Reactive Oxygen Species and Cell Signaling

Respiratory Burst in Macrophage Signaling

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Phagocytes such as neutrophils and macrophages produce reactive oxygen species (ROS) during phagocytosis or stimulation with a wide variety of agents through activation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase that is assembled at the plasma membrane from resident plasma membrane and cytosolic protein components. One of the subunits of the phagocyte NADPH oxidase is now recognized as a member of a family of NADPH oxidases, or NOX, present in cells other than phagocytes. Physiologic generation of ROS has been implicated in a variety of physiologic responses from transcriptional activation to cell proliferation and apoptosis. The increase in superoxide and hydrogen peroxide (H_2O_2) that results from stimulation of the NADPH oxidase is transient, in part due to the presence of the antioxidant enzymes, which return their concentrations to the prestimulation steady state level. Thus, the antioxidant enzymes may function in the "turn-off" phase of signal transduction by ROS. During its transient elevation, H_2O_2 may act as a modifier of key signaling enzymes through reversible oxidation of critical thiols. The rapid reaction of thiols with H_2O_2 when in their unprotonated state would provide a potential mechanism for the specificity that is necessary for physiologic cell signaling.

Keywords: protein tyrosine phosphatase; mitogen-activated kinase; transcription factors; superoxide; hydrogen peroxide

It has long been recognized that reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are produced in aerobic cells either as by-products during mitochondrial electron transport or by several oxidoreductases and metal-catalyzed oxidation of metabolites. Stimulated production of ROS was first described in phagocytic cells like neutrophils and macrophages and was named "the respiratory burst" due to the transient consumption of oxygen. The respiratory burst is performed by a multicomponent nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase (*see below*) and is critical for the bactericidal action of phagocytes. Recently, production of ROS has been demonstrated in a variety of cells other than phagocytes (1–3), and several studies have implicated ROS in physiologic signaling (4–6). Furthermore, novel NADPH oxidases related to the phagocyte enzyme, now called NOX proteins, have been identified (7). All together, these findings provide strong support to the emerging concept that $O_2^{\cdot-}$ and H_2O_2 are required participants of normal physiologic signaling in many cells, a process we will refer to here as redox signaling (for reviews *see* References 8–15).

Surprisingly, little attention has been given to the role of the NADPH oxidase in phagocyte signaling. Because the phagocyte NADPH oxidase and the NOX proteins are members of a large family of proteins, we propose that the macrophage version, in addition to its microbicidal role, may also participate in cell signaling. Although this brief review cannot cover all aspects of redox signaling, we will point out some important principles and summarize recent studies in macrophages.

REDOX SIGNALING AND ANTIOXIDANT ENZYMES

The effects of ROS in signaling have often been attributed to "a shift" in the redox potential of the cells. As pointed out by Schafer and Buettner, the glutathione disulfide (GSSG)/2 glutathione (GSH) ratio can serve as a good indicator of the cellular redox state (16). This ratio results primarily from a combination of the rates of H_2O_2 removal by GSH peroxidase and GSSG reduction by GSH reductase, regulating the GSH concentration. Thus, antioxidant enzymes play a critical role in the maintenance of the cellular reductive potential. H_2O_2 can be produced through $O_2^{\cdot-}$: dismutation or catalysis by the superoxide dismutases:

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

The enzyme-catalyzed reaction occurs at a near diffusion-limited rate. As a result, the steady state concentration of $O_2^{\cdot-}$ is estimated to be $\sim 10^{-11}$M (17). This suggests that $O_2^{\cdot-}$ would have to act within a very short radius of its site of production to play a role in signaling within a cell. This spatial restriction for $O_2^{\cdot-}$ reactivity may be advantageous, keeping with the general principle of signal transduction that restricted location of action provides specificity. Several different enzymes eliminate H_2O_2. Catalase, which is generally found only in peroxisomes, catalyzes a very rapid dismutation reaction:

$$2H_2O_2 \xrightarrow{Catalase} H_2O + O_2$$

GSH peroxidases, which are selenoproteins, are found in the cytosol and mitochondria. These enzymes reduce H_2O_2, using GSH and produce GSSG:

$$H_2O_2 + 2GSH \xrightarrow{Glutathione\ peroxidase} 2H_2O + GSSG$$

These enzymes can also reduce lipid hydroperoxides and peroxynitrite as well. Less commonly recognized is the fact that the peroxiredoxins catalyze the reduction of H_2O_2 by GSH or other thiols in a reaction similar to that catalyzed by the selenium in GSH peroxidases but contain reactive cysteine residues in their unprotonated form, i.e., thiolate (S^-) (18, 19). The reaction has three steps:

$$H_2O_2 + PS^- \rightarrow H_2O + PSO^-$$

$$PSO^- + RSH \rightarrow PSSR + OH^-$$

$$PSSR + RSH \rightarrow PS^- + RSSR + H^+$$

where PS^- represents the peroxiredoxin, PSO^- represents a sulfenate (SO^-) intermediate, and RSH and RSSR represent

(*Received in original form June 14, 2002; accepted in final form October 1, 2002*)

Supported by grant HL37556 from the National Institutes of Health.

