ybp1 was found to facilitate the activation of Yap1 by H_2O_2 (217). Whereas the role of Ybp1 is not entirely clear, the protein may function as a scaffolding protein, enhancing the interaction of Yap1 with Gpx3.

It is becoming clear that the ability to oxidize Yap1 in response to H_2O_2 is not unique to Gpx3. In *S. cerevisiae* strain Y700, derived from strain W303, the thioredoxin peroxidase Tsa1 is required for the activation of Yap1 (154) and appears to function independently of Ybp1. In addition, oxidation of Pap1, the Yap1 homologue in *S. pombe*, in response to 200 μ*M* H_2O_2 is mediated by Tpx1, a 2-Cys peroxiredoxin-type thioredoxin peroxidase (27, 220). Thus while Yap1 homologues are distributed amongst multiple yeast species, it appears that the peroxidase mediating the oxidation of the transcription factor in response to H_2O_2 is likely to vary with the species and even strain of yeast under study.

There are several unanswered questions concerning Yap1 activation. For example, does Cys310-Cys629 disulfide bond formation occur *in vivo*, and which peroxidase mediates this oxidative event. A particularly important question concerns the kinetics of Yap1 activation by H_2O_2 and subsequent deactivation, and the cellular alterations required for nuclear accumulation of Yap1 to occur. Gpx3 is a professional peroxidase, and as such would be expected to react with low levels of H_2O_2 with rapid kinetics, similar to the rate of reaction of OxyR with H_2O_2. However, relatively high (100–800 μ*M*) concentrations of H_2O_2 are required to activate Yap1 *in vivo*, in contrast to the complete activation of OxyR with just 5 μ*M* H_2O_2 applied to the cells. This observation implies that the deactivation of Yap1 by thioredoxin is relatively rapid, and that substantial Yap1 nuclear accumulation can occur only after a substantial portion of the cellular thioredoxin pool has been oxidized. If such is the case, then Yap1 would be best classified as a sensor of oxidative stress rather than as an example of H_2O_2 signaling. Thus an important question is whether Yap1 is oxidized to any significant extent in the presence of reduced thioredoxin, or as the data imply near complete oxidation of thioredoxin is required for Yap1 oxidation.

Based on our current understanding, the relevant reactions relating to the oxidation of Yap1 in *S. cerevisiae* are illustrated in Figure 6. H_2O_2 initially oxidizes the Cys36 of Gpx3 to the sulfenic acid (R-SOH). The Gpx3 sulfenic acid may lead to disulfide bond formation on Gpx3 or be reduced directly by GSH. Either the sulfenic acid form or the disulfide bond form of Gpx3 may be able to oxidize Yap1. It is currently not clear which oxidized form of wild-type Gpx3 directly oxidizes Yap1 *in vivo*, although kinetic analyses comparing rates of disulfide bond formation with rates of Yap1 oxidation could potentially clarify this issue. Both Yap1 and Gpx3 that have been oxidized to the disulfide bond form are readily reduced by thioredoxin. Thus, in cells, under resting conditions with an intracellular H_2O_2 concentration of < 1 μ*M*, reduced thioredoxin maintains Yap1 in the reduced state (Fig. 7). Under these conditions, Yap1 that enters the nucleus is readily exported by the Crm1 nuclear export receptor. With stress levels of H_2O_2 (> 10 μ*M* intracellular), the reduced thioredoxin pool is depleted by thioredoxin peroxidases, allowing Yap1 to become oxidized by Gpx3. Oxidized Yap1 is no longer exported from the nucleus by Crm1, resulting in the nuclear accumulation of Yap1, and the activation of Yap1-dependent genes.

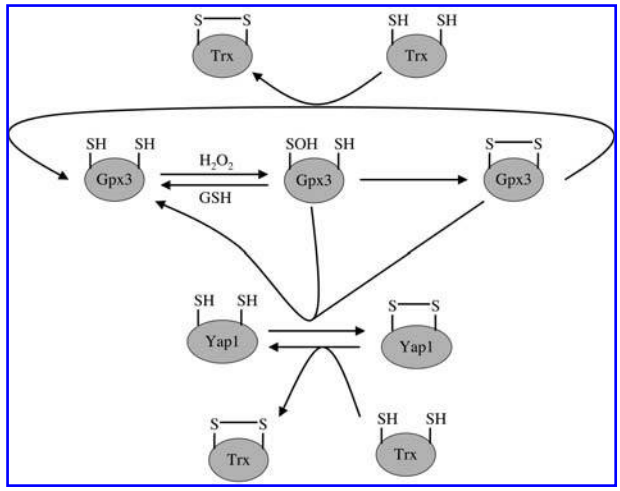

FIG. 6. Reactions relevant to the oxidation of Yap1 in *Saccharomyces cerevisiae*. H_2O_2 oxidizes the active-site Cys of glutathione peroxidase 3 (Gpx3) to the sulfenic acid. This Gpx3 sulfenic acid may be reduced by glutathione (GSH) or may result in the formation of a disulfide bond on Gpx3. Gpx3 with either the sulfenic acid or (possibly) the disulfide bond oxidizes two Cys residues on Yap1 to the disulfide bond. Both the Gpx3 disulfide bond and the Yap1 disulfide bond are reduced by thioredoxin (Trx).

Thus the Yap1 system in *S. cerevisiae* appears to be distinct from the OxyR system in prokaryotes. In the case of OxyR, sensing of low endogenous levels of H_2O_2 and response to oxidative stress are both accomplished by a single pathway, and in fact a single protein. In *S. cerevisiae* these response functions appear to likely be separated and performed by distinct pathways, with the Gpx3-Yap1 pathway responding to oxidative stress but likely not responding to variations in physiologic levels of H_2O_2. Whether yeast are able to sense and respond to intracellular levels of H_2O_2 below 1 μ*M*, and precisely how this H_2O_2 sensing may be occurring is currently unclear.

VI. SIGNALING WITH HYDROGEN PEROXIDE IN ANIMALS

A. The evidence supporting a role for hydrogen peroxide as a signaling agent in animals

Progress in uncovering the roles of H_2O_2 in animals has been much slower than with unicellular organisms. Although the field is highly active with an ever-increasing number of publications each week, many of the observations remain controversial. In fact it has yet to be established if mammalian cells routinely respond to stress levels of H_2O_2 with global upregulation of antioxidant enzymes as is seen in yeast and prokaryotes (71). Nonetheless, evidence supporting a role for low levels of H_2O_2 as a signaling agent has been steadily accumulating. Support for this idea principally de-

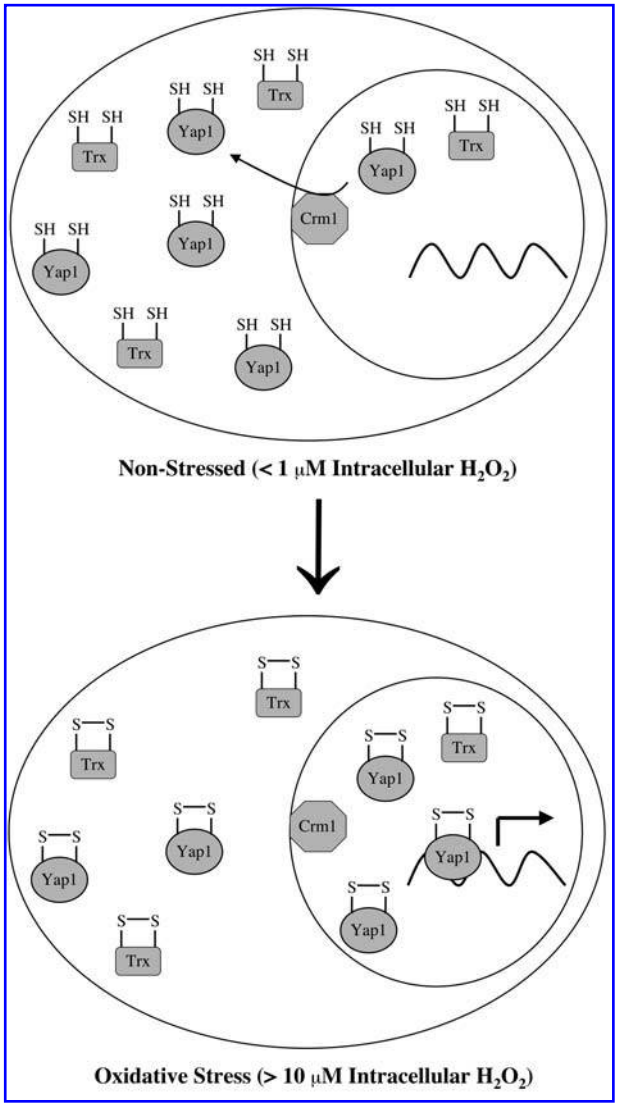

FIG. 7. Nuclear accumulation of Yap1 during mild oxidative stress. Under resting conditions with an intracellular H_2O_2 level below 1 μ*M*, thioredoxin (Trx) maintains Yap1 in the reduced state. Reduced Yap1 readily enters the nucleus but is efficiently exported out of the nucleus by Crm1, keeping nuclear Yap1 levels low. During mild oxidative stress with intracellular H_2O_2 levels above 10 μ*M*, the reduced Trx pool is transiently depleted, allowing Yap1 to become oxidized by Gpx3. Oxidized Yap1 is not efficiently exported from the nucleus by Crm1, allowing for nuclear accumulation of Yap1 and activation of Yap1-dependent genes.

