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Review

Hydrogen Peroxide Sensing and Signaling

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It is well established that oxidative stress is an important cause of cell damage associated with the initiation and progression of many diseases. Consequently, all air-living organisms contain antioxidant enzymes that limit oxidative stress by detoxifying reactive oxygen species, including hydrogen peroxide. However, in eukaryotes, hydrogen peroxide also has important roles as a signaling molecule in the regulation of a variety of biological processes. Here, we will discuss the molecular mechanisms by which hydrogen peroxide is sensed and the increasing evidence that antioxidant enzymes play multiple, key roles as sensors and regulators of signal transduction in response to hydrogen peroxide.

Introduction

Although hydrogen peroxide is better known for its cytotoxic effects, in recent years it has become established as an important regulator of eukaryotic signal transduction. Hydrogen peroxide is generated in response to various stimuli, including cytokines and growth factors (Figure 1), and is involved in regulating biological processes as diverse as immune cell activation and vascular remodeling in mammals (for a review see Geiszt and Leto [2004]) and stomatal closure and root growth in plants (Foreman et al., 2003; Laloi et al., 2004). The use of a cytotoxic chemical as a signaling molecule obviously has potential risks so it is no surprise that the generation of hydrogen peroxide is tightly regulated (for a review see Ushio-Fukai [2006a]). Similarly, the localization, expression, and activities of hydrogen peroxide-detoxifying antioxidant enzymes are also highly regulated. Indeed, regulation of antioxidant activity is not just of key importance in adaptation to the environment but also in modulating peroxide-signal transduction. In addition, recent studies have identified several peroxide-signaling mechanisms in which antioxidant enzymes are critically required as peroxide sensors. Thus, antioxidant enzymes are emerging as key regulators of the multitude of pathways that respond to hydrogen peroxide. Here we will discuss, with examples, the various molecular mechanisms for sensing hydrogen peroxide, focusing on the roles of antioxidant enzymes in hydrogen peroxide signal transduction. Studies in prokaryotes and in yeast have been particularly important in identifying molecular mechanisms for (1) sensing hydrogen peroxide and (2) tailoring the appropriate response. We will discuss the implications of these studies for peroxide signaling in multicellular eukaryotes.

Biological Sources of Hydrogen Peroxide

Most biological sources of hydrogen peroxide involve the spontaneous or catalytic breakdown of superoxide anions (O^{2-}), produced by the partial reduction of oxygen during aerobic respiration and following the exposure of cells to a variety of physical, chemical, and biological agents (Figure 1). For instance, it has long been established that phagocytic immune cells activate NADPH oxidase complexes to generate superoxide, and hence hydrogen peroxide, as a cytotoxic agent during the engulfment of microbes. However, recent work has shown that NADPH oxidases are not confined to phagocytic immune cells but are found in a variety of different cell and tissue types. In fact, the nonimmune cell, Nox family of NADPH oxidases has been linked with the generation of reactive oxygen species (ROS) as important signaling molecules in angiogenesis, inner ear development, and insulin signaling (for a review see Geiszt and Leto [2004]). Indeed, there is increasing evidence that various growth factors and cytokines, including PDGF, EGF, insulin, angiotensin II, and TNFα, generate hydrogen peroxide in target cells by stimulating the activation of NADPH oxidases (Geiszt and Leto, 2004; Park et al., 2004). Hydrogen peroxide is also generated as a signaling molecule by NADPH oxidase-independent mechanisms (Fay et al., 2006; Ali et al., 2006; Chiarugi et al., 2003; DeYulia et al., 2005) (Figure 1).

Hydrogen Peroxide Induces Diverse Biological Responses

In unicellular organisms an important response to increased levels of hydrogen peroxide is the increased production of antioxidants and repair proteins to allow adaptation to these oxidative conditions (Jamieson, 1998; Storz and Tartaglia, 1992). Similarly, antioxidant gene expression is activated by peroxides in certain cell types in multicellular organisms (An and Blackwell, 2003; Inoue et al., 2005; Sablina et al., 2005). However, in multicellular organisms, hydrogen peroxide can also activate signaling pathways to stimulate cell proliferation (Foreman et al., 2003; Geiszt and Leto, 2004), differentiation (Li et al.,

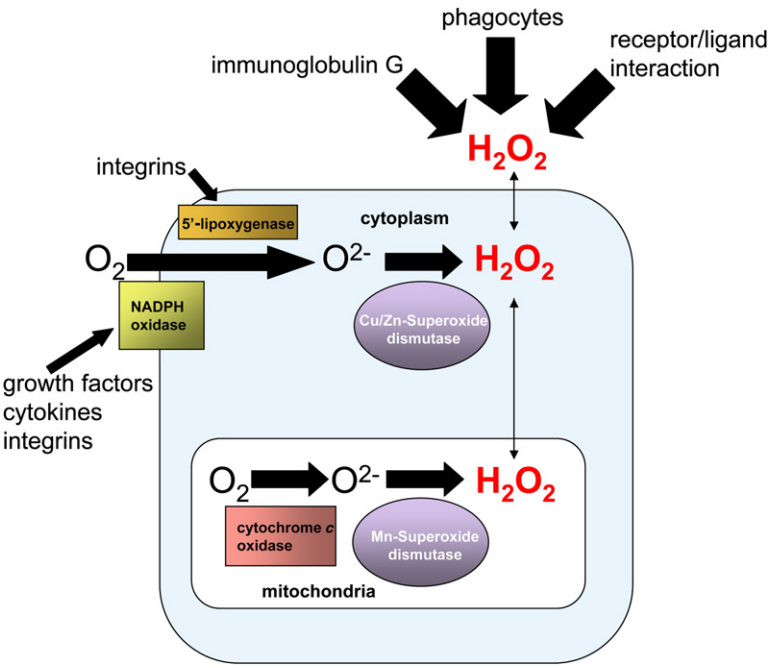

Figure 1. Eukaryotic Cells Are Exposed to Different Sources of Hydrogen Peroxide

Hydrogen peroxide can be produced extracellularly, for example by the immunoglobulin G-catalyzed oxidation of water, by receptor/ligand interactions, and by phagocytic immune cells. Superoxide anions, which are produced by the partial reduction of oxygen by cytochrome *c* oxidase in mitochondria, by membrane-associated NADPH oxidase, or by 5′-lipoxygenase in the cytoplasm, are rapidly converted to hydrogen peroxide by the action of cytoplasmic and mitochondrial superoxide dismutase enzymes. Growth factors, cytokines, and integrins stimulate the activation of NADPH oxidase and/or 5′-lipoxygenase. Hydrogen peroxide can diffuse across membranes as indicated by the finer arrows.