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Am J Respir Crit Care Med Vol 166. pp S4–S8, 2002
DOI: 10.1164/rccm.2206007
Internet address: www.atsjournals.org

the thiol and disulfide forms of a compound, which may be GSH, thioredoxin (Trx), or some other thiol.

We propose that the antioxidant enzymes may serve as turn-off enzymes in redox signaling. By analogy to the cyclic adenosine monophosphate second messenger signaling system, in which phosphodiesterase activity turns off signaling by decreasing cyclic adenosine monophosphate, the antioxidant enzymes would turn off signaling by decreasing their substrates. Thus, by restoring the low steady state ROS concentrations of oxidant second messengers, antioxidant enzymes cause the signal to be transient, another general characteristic of signal transduction. Further studies are warranted to test this point.

SPECIFICITY OF ROS REACTIONS

One potential mechanism for redox signaling could be via disulfide exchange between GSSG and protein thiols. However, there are countless protein thiols that could potentially undergo this reaction, thereby lacking specificity. The concept of specificity, one of the major characteristics of signaling pathways in general, is a challenge when it comes to the role of ROS in signaling, given that even their name implies high reactivity. Actually, H_2O_2 is unreactive with thiols in the absence of catalysis, usually by transition metals. Metal-catalyzed reactions or peroxynitrite can oxidize cysteine to either a sulfinic or sulfonic acid that are not easily reduced. However, H_2O_2 reacts readily with the far less common thiolate anion (-S^-) to form sulfenic acid (-SOH), which at physiologic pH ionizes to form a sulfenate (-SO^-) (pKa 6.1) (20). This intermediate can be easily reduced, making the reaction reversible, another important principle in signaling. The S^- is only found in proteins where the cysteine, which has a pKa around 8.5, hence significantly higher than the physiologic range, is situated in a positively charged electrostatic field provided by surrounding residues that allows dissociation to the S^- and stabilization of the structure. This has been well characterized while studying the mechanisms of catalysis by protein tyrosine phosphatases (PTP) (21). The presence in PTP of at least one reactive sulfhydryl that was essential for catalysis was hinted by the inhibition of PTP by alkylating agents. All PTP contain a highly conserved cysteine that resides in the PTP signature motif, HCXXGXXRST, and mutation of this cysteine residue results in loss of activity. This cysteine is in the form of a S^- and forms a thiophosphoenzyme intermediate during hydrolysis, acting as a nucleophile. The pKa of cysteine was measured to be around 4.67 in *Yersinia* PTP. The His residue, which is also conserved in all PTP and is adjacent to the cysteine in the active site, together with an α-helix that follows the phosphate-binding loop generate a dipole that contributes to the low pKa (22, 23). The active site of peroxiredoxins also provides the proper microenvironment for formation of S^-, as mentioned previously. The conserved catalytic site of the Trx family of proteins contains two cysteine residues, one of which is S^- that can form SO^-. The second cysteine then reacts with SO^- to form an intramolecular disulfide bridge. The intramolecular disulfide bond cannot be readily reduced by GSH. Instead, the enzyme, Trx reductase catalyzes an NADPH-dependent reduction of the disulfide to restore Trx to its reduced form:

$$H_2O_2 + Trx(SH)(S^-) \rightarrow H_2O + Trx(SH)(SO^-)$$

$$Trx(SH)(SO^-) \rightarrow TrxSS + OH^-$$

$$TrxSS + NADPH \xrightarrow{Trx\ Reductase} Trx(SH)(S^-) + NADP^+$$

Thus, the reaction with H_2O_2 being restricted to such proteins, specificity may ensue (21, 24–26).