rives from the following two observations: 1) The application of low physiologic levels of H_2O_2 stimulates proliferation and/or enhanced survival of mammalian cells, while the application of catalase inhibits proliferation and cell survival; 2) Mammalian cells generate O_2^- and/or H_2O_2 in response to numerous physiologic stimuli, and a group of enzymes, the NADPH oxidase (NOX) family, have been identified whose primary function appears to be to generate O_2^- and/or H_2O_2 in a regulated fashion. These two fundamental observations are being extended by two somewhat independent approaches in order to identify signaling pathways utilizing H_2O_2. These two approaches involve the identification and characterization of oxidation-dependent steps in receptor-mediated signal transduction pathways, and the identification and characterization of signaling pathways activated by the application of physiologic levels of H_2O_2. These four areas of research will be discussed.

B. Regulated formation of hydrogen peroxide in animals

Clearly for H_2O_2 to be serving as a signaling agent, it is necessary for it to be generated in a regulated fashion. Over the last 10 years it has become clear that numerous diverse stimuli stimulate mammalian cells to release O_2^- and/or H_2O_2, including growth factors, cytokines, vasoactive modulators, integrins, and biomechanical stimulation (126, 193, 205). In fact, it is becoming somewhat more difficult to find receptor-ligand interactions that do not stimulate O_2^-/H_2O_2 production. It is possible, that H_2O_2 is generated to some extent by the mitochondria and peroxisomes in eukaryotic cells (150, 185) and such metabolic generation of H_2O_2 could reflect the overall level of metabolic activity of these cells. As discussed above, one of the functions of the prokaryotic H_2O_2 sensor OxyR appears to be to sense metabolically derived H_2O_2 in addition to its role in sensing H_2O_2 from the environment. Furthermore, dysregulation, and specifically upregulation, of metabolism-derived H_2O_2 may play an important role in certain disorders such as aging (38, 175). However, over the last 10 years it has become clear that the cells of animals and plants intentionally generate O_2^- and H_2O_2 in response to stimuli, using multi-component NOX complexes whose primary function appears to be to generate these ROS (24, 117). These complexes were first identified in phagocytes, where they had been assigned the role of generating O_2^- during the respiratory burst, to aid in the killing of microorganisms (17, 84). However, over the last 10 years it has become clear that there are multiple isoforms of the key catalytic subunit of NOX complexes, and that these complexes are present in most if not all of the cells of animals, offering what could potentially be an ideal first component of highly-regulated signaling pathways utilizing H_2O_2.

The NOX complex most thoroughly characterized biochemically is the gp91phox (NOX2) containing NOX complex of phagocytes (28, 89, 116, 119, 219). This complex consists of a gp91phox catalytic subunit bound to multiple regulatory subunits, p22phox, p40phox, p47phox, p67phox, and the small GTP binding protein Rac. The gp91phox catalytic subunit is a transmembrane protein with a C-terminal cytoplasmic domain containing an NADPH binding site as well as bound FAD. The N-terminal portion of gp91phox consists of six membrane spanning helices to which are bound two iron-containing heme prosthetic groups. The path of electron flow in these enzymes is from NAPDH bound to the cytosolic domain, to FAD, to the heme prosthetic groups in the lipid bilayer, to oxygen to generate O_2^- at the opposite surface of the lipid bilayer. A very important aspect of NOX complexes is that they do not generate O_2^- in the cytosol. The O_2^- is formed on the opposite side of a lipid bilayer, thus either in the extracellular space or potentially within a membrane-bound vesicle or organelle.

Although it has been demonstrated that the immediate product from gp91phox containing NOX complexes is O_2^- (135), this has not been demonstrated equivocally for all of the gp91phox isoforms, and in fact some recent evidence suggests that some isoforms, particularly DUOX1 and DUOX2, may release H_2O_2 as the primary product rather than O_2^- (3). When O_2^- is the product, it can be readily converted to H_2O_2 by the extracellular form of superoxide dismutase, EC-SOD (Fig. 8) (79).

In humans there are known to be seven distinct gp91phox isoforms (116). Four of these isoforms, gp91phox (now referred to as NOX2), NOX1, NOX3, and NOX4, contain the classic domain architecture of gp91phox described above (Fig. 9). The NOX5 isoform contains an additional N-terminal cytoplasmic domain consisting of a calmodulin-like domain with four sites for binding calcium, allowing this isoform to be regulated by intracellular calcium levels. The final two isoforms, DUOX1 and DUOX2, have the domain architecture of NOX5 plus an additional membrane spanning helix at the N-terminus with an extracellular N-terminal domain that shows homology to heme-dependent peroxidases. Studies in which this isolated extracellular domain was overexpressed in *E. coli* have provided evidence that these domains do possesses peroxidase activity (76). It is generally believed that the primary function of the DUOX1 and DUOX2 isoforms is to facilitate oxidative biosynthesis (3), as described above.

The mechanisms of activation of NOX complexes are complex and best understood for the gp91phox of phagocytes (17, 89, 116). For gp91phox, activation involves at least three distinct components: phosphorylation of p47phox, Rac guanine nucleotide exchange, and metabolism of phospholipids (Fig. 10). In the resting state, the catalytic subunit, gp91phox, is bound to a second component, p22phox, in the plasma membrane and together comprise flavocytochrome-b_{558}. Under resting conditions the three regulatory components, p40phox, p47phox, and p67phox, are likely in a complex in the cytosol. Phosphorylation by multiple kinases of an autoinhibitory region on p47phox releases this region from two tandem SH3 domains on p47phox, allowing them to interact with a proline-rich sequence in the C-terminal tail of p22phox (101). Binding of the p40phox–p47phox–p67phox

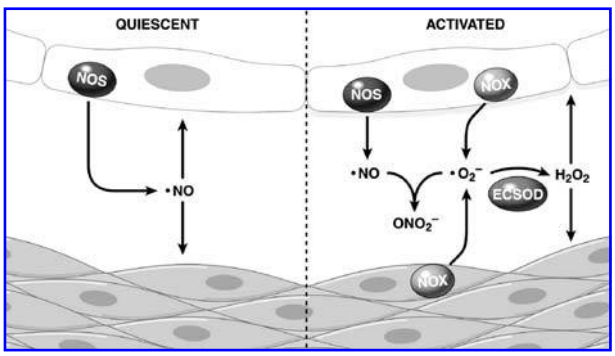

FIG. 8. The generation of H_2O_2 in the vessel wall. At quiescent sites in the vasculature, •NO generated predominantly by endothelial •NO synthase (NOS) diffuses into endothelial and smooth muscle cells to activate its primary sensor, the soluble isoform of guanylate cyclase. At activated sites in the vasculature, O_2^- is generated in the extracellular space by endothelial and smooth muscle cell NADPH oxidase (NOX) complexes. The O_2^- reacts with and inactivates •NO, forming peroxynitrite (ONO_2^-). By the action of extracellular superoxide dismutase (ECSOD), O_2^- is also converted into H_2O_2, which readily enters into endothelial and smooth muscle cells.

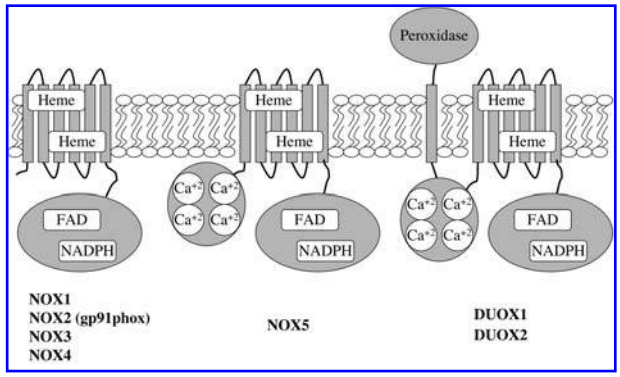

FIG. 9. Human isoforms of the NADPH oxidase catalytic subunit. In NOX1, NOX2 (gp91phox), NOX3, and NOX4 there is a C-terminal cytoplasmic domain that binds NADPH and FAD. In addition there are six membrane-spanning helices, which bind two heme moieties. In NOX5, there is an additional N-terminal cytoplasmic calmodulin-like calcium-binding domain. In the dual (NADPH) oxidases DUOX1 and DUOX2 the basic structure of NOX5 is extended by the presence of an additional membrane-spanning helix and an N-terminal extracellular domain with homology to heme-dependent peroxidases.