2006; Sauer et al., 2000), migration (reviewed in Ushio-Fukai [2006b]), or apoptosis (Cai, 2005; Gechev and Hille, 2005). An important question is what determines the biological response initiated by hydrogen peroxide in any given cell?

There is considerable variation between cells in the concentration of exogenous hydrogen peroxide required to initiate a particular biological response. For example, the concentration of hydrogen peroxide required to cause apoptotic cell death of mammalian cells can vary as much as 20-fold, depending on the cell type (Chen et al., 2005; Choi et al, 2006; Sablina et al., 2005). Moreover, it has been observed that different levels of hydrogen peroxide can induce distinct responses within a cell. For example, in the fission yeast *Schizosaccharomyces pombe*, distinct transcriptional responses are induced in response to low and high levels of hydrogen peroxide (Quinn et al., 2002) (see later). Similarly, recent studies in mammalian cells have revealed concentration-specific responses to hydrogen peroxide. For example, different patterns of p53-regulated gene expression are initiated in response to different levels of hydrogen peroxide; antioxidants are induced in response to low levels of hydrogen peroxide, where they lower ROS levels and hence protect the cell from DNA damage, while higher levels also stimulate the expression of pro-oxidants involved in apoptosis (Sablina et al., 2005) (Figure 2).

Hydrogen peroxide also has concentration-dependent effects upon the conjugation of mammalian proteins to the SUMO ubiquitin-like modifier. Whereas concentrations of hydrogen peroxide such as those generated by activated macrophages inhibit conjugation of SUMO and hence reduce sumoylation, at higher concentrations (e.g., 100 mM) deconjugation of SUMO is inhibited, causing an increase in the levels of sumoylation (Bossis and Melchior, 2006; Manza et al., 2004; Saitoh and Hinchey, 2000; Zhou et al., 2004) (Figure 2). Sumoylation is an important regulator of the localization, activity, and stability of many proteins, and thus the concentration-dependent regulation of this protein modification may be important to ensure the appropriate biological response to increasing levels of hydrogen peroxide. Currently the mechanisms for regulating concentration-dependent and cell-type-specific effects of hydrogen peroxide are, in most cases, unclear. However, here we will discuss evidence that suggests that the levels, localization, and/or activities of cellular antioxidants are important in determining which biological responses to hydrogen peroxide are initiated.

Regulation of Responses to Hydrogen Peroxide by Regulated Localization and Expression of Antioxidant Enzymes

Although, as a nonpolar molecule, hydrogen peroxide is able to diffuse across membranes, exogenously added hydrogen peroxide has been shown to be less effective at eliciting a signaling response than endogenously produced hydrogen peroxide (Choi et al., 2005). Indeed, NADPH oxidases are assembled and localized in a highly regulated manner suggesting that hydrogen peroxide signals are only transmitted a relatively short distance (for a review see Ushio-Fukai [2006a]). Consistent with this, Bossis and Melchior (2006) have reported compartmentalized effects of hydrogen peroxide on SUMO-conjugating enzymes, with enzymes located in the cytoplasm more susceptible to inactivation by hydrogen peroxide than those in the nucleus. As we will discuss below, the

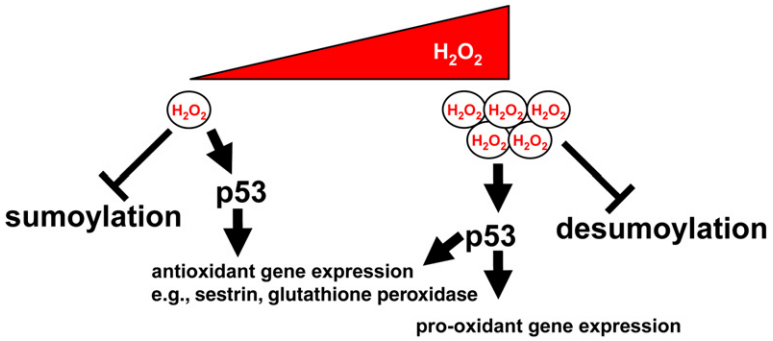

Figure 2. Concentration-Dependent Effects of Hydrogen Peroxide on Signaling in Mammals

In mammals, several signaling processes are differentially affected by increasing concentrations of hydrogen peroxide. For example, the level of sumoylation of proteins is determined by the level of hydrogen peroxide (Bossis and Melchior, 2006). Furthermore, the p53 transcription factor activates antioxidant genes at low, sublethal concentrations but additional, pro-oxidant target genes are also activated as hydrogen peroxide levels increase (Sablina et al., 2005).

presence of abundant enzymatic and nonenzymatic cellular antioxidants is also likely to limit the effects of hydrogen peroxide to close to its site of generation.

In addition to nonenzymatic antioxidants such as vitamins C and E, and carotenoids, cells contain a portfolio of antioxidant enzymes whose activities are directed at reducing hydrogen peroxide. These enzymes can be distinguished by their catalytic mechanisms, cellular localization, and regulation. The major antioxidant enzymes involved in the catalytic breakdown of peroxides are catalase, glutathione peroxidase, and thioredoxin peroxidase (peroxiredoxins). Catalase is a highly efficient enzyme that is widely expressed in peroxisomes, in which ROS levels are high, but may also be found in the cytosol or mitochondria. Whereas catalase utilizes a heme prosthetic group, the catalytic reduction of hydrogen peroxide by thioredoxin peroxidases involves the oxidation of catalytic cysteine residues (Figure 3). The catalytic mechanism of glutathione peroxidases involves the cyclic oxidation/reduction of catalytic cysteine or selenocysteine residues. Peroxiredoxins have been subdivided into classes based on protein similarities and the mechanism of reduction