A few years ago, Denu and Tanner demonstrated *in vitro* that the reaction of the S^- with low concentrations of H_2O_2 inhibited the PTP activity, which was restored by thiols, suggesting the formation of a sulfenic acid (-SOH) intermediate (21). This was later confirmed by *in vivo* studies (27). The predominant thiol reducing the sulfenic acid/sulfenate is most likely GSH as its cellular concentration ranges from 1 to 10 mM (Figure 1). Interestingly, the oxidation of PTP and reduction by GSH has the same chemistry as that of the peroxiredoxin reaction shown previously, and it will be important to further determine the role of these enzymes in redox signaling. A recent study by Meng and coworkers brought additional support for the role of PTP inhibition by H_2O_2 in physiologic signaling (28). The PTP, SHP-2, which has a Src homology 2 (SH2) domain, binds through this domain to a phosphotyrosine in the intracellular domain of the activated tyrosine kinase receptor on stimulation with platelet-derived growth factor, which is also known to induce ROS production. In this paper, they demonstrated that the specific, spatiotemporal, and reversible inhibition of SHP-2 was necessary for both downstream platelet-derived growth factor signaling and turn-off. Only the SHP-2 activity associated with the receptor was altered by H_2O_2 and activation of the same cells with epithelial growth factor, which also induces ROS production, did not result in SHP-2 inhibition. Thus, the reaction of S^- with H_2O_2 is specific, reversible, and directly connected to changes in the generation of H_2O_2, providing a mechanism consistent with a fundamental role in redox signaling.

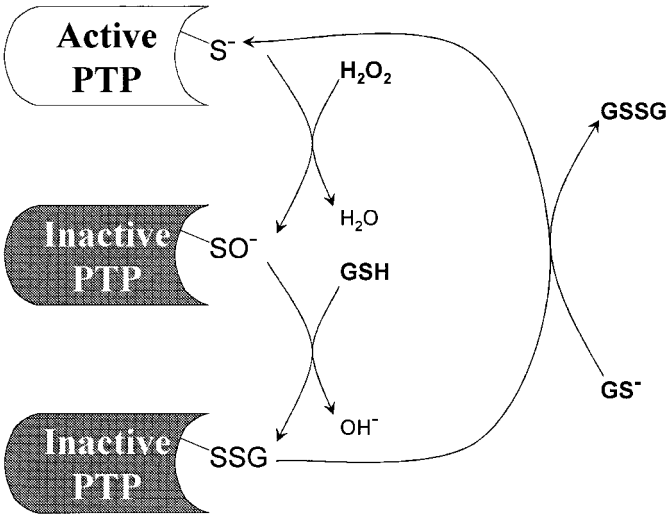

Figure 1. The active site *S^-* in PTPs can react with H_2O_2 to form *SO^-*. This form of the enzyme is catalytically inactive. *GSH* can react with *SO^-* to form a mixed disulfide that can then react with another GSH to form *GSSG* and restore the active form of the *PTP*. The reaction is analogous to that catalyzed by GSH-dependent peroxiredoxins.

ROS TARGETS

As mentioned previously, a large body of evidence has now indicated that endogenously produced ROS are participants of several signal transduction pathways in many cells. Except for the study mentioned previously, which suggests that the PTP is the main target for H_2O_2, the exact targets in the ROS-sensitive pathways have just begun to be identified. Here, we will only briefly mention pathways that are relevant to the section on redox signaling in macrophages (for more extensive reviews *see* References 8–14).

The first signaling components to be identified as redox sensitive were transcription factors. Nuclear factor-κB (NF-κB), a

critical transcription factor for the expression of inflammatory mediators, is sequestered in the cytosol in a complex with its inhibitor IκB. ROS have been demonstrated to induce activation of NF-κB, although the mechanisms are not clearly understood and may not involve a direct effect of ROS on NF-κB (for review, *see* Reference 29). Activator protein-1 is a transcription factor complex formed by homo- or heterodimerization of members of the Jun and Fos families of proteins (for review, *see* References 30 and 31). ROS can regulate activator protein-1 activity through several mechanisms and targets that may include the reversible S-glutathiolation of a single conserved cysteine residue, as demonstrated *in vitro* (32), the reversible redox regulation by Trx and the nuclear protein Ref1 (33). It can also be regulated through the c-Jun N-terminal kinase cascade. The c-Jun N-terminal kinases are part of the mitogen-activated protein kinase (MAPK) superfamily of serine/threonine kinases that also includes the extracellular signal-regulated kinases ERK1 and ERK2 and the $p38^{MAPK}$. All MAPKs are activated through a cascade of phosphorylation, also referred to as the MAPK core or module, and have the unusual particularity to require phosphorylation on both threonine and tyrosine residues within a TxY motif for full activation. One of the upstream kinases in the c-Jun N-terminal kinase and $p38^{MAPK}$ modules is the apoptosis-signal regulating kinase-1, which is maintained in an inactive state by bound reduced Trx. Oxidation of Trx by ROS releases apoptosis-signal regulating kinase-1, permitting its activation and downstream signaling (34–36). Thus, the apoptosis-signal regulating kinase-1/MAPK and the PTP are so far the best described signaling pathway/molecules for which the mechanism of action of ROS has been identified.