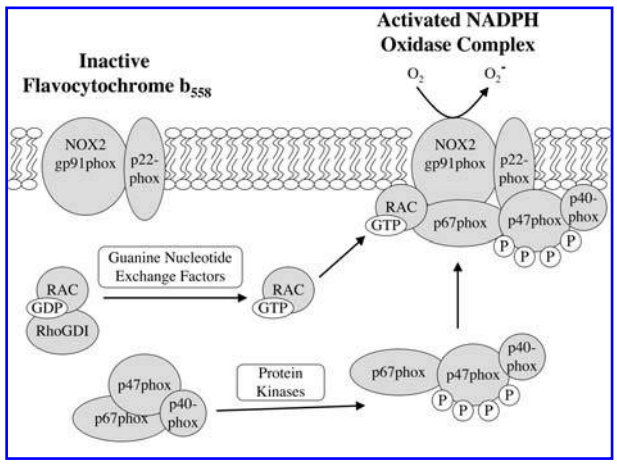

FIG. 10. Activation of the phagocytic NADPH oxidase complex. Activation of the gp91phox (NOX2) containing NOX complex of phagocytes involves phosphorylation of the cytoplasmic regulator p47phox, with the translocation of the cytoplasmic p47phox, p67phox, and p40phox regulatory components to the plasma membrane to interact with flavocytochrome-b_{558}, which is composed of gp91phox and p22phox. Activation of the complex also involves guanine nucleotide exchange on the GTP-binding protein RAC stimulated by guanine nucleotide exchange factors. Guanine nucleotide exchange on RAC is associated with release of RhoGDI and translocation of RAC from the cytosol to the NOX complex at the plasma membrane.

complex to flavocytochrome-b_{558} allows p67phox to interact with the cytosolic domain of gp91phox, enhancing the rate of reduction of FAD by NADPH. The precise role of p40phox remains controversial. However, phosphatidylinositols generated by phosphatidylinositol 3-kinase do bind to phox homology (PX) domains on both p40phox and p47phox at the plasma membrane, facilitating NOX complex formation. Finally Rac complexed with GDP is retained in the cytoplasm in an inactive form bound to Rho guanine nucleotide dissociation inhibitor (RhoGDI), which binds to and masks a geranyl-geranyl lipid modification on the C-terminus of Rac (130). Activation of guanine nucleotide exchange factors facilitates the release of GDP and the binding of GTP by Rac, which promotes dissociation of Rac from RhoGDI and the association of Rac with the plasma membrane. Activated Rac binds to p67phox facilitating the formation of the active NOX complex.

Our understanding of the actual mechanisms of activation for the other isoforms is less clear. There appears to be distinct groups of cytosolic regulatory proteins for the different gp91phox isoforms. For NOX1 this consists of the p47phox homologue NOXO1 and the p67phox homologue NOXA1 (19). However, unlike for p47phox, NOXO1 appears to be bound to p22phox-NOX1 constitutively (47). The precise mechanisms for the regulation of NOX3 and NOX4 in cells are less clear (48, 116, 213). NOX5, DUOX1, and DUOX2 are regulated by intracellular calcium levels (20, 62). The smaller membrane component, p22phox, is required for complexes containing NOX1, NOX2, NOX3, and NOX4, but not for NOX5 (107).

Thus there is a significant amount of complexity in the regulation of the activation of NOX complexes. The activation of these complexes clearly involves tightly controlled processes, enabling the cell not to inadvertently waste valuable reducing equivalents in the needless generation of potentially harmful ROS. These enzyme complexes could serve to generate H_2O_2 in a regulated fashion for the purposes of signal transduction.

C. *Effects of physiologic levels of hydrogen peroxide on cellular proliferation and survival*

Some of the most compelling evidence for H_2O_2 serving as a signaling agent in animals has derived from the effects of adding low concentrations of H_2O_2 directly to cells in culture. In an early study, thymidine incorporation by primary human dermal fibroblasts was increased within 4 hr in response to 1 μ*M* H_2O_2 (146). In a seminal report, 1 μ*M* H_2O_2 was shown to increase the proliferation rate of proliferating hamster kidney BHK-21/C13 fibroblasts, as assessed by counting cells at multiple time points (36). More recently it was shown that H_2O_2 increased both the viability and mitotic index of cultured rat L6C5 myoblasts, at 1 hr after treatment, and the effects were maximal with 5 μ*M* H_2O_2 (39). Low concentrations of H_2O_2 stimulated proliferation of rabbit TOTL-86 lens epithelial cells, and the effect was maximal with 1 μ*M* H_2O_2 (153). Both thymidine incorporation and proliferation (cell number) were increased by the addition of 10 μ*M* H_2O_2 to human U373-MG astrocytoma cells (11). Proliferation of primary human tracheal smooth muscle cells was enhanced by the application of 1 μ*M* H_2O_2 (157). The proliferation of primary human endothelial cells was enhanced by 1–10 μ*M* H_2O_2 (170). DNA synthesis was stimulated in Eahy926 endothelial cells with a maximal effect at 1 μ*M* H_2O_2 (74), and 5–10 μ*M* H_2O_2 stimulated DNA synthesis in primary mouse hepatocytes (125). In addition, 1 μ*M* H_2O_2 was shown to stimulate tube formation by cultured primary bovine aortic endothelial cells in an *in vitro* model of angiogenesis (235, 236). Application of catalase inhibited this H_2O_2-stimulated angiogenesis *in vitro* (235). Likewise application of catalase inhibited angiogenesis *in vivo* in the chicken embryo (169), and the overexpression of catalase was demonstrated to inhibit angiogenesis *in vivo* in a mouse dermal wound healing model (180).

In considering these observations, it is important to stress the distinction between these effects and those of oxidative stress resulting from the application of supraphysiologic levels of H_2O_2 (Fig. 11). As discussed above, the application of H_2O_2 at concentrations greater than 10 μ*M* will result in greater than 1 μ*M* H_2O_2 inside the cell, and will thus result in an oxidative stress to the cell. It has been observed that the application of supraphysiologic levels of H_2O_2 in the range of 30–200 μ*M* may in fact stimulate DNA synthesis to some extent in some cells and may even enhance survival, especially in response to a later more severe oxidative stress (16, 60, 80, 176, 224). However, the primary direct effect of these supraphysiologic levels of H_2O_2 is that of impaired proliferation and/or decreased viability, and these treatments do not directly stimulate cellular proliferation (5, 193). Likewise, enhanced cellular proliferation in response to low (< 10 μ*M*) H_2O_2 is to date an observation limited to animal cells, and even more precisely limited to vertebrate cells. This phenomenon has not been demonstrated in bacteria or yeast. Although enhanced survival in response to transient application of stress levels (30–200 μ*M*) of H_2O_2 has been observed in both bacteria and yeast, particularly in the setting of a subse-

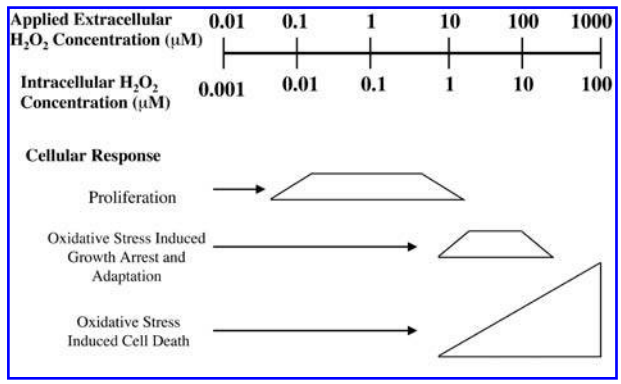

FIG. 11. Vertebrate cellular responses to applied H_2O_2. The application of low concentrations (0.1–7 μ*M*) of H_2O_2 to cultured cells results in intracellular H_2O_2 levels of ~0.01–0.07 μ*M* and directly stimulates cell proliferation. Higher levels of H_2O_2, typically 20–200 μ*M*, stimulate initially growth arrest, which may be followed by growth promoting adaptation to oxidative stress. Increasing amounts of cell death occur with applied concentrations of $H_2O_2 \geq 10$ μ*M*. The precise transition points for these cellular responses may vary due to cell type and culture conditions.

quent more severe oxidative stress (52, 59, 68). It is also important to point out, that a mitogenic response to low levels of H_2O_2 may not be observed by cells in culture that are already producing relatively large quantities of H_2O_2 (120). This may be particularly problematic when studying transformed cells, which often produce more H_2O_2 than is produced by primary cells (167, 200).

