REVIEW ARTICLE

NADPH Oxidase: An Update

By Bernard M. Babior

THE NADPH OXIDASES are a group of plasma membrane–
associated enzymes found in a variety of cells of mesoder-
mal origin. The most thoroughly studied of these is the
leukocyte NADPH oxidase, which is found in professional
phagocytes and B lymphocytes. It catalyzes the production of
superoxide (O$_2^-$) by the one-electron reduction of oxygen,
using NADPH as the electron donor:

$$2 \text{O}_2 + \text{NADPH} \rightarrow 2 \text{O}_2^- + \text{NADP}^+ + \text{H}^+$$

The O$_2^-$ generated by this enzyme serves as the starting
material for the production of a vast assortment of reactive
oxidants, including oxidized halogens, free radicals, and singlet
oxygen. These oxidants are used by phagocytes to kill invading
microorganisms, but they also cause a lot of what the military
would call “collateral damage” to nearby tissues, so their
production has to be tightly regulated to make sure they are only
generated when and where required.

In the 40 years since Sbarra and Karnovsky first reported
findings suggesting the existence of such an enzyme in neutro-
phils, a great deal has been learned about the leukocyte oxidase.
Research over this period of time has shown that the core
enzyme comprises five components: p40$^{\text{PHOX}}$ (PHOX for PHago-
cyte Oxidase), p47$^{\text{PHOX}}$, p67$^{\text{PHOX}}$, p22$^{\text{PHOX}}$, and gp91$^{\text{PHOX}}$. In the
resting cell, three of these five components—p40$^{\text{PHOX}}$, p47$^{\text{PHOX}}$
and p67$^{\text{PHOX}}$—exist in the cytosol as a complex. The other two
components—p22$^{\text{PHOX}}$ and gp91$^{\text{PHOX}}$—are located in the mem-
branes of secretory vesicles* and specific granules, where they
occur as a heterodimeric flavohemoprotein known as cyto-
chrome b$_{558}$. Separating these two groups of components by
distributing them between distinct subcellular compartments
 guarantees that the oxidase is inactive in the resting cell.

When the resting cell is exposed to any of a very wide variety
of stimuli, the cytosolic component p47$^{\text{PHOX}}$ becomes heavily
phosphorylated and the entire cytosolic complex migrates to the
membrane, where it associates with cytochrome b$_{558}$ to as-
semble the active oxidase (Fig 1). The assembled oxidase is
now able to transfer electrons from the substrate to oxygen by
means of its electron-carrying prosthetic groups—its flavin and
then (according to most investigators, but not me) its heme

*Secretory vesicles are small intracellular vesicles that fuse rapidly
with plasma membrane to discharge their contents to the exterior (or to
the lumen of a phagosome) in response to appropriate stimuli.

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†The turnover number is the number of molecules of product made
each second by one molecule of enzyme.
production, the observed turnover number is equivalent to a rate of \( \text{O}_2^- \) production of about 0.2 nmol/min/10^6 cells, only 2% of the rate seen with maximally activated whole neutrophils. On the other hand, a rate of heme reduction fast enough to account for \( \text{O}_2^- \) production by whole cells was calculated from observed levels of reduction of the partly reduced flavin and heme in the working enzyme. However, this calculation depended on the assumption that the observed levels of reduction were steady-state levels; but it was later shown that this assumption was not in accord with experimental findings \(^6\) (see also ref \(^7\)). Moreover, earlier studies under anerobic conditions had shown that both in whole cells and in a cell-free system, the actual rate of reduction of the cytochrome b_{558} heme by NADPH was only about 0.1% of the rate of \( \text{O}_2^- \) production by the identical systems operating in air.\(^8\)\(^,\)\(^9\) Because a multistep reaction can go no faster than its slowest step, the rate of \( \text{O}_2^- \) production by neutrophils could be no greater than a few pmol/min/10^6 cells if the heme were an obligatory intermediate in electron transfer. Therefore, it is difficult for me to grasp the logic of the widely held belief that a heme residue of cytochrome b_{558} participates at all in electron transport by the leukocyte NADPH oxidase, much less that it is the terminal electron carrier. Evidence for heme participation such as the recent demonstration that exposure of the oxidase to an activating agent changes the spin state of the iron in cytochrome b_{558} from low-spin hexacoordinate to high-spin pentacoordinate,\(^10\) though of great interest, is indirect and can be interpreted in many ways. Kinetic competence is the gold standard, and there is no escape from the fact that the rate of reduction of the heme is far too slow for it to participate in any meaningful way as an electron carrier for the oxidase.