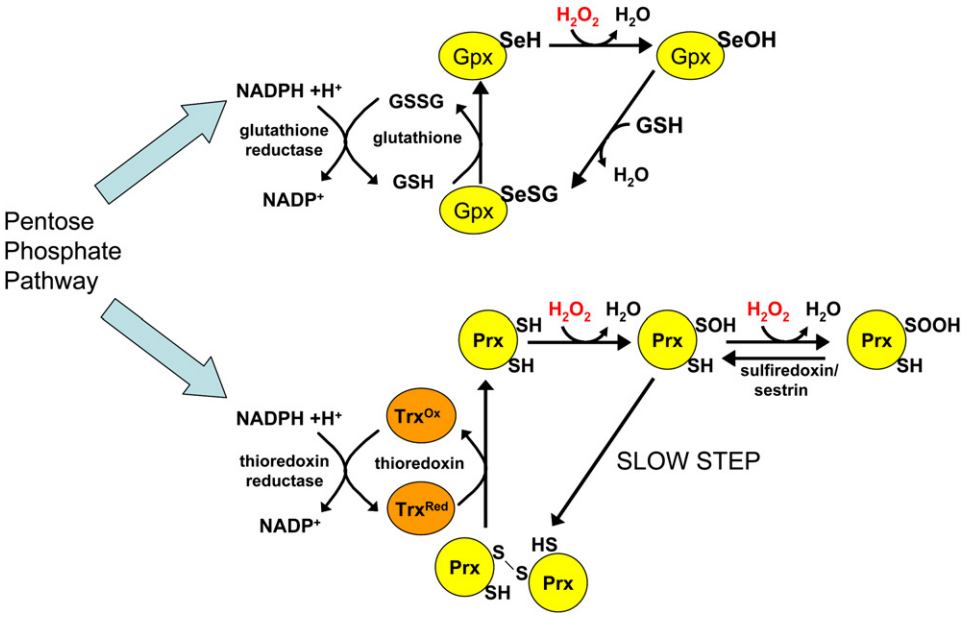

Figure 3. The Redox-Cycling Reactions Involved in the Catalytic Removal of Hydrogen Peroxide by the Glutathione Peroxidase and Thioredoxin Peroxidase Systems

The catalytic reduction of hydrogen peroxide by glutathione peroxidases (Gpx) and thioredoxin peroxidases involves the oxidation of catalytic thiol groups on selenocysteine (SeH) or cysteine (SH) residues in glutathione peroxidases and cysteine residues in thioredoxin peroxidases/peroxiredoxins. The recycling of oxidized peroxidase enzymes involves oxidation of reduced glutathione (GSH) or thioredoxin (Trx). The reduction of oxidized glutathione and thioredoxin are coupled to NADPH by glutathione reductase or thioredoxin reductase, respectively. The pentose phosphate pathway provides NADPH for reduction, and hence antioxidant capacity is also linked to cellular metabolism. One class of peroxiredoxin, the typical 2-Cys Prx (Prx), contains two highly conserved cysteine residues that are involved in the thioredoxin-coupled catalytic reduction of hydrogen peroxide. In the first step the peroxidatic cysteine residue becomes oxidized to sulfenic acid (SOH), then forms a disulfide bond with the resolving cysteine residue on the partner protein in the homodimer preventing further oxidation. However, in eukaryotic 2-Cys Prx disulfide bond formation is slow and as a result the SOH form of the peroxidatic cysteine residue is sensitive to further oxidation to the sulfinic acid (SOOH) derivative which can be reduced by either sulfiredoxin or sestrin enzymes.

of the oxidized protein (for a review see Wood et al. [2003c]). For example, the typical 2-cysteine peroxiredoxins (2-Cys Prx) contain two highly conserved cysteine residues, which are both involved in the thioredoxin-coupled catalytic reduction of hydrogen peroxide (Figure 3). Enzymes with glutathione or thioredoxin peroxidase activity have been identified in various cellular locations. For example, eukaryotic cells express distinct genes encoding mitochondrial and cytoplasmic thioredoxin, thioredoxin reductase, and thioredoxin peroxidase enzymes (Park et al., 2000b; Wood et al., 2003c) and cells of vertebrates express distinct genes encoding cytosolic or secreted glutathione peroxidase enzymes (for a review see Brigelius-Flohe [1999]). The presence of differentially compartmentalized, and even secreted (Haridas et al., 1998), peroxide-decomposing enzymes suggests that it is important that these enzymes are localized appropriately to protect against hydrogen peroxide. However, this compartmentalization also has important consequences for hydrogen peroxide signaling; because hydrogen peroxide diffuses through membranes, the compartmentalization of antioxidant enzymes provides a mechanism for restricting increases in hydrogen peroxide to a particular cellular compartment. Indeed, there is increasing evidence that the localization of specific antioxidants influences the cellular response to hydrogen peroxide by controlling local levels. For example, reducing the levels of a mitochondrion-specific typical 2-Cys Prx, PrxIII, that removes hydrogen peroxide generated in the mitochondria in response to TNFα or staurosporine, increases the sensitivity of cells to TNFα or staurosporine-induced apoptosis (Chang et al., 2004). Conversely, the growth-promoting increase in hydrogen peroxide produced following interaction of the hemapoietic growth factor GM-CSF with its cell surface receptor was inhibited by overexpression of peroxiredoxin or catalase at the cell surface (DeYulia et al., 2005).

In yeast, there is evidence that the localization of antioxidant enzymes may be affected by growth conditions or following implementation of a distinct growth program (Petrova et al., 2004; Urban et al., 2005; Shin et al., 2005). Potentially this may have important implications for the responses of these cells to hydrogen peroxide. In mammals, the redistribution of the typical 2-Cys Prx, PrxII, to colocalize with activated PDGF receptors (Choi et al., 2005) or phospholipase D1 (Xiao et al., 2005) suggests that localizing PrxII to the intracellular site of hydrogen peroxide generation is important for switching off peroxide-signaling events (Choi et al., 2005).

In bacteria and unicellular eukaryotes, the induced expression of detoxifying enzymes in response to hydrogen peroxide plays an important role in protecting the cell against oxidative damage (Gasch et al., 2000; Chen et al., 2003; Smith et al., 2004; Zheng et al., 2001). Indeed, induction of antioxidants in response to a sublethal dose of hydrogen peroxide allows cells to adapt to, and thus survive, subsequent exposure to a much larger dose (Jamieson, 1998). In mammals, basal levels of antioxidants

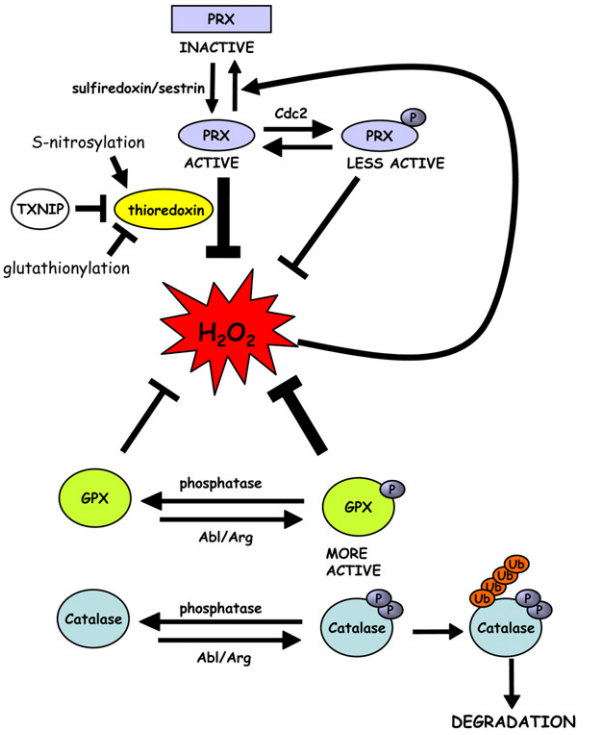

Figure 4. Examples of Mechanisms of Posttranslational Regulation of Cellular Antioxidant Activity
The activities of several eukaryotic antioxidant enzymes, such as 2-Cys peroxiredoxins (PRX), thioredoxin, glutathione peroxidase (GPX), and catalase, are regulated by oxidation, phosphorylation (P), ubiquitination (Ub), and/or protein-protein interactions (e.g., thioredoxin with TXNIP).