A complementary line of investigation has been to elucidate the effects of removing endogenous H_2O_2 by the application or overexpression of catalase. Numerous studies have now shown that catalase indeed inhibits mammalian cell proliferation and in some cases even stimulates cell death. Overexpression of catalase in rat aortic smooth muscle cells was shown to decrease both DNA synthesis and actual proliferation rates in these cells (33). In addition, the catalase overexpressing cells showed increased rates of apoptosis. Application of extracellular catalase was shown to inhibit both proliferation and DNA synthesis in transformed Rat-1 fibroblasts (172). Likewise overexpression of catalase was shown to inhibit DNA synthesis and proliferation in human aortic endothelial cells, and to promote apoptosis in these cells (240). Treatment of primary rat pneumocytes with catalase both decreased proliferation and decreased the level of phosphorylated cyclin-dependent kinase 2 (Cdk2) at 5 hr after treatment (174). Treatment with catalase also inhibited proliferation and reduced expression of Cyclin D1 at 4 hr after treatment in M1619 malignant melanoma cells (31). However, the precise mechanism by which catalase reduces cell proliferation is unclear. Catalase does not appear to result in a discrete block of the cell cycle at a specific cell-cycle checkpoint (172). Some secondary effects of the overexpression or addition of catalase have been reported. Overexpression or extracellular addition of catalase does result in the upregulation of cyclooxygenase-2 mRNA and protein in vascular smooth muscle cells (33, 46). In addition, application of catalase also transiently stimulates activation of the c-JUN N-terminal kinase JNK1 in transformed Rat-1 cells (172). These results suggest that catalase may induce a hypo-peroxide stress in the cells.

A third line of evidence linking H_2O_2 to cellular proliferation derives from studies correlating cellular H_2O_2 production with proliferation and even neoplastic transformation. In general, in mammalian cells, ROS production is greater in proliferating subconfluent cultures compared with confluent cells (23, 75, 158). Interestingly, induction of DNA synthesis by 10 μM H_2O_2 was observed in dense but not sparse cultures of fibroblasts, in which the dense cultures were producing significantly less ROS than the sparse cultures (158). Human tumor cell lines produce relatively large quantities of H_2O_2, in some cases approaching the amounts produced by activated neutrophils (200). In fact the actual rates of H_2O_2 production have been shown to correlate with proliferation rates in multiple mammalian epithelial cell lines (167). A provocative report indicated that overexpression of the O_2^- producing NOX1 catalytic subunit of the NOX complexes resulted in transformation of NIH3T3 cells (197). Of course, as discussed above, modulation of NOX activity could potentially alter not only H_2O_2 levels, but also O_2^-, $^{\bullet}NO$, and peroxynitrite levels. The enhanced proliferation rate resulting from NOX1 overexpression was inhibited by the overexpression of catalase (10), consistent with, but not definitively indicative of, H_2O_2 mediating the pro-growth effects of NOX1 upregulation. As discussed above, overexpression of catalase can potentially alter O_2^-, $^{\bullet}NO$, and peroxynitrite levels in addition to H_2O_2 levels. Subsequent follow-up revealed that the transformation induced by NOX1 upregulation was dependent on the presence of Ras mutations in these cells (116). However, small interfering RNAs targeting NOX1 do prevent Ras-mediated transformation (142). Similarly, small interfering RNAs or anti-sense oligonucleotides targeting catalytic or regulatory components of the NADPH oxidase complexes inhibit proliferation in multiple mammalian cells types (30, 116, 183). These studies demonstrate that the ROS generated by NOX complexes have pro-growth effects and are likely important for oncogenic transformation, but may not be sufficient on their own to stimulate transformation.

D. Oxidation-dependent steps in receptor-mediated signal transduction

Given that numerous physiologic ligand-receptor interactions result in the generation of O_2^-/H_2O_2 by mammalian cells, one approach aimed at elucidating H_2O_2 signaling pathways has been to identify oxidation-dependent steps in receptor-mediated signaling pathways (Fig. 12). This approach has generally involved applying H_2O_2 scavengers of varying specificity along with the ligand to inhibit the oxidation-dependent step following ligand-receptor interaction, and also

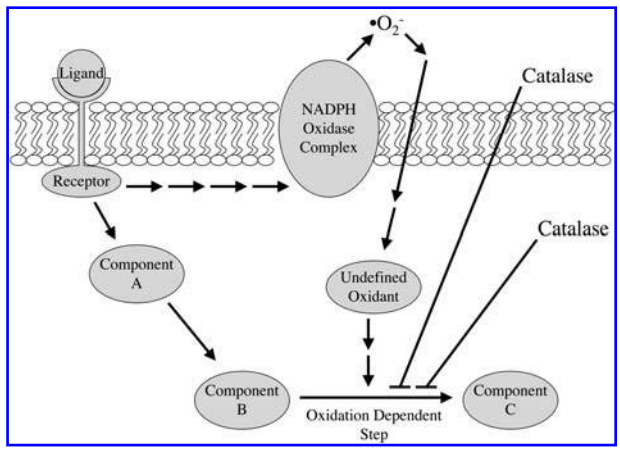

FIG. 12. Properties of oxidation-dependent steps in receptor-mediated signaling pathways. Depicted is a general schematic for oxidation-dependent steps in receptor-mediated signaling pathways. The binding of a ligand to a receptor activates downstream components: Component A, Component B, and Component C. The ligand/receptor interaction also activates O_2^- production from NOX complexes. A step is identified, in this case the activation of Component C, which is dependent on the presence of an undefined oxidant. The step is inhibited by downregulation or inhibition of NOX complexes, and is also inhibited by either the extracellular addition of catalase or the overexpression of catalase. In the absence of ligand, application of physiologic levels of H_2O_2 does not activate Component C, but application of supraphysiologic levels of H_2O_2 may mimic the ligand-induced activation of Component C.

applying H_2O_2 in the absence of ligand to mimic the effects of the ligand. To date, this latter effect has inevitably required supraphysiologic levels of H_2O_2 (typically 0.1–2 m*M*) and is not achieved by the application of physiologic levels of H_2O_2 (<10 μ*M* extracellular). Using these approaches oxidation-dependent steps have been implicated in the signaling pathways for numerous ligand-receptor interactions. We will discuss some of the more extensively studied systems, which involve the oxidation of Ras and the oxidation of thiolate-dependent phosphatases.

E. Oxidation of ras in angiotensin II and thrombin-mediated signal transduction

One of the more extensively studied signaling pathways with an oxidation-dependent step is that of angiotensin II (AngII) activation of p38 MAP kinase in vascular smooth muscle cells (Fig. 13). AngII acting through the G-protein coupled AT_1 receptor is a major factor stimulating the growth of vascular smooth muscle cells, and many of the pro-growth effects of AngII are believed to be secondary to the activation of MAP kinases (212). AngII also stimulates the release of O_2^-/H_2O_2 from vascular smooth muscle cells (88), and scavenging of $^{\bullet}NO$ by O_2^- released in response to AngII is an important mechanism for impaired vasodilation in the vasculature. AngII stimulated generation of reactive oxygen species as measured by DCF oxidation is inhibited by anti-sense RNA to the p22phox component of the NOX complexes (218, 239). Treatment of vascular smooth muscle cells with AngII leads to the rapid phosphorylation and activation of p38 MAP kinase (214). Overexpression of catalase inhibits both AngII induced increases in p38 MAP kinase phosphorylation as well as smooth muscle cell growth (214, 239). In the absence of AngII, supraphysiologic levels (100–200 μ*M*) of H_2O_2 stimulate the phosphorylation of p38 MAP kinase (214). More recently it was determined that AngII stimulates both the activation of Ras, and the formation of an oxidized form of Ras, in which Cys118 is in a mixed disulfide with glutathione (Ras-SSG) (1). The formation of Ras-SSG is also stimulated by the application of supraphysiologic levels of H_2O_2 (250 μ*M*) in the absence of AngII. A previous quantitative analysis showed that Ras is not activated by the application of 1–10 μ*M* H_2O_2 to cells and is only modestly activated by the application of 1 m*M* H_2O_2 (118). Overexpression of catalase inhibits both the activation of Ras, and the formation of Ras-SSG in response to AngII, and overexpression of the C118S mutant of Ras inhibits both AngII stimulated phosphorylation of p38 MAP kinase as well as AngII stimulated cell growth (1).

