Cellular Regulation by Hydrogen Peroxide

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Abstract. Substantial evidence suggests that the transient production of H$_2$O$_2$ is an important signaling event triggered by the activation of various cell surface receptors. Understanding the intracellular messenger function of H$_2$O$_2$ calls for studies of how receptor occupation elicits the production of H$_2$O$_2$, what kinds of molecules are targeted by the produced H$_2$O$_2$, and how H$_2$O$_2$ is eliminated after the completion of its mission. Recent studies suggest that growth factor–induced H$_2$O$_2$ production requires the activation of PtdIns 3-kinase. The essential role of PtdIns 3-kinase is likely to provide PI(3,4,5)P$_3$ that recruits and activates a guanine nucleotide exchange factor of Rac, which is required for the activation of NADPH oxidase. The targets of H$_2$O$_2$ action include proteins that contain a reactive Cys residue. Thus, H$_2$O$_2$ produced in response to growth factor causes inactivation of protein tyrosine phosphatases in various cells by oxidizing specifically the catalytic Cys. These results, together with other observations, indicate that the activation of a receptor tyrosine kinase per se by binding of the corresponding growth factor might not be sufficient to increase the steady-state level of protein tyrosine phosphorylation in cells. Rather, the concurrent inhibition of protein tyrosine phosphatases by H$_2$O$_2$ might also be required. Peroxiredoxins, members of a newly discovered family of peroxidases, efficiently reduced the intracellular level of H$_2$O$_2$ produced in the cells stimulated with various cell surface ligands. Furthermore, the activity of peroxiredoxin enzymes seems to be regulated via protein phosphorylation as in the case of many other intracellular messenger metabolizing enzymes.

Incomplete reduction of O$_2$ during respiration produces superoxide anion (O$_2^-·$), which is spontaneously or enzymatically dismutated to H$_2$O$_2$. Many cell types, furthermore, produce low levels of O$_2^-·$ and H$_2$O$_2$ in response to a variety of extracellular stimuli, including cytokines (TGF-$β_1$, TNF-$α$, and IL), peptide growth factors (PDGF, EGF, VEGF, bFGF, and insulin), the agonists of heterotrimeric G protein–coupled receptors (GPCR; angiotensin II, thrombin, lysophosphatidic acid, sphingosine 1-phosphate, histamine, and bradykinin), and shear stress (1). The addition of exogenous H$_2$O$_2$ or the intracellular production in response to receptor stimulation affects the function of various proteins, including protein kinases, protein phosphatases, transcription factors, phospholipases, ion channels, and G proteins (1). Given that H$_2$O$_2$ is a small, diffusible, and ubiquitous molecule that can be synthesized and destroyed rapidly in response to external stimuli, it meets all of the important criteria for an intracellular messenger, and H$_2$O$_2$ is now recognized as a ubiquitous intracellular messenger under subtoxic conditions (1–4). Understandably, there has long been skepticism about the messenger role of H$_2$O$_2$, partly because it seemed illogical for nature to use a dangerous molecule such as H$_2$O$_2$ for such a crucial function. However, if one considers that nitric oxide (NO), itself a reactive radical, is easily converted to the more reactive OONO while also functioning as an established intracellular messenger, then the proposed H$_2$O$_2$ function is not implausible. Moreover, recent evidence that specific inhibition of H$_2$O$_2$ generation results in a complete blockade of signaling by PDGF, EGF, and angiotensin II is a strong indication that H$_2$O$_2$ serves in a messenger role (5–7). However, the mechanisms by which H$_2$O$_2$ is produced and mediates receptor signaling have not been well characterized.

Receptor-Mediated H$_2$O$_2$ Production

The mechanism of the receptor-mediated generation of H$_2$O$_2$ has been studied extensively in phagocytic cells, in which O$_2^-·$ (and thus H$_2$O$_2$) is produced via the reduction of O$_2$ by a complicated NADPH oxidase complex (8). Although it had remained unclear for many years whether nonphagocytic cells contain a NADPH oxidase system similar to that of phagocytic cells, several new types of NADPH oxidase were identified in nonphagocytic cells, and some of these enzymes seemed to be responsible for H$_2$O$_2$ production in response to GPCR ligands and peptide growth factors (9). Although H$_2$O$_2$ is also known to be generated by xanthine oxidase, NO synthase, and cytochrome P450, as well as in the mitochondria, there is no convincing evidence linking them to cell surface receptor activation. Evidence suggests, however, that lipooxygenases, which convert arachidonic acid to lipid peroxides, are activated in cells treated with certain cytokines (10).