are undoubtedly important in the maintenance of homeostatic conditions and protection of cells against the damaging effects of oxidative stress (Neumann et al., 2003; Lee et al., 2003, 2005). However, in multicellular organisms, the increased expression of antioxidant enzymes is not a universal response of all cells to hydrogen peroxide (An and Blackwell, 2003; Desaint et al., 2004). This suggests that in some cells it may be more important that antioxidant levels are set appropriately for hydrogen peroxide to signal cell division, apoptosis, differentiation, or migration than increase to allow survival of damaging levels.

Regulation of Hydrogen Peroxide Signaling by Posttranslational Regulation of Antioxidant Enzymes

Several antioxidant enzymes undergo posttranslational modifications that regulate the peroxide-detoxifying activity of the enzyme (Figure 4). The evolution of mechanisms that regulate cellular antioxidant activity suggests the critical importance of hydrogen peroxide signaling events in the control of biological processes. Here, we will describe some examples in which posttranslational regulation of antioxidant activity has been shown to have an

important effect upon hydrogen peroxide-regulated signaling pathways.

Interestingly, it has been suggested that the regulation of tyrosine phosphorylation of catalase provides a mechanism for initiating distinct responses to low and high levels of peroxide (Cao et al., 2003c). In particular, it has been proposed that the activation of the c-Abl and Arg tyrosine kinases by low levels of peroxide leads to increased phosphorylation of catalase, increasing activity and thus protecting cells against peroxide. However, at higher levels of peroxide, c-Abl and Arg dissociate from catalase and catalase activity is reduced, either by dephosphorylation or by ubiquitination and degradation of phosphorylated catalase, allowing the initiation of apoptosis (Cao et al., 2003a, 2003b, 2003c) (Figure 4). Conversely, the thioredoxin peroxidase activity of a mammalian typical 2-Cys Prx is inhibited by direct phosphorylation by the Cdc2 cyclin-dependent kinase (Cdk) during mitosis (Chang et al., 2002) (Figure 4). As yet the precise role of this Cdk-dependent phosphorylation in cell-cycle control remain unclear, but intriguingly it suggests that a transient increase in hydrogen peroxide may be required for efficient mitosis.

The regulated inactivation of typical 2-Cys Prx by oxidation of a catalytic cysteine is also emerging as an important mechanism for regulating the response of eukaryotic cells to hydrogen peroxide. Although their catalytic efficiency is lower than catalase, these ubiquitous enzymes are so abundant (up to ~1% of total protein in some cells) and have such a high affinity (typically around 10 μM) for hydrogen peroxide that they provide an important protective barrier against hydrogen peroxide. However, biochemical studies of eukaryotic typical 2-Cys Prx revealed that their thioredoxin peroxidase activity is inactivated by "overoxidation" of the peroxidatic cysteine to sulfinic acid at relatively low concentrations of hydrogen peroxide (Rabilloud et al., 2002; Wagner et al., 2002; Koo et al., 2002). This increased susceptibility to overoxidation was linked to the presence of specific amino acids, inserted near the C terminus, that slow the rate of formation of the disulfide between the peroxidatic and resolving cysteines of typical 2-Cys Prx, thus increasing the chance of further oxidation of the sulfenic intermediate by hydrogen peroxide (Wood et al., 2003b) (Figures 3 and 4). The susceptibility of eukaryotic typical 2-Cys Prx to an apparently irreversible overoxidation event was intriguing and raised important questions about their role in responses to hydrogen peroxide. However, at the same time studies by Rhee and colleagues made the surprising discovery that this overoxidation of the peroxidatic cysteine is actually reversible (Woo et al., 2003). Indeed, studies in budding yeast identified a conserved enzyme, sulfiredoxin, which reverses this overoxidation (Biteau et al., 2003) (Figures 3 and 4). Mammals also contain sestrin proteins, unrelated to sulfiredoxins, which are capable of reversing the overoxidation of typical 2-Cys Prx (Budanov et al., 2004) (Figures 3 and 4). Interestingly, both sulfiredoxin and sestrin gene expression is regulated in response to oxidative stress (Biteau et al., 2003; Bozonet et al., 2005; Budanov et al., 2002; Vivancos et al., 2005), providing a further layer of regulation for the thioredoxin peroxidase activity of 2-Cys Prx.

The high abundance of typical 2-Cys Prx in some eukaryotic cell types suggested that overoxidation of the peroxidatic cysteine may be an important mechanism by which antioxidant capacity is integrated with responses to increased levels of hydrogen peroxide (Wood et al., 2003b). Indeed, recent studies in *S. pombe* have revealed that the sensitivity of the 2-Cys Prx, Tpx1, to oxidation and inactivation of its thioredoxin peroxidase activity (Koo et al., 2002) acts as a molecular switch to allow distinct transcriptional responses to low and high levels of hydrogen peroxide (Bozonet et al., 2005; Vivancos et al., 2005) (Figure 5). In *S. pombe*, the transcriptional response of cells to low levels of hydrogen peroxide is dependent on the AP-1-like transcription factor Pap1, while the Sty1 (also known as Spc1 and Phh1) stress-activated protein kinase (SAPK)-regulated transcription factor, Atf1, plays a more important role at higher levels of hydrogen peroxide (Quinn et al., 2002) (Figure 5). Pap1 is activated by oxidation of specific cysteine residues, which results in the nuclear accumulation of the protein. Importantly, the hydrogen peroxide-induced oxidation of Pap1 is dependent on the thioredoxin peroxidase-active form of Tpx1 (Figure 5). Consequently, these data suggest that when Tpx1 is oxidized at high concentrations of hydrogen peroxide, this prevents oxidation of Pap1 but allows hydrogen peroxide to accumulate where it stimulates the activation of Sty1/Atf1 and the expression of Atf1-dependent genes (Figure 5). Hence, the oxidation/reduction status of Tpx1, which is influenced by the levels of Tpx1, the Srx1 sulfiredoxin, and hydrogen peroxide, determines the precise balance of activation of two independent pathways (Bozonet et al., 2005; Vivancos et al., 2005) (Figure 5). Intriguingly, truncated forms of Tpx1, which are less sensitive to oxidative inactivation of their thioredoxin peroxidase activity, have been isolated from cells (Koo et al., 2002), and a typical 2-Cys Prx isolated from erythrocytes is sensitive to proteolytic digestion by calpain in vitro (Schröder et al., 1998). Together these studies suggest that proteolytic processing of 2-Cys Prx may add yet another layer of regulation to hydrogen peroxide signaling.

