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Cellular Regulation by Hydrogen Peroxide

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Abstract. Substantial evidence suggests that the transient production of H_2O_2 is an important signaling event triggered by the activation of various cell surface receptors. Understanding the intracellular messenger function of H_2O_2 calls for studies of how receptor occupation elicits the production of H_2O_2, what kinds of molecules are targeted by the produced H_2O_2, and how H_2O_2 is eliminated after the completion of its mission. Recent studies suggest that growth factor–induced H_2O_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_2O_2 action include proteins that contain a reactive Cys residue. Thus, H_2O_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_2O_2 might also be required. Peroxiredoxins, members of a newly discovered family of peroxidases, efficiently reduced the intracellular level of H_2O_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 ($O_2^{\cdot -}$), which is spontaneously or enzymatically dismutated to H_2O_2. Many cell types, furthermore, produce low levels of $O_2^{\cdot -}$ and H_2O_2 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 H_2O_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_2O_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_2O_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_2O_2, partly because it seemed illogical for nature to use a dangerous molecule such as H_2O_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_2O_2 function is not implausible. Moreover, recent evidence that specific inhibition of H_2O_2 generation results in a complete blockage of signaling by PDGF, EGF, and angiotensin II is a strong indication that H_2O_2 serves in a messenger role (5–7). However, the mechanisms by which H_2O_2 is produced and mediates receptor signaling have not been well characterized.

Receptor-Mediated H_2O_2 Production

The mechanism of the receptor-mediated generation of H_2O_2 has been studied extensively in phagocytic cells, in which $O_2^{\cdot -}$ (and thus H_2O_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 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 H_2O_2 production in response to GPCR ligands and peptide growth factors (9). Although H_2O_2 is also known 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 H_2O_2-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 H_2O_2, suggesting the involvement of gp91 *phox*-like protein (11,12).

We studied the mechanism of PDGF-mediated generation of H_2O_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 (Tyr^{579} and Tyr^{581}), PtdIns 3-kinase (PI3K; Tyr^{740} and Tyr^{751}), GAP (Tyr^{771}), SH2 domain–containing protein tyrosine phosphatase-2 (SHP-2; Tyr^{1009}), and PLC-γ1 (Tyr^{1021}). Experiments with a series of PDGF-βR mutants suggested that only the PI3K binding site was alone sufficient for PDGF-induced H_2O_2 production (12). The effect of PDGF on H_2O_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_2O_2 production (12). These results suggest that a product of PI3K is required for PDGF-induced production of H_2O_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_2O_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_2O_2 production was significantly reduced when βPix expression was reduced by RNA interference in several different cell lines.

H_2O_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_2O_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_2O_2 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 H_2O_2 than is the cysteine thiolate anion ($Cys\text{-}S^-$), few proteins might be expected to possess a cysteine residue that is vulnerable to oxidation by H_2O_2 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 H_2O_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-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 H_2O_2, and this modification can be reversed by incubation with thiol compounds such as dithiothreitol and reduced glutathione. These observations suggested that PTP might undergo H_2O_2-dependent inactivation in cells. However, such evidence was not available until we demonstrated the ability of intracellularly produced H_2O_2 to oxidize PTP-1B in EGF-stimulated A431 cells by taking advantage of the fact that

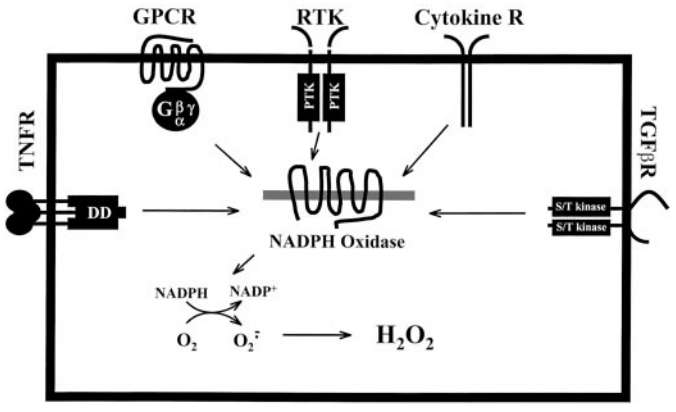

Figure 1. Various cell surface receptors might produce H_2O_2 by activating NADPH oxidase isoforms.

H_2O_2 and [^{14}C]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 H_2O_2 is reversible in cells. We also showed that the essential residue Cys^{215} was oxidized to cysteine sulfenic acid (Cys-SO_2H).

Insulin stimulation also induces the production of intracellular H_2O_2. 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 H_2O_2 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 [^{32}P]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 H_2O_2 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 H_2O_2 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 H_2O_2 and the subsequent reactivation of PTP by electron donors. The proposed role of H_2O_2 in growth factor–induced protein tyrosine phosphorylation is depicted in Figure 2.