Among those H$_2$O$_2$-generating enzymes, the best studied in...
relation to receptor stimulation is NADPH oxidase in phagocytic cells. The multisubunit NADPH oxidase complex consists of the catalytic moiety gp91 phox and several other regulatory proteins, including the small GTPase Rac (8). Nonphagocytic cells generate reactive oxygen species (ROS) at much lower levels compared with phagocytic cells. Overexpression of wild-type Rac1 or a constitutively active form of Rac1 (Rac1V12) in fibroblasts resulted in increased production of H$_2$O$_2$, suggesting the involvement of gp91 phox-like protein (11,12).

We studied the mechanism of PDGF-mediated generation of H$_2$O$_2$. The binding of PDGF to its receptors results in receptor autophosphorylation on specific tyrosine residues. These phosphotyrosine residues initiate cellular signaling by acting as high-affinity binding sites for the SH2 domains of various effector proteins. In the PDGF-β receptor, seven autophosphorylation sites have been identified as specific binding sites for Src (Tyr579 and Tyr581), PtdIns3-kinase (PI3K; Tyr740 and Tyr751), GAP (Tyr777), SH2 domain–containing protein tyrosine phosphatase-2 (SHP-2; Tyr1006), and PLC-γ1 (Tyr1024). Experiments with a series of PDGF-βR mutants suggested that only the PI3K binding site was alone sufficient for PDGF-induced H$_2$O$_2$ production (12). The effect of PDGF on H$_2$O$_2$ generation was blocked by the PI3K inhibitors LY294002 and wortmannin or by overexpression of Rac1N17. Furthermore, expression of the membrane-targeted p110 subunit (p110-CAAX) of PI3K was sufficient to induce H$_2$O$_2$ production (12). These results suggest that a product of PI3K is required for PDGF-induced production of H$_2$O$_2$ in nonphagocytic cells and that Rac1 mediates signaling between the PI3K product and the putative NADPH oxidase.

Our observation that Rac1N17 blocked the Y740|751 receptor-induced generation of H$_2$O$_2$ indicates that Rac1 acts downstream of PI3K in the signaling pathway that leads to activation of NADPH oxidase. Moreover, signaling by this pathway seems to be independent of activation of GAP, SHP-2, and PLC-γ1. Additional evidence suggests that Rac functions downstream of PI3K (13). Thus, the exchange of Rac-bound GDP for GTP catalyzed by guanine nucleotide exchange factors (GEF) is stimulated by PI(3,4,5)P$_3$, a product of the action of PI3K. A family of GEF proteins that mediate the activation of Rac-related proteins has been identified. All members of this family, including Vav, Sos, and βPix, contain a pleckstrin homology domain that binds inositol-containing phospholipids such as PI(4,5)P$_2$ and PI(3,4,5)P$_3$ (13,14). Our unpublished results indicate that βPix might be the Rac GEF responsible for the activation of an NADPH oxidase. Thus, βPix physically associated with the NADPH oxidase protein, and PDGF-induced H$_2$O$_2$ production was significantly reduced when βPix expression was reduced by RNA interference in several different cell lines.

H$_2$O$_2$ is also produced in response to many GPCR ligands and cytokines. Essentially no mechanistic studies have been done with these ligands. Given that the phagocytic NADPH oxidase is activated in response to GPCR ligands such as thrombin and fMetLeuPhe and cytosolic regulatory components of the oxidase are extensively regulated by various kinases and adaptor proteins, GPCR and cytokine receptors are also coupled to NADPH oxidase isoforms (Figure 1). In the next few years, a flurry of research activities are expected to elucidate the activation mechanisms of those NADPH oxidases in response to various ligands.