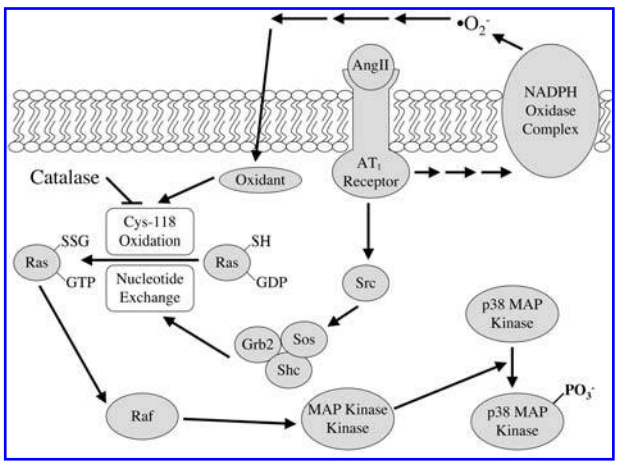

FIG. 13. Model of an oxidation-dependent step in AngII signaling. AngII interacting with the AT_1 receptor leads to the activation of Src tyrosine kinase, which promotes guanine nucleotide exchange on Ras via the action of Grb2, Shc, and Sos. Activation of Ras leads to the eventual phosphorylation of p38 MAP kinase. The AngII interaction with the AT_1 receptor also stimulates extracellular O_2^- generation from NOX complexes. The activation of Ras is dependent on the oxidation of Ras Cys118 by an undefined oxidant that is likely derived from NOX-generated O_2^-. Oxidation of Ras is blocked by the overexpression of catalase.

Thus there is at least one oxidation-dependent step modulating the activation of p38 MAP kinase in response to AngII in vascular smooth muscle cells. The current evidence indicates that one such oxidation-dependent step in this pathway is the oxidation of Cys118 of Ras. However, many questions remain concerning the role of Ras oxidation in AngII signaling. It is currently not clear what fraction of activated Ras is represented by Ras-SSG, or if this oxidized form of Ras is the major oxidized form in the cells. Nor is it precisely clear how Cys118 oxidation on Ras is related to classical activation of the protein by guanine nucleotide exchange. Although, in Ras, Cys118 is located within the nucleotide binding NKCD motif, and in *in vitro* studies, oxidation of Cys118 enhances release of GDP (95, 96). More importantly, the nature of the oxidant oxidizing Ras-Cys118 is not precisely clear. Although the precise rate constant for the reaction of Ras with H_2O_2 has not been reported, the application of supraphysiologic levels of H_2O_2 are required to oxidize and/or activate Ras in cells, implying that this rate of reaction is relatively slow. Likewise, *in vitro* studies have revealed that treatment of Ras with either $^{\bullet}NO$ in the presence of oxygen or high levels of O_2^- do stimulate the dissociation of GDP from wild-type Ras but not the C118S mutants, while H_2O_2, even at the markedly high concentration of 300 m*M*, had no effect on GDP dissociation (95, 96). The only substantial evidence implicating H_2O_2 as the oxidant in the AngII-p38 MAP kinase pathway is the inhibition of the pathway by catalase. However as outlined above, catalase may have effects on cells beyond scavenging H_2O_2, such as decreasing $^{\bullet}NO$ and/or peroxynitrite, and dramatically lowering the steady-state H_2O_2 level may affect cells well beyond the pathways in which H_2O_2 is directly involved.

A similar pathway is that of thrombin stimulation of p38 MAP kinase phosphorylation in vascular smooth muscle cells. Thrombin, a component of the coagulation cascade, stimulates smooth muscle cell proliferation (138). Thrombin also stimulates release of H_2O_2 and O_2^- from vascular smooth muscle cells, and thrombin-induced proliferation is inhibited by application of extracellular catalase (161). As with AngII, thrombin has been shown to stimulate the phosphorylation of p38 MAP kinase in vascular smooth muscle cells, and inhibition of p38 MAP kinase inhibits thrombin-induced proliferation (105). In

addition, thrombin-induced phosphorylation of p38 MAP kinase is inhibited by the overexpression of a dominant-negative form of Ras, implying that as for AngII, thrombin stimulated phosphorylation of p38 MAP kinase involves the activation of Ras (105). Thrombin-stimulated phosphorylation of p38 MAP kinase was also shown to be inhibited by the application of catalase extracellularly (221), or by overexpression of oligonucleotides anti-sense to the p22phox regulatory component of the NOX complexes (29). Utilizing vascular smooth muscle cells from mice with partial deficiency of copper-zinc superoxide dismutase (SOD1+/−), it was demonstrated that a reduction in SOD1 levels actually enhances thrombin-stimulated cell growth and thrombin-stimulated p38 MAP kinase phosphorylation (131), suggesting that a O_2^--derived oxidant other than H_2O_2 is responsible for the effect. Thus there is growing evidence that the phosphorylation of p38 MAP kinase in response to activation of G-protein coupled receptors in vascular smooth muscle cells involves an oxidation-dependent step consistent with the oxidation of Ras at Cys118. However, the majority of evidence currently available suggests that this Ras-Cys118 oxidation is mediated by an oxidant distinct from H_2O_2.

F. The role of an oxidative event in the regulation of thiolate-dependent phosphatases

There is growing evidence that mammalian thiolate-dependent phosphatases (TDPs) may represent an oxidation-dependent step in multiple signal transduction pathways. TDPs include tyrosine phosphatases, dual-specificity phosphatases, and lipid phosphatases, and there are approximately 103 distinct TDPs in humans (2). This hypothesis was initially formulated for protein tyrosine phosphatases (PTPs) based on the observation of enhanced tyrosine phosphorylation upon addition of supraphysiologic levels (0.1–3 m*M*) of H_2O_2 to cells (92, 93, 198). As discussed above, the thiolate moieties at the active sites of TDPs will react with H_2O_2 with a modestly enhanced rate compared to that of the reaction of most typical protein Cys thiol moieties with H_2O_2 to generate the sulfenic acid (42, 70, 190). The sulfenic acid then typically reacts with a second nucleophile, the nature of which is dependent on the specific environment of the individual enzyme's active site (Fig. 14). The sulfenic acid reacts with a backbone amide nitrogen to generate the sulfenyl-amide in PTP-1B (182, 216), and with a second Cys-thiol to generate a

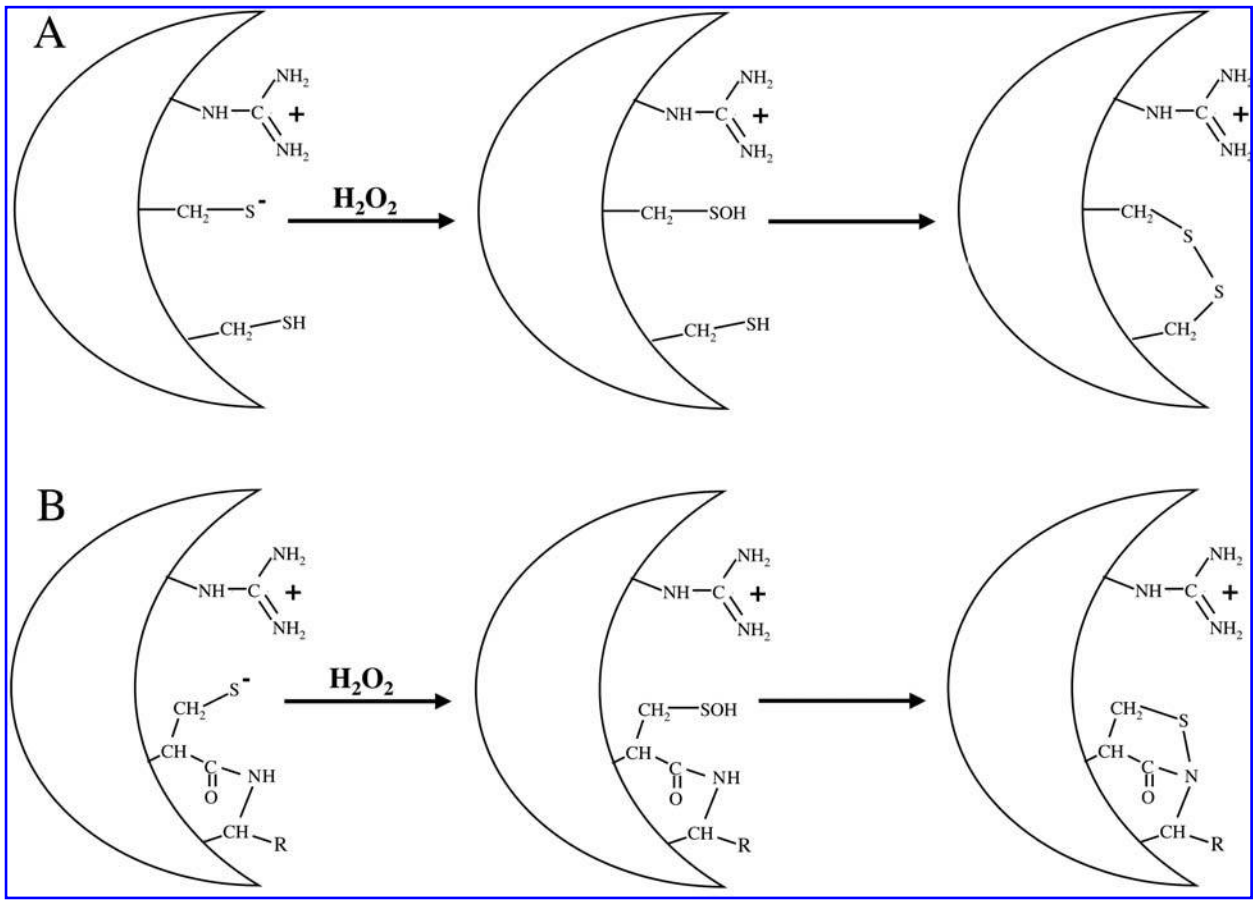

FIG. 14. *In vitro* reactions of thiolate-dependent phosphatases with H_2O_2. The active sites of thiolate-dependent phosphatases contain an activated Cys-thiolate moiety due to the presence of a nearby Arg residue. The thiolates react relatively slowly (10–100 $M^{-1}s^{-1}$) with H_2O_2 to generate the sulfenic acid (R-SOH). In the case of the dual specificity phosphatase Cdc25B, the sulfenic acid reacts with a second thiol to generate a disulfide bond (**A**). In the case of PTP-1B, the sulfenic acid reacts with an adjacent backbone amide to generate the sulfenyl-amide (**B**).

disulfide bond in the Cdc25B dual specificity phosphatase (35), PTEN (123), RPTPα (215), and LMW-PTP (42). So these observations raise the question as to whether TDPs serve as sensors for H_2O_2 in mammals.