The foregoing considerations apply only to the heme of the flavocytochrome. There is universal agreement that its flavin is an electron carrier. Oxidase activity is lost when FAD is removed from the enzyme, and restored when FAD is added back.\(^11\) The oxidase is inhibited by flavin antagonists such as deaza-FAD\(^12\) and diphenylene iodonium\(^13\) (in contrast to the lack of effect of heme antagonists such as CN\(^-\), N\(_3\)\(^-\), CO, and butyl isonitrile\(^14\)). In model systems, \( \text{O}_2^- \) is produced by the reaction between oxygen and reduced flavins. Finally, recent experiments demonstrating that under special conditions purified cytochrome b_{558} alone catalyzes \( \text{O}_2^- \) production from NADPH and oxygen\(^15\)\(^,\)\(^16\) leaves little doubt that the flavin of cytochrome b_{558} is an electron carrier for the leukocyte NADPH oxidase.

The ability of the leukocyte NADPH oxidase to use artificial electron acceptors was discovered some time ago by Green and Wu.\(^17\) Recently, iodonitrotetrazolium violet (INT) was added to the list of oxidants that could accept electrons from the oxidase.\(^18\)\(^,\)\(^19\) Partial purification of the INT-reducing activity from activated neutrophil membranes showed that it contained cytochrome b_{558}, and that its activity was dependent on cytosol and FAD.\(^19\) INT reduction persisted under anerobic conditions, indicating that the dye accepted electrons directly from the enzyme.

Chronic granulomatous disease (CGD) is an inherited disorder characterized by the failure of \( \text{O}_2^- \) production by phagocytes, resulting in a marked increase in the susceptibility of affected patients to bacterial and fungal infections. The disease is caused by a mutation that results in the loss or inactivation of one of the core subunits of the oxidase (CGD due to a mutation affecting p40\(^{PHOX}\)) has not yet been reported). Inactivating mutations (Table \(1\)) have provided important information concerning the mechanism of the oxidase. Several recent reports have identified new mutations leading to inactive gp91\(^{PHOX}\). These include gp91\(^{PHOX}\) A57E,\(^20\) E309K, C537R, P339H, and \( \Delta K315\) and gp91\(^{PHOX}\) \( \Delta F215 \) or \( \Delta F216 \). Cells containing gp91\(^{PHOX}\) \( \Delta F215 \) or \( \Delta F216 \) have normal amounts of gp91\(^{PHOX}\) but show no trace of the cytochrome b_{558} spectrum. This finding suggests that one or both of these phenylalanine

| Table 1. Natural Mutations That Inactivate the Leukocyte NADPH Oxidase With Retention of the Mutated Protein |
|--------------------------------------------------|-------------------|
| **Oxidase Subunit** | **Mutation** |
| gp91\(^{PHOX}\) | P415H |
| gp91\(^{PHOX}\) | D500G |
| gp91\(^{PHOX}\) | A57E |
| gp91\(^{PHOX}\) | R545 |
| gp91\(^{PHOX}\) | E309K |
| gp91\(^{PHOX}\) | C537R |
| gp91\(^{PHOX}\) | P339H |
| gp91\(^{PHOX}\) | QKT(507-509)HIWA |
| gp91\(^{PHOX}\) | \( \Delta F215 \) or \( \Delta F216 \) |
| p22\(^{PHOX}\) | P156Q |
| p67\(^{PHOX}\) | \( \Delta K58 \) |
residues are essential for the binding of the cofactors to the cytochrome.