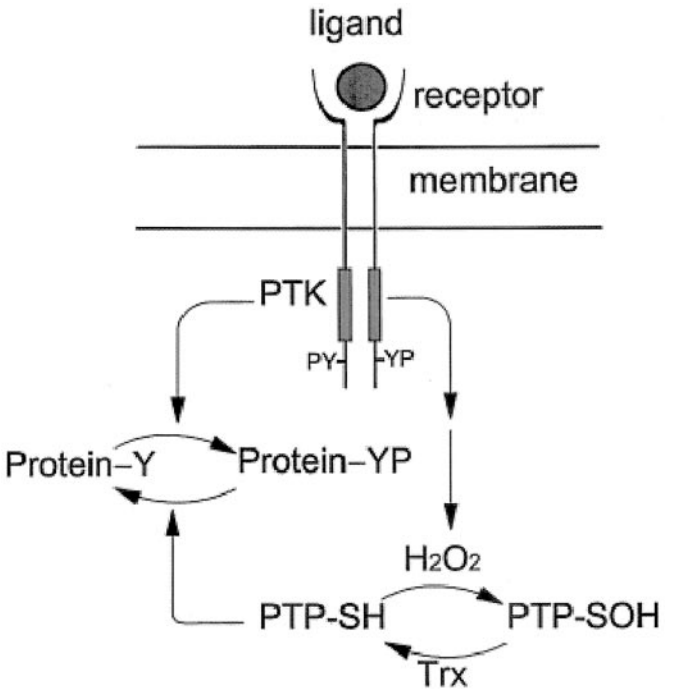

Figure 2. Role of H_2O_2-dependent oxidation of a PTP in growth factor–induced tyrosine phosphorylation.

Elimination of Intracellular H_2O_2 via Peroxiredoxins

In general, elimination as well as production of intracellular messengers is highly controlled process. This would seem especially true for H_2O_2, which is readily converted to deleterious hydroxyl radicals. Enzymes that are capable of eliminating H_2O_2 include peroxiredoxin (Prx) in addition to the two conventional enzymes catalase and glutathione peroxidase. Prx is a novel family of peroxidases that reduce H_2O_2 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 Cys^{52} 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 Cys^{173} in mammalian Prx I and II (21). The catalytic mechanism of Prx enzymes has been well established (Figure 3). The conserved N-terminal Cys^{52}-SH is selectively oxidized by H_2O_2 to cysteine sulfenic acid (Cys-SOH), which then reacts with Cys^{173}-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 H_2O_2 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 H_2O_2.

Prx I contains a consensus site (Thr^{90}-Pro-Lys-Lys^{93}) for phosphorylation by cyclin-dependent kinases (Cdk). We re-

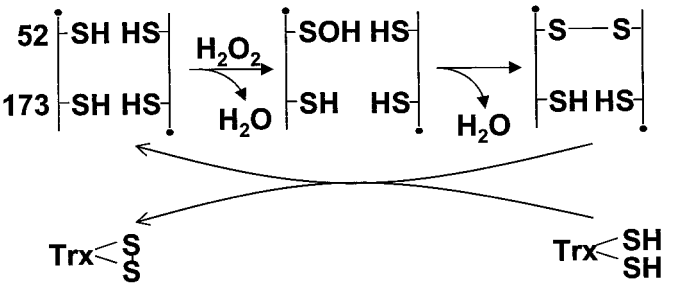

Figure 3. Reaction mechanism of peroxiredoxin.

cently showed that Prx I and II can be phosphorylated specifically at 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_2O_2 eliminating enzymes catalase, glutathione peroxidase, and peroxiredoxin are regulated through posttranslational modification.

Conclusion

A growing body of evidence now qualifies H_2O_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_2O_2 is generated in response to receptor stimulation and which molecules are direct targets of the H_2O_2 messenger function. Here we described how H_2O_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_2O_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_2O_2. In addition, these components are expected to be subject to control by other second messenger–generating cascades.

We proposed that H_2O_2 propagates its signal by oxidizing active site cysteines of PTP that are sensitive to oxidation by H_2O_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_2O_2 through oxidation of their H_2O_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_2O_2 is also regulated through the fine control of processes involved in the production as well as elimination.