The recent revelation that p53 is involved in the initiation of distinct transcriptional responses to different levels of hydrogen peroxide (Figure 2) (Sablina et al., 2005) is reminiscent of the 2-Cys Prx-regulated responses to different levels of hydrogen peroxide observed in *S. pombe* (Figure 5) (Quinn et al., 2002; Bozonet et al., 2005; Vivancos et al., 2005). In addition, as discussed above, different levels of hydrogen peroxide cause distinct changes in the pattern of protein sumoylation (Bossis and Melchior, 2006) (Figure 2). Hence, it is tempting to speculate that the sensitivity of typical 2-Cys Prx to increasing levels of hydrogen peroxide may also be involved in determining the appropriate level of p53-dependent gene expression and protein sumoylation in mammals (Figure 2). Indeed,

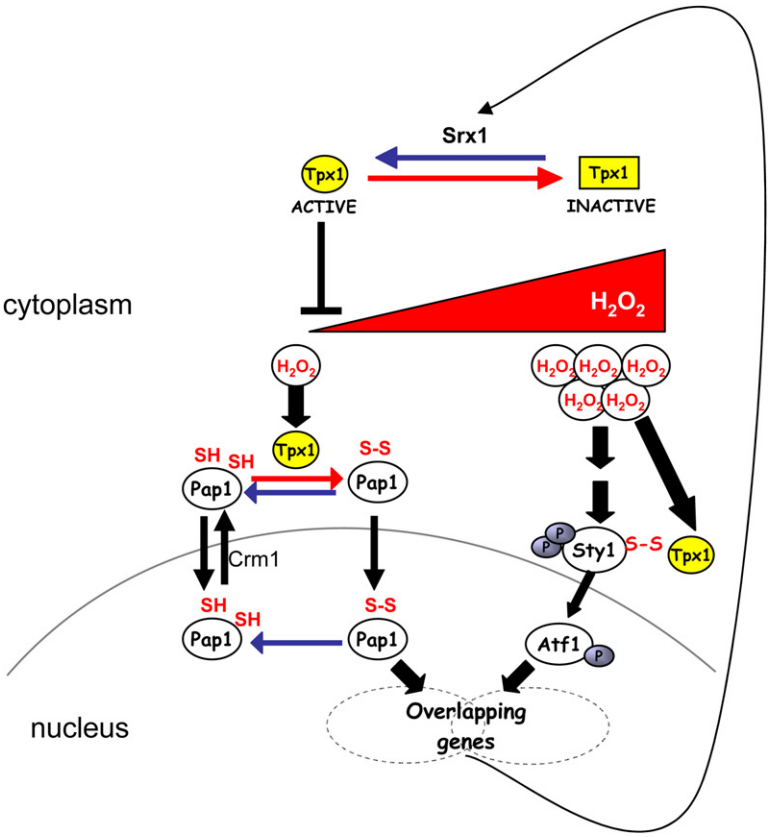

Figure 5. Regulation of Stress-Induced Gene Expression in *S. pombe* in Response to Different Levels of Hydrogen Peroxide

In this model the 2-Cys Prx Tpx1 is proposed to act as both a sensor and a floodgate to allow activation of Pap1 by low levels of hydrogen peroxide and increased activation of Sty1 (and hence Atf1) at increasing levels of hydrogen peroxide. The thioredoxin peroxidase activity of Tpx1 is required for the hydrogen peroxide-induced oxidation and nuclear accumulation of Pap1. At high concentrations of hydrogen peroxide the thioredoxin peroxidase activity of Tpx1 is inhibited, preventing activation of Pap1 but allowing increased activation of Sty1/Atf1. Sulfiredoxin (Srx1) restores the thioredoxin peroxidase activity of Tpx1, allowing Tpx1-dependent removal of hydrogen peroxide and activation of Pap1. Red arrows indicate oxidation reactions, blue arrows indicate reduction reactions.

studies in mammalian cells have suggested that 2-Cys Prx do play important roles in determining the threshold of hydrogen peroxide involved in activating specific pathways. For example, the inhibition of PrxII enhances the hydrogen peroxide-mediated activation of the JNK and p38 SAPK pathways and prolongs activation of the PDGF receptor but reduces activation of the ERK mitogen-activated protein kinase (MAPK) (Choi et al., 2005; Kang et al., 2004). However, it is still unclear what role, if any, overoxidation and inhibition of the thioredoxin peroxidase activity of PrxII has in the regulation of these responses. Although the sulfinic derivative of PrxII has been detected in cells exposed to TNFα, suggesting that sufficient hydrogen peroxide may be generated as a signaling molecule to cause in vivo oxidation of a 2-Cys Prx (Rabilloud et al., 2002), there is no evidence that 2-Cys Prx are inactivated by overoxidation in response to mitogenic stimuli (Choi et al., 2005; Phalen et al., 2006). However, it remains possible that a proportion of the 2-Cys Prx located close to the source of the hydrogen peroxide is transiently inactivated but difficult to detect in the large cellular pool of 2-Cys Prx. Indeed, the intracellular levels and relative localization of 2-Cys Prx and sulfiredoxin/sestrin proteins will also potentially give rise to pools of more sensitive/resistant 2-Cys Prx. Thus, the role of overoxidation of 2-Cys Prx and its regulation by sulfiredoxins in peroxide signaling merits further investigation.