SIGNALING BY ROS IN MACROPHAGES

It took several years after the discovery of the production of ROS by phagocytes by Babior and coworkers (37) to uncover the characteristics of the responsible enzyme. It is now clear that the physical separation of the NADPH oxidase components between the cytosol and the plasma membrane in the nonstimulated cells prevents its activation unless phagocytosis or soluble stimuli trigger assembly. The cytosolic complex composed of a $p47^{phox}$/$p67^{phox}$/$p40^{phox}$, and the small GTPase Rac1/Rac2 separately move to the plasma membrane on stimulation and join with the membrane-bound flavocytochrome subunits, $gp91^{phox}$ and $p22^{phox}$ to form the active oxidase complex. This NADPH oxidase has the unusual characteristic of using an electron from cytosolic NADPH to reduce extracellular O_2 to $O_2^{\cdot -}$:

$$NADPH + 2O_2 \xrightarrow{NADPH\ oxidase} NADP^+ H^+ + 2O_2^{\cdot -}$$

Nonenzymatic $O_2^{\cdot -}$ dismutation to H_2O_2 is rapid, but superoxide dismutases accelerate the reaction by 10^4-fold. Although extracellular superoxide dismutase is found in some locations in the body, most $O_2^{\cdot -}$ produced by the respiratory burst is converted to H_2O_2 by nonenzymatic dismutation outside of cells. H_2O_2 is highly diffusible and relatively unreactive and so can rapidly enter cells, even if produced extracellularly. Several studies have also recently suggested, mostly in neutrophils, that intracellular assembly of the oxidase occurs, resulting in intracellular $O_2^{\cdot -}$ and H_2O_2 production (38).

The production of ROS by phagocytes has been mainly studied in the context of bacterial killing. Proof of their importance in this process is offered by nature with the genetic disease, chronic granulomatous disease, where the lack of NADPH oxidase results in lack of ROS production and poor clearance of many bacterial and fungal pathogens. Nevertheless, macrophages, in particular alveolar macrophages (AM), are far less potent than neutrophils and eosinophils at producing ROS, and they may need to acquire an activated phenotype to upregulate their bactericidal capability. Redox signaling may help orchestrating the inflammatory response by inducing the synthesis of cytokines that affect macrophages and induce neutrophil influx. In fact, several studies have now suggested that ROS can regulate the production of cytokines in macrophages through mechanisms that are dependent on NF-κB. In Kupffer cells, the resident macrophage in the liver, production of tumor necrosis factor-α was regulated by a ROS-activated NF-κB pathway (39). Lipopolysaccharide, which stimulates the production of tumor necrosis factor α, induced the production of ROS via a pathway dependent on Rac1 and the activation of NF-κB through IκB kinases in RAW 264.7 cells (40). Various known stimulants of the NADPH oxidase were also shown to trigger NF-κB activation such as silica in mouse peritoneal macrophages (41, 42) or ADP and phorbol myristate acetate in rat AM and the J774.1 mouse monocyte/macrophage cell line (43). Furthermore, in primary AM and in RAW 264.7, the production of ROS by silica resulted in downstream signaling and production of tumor necrosis factor-α, which was inhibited by superoxide dismutase and catalase (44). These effects may differ with stimulus and cell types as adherence to plastic and exposure to particulates stimulated the respiratory burst in human AM but did not induce NF-κB activation (45). It was recently demonstrated that pulmonary NF-κB activation was altered in a knockout mouse model lacking $p47^{phox}$ and thereby deficient in functional NADPH oxidase and ROS production in phagocytes (46), indicating the importance of this pathway in lungs and the utility of such mouse models to study the effects on ROS. Activator protein-1 was also activated in rat AM under hyperoxia, which is known to increase ROS levels (47).