Cytosolic Components

p47\(\text{PHOX}\) is the subunit chiefly responsible for transporting the cytosolic complex from the cytosol to the membrane during oxidase activation. This is evident because neutrophils that lack p47\(\text{PHOX}\) are unable to transfer p67\(\text{PHOX}\) from the cytosol to the membrane during activation, although p67\(\text{PHOX}\)-deficient neutrophils transfer p47\(\text{PHOX}\) in a normal fashion\(^{23}\). Before the cytosolic oxidase components can be transferred to the membrane, however, p47\(\text{PHOX}\) must be extensively phosphorylated. This phosphorylation is one of the characteristic events of oxidase activation. p47\(\text{PHOX}\), however, is not absolutely indispensable for oxidase activity. Even though its deficiency results in a detergent-activated cell-free system containing purified cytochrome b\(_{558}\) and recombinant cytosolic factors, p47\(\text{PHOX}\) can be omitted as long as the system contains high concentrations of p67\(\text{PHOX}\) and Rac2\(^{24,25}\). The effect of p47\(\text{PHOX}\) is to tighten by nearly 100-fold the binding of each of the other cytosolic proteins to the assembled oxidase.

The function of p67\(\text{PHOX}\) has been a mystery. Unlike p47\(\text{PHOX}\), p67\(\text{PHOX}\) is absolutely required for oxidase activity. When incubated with membranes in the detergent-activated cell-free system, a cytosol lacking p47\(\text{PHOX}\) but containing p67\(\text{PHOX}\) was able to support INT reduction\(^{18}\). Furthermore, p67\(\text{PHOX}\) facilitated electron transfer to the flavin of cytochrome b\(_{558}\) in the absence of p47\(\text{PHOX}\)\(^{26}\). When p47\(\text{PHOX}\) was also present, the system was said to allow electron transfer “to proceed beyond the flavin center to the heme in cytochrome b\(_{245}\)” and thence to oxygen,” a claim with which I am obviously not in full agreement. We recently furnished some evidence as to the possible function of p67\(\text{PHOX}\) in oxidase activity by showing that this subunit contained a catalytically essential binding site for NADPH\(^{27}\). However, there is evidence that an NADPH binding site also exists on cytochrome b\(_{558}\).28,29 The relationship between these two NADPH binding sites remains to be determined, though it should be noted that cytochrome b\(_{558}\) alone catalyzes active O\(_2^-\) production using NADPH as the reductant\(^{28}\), while catalysis of NADPH dehydrogenation by p67\(\text{PHOX}\) has to date not been demonstrated.

A CGD patient with a functionally significant mutation of p67\(\text{PHOX}\) has recently been described\(^{30}\). This mutant, p67\(\text{PHOX}\) ΔK58, loses its interaction with Rac, and when phagocytes containing the p67\(\text{PHOX}\) mutant are activated, the cytosolic complex fails to translocate to the membrane. These results imply that Rac, like p47\(\text{PHOX}\), is involved in the translocation of the cytosolic complex during oxidase activation.

The function of p40\(\text{PHOX}\) has recently been examined\(^{31}\). Oxidase activity can be established in K562 cells by transfecting them with plasmids that express recombinant p47\(\text{PHOX}\), p67\(\text{PHOX}\), and p40\(\text{PHOX}\)\(^{32}\). Cells that express p40\(\text{PHOX}\) along with other recombinant oxidase components produce only about half the amount of O\(_2^-\) generated by cells not expressing p40\(\text{PHOX}\). Similarly, adding p40\(\text{PHOX}\) to a cell-free detergent-dependent oxidase activating system inhibits O\(_2^-\) production. These results suggest that p40\(\text{PHOX}\) is an inhibitory oxidase subunit. On the other hand, interfering with the binding of p40\(\text{PHOX}\) to p67\(\text{PHOX}\) reduces by 50% the production of O\(_2^-\) in a detergent-dependent cell-free system, a result implying that p40\(\text{PHOX}\) is a stimulatory subunit\(^{33}\). Clearly, more research on this question is needed.

Location of the Oxidase

As discussed above, the activated NADPH oxidase is associated with the phagocyte membranes. Recent studies have indicated how the oxidase is distributed in the membrane, and on which membranes it is located. Immunoelectron microscopy showed that cytosolic oxidase components were grouped together with Rac on the inner face of neutrophil plasma membranes in 3- to 10-nm clusters\(^{34}\) (Fig 2). In a study of exceptional interest, Kobayashi et al\(^{35}\) used cytochemical staining to show that O\(_2^-\) production in phorbol-stimulated neutrophils initially took place in small alkaline phosphatase-containing cytoplasmic vesicles (Fig 3). These vesicles then fused together, forming larger vesicles that then merged with the plasma membrane. After a time, O\(_2^-\)-producing vesicles containing a marker of extracellular fluid appeared within the neutrophils. These results strongly imply that in resting cells, the membrane-associated oxidase components are located exclusively in intracellular organelles (secretory vesicles as shown here, and specific granules as demonstrated earlier\(^{36}\)), and that the active oxidase in the plasma membrane is delivered there by membrane fusion events. They further imply that vesicles whose membranes contain the active oxidase cycle between the interior of the cell and the plasma membrane.