Thioredoxin is an oxidoreductase that is involved in the reduction of many proteins, including thioredoxin peroxidase enzymes. The catalytic reduction of substrates by thioredoxin involves the formation of a disulfide bond between the two catalytic cysteines (positions 32 and 35 in mammals), which is subsequently reduced by NADPH (Figure 2). The oxidoreductase activity of mammalian thioredoxin is regulated by oxidative modifications of two other cysteines and interaction with an inhibitor protein TXNIP (also known as VDUP-1 or TBP-2) (Nishiyama et al., 1999; Casagrande et al., 2002; Haendeler et al., 2002) (Figure 4). TXNIP mRNA has a very short half-life, hence the regulation of TXNIP gene expression in response to various signals, including hyperglycemia (induction) and PDGF and biomechanical stress (repression), allows rapid modulation of thioredoxin activity (for a review see World et al. [2006]). Indeed, regulation of TXNIP levels seems to have evolved as an important mechanism for regulating thioredoxin and thioredoxin-dependent signaling. For example, high levels of TXNIP expression are associated with increased susceptibility to apoptosis whereas downregulation of TXNIP has a protective effect through the associated increase in thioredoxin activity (Wang et al., 2002; Yamawaki et al., 2005). Indeed, the protective effect of physiological blood flow on endothelial cells has been proposed to be mediated by the associated downregulation of TXNIP in response to fluid shear stress

(Yamawaki et al., 2005). Regulation of thioredoxin activity will affect the activity of thioredoxin peroxidase in removing hydrogen peroxide. Conversely, as one of the most abundant substrates for the oxidoreductase activity of thioredoxin, regulation of thioredoxin peroxidase activity is also likely to play an important part in influencing the activity of thioredoxin toward other substrates. For example, inactivation of 2-Cys Prx by phosphorylation or overoxidation is likely to increase the availability of thioredoxin to regulate other target proteins. Future studies of the complex relationships between the regulation of thioredoxin, thioredoxin peroxidase, and thioredoxin reductase are thus likely to be important for understanding how cells elicit the appropriate biological response to hydrogen peroxide.

Molecular Mechanisms of Hydrogen Peroxide Signaling

For hydrogen peroxide to behave as a signaling molecule it must first evade decomposition by antioxidants and then influence the activity of hydrogen peroxide-sensitive signaling proteins. Certain features of proteins render them susceptible to oxidation by hydrogen peroxide. For example, any protein containing a deprotonated cysteine residue is susceptible to oxidation by hydrogen peroxide. The cysteine residues of most cytosolic proteins are protonated, due to the low pH of the cytosol, and therefore unable to react with/sense hydrogen peroxide. This is critical for the utilization of hydrogen peroxide as a signaling molecule, as it enables specific targeting of proteins containing deprotonated cysteine residues. It is thus an essential feature of most hydrogen peroxide sensor proteins that they contain cysteine residues with a low pKa. In the case of proteins that are susceptible to oxidation by hydrogen peroxide, the deprotonated cysteine residue may have evolved specifically to allow detection of hydrogen peroxide and/or be required for the activity of the protein. For example, deprotonated thiol groups are utilized as reactive species in the catalytic mechanisms of a multitude of enzymes, thus rendering many of them susceptible to inactivation by oxidation. Indeed, exposure to ROS leads to reversible oxidation of thiol groups of key cysteine residues in many proteins, including transcriptional regulators, kinases, phosphatases, structural proteins, metabolic enzymes, and SUMO ligases (Table 1). In many cases these redox changes are associated with altered activities, and an effect on a signaling pathway or cellular process has been described (Table 1). For example, in bacteria, the mechanisms of hydrogen peroxide sensing and signal transduction underlying hydrogen peroxide-induced changes in gene expression are contained in transcriptional regulators that are directly oxidized in response to peroxide. Perhaps the best characterized of these is the transcriptional activator OxyR, which is directly oxidized in response to hydrogen peroxide. Although both oxidized and reduced OxyR are able to bind DNA, only the oxidized form of OxyR can activate transcription of antioxidant genes (Storz et al., 1990). The critical event in the activation of OxyR by hydrogen peroxide appears to be the oxidation of cysteine 199 to a sulfenic acid derivative (Kim et al., 2002; Storz et al., 1990). Although formation of the sulfenic acid derivative may be sufficient for transcriptional activation (Kim et al., 2002), it is generally accepted that this is followed by formation of a disulfide bond between cysteines 199 and 208 (Lee et al., 2004; Zheng et al., 1998). This is supported by the fact that disulfide bonds are more stable than sulfenic acids and also by structural data (Choi et al., 2001). A negative feedback loop involving the OxyR-regulated expression of the gene encoding glutaredoxin1 allows the reduction and deactivation of OxyR (Zheng et al., 1998; Aslund et al., 1999).

In eukaryotes, protein tyrosine phosphatases (PTPs) have a central role in controlling signaling events initiated in response to many stimuli, including growth factors and cytokines. For example, PTP-catalyzed dephosphorylation of tyrosine residues inactivates MAPKs and growth factor receptors and activates Cdks and nonreceptor tyrosine kinases. The use of a deprotonated cysteine residue in the catalytic dephosphorylation of substrates renders PTPs susceptible to inactivation by hydrogen peroxide. Although the nature of the oxidized derivative varies, the reversible sequestration of the key catalytic cysteine residue into an oxidized form regulates several different families of phosphatases. For example, the catalytic cysteine residue in PTP1B becomes oxidized to a cyclic sulfenamide derivative upon exposure of cells to hydrogen peroxide (Salmeen et al., 2003; van Montfort et al., 2003). However, in the case of other PTPs, for example Cdc25C, LMW-PTP, and the PTEN PIP_3 phosphatase, disulfide bond formation with a nearby cysteine residue protects the catalytic cysteine residue from further potentially irreversible oxidation. There is now substantial evidence that hydrogen peroxide generated in response to a range of growth factors and cytokines behaves as a second messenger to oxidize and thus inhibit the activity of PTPs. The regulation of PTPs by oxidation has been the subject of several excellent recent reviews, for example Cho et al. (2004) and Tonks (2005).