The ERK pathway was one of the first signaling pathways for which the link between the extracellular ligand and the nucleus was described. The signaling module of the ERK pathway is composed of ERK1/2, the dual-specificity kinases MEK1/2, and isoforms of Raf and is principally activated by hormones and growth factors (48). Exogenous H_2O_2 activates ERK1 and ERK2 in many cell types, although this activation appears to be cell type specific (49–52). Further studies showed that increased intracellular production of ROS also activated the ERK (53). In NR8383 rat AM, treatment with menadione, which induces the production of ROS through a mechanism presumably independent of the NADPH oxidase, activated ERK1, ERK2, and $p38^{MAPK}$ (54). Other studies have concentrated on the possible role of the ROS produced by the NADPH oxidase/respiratory burst. Many stimuli that trigger the respiratory burst in phagocytes induce the activation of the ERK (55). Thus, ERK activation may be a consequence of the burst or may play a role in the assembly of the NADPH oxidase. The results for the latter have been controversial, with both a role and a lack thereof proposed in studies done mainly in neutrophils. In rat AM, ADP stimulation of the respiratory burst occurs in the absence of ERK activation (56). This indicates that, at least in these cells, assembly of the NADPH oxidase may be independent of ERK activation. These data also suggest that endogenous production of H_2O_2 stimulated by the burst is not sufficient to induce ERK activation in these cells. However, we showed that H_2O_2 was necessary for ERK activation if the burst was stimulated by zymosan-activated serum, a source of C5a, as catalase but not superoxide dismutase almost completely abrogated the tyrosine phosphorylation and activation of the ERK1 and ERK2 (57). The mechanism involved here is still unclear, although the H_2O_2 target appears to be upstream of the ERK, as activation of MEK1/2 was also prevented by catalase. In a recent study using NR8383 cells, which recapitulate the results observed in primary AM, we showed that vanadate, a well-known inhibitor of PTP

could relieve the catalase block, suggesting that a PTP, inhibited either by H_2O_2 or vanadate may be involved in ERK activation (58). Although clearly showing that ROS, particularly H_2O_2, can act as second messengers and modulate the physiologic responses of macrophages, these reports do not address the mechanisms by which NF-κB or the ERK pathway are activated. Identifying the targets of ROS and the chemical modification they imposed will be required in the future. Present studies tend to indicate that thiol chemistry will be a predominant mechanism. Alteration through an essential cysteine of the small GTPase Ras, which links receptor activation to the ERK pathway has been proposed as a mechanism for ERK activation by ROS (59), although this was actually best demonstrated with nitric oxide, another small free radical molecule that plays a role in redox signaling (60, 61).

One of the fascinating questions in lung biology in the next few years will be to determine the role that redox signaling in AM or in other cells of the lung might play in regulating lung inflammation in response to bacterial challenge or other types of challenges. This is a daunting task, as the inflammatory response requires the tight regulation and interaction of many cell types and that injury and disease also result from chronic or overwhelming inflammation. The AM has been thought as a critical regulator of this process, in part because of its ability to phagocytose and kill bacteria but also to synthesize inflammatory mediators such as leukotriene B_4 and numerous cytokines, among which are tumor necrosis factor-α, interferon-γ, interleukin-6, interleukin-12, monocyte chemoattractant protein-1, and the macrophage inflammatory protein (MIP) family (62). Depletion of AM in lipopolysaccharide-challenged mice resulted in decrease in NF-κB activation, cytokine generation, and neutrophil influx, supporting the central role of AM (63). There are also an increasing number of data indicating that the products synthesized by the AM participate in their activated phenotype and that in their absence, AM are not able to either phagocytose or kill a number of bacteria. Human AM show very low rates of *Streptococcus pneumoniae* ingestion and killing *in vitro*, which has suggested that the role of AM in that case is to provide a rapid proinflammatory signal after ingestion of a few bacteria. Leukotriene B_4-null mice had enhanced lethality to *Klebsellia pneumoniae* due to decreased AM phagocytic and bactericidal activities (64). The use of various knockout mouse models will certainly help in delineating the role of each compartment of the inflammatory response. Just to name a few more of such studies, GM/CSF$^{-/-}$ null mice develop lung pathology and their AM were impaired in their $O_2^{\cdot -}$ production and bacterial capability after challenge with group B *Streptococcus*, supporting the role of GM-CSF *in vivo* in modulating the AM phenotype (65). MIP1α was also shown to play a similar role in MIP1α$^{-/-}$ after challenge with *K. pneumoniae* (66). Compounding the difficulty, other cells such as endothelial cells and neutrophils may also play a role and the use of mouse models deficient in the phagocyte NADPH oxidase (p47$^{phox-/-}$ and gp91$^{phox-/-}$) may help understanding the role of ROS derived from neutrophils and other cells, including endothelial cells (67, 68). We speculate that redox signaling will play an important role in the regulation of the inflammatory response and ultimately in development of lung disease and are looking forward to seeing exciting new research either proving or disproving this hypothesis.

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