Inhibitors

Sulphhydril groups are important for the function of the leukocyte NADPH oxidase. Recently, naturally occurring sul-
Hydral blockers have been found to inhibit the oxidase. The aldehyde 4-hydroxynonenal inhibited the oxidase with an IC₅₀ of 19 µmol/L. Inhibition was reversed with dithiothreitol, suggesting that the blockade of -SH groups is responsible for the action of the aldehyde. Inhibition by 4-hydroxynonenal is of physiological significance because the aldehyde is a major product of lipid peroxidation, and therefore will be present in tissues undergoing oxidative attack.

Nitric oxide (NO) also inhibits the oxidase, but it works by preventing the assembly of the oxidase during activation. It has no effect on the fully active oxidase, and does not interact with either the flavin or the hemes on cytochrome b₅₅₈.
Nitrosothiols (RSNO) also inhibit oxidase activation, preventing translocation of the cytosolic oxidase components p47\textsuperscript{PHOX} and p67\textsuperscript{PHOX} through an action on the membrane.\textsuperscript{39} The effect of nitrosothiols can be reversed by mercaptoethanol. It is likely that both NO and RSNO act by combining with cysteine-SH groups to form nitrosothiols on components of the oxidase.

It has recently been reported that PR-39, an antimicrobial peptide from neutrophils, is able to inhibit the NADPH oxidase by binding to the SH3 domains of p47\textsuperscript{PHOX}, thereby blocking the interaction between p47\textsuperscript{PHOX} and p22\textsuperscript{phox} that normally takes place during oxidase activation.\textsuperscript{39} This 39-amino acid peptide is very unusual in that it contains 10 arginines, 19 prolines, and no acidic residues. A role for this peptide in the regulation of oxidase activity has been proposed, but evidence supporting this proposal is scanty. It is true that exposure of neutrophils to PR-39 inhibits $O_2^-$ production in response to phorbol myristate acetate, but nonspecific toxicity of this peptide was not ruled out.

The Oxidase as a Battery

The phagocyte secretes $O_2^-$ as the anion, leaving behind the proton produced in the $O_2^-$-forming reaction. In accord with this finding are observations showing that the leukocyte NADPH oxidase is an electrogenic enzyme, and that its activation is associated with the opening of a channel through which the protons left behind can leave the cell before they shut down the enzyme.\textsuperscript{41,44} In a recent publication, the existence of an inward current associated with the activation of the oxidase has been directly measured by patch clamping,\textsuperscript{45} confirming earlier work by O.T.G. Jones and associates.\textsuperscript{41} In the discussion of their report, these investigators postulate that the electric current per se may be directly related to the function of the oxidase—ie, microbial killing. They further state that “the importance [of $O_2^-$] in microbial killing is unclear.” This statement is rather surprising in view of the very large body of evidence showing that this $O_2^-$ is the precursor of the powerful oxidants the phagocytes use as microbicidal agents.

Activation of the oxidase. During oxidase activation, the cytosolic oxidase subunits are transferred to the membrane, where they bind to the membrane-associated oxidase components (cytochrome b$_{558}$ and possibly Rap1A) to assemble the active enzyme. Translocation is preceded by the phosphorylation of certain oxidase-related proteins, some identified and some not. Low-molecular-weight guanine nucleotide binding proteins (GNBP) are also involved in oxidase activation, but the nature of their participation is unknown.