The peroxide-sensing mechanisms described above involve the reversible oxidation of deprotonated cysteine residues. However, hydrogen peroxide also reacts readily with Fe^{2+}, and consequently the Fe^{2+}-containing cofactors found in some proteins are potentially susceptible to oxidation. Indeed, a non-thiol mechanism involving the metal-catalyzed oxidation of histidine residues is important for hydrogen peroxide sensing by the PerR transcriptional repressor in *Bacillus subtilis* (Lee and Helmann, 2006). PerR contains two metal binding sites: a structural Zn^{2+} binding site and a regulatory Fe^{2+} binding site (Herbig and Helmann, 2001; Mongkolsuk and Helmann, 2002). PerR containing Fe^{2+} at the regulatory site binds DNA, repressing the expression of target genes encoding antioxidant enzymes, such as catalase (Herbig and Helmann, 2001). In response to hydrogen peroxide, derepression of PerR-regulated gene expression is achieved by the

Table 1. Examples of Redox-Regulated Proteins

Type of Protein	Protein	Effect of Thiol Modification	Reference
Transcription Factor	c-Jun/c-Fos	Inhibits DNA binding	Abate et al., 1990
	OxyR	Activation	Storz et al., 1990
	OhrR	Inhibits repressor	Fuangthong and Helmann, 2002
	Hsf1	Activation	Ahn and Thiele, 2003; Manalo et al., 2002
	Nrf-2/Keap-1	Prevents ubiquitin-mediated degradation of Nrf-2	Eggler et al., 2005; Kobayashi et al., 2006
	Bach1	Inactivates repressor	Ishikawa et al., 2005
Kinase	JNK	Inhibition	Park et al., 2000a
	Sty1 (Spc1, Phh1)	Activation	Veal et al., 2004
	MEKK1 (MAPKKK)	Inhibition	Cross and Templeton, 2004
	IKKbeta	Inhibition	Reynaert et al., 2004
	PKA RI (regulatory subunit)	Activation	Brennan et al., 2006
	PKA C (catalytic subunit)	Inhibition	Humphries et al., 2002, 2005
	Src tyrosine kinase	Activation	Giannoni et al., 2005
Phosphatases	LMW-PTPs	Inactivates	Chiarugi et al., 2001
	PTEN	Inactivates	Lee et al., 2002
	Cdc25C	Inactivates	Savitsky and Finkel, 2002
	PTP1B	Inactivates	Salmeen et al., 2003; van Montfort et al., 2003
Ion Channels	Ryanodine receptors	Activation	Reviewed in Hidalgo (2005)
	K(ATP) channels (ATP-sensitive potassium channels)	Activation	Avshalumov and Rice, 2003; Bao et al., 2005
Other	Serotonin N-acetyl transferase	Inhibition	Tsuboi et al., 2002
	RNase H1	Inactivates	Lima et al., 2003
	GAPDH	Inactivates	Molina y Vedia et al., 1992; Shenton and Grant, 2003
	SUMO E1 subunit Uba2/ E2-conjugating enzyme Ubc9	Inhibits sumoylation	Bossis and Melchior, 2006

Fe^{2+}-catalyzed oxidation of two Fe^{2+}-coordinating histidine residues in PerR (Lee and Helmann, 2006). The critical involvement of Fe in this mechanism means that the response of PerR to hydrogen peroxide is modulated by the levels of both hydrogen peroxide and Fe. As the toxicity of hydrogen peroxide is largely mediated by hydroxyl radicals generated by the Fe^{2+}-catalyzed Fenton reaction, this means that PerR is ideally adapted to allow increased antioxidant expression when cellular Fe levels are high. Another interesting feature of this regulation is that, in contrast to the reversible oxidation of thiol groups responsible for the regulation of other peroxide-sensitive signaling proteins, the oxidation of histidine residues in PerR is apparently irreversible. Although PerR is the only current example of either metal-catalyzed oxidation or histidine oxidation playing a key regulatory role in hydrogen peroxide sensing (Lee and Helmann, 2006), these studies may potentially have implications for the regulation of other Fe^{2+} binding proteins by hydrogen peroxide.

Antioxidant-Coupled Hydrogen Peroxide-Sensing Mechanisms

The sensitivity of cysteine residues to oxidation in any given protein will be determined by a combination of the pKa and the local environment of the cysteine residue, such as pH and the proximity of antioxidants/other peroxide-sensitive proteins. Indeed, as discussed previously, the removal of hydrogen peroxide by antioxidant enzymes renders them an important barrier to hydrogen peroxide signaling. However, the high affinity of some peroxidase enzymes, such as typical 2-Cys Prx, for hydrogen peroxide also makes them ideally suited for hydrogen peroxide sensing. Interestingly, in eukaryotes several hydrogen peroxide-sensing/signaling mechanisms have evolved in

which antioxidant enzymes have been co-opted as hydrogen peroxide sensors. In the specific examples outlined below the initial hydrogen peroxide-sensing event is the oxidation of an antioxidant enzyme, which then leads to subsequent changes in the activity of associated component(s) of the signaling pathway.

In the budding yeast *S. cerevisiae*, Yap1 is a member of a subfamily of AP-1-like transcription factors, which includes Pap1 in *S. pombe*, that are important for the regulation of oxidative stress-induced gene expression (Fernandes et al., 1997; Kuge et al., 1997). Under homeostatic conditions Yap1, like Pap1, is cytoplasmic by virtue of Crm1-dependent nuclear export. However, following exposure of cells to hydrogen peroxide, specific cysteine residues located in cysteine-rich domains (CRDs) of Yap1 are oxidized, disrupting the interaction of Yap1 with Crm1, and hence Yap1 accumulates in the nucleus (Delaunay et al., 2000). Although some of these cysteine residues are sensitive to direct modification by a number of oxidative stress-causing agents (Azevedo et al., 2003), unlike OxyR, Yap1 does not appear to be directly oxidized in response to hydrogen peroxide. Instead, the hydrogen peroxide-induced oxidation of Yap1 requires at least two other proteins: the Gpx3 (also known as Orp1) peroxidase, which utilizes thioredoxin as an electron donor rather than glutathione (Delaunay et al., 2002), and a second protein, Ybp1 (Veal et al., 2003). In response to hydrogen peroxide, cysteine 598 of Yap1 forms an intermolecular disulfide bond with cysteine 36 of Gpx3 which is then resolved into an intramolecular disulfide bond between cysteines 303 and 598 of Yap1 (Delaunay et al., 2002). A second disulfide bond also forms between cysteines 310 and 629 in Yap1 (Wood et al., 2003a). Although Ybp1 forms a complex with Yap1 and is essential for the Gpx3-dependent oxidation of Yap1, its role in the oxidation mechanism is unknown (Veal et al., 2003). Interestingly, a widely used laboratory strain of *S. cerevisiae* that lacks a fully functional Ybp1 employs an alternative 2-Cys Prx-dependent mechanism for hydrogen peroxide-induced oxidation of Yap1 (Okazaki et al., 2005). Moreover, in *S. pombe* it is the typical 2-Cys Prx Tpx1, rather than the Gpx3 ortholog Gpx1, that is required for the peroxide-induced oxidation of Pap1 (Bozonet et al., 2005; Vivancos et al., 2005) (Figure 5). It will be interesting to explore whether other eukaryotic thioredoxin peroxidases also have roles in directing the activation of peroxide-signaling proteins by oxidation.