**Protein Targets of H$_2$O$_2$**

Unlike other second messenger molecules, ROS are too simple structurally to be recognized specifically by a protein. It thus is unlikely that the modulation of protein functions by ROS is mediated by their reversible binding to proteins. H$_2$O$_2$ is, however, a mild oxidant that can oxidize Cys residues in proteins. Because the pH of the sulfhydryl group of most cysteine residues is approximately 8.5 and because Cys-SH is less readily oxidized by H$_2$O$_2$ than is the cysteine thiolate anion (Cys-S$^-$), few proteins might be expected to possess a cysteine residue that is vulnerable to oxidation by H$_2$O$_2$ in cells (15). However, certain protein cysteine residues have low $pK_a$ values and exist as thiol anions at neutral pH because of nearby positively charged amino acid residues that are available for interaction with the negatively charged thiolate. Proteins with low-$pK_a$ cysteine residues can be the targets of specific oxidation by H$_2$O$_2$, and such oxidation can be reversed by thiol donors such as GSH, glutaredoxin, and thioredoxin (Trx).

Proteins with low-$pK_a$ cysteine residues include protein tyrosine phosphatases (PTP). All PTP contain an essential cysteine residue ($pK_a$, 4.7 to 5.4) in the signature active site motif, His-Cys-X-X-Gly-X-X-Ser/Thr (where X is any amino acid), that exists as a thiolate anion at neutral pH (16). This thiolate anion contributes to formation of a thiol-phosphate intermediate in the catalytic mechanism of PTP. The active site cysteine was shown to be oxidized in vitro by various oxidants, including H$_2$O$_2$, and this modification can be reversed by incubation with thiol compounds such as diithiothreitol and reduced glutathione. These observations suggested that PTP might undergo H$_2$O$_2$-dependent inactivation in cells. However, such evidence was not available until we demonstrated the ability of intracellularly produced H$_2$O$_2$ to oxidize PTP-1B in EGF-stimulated A431 cells by taking advantage of the fact that

![Figure 1](image-url)
H₂O₂ and [¹⁴C]iodoacetic acid selectively and competitively react with cysteine residues that exhibit a low pKₐ (17). We observed that the amount of oxidatively inactivated PTP-1B was maximal (approximately 40%) 10 min after exposure of cells to EGF and returned to baseline values by 40 min, suggesting that the oxidation of this phosphatase by H₂O₂ is reversible in cells. We also showed that the essential residue Cys²¹⁵ was oxidized to cysteine sulfenic acid (Cys-SO₂H).

Insulin stimulation also induces the production of intracellular H₂O₂. Goldstein's laboratory demonstrated the reversible oxidation of PTP-1B in insulin-stimulated cells by directly measuring the catalytic activity of PTPase activity in cell homogenates under strictly anaerobic conditions (18). Approximately 60% of total cellular PTPase activity was found to be reversibly inactivated in 3T3-L1 adipocytes and hepatoma cells stimulated with insulin. Another method was developed in Tonks's laboratory to reveal reversible oxidation of PTP in cells (19). This method is based on the fact that those PTP with the oxidized Cys-SOH at their active site are resistant to alkylation by iodoacetic acid and can be reactivated by treatment with dithiothreitol, whereas any PTP that had not been oxidized by H₂O₂ in the cell became irreversibly inactivated by the alkylation. Using an in-gel assay in which the protein bands corresponding to reactivated PTP were detected by hydrolysis of [³²P]phosphate-labeled substrate, Tonks's laboratory showed that several PTP, including SHP-2 and PTP-1B, were reversibly oxidized in Rat-1 cells treated with PDGF.

These results, together with the observation that increased levels of PDGF-, EGF-, or insulin-induced protein tyrosine phosphorylation requires H₂O₂ production (5,6,18,19), indicate that the activation of receptor protein tyrosine kinase per se by binding of the corresponding growth factor may not be sufficient to increase the steady-state level of protein tyrosine phosphorylation in cells. Rather, the concurrent inhibition of PTP by H₂O₂ may also be required. This suggests that the extent of autophosphorylation of receptor protein tyrosine kinases and their substrates would return to basal values after degradation of H₂O₂ and the subsequent reactivation of PTP by electron donors. The proposed role of H₂O₂ in growth factor–induced protein tyrosine phosphorylation is depicted in Figure 2.

