

Cellular Regulation by Hydrogen Peroxide

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Abstract. Substantial evidence suggests that the transient production of H₂O₂ is an important signaling event triggered by the activation of various cell surface receptors. Understanding the intracellular messenger function of H₂O₂ calls for studies of how receptor occupation elicits the production of H₂O₂, what kinds of molecules are targeted by the produced H₂O₂, and how H₂O₂ is eliminated after the completion of its mission. Recent studies suggest that growth factor-induced H₂O₂ production requires the activation of PtdIns 3-kinase. The essential role of PtdIns 3-kinase is likely to provide PI(3,4,5)P₃ that recruits and activates a guanine nucleotide exchange factor of Rac, which is required for the activation of NADPH oxidase. The targets of H₂O₂ action include proteins that contain a reactive Cys residue. Thus, H₂O₂ 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 H₂O₂ might also be required. Peroxiredoxins, members of a newly discovered family of peroxidases, efficiently reduced the intracellular level of H₂O₂ 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 O₂ during respiration produces superoxide anion (O₂^{•-}), which is spontaneously or enzymatically dismutated to H₂O₂. Many cell types, furthermore, produce low levels of O₂^{•-} and H₂O₂ in response to a variety of extracellular stimuli, including cytokines (TGF-β1, 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ is now recognized as a ubiquitous intracellular messenger under subtoxic conditions (1–4). Understandably, there has long been skepticism about the messenger role of H₂O₂, partly because it seemed illogical for nature to use a dangerous

molecule such as H₂O₂ 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 H₂O₂ function is not implausible. Moreover, recent evidence that specific inhibition of H₂O₂ generation results in a complete blockage of signaling by PDGF, EGF, and angiotensin II is a strong indication that H₂O₂ serves in a messenger role (5–7). However, the mechanisms by which H₂O₂ is produced and mediates receptor signaling have not been well characterized.

Receptor-Mediated H₂O₂ Production

The mechanism of the receptor-mediated generation of H₂O₂ has been studied extensively in phagocytic cells, in which O₂^{•-} (and thus H₂O₂) is produced via the reduction of O₂ by a complicated NADPH oxidase complex (8). Although it had remained unclear for many years whether nonphagocytic cells contain an 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 H₂O₂ production in response to GPCR ligands and peptide growth factors (9). Although H₂O₂ 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 lipoxygenases, which convert arachidonic acid to lipid peroxides, are activated in cells treated with certain cytokines (10).

Among those H₂O₂-generating enzymes, the best studied in

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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 H₂O₂, suggesting the involvement of gp91 *phox*-like protein (11,12).

We studied the mechanism of PDGF-mediated generation of H₂O₂. 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), PtdIns 3-kinase (PI3K; Tyr⁷⁴⁰ and Tyr⁷⁵¹), GAP (Tyr⁷⁷¹), SH2 domain-containing protein tyrosine phosphatase-2 (SHP-2; Tyr¹⁰⁰⁹), and PLC- γ 1 (Tyr¹⁰²¹). Experiments with a series of PDGF- β R mutants suggested that only the PI3K binding site was alone sufficient for PDGF-induced H₂O₂ production (12). The effect of PDGF on H₂O₂ 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 H₂O₂ production (12). These results suggest that a product of PI3K is required for PDGF-induced production of H₂O₂ 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 H₂O₂ 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- γ 1. 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)P₃, 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 β Pix, contain a pleckstrin homology domain that binds inositol-containing phospholipids such as PI(4,5)P₂ and PI(3,4,5)P₃ (13,14). Our unpublished results indicate that β Pix might be the Rac GEF responsible for the activation of an NADPH oxidase. Thus, β Pix physically associated with the NADPH oxidase protein, and PDGF-induced H₂O₂ production was significantly reduced when β Pix expression was reduced by RNA interference in several different cell lines.

H₂O₂ 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 ki-

nases 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 H₂O₂

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. H₂O₂ is, however, a mild oxidant that can oxidize Cys residues in proteins. Because the pK_a of the sulfhydryl group of most cysteine residues is approximately 8.5 and because Cys-SH is less readily oxidized by H₂O₂ than is the cysteine thiolate anion (Cys-S⁻), few proteins might be expected to possess a cysteine residue that is vulnerable to oxidation by H₂O₂ in cells (15). However, certain protein cysteine residues have low pK_a values and exist as thiolate 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 H₂O₂, 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-Arg-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 H₂O₂, and this modification can be reversed by incubation with thiol compounds such as dithiothreitol and reduced glutathione. These observations suggested that PTP might undergo H₂O₂-dependent inactivation in cells. However, such evidence was not available until we demonstrated the ability of intracellularly produced H₂O₂ to oxidize PTP-1B in EGF-stimulated A431 cells by taking advantage of the fact that