Further support for the role of TDPs in H_2O_2 sensing has come from the observation of transient inactivation of particular TDPs following receptor activation (Fig. 15). This inhibition appears to involve the reversible oxidative modification of the active-site cysteine thiolate, and is readily reversed by DTT. Along this line, epidermal growth factor (EGF) was shown to stimulate the transient inhibition of PTP1B in A431 human epidermoid carcinoma cells (122). Insulin has been shown to stimulate the inhibition of PTP1B in HepG2 cells and 3T3-L1 adipocytes, and this phosphatase inhibition was prevented by the addition of catalase (133). Insulin was also shown to stimulate the inhibition of the PTPs PTP1B and TC45 in Rat-1 cells (140). Platelet-derived growth factor (PDGF) was shown to stimulate the oxidation and inhibition of the PTP SHP-2 in Rat-1 and NIH 3T3 cells (141, 163), and of LMW-PTP in NIH3T3 cells and C2C12 myoblasts (49). TNF-α was shown to stimulate the oxidation and inhibition of JNK tyrosine phosphatases in IKKβ-deficient fibroblasts (103). B-lymphocyte antigen receptor activation in A20 cells also leads to a transient inactivation of PTPs, which is inhibited by overexpressing either Prx-I or catalase (188). PTEN was demonstrated to be oxidized by addition of EGF to HeLa cells, addition of PDGF to NIH3T3 cells, and addition of insulin to HEK293 cells (115).

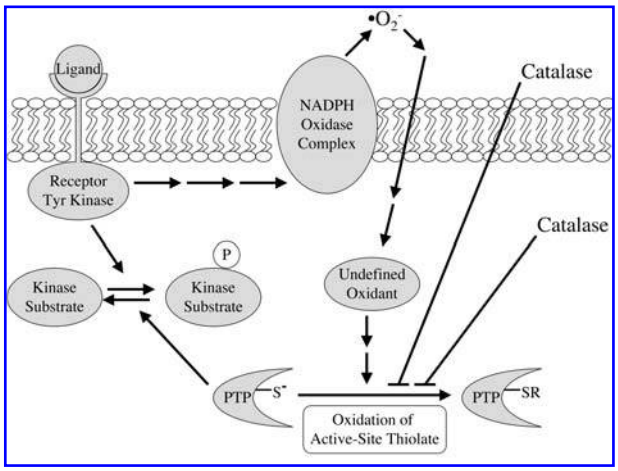

FIG. 15. Model for the oxidation of protein tyrosine phosphatases during growth factor-mediated signaling. The binding of growth factor ligands to receptor Tyr kinases facilitates the Tyr phosphorylation of kinase substrates. PTPs downregulate the pathway by dephosphorylating the phosphorylated kinase substrate. Receptor activation also stimulates extracellular generation of O_2^- by NOX complexes. Oxidation of the active-site thiolates of PTPs by an undefined oxidant inhibits their activity promoting kinase substrate phosphorylation. The oxidation of the PTP is inhibited by either the extracellular addition of catalase or the overexpression of catalase, but PTP oxidation does not occur following the application of physiologic levels of H_2O_2.

The active site thiolates in TDPs react with H_2O_2 with second order rate constants of 10–100 M^{-1}-s^{-1} (42, 70, 190), comparable to the rate constants for the reaction of most nonperoxidase thiolate-dependent enzymes. However this rate of reaction is 3 to 6 orders of magnitude slower than the reaction of H_2O_2 with peroxidases, catalases, and the bacterial H_2O_2 sensor OxyR. The slow rate of reaction of TDPs with H_2O_2 explains why the application of physiologic levels (< 10 μ*M*) of H_2O_2 to cells neither inhibits these enzymes nor enhances tyrosine phosphorylation (51, 123, 141, 164).

Given the remarkably slow rate of reaction of TDPs with H_2O_2, and the apparent inability of TDPs to react with physiologic levels of H_2O_2, an obvious consideration would be that these enzymes could be oxidized secondarily in response to H_2O_2 by a peroxidase, and thus be functioning as indirect sensors of H_2O_2 as discussed above. One potential peroxidase for this role would be peroxiredoxin II (Prx-II). However, gene deletion of Prx-II enhances, rather than inhibits total Tyr phosphorylation simulated by PDGF or EGF in mouse embryonic fibroblasts (51). Comparison of these Prx-II deficient mouse embryonic fibroblasts transfected with either Prx-II or control plasmids, revealed less inactivation of Tyr phosphatases following PDGF treatment in the Prx-II transfected group. Thus Prx-II at least appears not to mediate oxidative TDP inactivation.

Although TDPs appear to be transiently inhibited in an oxidative fashion following receptor activation, many questions remain. First and foremost is the actual identity of the oxidant reacting with the TDPs. There is growing evidence that the oxidant may in fact be a •NO-derived oxidant such as a nitrosothiol or peroxynitrite rather than H_2O_2. Both of these species in fact react with the thiolate moieties of TDPs much faster than does H_2O_2 (127, 201). In CHO cells, the inhibition of transfected PTPs SHP-1 and SHP-2 by supraphysiologic levels (90 μ*M*) of H_2O_2 was found to be inhibited by the •NO synthase inhibitor L-NAME (21). Indicating that even at these supraphysiologic levels, H_2O_2 is unable to react in any substantial amount directly with TDPs. The study also implies that a •NO-derived oxidant could potentially mediate TDP inhibition *in vivo*. In a few instances, as outlined above, receptor stimulated inhibition of TDPs was prevented by the application or overexpression of catalase. This observation has been interpreted as indicating H_2O_2 to be directly involved. However, as outlined above, cells in culture lack their physiologic •NO sink, namely the hemoglobin of red blood cells, and in such situations catalase is able to metabolize •NO (34). Additionally, marked reduction of H_2O_2 by catalase can lead to reduced steady-state levels of superoxide inhibiting peroxynitrite formation (137). Thus it cannot be claimed with certainty that the effects of catalase are solely due to the removal of H_2O_2.

If H_2O_2 is indeed the oxidant acting either directly on TDPs or indirectly via peroxidases, then one possibility would be that these enzymes are only transiently sensitive to physiologic levels of H_2O_2 following receptor ligation; however, currently no data has been provided indicating this to be the case. The localized generation of high levels of H_2O_2 has been proposed as an additional possibility (51). However, currently no enzyme has been identified that generates high levels of H_2O_2 at specific cytosolic locations in a regulated

fashion. The NOX complexes generate O_2^- (and possibly H_2O_2 directly) either extracellularly or perhaps within cytoplasmic vacuoles or organelles. In addition, the remarkably rapid diffusion of H_2O_2 would appear to more than adequately prevent the stabilization of a markedly elevated concentration of H_2O_2 at a specific cytosolic location. Also, it would appear difficult to explain the inhibitory effects of extracellular catalase if TDP oxidation was due to a sequestered intracellular pool of H_2O_2.

The transient oxidative inactivation of TDPs following receptor activation is an interesting phenomenon with important implications for mammalian signal transduction. However, as outlined above several issues need to be clarified before it will be generally accepted that TDPs function as sensors for H_2O_2. The majority of evidence suggests that a reactive species distinct from H_2O_2 mediates the oxidative inactivation of TDPs.