References

1. Rhee SG, Bae YS, Lee SR, Kwon J: Hydrogen peroxide: A key messenger that modulates protein phosphorylation through cysteine oxidation. *Science's stke.* Available at: www.stke.sciencemag.org/cgi/content/full/OC_sigtrans; 2000/53/pe1
2. Rhee SG: Redox signaling: Hydrogen peroxide as intracellular messenger. *Exp Mol Med* 31: 53–59, 1999
3. Finkel T: Oxygen radicals and signaling. *Curr Opin Cell Biol* 10: 248–253, 1998
4. Suzuki YJ, Ford GD: Redox regulation of signal transduction in cardiac and smooth muscle. *J Mol Cell Cardiol* 31: 345–353, 1999
5. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T: Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* 270: 296–299, 1995
6. Bae YS, Kang SW, Seo MS, Baines IC, Tekle E, Chock PB, Rhee SG: Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem* 272: 217–221, 1997
7. Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K, Griendling KK: Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. *J Biol Chem* 274: 22699–22704, 1999
8. Babior BM: NADPH oxidase: An update. *Blood* 93: 1464–1476, 1999
9. Lambeth JD: Nox/Duox family of nicotinamide adenine dinucleotide (phosphate) oxidases. *Curr Opin Hematol* 9: 11–17, 2002
10. Woo CH, Eom YW, Yoo MH, You HJ, Han HJ, Song WK, Yoo YJ, Chun JS, Kim JH: Tumor necrosis factor-alpha generates reactive oxygen species via a cytosolic phospholipase A2-linked cascade. *J Biol Chem* 275: 32357–32362, 2000
11. Sundaresan M, Yu ZX, Ferrans VJ, Sulciner DJ, Gutkind JS, Irani K, Goldschmidt-Clermont PJ, Finkel T: Regulation of reactive-oxygen-species generation in fibroblasts by Rac1. *Biochem J* 318: 379–382, 1996
12. Bae YS, Sung JY, Kim OS, Kim YJ, Hur KC, Kazlauskas A, Rhee SG: Platelet-derived growth factor-induced H(2)O(2) production requires the activation of phosphatidylinositol 3-kinase. *J Biol Chem* 275: 10527–10531, 2000
13. Rameh LE, Cantley LC: The role of phosphoinositide 3-kinase lipid products in cell function. *J Biol Chem* 274: 8347–8350, 1999
14. Leevers SJ, Vanhaesebroeck B, Waterfield MD: Signalling through phosphoinositide 3-kinases: The lipids take centre stage. *Curr Opin Cell Biol* 11: 219–225, 1999
15. Kim JR, Yoon HW, Kwon KS, Lee SR, Rhee SG: Identification of proteins containing cysteine residues that are sensitive to oxidation by hydrogen peroxide at neutral pH. *Anal Biochem* 283: 214–221, 2000
16. Denu JM, Dixon JE: Protein tyrosine phosphatases: Mechanisms of catalysis and regulation. *Curr Opin Chem Biol* 2: 633–641, 1998
17. Lee SR, Kwon KS, Kim SR, Rhee SG: Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem* 273: 15366–15372, 1998
18. Mahadev K, Zilbering A, Zhu L, Goldstein BJ: Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. *J Biol Chem* 276: 21938–21942, 2001
19. Meng TC, Fukada T, Tonks NK: Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Mol Cell* 9: 387–399: 2002
20. Chae HZ, Robison K, Poole LB, Church G, Storz G, Rhee SG: Cloning and sequencing of thiol-specific antioxidant from mammalian brain: Alkyl hydroperoxide reductase and thiol-specific

antioxidant define a large family of antioxidant enzymes. *Proc Natl Acad Sci U S A* 91: 7017–7021, 1994
21. Rhee SG, Kang SW, Chang TS, Jeong W, Kim K: Peroxiredoxin, a novel family of peroxidases. *IUBMB Life* 52: 35–41, 2001
22. Chae HZ, Uhm TB, Rhee SG: Dimerization of thiol-specific antioxidant and the essential role of cysteine 47. *Proc Natl Acad Sci U S A* 91: 7022–7026, 1994
23. Chae HZ, Chung SJ, Rhee SG: Thioredoxin-dependent peroxide reductase from yeast. *J Biol Chem* 269: 27670–27678, 1994
24. Zhang P, Liu B, Kang SW, Seo MS, Rhee SG, Obeid LM: Thioredoxin peroxidase is a novel inhibitor of apoptosis with a mechanism distinct from that of Bcl-2. *J Biol Chem* 272: 30615–30618, 1997
25. Kang SW, Chae HZ, Seo MS, Kim K, Baines IC, Rhee SG: Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha. *J Biol Chem* 273: 6297–6302, 1998
26. Jin DY, Chae HZ, Rhee SG, Jeang KT: Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation. *J Biol Chem* 272: 30952–30961, 1997
27. Kang SW, Baines IC, Rhee SG: Characterization of a mammalian peroxiredoxin that contains one conserved cysteine. *J Biol Chem* 273: 6303–6311, 1998
28. Chang TS, Jeong W, Choi SY, Yu S, Kang SW, Rhee SG: Regulation of peroxiredoxin I activity by Cdc2-mediated phosphorylation. *J Biol Chem* 277: 25370–25376, 2002