The mechanism or mechanisms by which Yap1 activation/oxidation is reversed are unclear. However, thioredoxin is capable of reducing oxidized Yap1 in vitro and, moreover, loss of thioredoxin function results in the constitutive nuclear accumulation of Yap1 in vivo (Delaunay et al., 2000). Thus, these data suggest that a negative feedback loop involving the oxidation state of thioredoxin plays a key role in regulating Yap1. Indeed, as an oxidoreductase, thioredoxin is involved in regulating the oxidation state and activities of several proteins, for example NF-κB (for a review see Powis and Montfort [2001]). In addition, thioredoxin directly regulates the activation of specific signal transduction proteins through hydrogen peroxide-sensitive noncovalent interactions. One of the best understood hydrogen peroxide-sensing mechanisms, in which thioredoxin is directly involved, is the activation of the mammalian ASK1 MAPKKK (Saitoh et al., 1998). ASK1 is activated in response to a variety of stimuli, including TNFα, glucose deprivation, Ca^{2+}, and ER stress (for a review see Hayakawa et al. [2006]). Once active, ASK1 phosphorylates and thus activates the MAPKK in both the p38 and JNK SAPK pathways. Reduced thioredoxin binds to the N-terminal region of ASK1, preventing activation by autophosphorylation of a key threonine residue in the kinase domain. However, following exposure of cells to stress or cytokine-induced increases in hydrogen peroxide, an intramolecular disulfide bond forms between the two catalytic cysteine residues in thioredoxin. This oxidation of thioredoxin disrupts its interaction with ASK1, allowing ASK1 to become activated through autophosphorylation (Liu et al., 2000) (reviewed in Hayakawa et al. [2006]). The thioredoxin inhibitor TXNIP competes with ASK1 for binding to thioredoxin (Yamawaki et al., 2005). Thus, high levels of TXNIP increase the sensitivity of ASK1 to TNFα-induced, hydrogen peroxide-mediated activation by two mechanisms: (1) by reducing the antioxidant capacity of the cell by inhibiting thioredoxin and (2) by inhibiting the interaction of ASK1 with reduced thioredoxin.

Multiple mechanisms have been identified that lead to hydrogen peroxide-induced activation of p38/JNK SAPKs. For example, in addition to dissociation of oxidized thioredoxin from the ASK1 MAPKKK, mammalian JNK is activated by hydrogen peroxide via direct oxidation and inactivation of the MAPK phosphatase (Kamata et al., 2005) and by peroxide-induced dissociation of the kinase inhibitor GSTpi (Adler et al., 1999). In *S. pombe*, a two-component signaling pathway is required for hydrogen peroxide-dependent activation of the MAPKKKs in the Sty1 SAPK pathway (Nguyen et al., 2000; Buck et al., 2001). In addition, the typical 2-Cys Prx Tpx1 is required for the activation of Sty1 by hydrogen peroxide but not by other stress conditions (Veal et al., 2004). Although the loss of an important peroxide-detoxifying enzyme leading to reduced peroxide signaling is counterintuitive, this suggests that Tpx1 has an important role as a peroxide sensor in addition to its thioredoxin peroxidase activity. Indeed, analysis of mutant versions of Tpx1 suggests that the peroxide-induced activation of Sty1 does not require the thioredoxin peroxidase activity (Veal et al., 2004). The formation of hydrogen peroxide-induced intermolecular disulfide bonds between conserved cysteine residues in Sty1 and Tpx1 suggests that Tpx1 is directly involved in regulating the activation of Sty1, although the exact mechanism by which this oxidation allows activation of Sty1 is unclear. Excitingly, recent studies in macrophage-derived foam cells have shown that a mammalian 2-Cys Prx, Prx1, is required for hydrogen peroxide-induced activation of p38 SAPK, suggesting that this peroxide-sensing mechanism is conserved in

mammals (Conway and Kinter, 2006). These studies raise the intriguing possibility that 2-Cys Prx have other thioredoxin peroxidase-independent roles in responses to hydrogen peroxide. Indeed, typical 2-Cys Prx also appear to have a peroxide-induced thioredoxin peroxidase-independent chaperone activity that is important in protecting cells against the toxic effects of hydrogen peroxide (Jang et al., 2004; Moon et al., 2005).

Conclusions and Perspectives

Increased levels of hydrogen peroxide in cells can result in oxidative stress and cause cellular damage. Indeed, such damage is associated with the initiation and progression of many diseases, including neurodegenerative disorders, diabetes, atherosclerosis, and cancer. However, studies in higher eukaryotes have revealed that hydrogen peroxide is also used as a signaling molecule to regulate many different cellular processes. Hence, hydrogen peroxide can have both positive (signaling) and negative (damage) effects depending on the level of hydrogen peroxide and the cell type under investigation. In each case it is important that the appropriate response is initiated. Indeed, it is likely that inappropriate peroxide signaling also contributes to the development of many disease states such as atherosclerosis, cardiac hypertrophy, and inflammation. The association of oxidative stress with disease and the aging process has led to great interest in utilizing antioxidants to protect against oxidative stress-induced damage. However, dietary antioxidants have had mixed success in protecting against damage and disease. This has been partly attributed to the difficulty in ensuring that an adequate and appropriate level of these antioxidants reaches the site of generation of the ROS. However, the studies described in this review suggest that treatments that pharmacologically raise the levels of intracellular antioxidants are also likely to disrupt important hydrogen peroxide-induced signaling pathways. This should be taken into consideration in the design of future strategies to treat and prevent oxidative stress-associated cell damage and disease.

Studies of hydrogen peroxide signaling have concentrated on identifying the mechanisms regulating the generation of hydrogen peroxide, and this is certainly an important growing area of research. However, as large-scale studies identify increasing numbers of proteins that are sensitive to thiol oxidation it will also be important to determine which of these are oxidized under physiological conditions, such as by hydrogen peroxide generated in response to growth factors. Furthermore, since the local antioxidant capacity determines the susceptibility of a cellular target to oxidative damage or to hydrogen peroxide signaling, it will be equally important to determine the impact of regulation of antioxidant enzymes on peroxide-sensing/signaling events. The studies described here, in particular those detailing the complex relationships between thioredoxin/thioredoxin peroxidase enzymes and hydrogen peroxide signaling, have provided some insight into how a multicellular organism coordinates appropriate cellular responses to hydrogen peroxide. In the future, approaches that take into account a multitude of factors, including the site of generation of hydrogen peroxide, the influence of antioxidants and membrane composition on its half-life and diffusion, pH, and Fe^{2+} levels, together with the localization and protein-protein interactions of the hydrogen peroxide sensors, will ultimately be required in order to answer outstanding questions in hydrogen peroxide signaling.

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