G. *Signal transduction stimulated by physiologic levels of hydrogen peroxide in animals*

Given the general lack of direct H_2O_2 responsiveness of the oxidation-dependent steps associated with receptor-mediated signal transduction discussed above, an alternative approach to understand H_2O_2 signaling in mammals has been to focus on the signaling events actually stimulated by the same low physiologic concentrations of H_2O_2 that stimulate cell growth and proliferation. In taking this approach one must be very cognizant of the concentration of H_2O_2 applied to the cells. As discussed above, oxidative stress resulting in growth arrest and cell death will begin to occur in some settings with just 0.7 μ*M* intracellular H_2O_2. Thus a true H_2O_2-sensing pathway accounting for the mitogenic effects of H_2O_2, should be substantially if not maximally activated with < 10 μ*M* extracellular H_2O_2. One should also take into account the importance of not stressing the cells by serum starvation, prior to the addition of H_2O_2. This nonphysiologic stress itself induces oxidant generation by the cells and may facilitate the activation of stress response pathways in response to what would otherwise be a growth-promoting dose of H_2O_2. These considerations are for intact cells for which the intracellular level of H_2O_2 is expected to be one order of magnitude lower than the extracellular concentration. When assessing broken-cell preparations, cell extracts and purified proteins, it would be desirable for the *maximal* effect to be achieved with < 1 μ*M* H_2O_2 in order to consider the response as being regulated by physiologic levels of H_2O_2.

The low concentrations of H_2O_2 that stimulate proliferation of mammalian cells have been demonstrated to stimulate the induction of specific genes and the up-regulation of specific proteins. In human monocytes, 1–10 μ*M* H_2O_2 was demonstrated to stimulate increased mRNA levels for chemokine receptors CCR2, CCR5, and CXCR4 at 4 hr, and the effect was essentially maximal at 10 μ*M* H_2O_2 (181). In human endothelial hybridoma EaHy926 cells, application of 10 μ*M* H_2O_2 stimulated increased protein levels of p22phox at 3 hr, and the effect appeared to be maximal with 10 μ*M* H_2O_2 (74). Correspondingly, the proliferation induced by 10 μ*M* H_2O_2 in these cells was markedly inhibited by transfection of a p22phox anti-sense vector. In vascular endothelial cells, 1–10 μ*M* H_2O_2 stimulates the upregulation of macrophage colony stimulating factor mRNA at 24 hr, and the effect is maximal with 10 μ*M* H_2O_2 (98). In cardiac fibroblasts, 0.5–5 μ*M* H_2O_2 stimulates the downregulation of multiple procollagen mRNAs, the upregulation of fibronectin mRNA, and the up-regulation of matrix metalloproteinase activity at 24 hr (189). In cultured rat pancreatic islets, 5 μ*M* H_2O_2 increased c-Myc mRNA at 18 hr (77).

Given that the cellular response to physiologic levels of H_2O_2 is predominantly enhanced proliferation, not surprisingly, the most proximal effects of these treatments on cellular proteins have been localized to the nucleus (Fig. 16). In murine NSF/N1.H7 myeloid cells, 2.5–10 μ*M* H_2O_2 stimulates degradation of the cyclin dependent kinase (Cdk) inhibitor p27, and an increase in Cdk2 activity at 30 min following treatment, and the effects appear to be maximal with 10 μ*M* H_2O_2 (69). The Cdk inhibitor p21 is not affected by this treatment. Likewise, treatment of primary rat pneumocytes with catalase both decreases proliferation and decreases Cdk2 activation (as assessed by Thr160 phosphorylation status) at 5 hr after treatment (174). Treatment with catalase was also shown to inhibit proliferation and reduce expression of Cyclin D1 at 4 hr after treatment in M1619 malignant melanoma cells (31). An additional link between Cdks and H_2O_2 was revealed with the demonstration that multiple mammalian peroxiredoxins (particularly Prx-I) are phosphorylated by Cdks *in vitro* resulting in the inhibition of their activity (45). Such a process could potentially allow for the fine-tuning of H_2O_2 levels during specific stages of the cell cycle.

Utilizing a two-dimensional protein electrophoresis based screen, it was determined that low concentrations (1–8 μ*M*) of H_2O_2 stimulate the hyperphosphorylation of the nuclear pre-mRNA binding protein hnRNP-C in primary endothelial cells (192). This effect was maximal with 5–8 μ*M* H_2O_2, and was

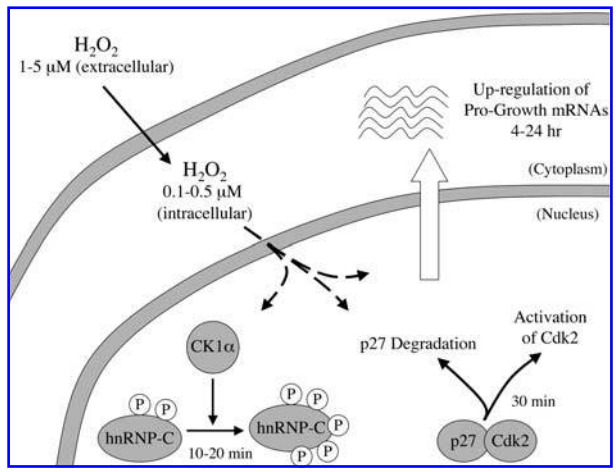

FIG. 16. Biochemical responses to physiologic levels of H_2O_2. The application of 1–5 μ*M* H_2O_2 to cells results in intracellular H_2O_2 levels of 0.1–0.5 μ*M*, stimulating CK1α-mediated phosphorylation of hnRNP-C at 10–20 min, the degradation of p27 and activation of Cdk2 at 30 min, and the upregulation of pro-growth mRNA transcripts at 4–24 hr.

observed without serum starvation. Phosphate mapping of endogenous protein revealed hnRNP-C to be basally phosphorylated at Ser247 and Ser286 (based on hnRNP-C1 numbering), and the low concentrations of H_2O_2 were found to stimulate additional phosphorylations at Ser240 and the stretch of four contiguous Ser residues Ser225-Ser228 (194). A protein kinase capable of phosphorylating these H_2O_2-stimulated sites of phosphorylation was purified and identified as protein kinase CK1α (106). Importantly, treatment of cells with a protein kinase CK1 inhibitor inhibited H_2O_2-stimulated hyperphosphorylation of hnRNP-C, and also reduced the basal level of hnRNP-C hyperphosphorylation, similar to the effects of adding catalase to the cells (106, 192). These results suggest that there is a tonic level H_2O_2-stimulated, CK1α-mediated hyperphosphorylation of hnRNP-C in cultured human endothelial cells.

The precise function of hnRNP-C is not clear (222). The protein is present at high levels in the nucleus of mammalian cells and likely binds most if not all of the pre-mRNA transcripts as they are synthesized. However, hnRNP-C is predominantly a nuclear-restricted protein, and must release processed mRNAs for export to the cytosol. Steady-state RNA binding studies with phosphoserine-mimicking Ser to Glu mutants of hnRNP-C revealed that the H_2O_2-stimulated hyperphosphorylation of hnRNP-C substantially reduces the protein's affinity for mRNA, potentially aiding in mRNA export (106). Interestingly, yeast which lack hnRNP-C and also lack a true functional orthologue for protein kinase CK1α, have not been demonstrated to display a true mitogenic response to low concentrations of H_2O_2. Also heterologous expression of hnRNP-C in yeast is lethal due to the accumulation of hnRNP-C in the nucleus and the retention of mRNA transcripts there (202). Yeast, lacking a true orthologue for protein kinase CK1α, appear to lack the regulatory machinery required for hnRNP-C to release transcripts for export to the cytosol. Deficiency of hnRNP-C in mice is lethal at the egg cylinder stage and results in impaired growth and differentiation of the isolated stem cells (225). Consistent with a role in proliferation, hnRNP-C is particularly hyperphosphorylated during mitosis, and protein kinase CK1α entry into the nucleus is required for cell cycle progression (90, 166). Thus although our understanding of the CK1α/hnRNP-C pathway is currently incomplete, the available evidence indicates that this is a pro-growth pathway specific to vertebrates, that is activated by physiologic levels of H_2O_2.

Another focus of research has been on the alteration of cytoplasmic calcium levels in response to low concentrations of H_2O_2. Certainly numerous studies have demonstrated that supraphysiologic levels (>10 μ*M*) of H_2O_2 result in alterations in cytoplasmic calcium levels, and the mechanism and relevance of this phenomenon are unclear. However, a few studies have indicated that H_2O_2 at physiologic levels has effects on the regulation of cytoplasmic calcium. In rat hippocampal slices, within 10 min of application, 1 μ*M* H_2O_2 was observed to enhance the phenomenon of long-term potentiation (104). This effect was inhibited both by inhibitors of the calcium activated phosphatase calcineurin and by inhibition of L-type voltage-dependent calcium channels. In human aortic endothelial cells, 3–10 μ*M* H_2O_2 was reported to enhance the ability of inositol triphosphate to stimulate calcium release from internal stores, and the effect was near maximal with 10 μ*M* H_2O_2 (100). In the absence of inositol triphosphate, H_2O_2 did not appear to affect cytoplasmic calcium levels. Also in human aortic endothelial cells, histamine-stimulated calcium oscillations were prevented by expression of a dominant-negative form of Rac1 (99). In this setting of a dominant-negative Rac1, 10 μ*M* H_2O_2 stimulated calcium oscillations in the presence of histamine, but had no effect in the absence of histamine. The 10 μ*M* H_2O_2 had no effect on inositol triphosphate levels in these cells. Finally, the application of 1 μ*M* H_2O_2 was shown to decrease internal calcium concentrations during titanic contraction of mouse skeletal muscle fibers (4). These studies indicate that the application of low physiologic levels of H_2O_2 may play a role in modulating calcium-levels in different cell types. However, in all of these situations, H_2O_2 was not functioning independently, but rather modifying the response to an independent modulator of calcium levels.