Elimination of Intracellular H₂O₂ via Peroxiredoxins

In general, elimination as well as production of intracellular messengers is highly controlled process. This would seem especially true for H₂O₂, which is readily converted to deleterious hydroxyl radicals. Enzymes that are capable of eliminating H₂O₂ include peroxiredoxin (Prx) in addition to the two conventional enzymes catalase and glutathione peroxidase. Prx is a novel family of peroxidases that reduce H₂O₂ and alkylhydroperoxides with the use of reducing equivalents provided by Trx (20,21). Prx members exist as homodimers, with two monomers arranged in a head-to-tail manner, and are enzymes without any prothetic group (22). The only redox-active residue is cysteine, which is conserved for all Prx enzymes and corresponds to Cys⁵² in mammalian Prx I and II. The majority of Prx enzymes, including four (Prx I to IV) of six mammalian Prx, contain an additional conserved Cys that corresponds to Cys¹⁷³ in mammalian Prx I and II (21). The catalytic mechanism of Prx enzymes has been well established (Figure 3). The conserved N-terminal Cys⁵²-SH is selectively oxidized by H₂O₂ to cysteine sulfenic acid (Cys-SOH), which then reacts with Cys¹⁷³-SH of the other subunit to produce an intermolecular disulfide. The disulfide is then specifically reduced by Trx but not by glutaredoxin or GSH (23). When overexpressed in various cells, Prx enzymes efficiently reduced the intracellular level of H₂O₂ produced in the cells stimulated with PDGF or TNF-α, inhibited NF-κB activation induced by TNF-α, and blocked the apoptosis induced by ceramide (24–27), indicating that Prx enzymes serve as a component of signaling cascades by removing H₂O₂.

Prx I contains a consensus site (Thr⁹⁰-Pro-Lys-Lys⁹³) for phosphorylation by cyclin-dependent kinases (Cdk). We re-
ently showed that Prx I and II can be phosphorylated specifically at Thr^{90} by several Cdk, including Cdc2 kinase (28). Prx I phosphorylation at Thr^{90} caused a decrease in peroxidase activity by >80%. Using antibodies specific to Prx I phosphorylated at Thr^{90} and HeLa cells arrested at various stages of cell cycle, we showed that Prx I phosphorylation at Thr^{90} occurred in parallel to the activation of Cdc2 kinase. Prx I phosphorylation was observed in cells in mitotic phase but not in interphase, despite that Prx I can be phosphorylated by other Cdk isoforms in vitro. This is probably because Prx I, a cytosolic protein, can encounter activated Cdk only after the nuclear envelope breaks down during mitosis and because Cdc2 kinase is the Cdk that is activated in mitotic phase. Both the in vitro and in vivo phosphorylation of Prx I at Thr^{90} was inhibited by roscovitine, an inhibitor of Cdk. Prx II also can be phosphorylated, albeit more weakly than is Prx I, by Cdc2 kinase in vitro. Prx II is also a cytosolic protein. Therefore, Cdc2 kinase–dependent phosphorylation and inactivation of Prx II are likely to occur in mitosis.

Although physiologic significance of Prx phosphorylation is not clear, our results clearly demonstrated that peroxidase activity of Prx I is regulated through phosphorylation. This was the first example that any of the H_{2}O_{2} eliminating enzymes catalase, glutathione peroxidase, and peroxiredoxin are regulated through posttranslational modification.

**Conclusion**

A growing body of evidence now qualifies H_{2}O_{2} to join the ranks of cAMP, Ca_{2+}, inositol 1,4,5-trisphosphate, and NO in its role as an intracellular messenger. However, unlike the other second messengers, very little is known about the pathway by which H_{2}O_{2} is generated in response to receptor stimulation and which molecules are direct targets of the H_{2}O_{2} messenger function. Here we described how H_{2}O_{2} production by PDGF involves signaling molecules such as PI3K, Rac1, Rac GEF, and NADPH oxidase. Given that stimulation of numerous (cytokine, growth factor, G protein-coupled) receptors induces H_{2}O_{2} production, various protein kinases and phosphatases, heterotrimeric and small G proteins, adaptor proteins, and scaffolding proteins are likely to be involved in the production of H_{2}O_{2}. In addition, these components are expected to be subject to control by other second messenger–generating cascades.

We proposed that H_{2}O_{2} propagates its signal by oxidizing active site cysteines of PTP that are sensitive to oxidation by H_{2}O_{2} because their pK_{a} is lower than those of other cysteines. In addition, many protein kinases, transcriptional factors, and ion channels are controlled by H_{2}O_{2} through oxidation of their H_{2}O_{2}-sensitive cysteine residues. Our result that Prx I and Prx II can be regulated through Cdc2 kinase–dependent phosphorylation is consistent with the thesis that intracellular concentration of H_{2}O_{2} is also regulated through the fine control of processes involved in the production as well as elimination.

**References**

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