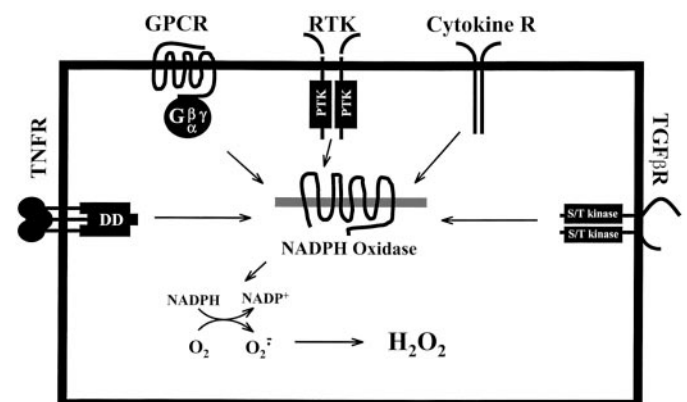


Figure 1. Various cell surface receptors might produce H₂O₂ by activating NADPH oxidase isoforms.

H₂O₂ and [¹⁴C]iodoacetic acid selectively and competitively react with cysteine residues that exhibit a low pK_a. (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 prosthetic group (22). The only redox-active residue is cysteine, which is conserved for all Prx enzymes and

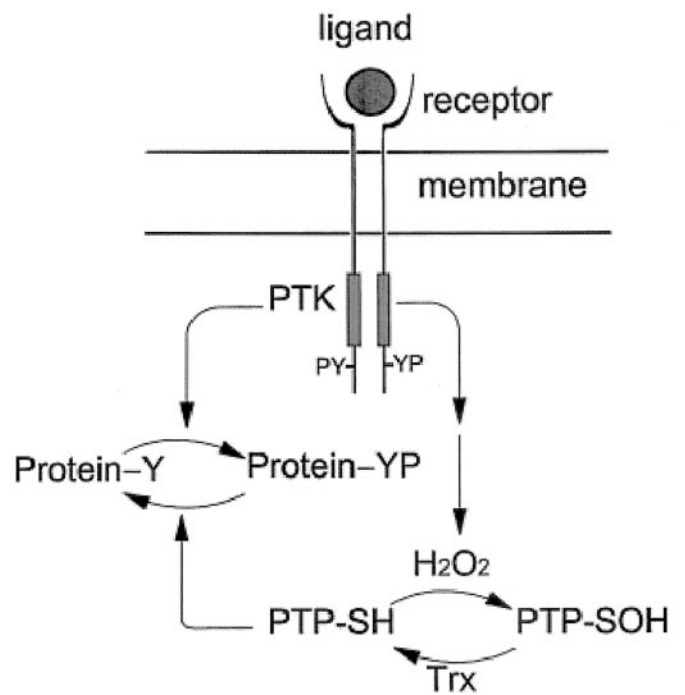


Figure 2. Role of H₂O₂-dependent oxidation of a PTP in growth factor-induced tyrosine phosphorylation.

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- κ B 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-

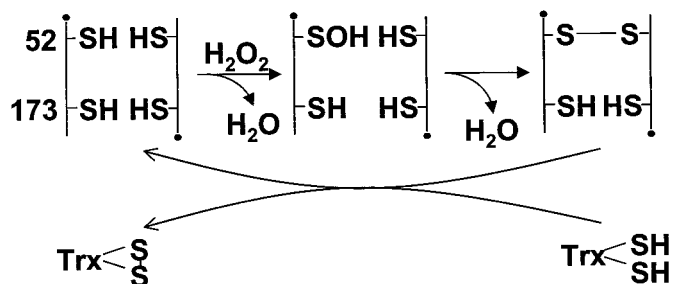


Figure 3. Reaction mechanism of peroxiredoxin.

cently showed that Prx I and II can be phosphorylated specifically at Thr⁹⁰ by several Cdk, including Cdc2 kinase (28). Prx I phosphorylation at Thr⁹⁰ caused a decrease in peroxidase activity by >80%. Using antibodies specific to Prx I phosphorylated at Thr⁹⁰ and HeLa cells arrested at various stages of cell cycle, we showed that Prx I phosphorylation at Thr⁹⁰ 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 Thr⁹⁰ 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 H₂O₂ eliminating enzymes catalase, glutathione peroxidase, and peroxiredoxin are regulated through posttranslational modification.

Conclusion

A growing body of evidence now qualifies H₂O₂ to join the ranks of cAMP, Ca₂₊, 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 H₂O₂ is generated in response to receptor stimulation and which molecules are direct targets of the H₂O₂ messenger function. Here we described how H₂O₂ 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 H₂O₂ 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 H₂O₂. In addition, these components are expected to be subject to control by other second messenger-generating cascades.

We proposed that H₂O₂ propagates its signal by oxidizing active site cysteines of PTP that are sensitive to oxidation by H₂O₂ because their pK_a is lower than those of other cysteines. In addition, many protein kinases, transcriptional factors, and ion channels are controlled by H₂O₂ through oxidation of their H₂O₂-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 H₂O₂ is also regulated through the fine control of processes involved in the production as well as elimination.

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