VII. SIGNALING WITH HYDROGEN PEROXIDE IN PLANTS

Our current understanding of the potential role of H_2O_2 as a signaling agent in plants is very similar to that in animals. Plants are subject to an additional potential source of ROS, the chloroplast with its photosynthetic machinery. However metabolic modeling based on the calculated concentrations of antioxidant enzymes and kinetic rate constants revealed the calculated steady-state concentration of H_2O_2 in chloroplasts to be 0.37 μ*M*, remarkably similar to the determined physiologic levels of H_2O_2 in both animals and microbes (168). Thus, like animals, the physiologic intracellular level of H_2O_2 in plants is below 1 μ*M*. However, unlike the case for animals, there has yet to be demonstrated a physiologic cellular response of plant cells to applied concentrations of H_2O_2 in the range of 1–10 μ*M*; specifically, it has not been demonstrated that the application of these low concentrations of H_2O_2 stimulate plant cell growth or proliferation. Another important difference between plant and animal cells is the presence of the plant cell wall, with its numerous oxidative cross-links. As discussed above, H_2O_2 likely plays a key role as a substrate in the oxidative biosynthesis and remodeling of the plant cell wall.

Not dissimilar from animals, numerous biotic and abiotic stresses do stimulate the release of O_2^- and/or H_2O_2 from plant cells (8, 63). The precise role of H_2O_2 in these stress responses has not been precisely defined, but possible functions would include cell wall structural modulation, direct pathogen cytotoxicity, and signal transduction. The source for the H_2O_2 during these stress responses appears to derive principally from homologues of the catalytic components of the mammalian NOX complex. In fact there appear to be as many as ten distinct genes coding for such NOX homologues in *Arabidopsis* (114, 211), compared with only seven in humans. Thus as in animals, the stimulated generation of H_2O_2 by regulated NOX-type complexes suggests that H_2O_2 could be functioning as a signaling agent in plants, and along this line oxidation-dependent steps in signal transduc-

tion pathways are being reported in plants as discussed above for animals.

One of the more defined oxidation-dependent signaling pathways studied in plants is that of abscisic acid (ABA) induced stomatal closure (73, 108). Under conditions of low water, plants generate the phytohormone ABA, which stimulates stomatal guard cells to close. Stomatal guard cell closure occurs in response altered ion channel activity that results in elevated levels of cytoplasmic calcium. ABA stimulated the production of reactive oxygen species by *Arabidopis* guard cells, and supraphysiologic levels (5 m*M*) of H_2O_2 were demonstrated to increase cytoplasmic calcium levels in these cells by the activation of plasma membrane cation channels (162). Inactivation of the plant NOX catalytic subunits atrboh-D and atrboh-F was shown to prevent ABA-induced stomatal closure, ABA-induced ROS formation, and activation of plasma membrane calcium channels in guard cells, but had no effect on stomatal closure induced by supraphysiologic levels (0.1–0.5 m*M*) of H_2O_2 (114). In the pea *Pisum sativum* L., stomatal closure in response to ABA was reported to be inhibited by the application of catalase (73). Supraphysiologic levels (0.1–0.5 m*M*) of H_2O_2 were found to directly activate plant guard cell plasma membrane calcium channels in intact cells (108). In addition the PTP ABI2, which is thought to function as a negative modulator of ABA signaling, was shown to be inhibited *in vitro* upon treatment with supraphysiologic levels (30–300 μ*M*) of H_2O_2 (139). Importantly, ABI2 was not inhibited *in vitro* by treatment with 3–10 μ*M* H_2O_2, which is 10-fold higher than the physiologic intracellular level of H_2O_2. Thus ABA-induced stomatal closure has many parallels with oxidation-dependent steps in mammalian receptor-mediated signal transduction pathways. There is generation of ROS by regulated NOX-type complexes, the process is inhibited by catalase, and mimicked by supraphysiologic levels of H_2O_2. Thus it is likely that there are oxidation-dependent steps in ABA-mediated signaling, possibly including oxidation of ABI2 or a plasma membrane calcium channel. However, for the reasons outlined above for signaling in animal cells, the data do not yet definitively indicate that H_2O_2 itself is the oxidant mediating these effects.

It has been demonstrated that the application of physiologic levels of H_2O_2, inactivates potassium channels in *V. faba* guard cells (108). H_2O_2 was found to inhibit both inward and outward rectifying-potassium currents in intact guard cells, and the effect was near maximal with just 1 μ*M* H_2O_2. This inhibition of the potassium currents was maximal 10–20 min after the application of H_2O_2, implying that the channels themselves are likely not the direct sensors of the H_2O_2, but downstream components of signaling pathways. This effect on potassium currents is similar to the hyperphosphorylation of hnRNP-C in human endothelial cells, which occurs in 10–20 min in response to physiologic levels of H_2O_2. The H_2O_2-stimulated inhibition of potassium currents in guard cells does not appear to account for oxidation-dependent steps in ABA signaling pathways, since it is commonly felt that there is activation of outward-rectifying potassium currents in response to ABA (184). However, the observation that the application of physiologic levels of H_2O_2 leads to the inhibition of potassium channels suggests that H_2O_2 may indeed be functioning as a signaling messenger in plants.

VIII. CONCLUSIONS AND FUTURE DIRECTIONS

The roles played by H_2O_2 in biological processes continue to be elucidated. While clearly important for oxidative stress and oxidative biosynthesis, evidence continues to accumulate that H_2O_2 functions as a signaling agent in higher organisms, particularly vertebrates. Vertebrate cells clearly possess the biochemical machinery for generating H_2O_2 in a regulated fashion, and have been shown to display proliferative responses to physiologic levels of H_2O_2. In addition these same physiologic levels of H_2O_2 also stimulate rapid biochemical changes in the nuclei of these cells, such as the hyperphosphorylation of hnRNP-C and the activation of Cdk2. Although analysis of the biochemistry of peroxidases, catalases, and the prokaryotic H_2O_2 sensor OxyR has given us much insight into how a vertebrate H_2O_2 sensor may function, the actual identity of the vertebrate H_2O_2 sensor(s) remains unclear. Continued characterization of biochemical alterations induced by physiologic levels of H_2O_2 will likely continue to yield enlightening information in this regard. This general approach was successfully utilized in identifying the mammalian $^{\bullet}NO$ sensor sGC and the prokaryotic H_2O_2 sensor OxyR. It will also be important to gain a better understanding concerning the relationship of H_2O_2 to the numerous oxidation-dependent steps in receptor-mediated signaling pathways now being uncovered. Clearly for this to occur, there needs to be an elucidation of both the specific nature of the oxidants involved in these process and the mechanisms by which the oxidants are synthesized. It will also be important to understand more completely the nature of the oxidative processes that are actually occurring *in vivo* during receptor-mediated signaling, and the ability of any putative causative oxidants to react in this fashion in a physiologically relevant manner.

Thus this is a very exciting time for the field of H_2O_2 signaling. Due to the enormous implications for the pathogenesis of human diseases ranging from atherosclerosis to cancer, there is intense interest from the scientific community in furthering our understanding of H_2O_2 as a signaling agent. However, it is clear that the majority of the work is still ahead of us. It is likely that in the upcoming years significant advancements will be made in the field, greatly furthering our understanding of this process.

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ABBREVIATIONS

ABA, abscisic acid; AngII, angiotensin II; Cdk, cyclin-dependent kinase; c-CRD, C-terminal cysteine rich domain; cGPx3, Yeast GPx3 with an insert found in mammalian cytosolic GPx enzymes; DCF, 2′,7′-dichlorofluorescin diacetate; EGF, epidermal growth factor; Gpx, glutathione peroxidase; hnRNP, heterogeneous nuclear ribonucleoprotein;

JNK, c-JUN N-terminal kinase; n-CRD, N-terminal cysteine rich domain; NOX, NADPH oxidase; PDGF, platelet-derived growth factor; PHGPx, phospholipid hydroperoxide glutathione peroxidase; Prx, peroxiredoxin; PTP, protein tyrosine phosphatases; PX, phox homology; Ras-SSG, Ras with Cys118 in a mixed disulfide with glutathione; RhoGDI, Rho guanine nucleotide dissociation inhibitor; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; SOD1, copper-zinc superoxide dismutase; TDP, thiolate-dependent phosphatases; TSI, transition-state intermediate.

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