Protein-Protein Interactions Among the Oxidase Subunits

Examination of interactions between the various subunits of the oxidase is a very active field of investigation, and a remarkable number of studies on this subject have appeared in the published literature in the last few years.\textsuperscript{2,30,31,33,34,46-42} These studies have shown that in both the resting and active oxidases, the various subunits are associated with each other in the form of well-defined complexes. Resting cytosol contains an $\sim$250-kD complex of undetermined stoichiometry comprising the three oxidase subunits p47\textsuperscript{PHOX}, p67\textsuperscript{PHOX}, and p40\textsuperscript{PHOX}. Activation of the oxidase brings this cytosolic complex to the membrane, where it associates with cytochrome b$_{556}$, a membrane-bound dimer containing gp91\textsuperscript{PHOX} and p22\textsuperscript{phox} that in turn is probably interacting with Rap1A. A very large amount of effort has been expended in mapping the protein-protein interactions responsible for the formation of these complexes. An earlier review\textsuperscript{43} listed those protein-protein interactions that had been reported as of 1996. Since then, many more pairs of interactions have been mapped in whole or in part.

The major interactions among the cytosolic subunits that have been mapped to date are illustrated in Fig 4. The pairwise interactions themselves are well established, but the proposed allocation of the various interactions to the resting versus the activated state is strictly hypothetical, although it is based on evidence that rearrangements of the cytosolic complex take place during oxidase activation\textsuperscript{64,65} and on the identification of interactions that are required for translocation of the complex to the membrane. Of particular interest are the interactions between the C-terminal proline-rich SH3 binding domain of p47\textsuperscript{PHOX} and the SH3 domains of both p47\textsuperscript{PHOX} and p40\textsuperscript{PHOX} in the resting complex. The interaction of p47\textsuperscript{PHOX} with itself is thought to be responsible for the inability of resting p47\textsuperscript{PHOX} to bind to cytochrome b$_{556}$, the membrane-associated electron-
PHOX, the C-terminal proline-rich domain of p47^PHOX^ carrying component of the oxidase. During activation, however, the C-terminal proline-rich domain of p47^PHOX^ is occupied by the C-terminal SH3 domain of p67^PHOX^, an interaction that is required for translocation. This presumably means that this proline-rich domain separates from the p47^PHOX^ SH3 domain that it occupies in the resting state, and perhaps from the SH3 domain of p46^PHOX^ as well. Another interaction required for translocation is the one between the C-terminal SH3 domain of p47^PHOX^ and the N-terminal proline-rich domain of p67^PHOX^; in the diagram this is shown only in the activated complex, but it could be present in the resting complex as well. Finally, the N-terminal SH3 domain of p47^PHOX^, freed from its interaction with the distal p47^PHOX^ proline-rich domain, binds to the C-terminal portion of p67^PHOX^. But in the active oxidase, this p47^PHOX^ SH3 domain has to interact with p22^PHOX^. Maybe oxidase activation is a multi-step process, one step consisting of the rearrangements shown in the figure, and a subsequent step involving an exchange at the N-terminal SH3 domain of p47^PHOX^ in which the C-terminal portion of p67^PHOX^ is swapped for the proline-rich domain of p22^PHOX^. The possibility that oxidase activation is a multi-step process is also implied by experiments discussed below in the section on protein phosphorylation. These experiments suggest that oxidase activation can be dissected into three successive events: partial phosphorylation of p47^PHOX^, translocation of p47^PHOX^ and the other cytosolic oxidase components to the membrane, and then final phosphorylation of p47^PHOX^ coupled to the acquisition of catalytic activity by the enzyme. I must emphasize, however, that the foregoing discussion of the changes in protein-protein interactions undergone by the cytosolic complex during oxidase activation, though consistent with current findings, is pure speculation.

Although interactions among the cytosolic subunits, and between the cytosolic subunits and p22^PHOX^, are reasonably well defined, interacting domains between gp91^PHOX^ and the remainder of the oxidase subunits are uncertain. Phage display libraries have identified certain p47^PHOX^ peptides that recognize gp91^PHOX^ and the examination of hydrophobic domains^67^ has identified certain peptides in gp91^PHOX^ that could be important in oxidase activity. At relatively high concentrations, these peptides inhibit oxidase activity in the sodium dodecyl sulfate (SDS)-dependent cell-free oxidase activating system and in electrophorgeramnized neutrophils. However, peptide inhibition studies can be deceptive. Illustrating this is work carried out on a peptide from the C-terminus of gp91^PHOX^. For a number of years, an interaction involving seven residues near the C-terminus of gp91^PHOX^ (RGVHFIF) was thought to be of considerable functional significance because a peptide with this sequence was found to be a potent inhibitor of oxidase activity in an SDS-dependent cell-free oxidase activating system. Recently, however, it was shown that mutations in this sequence had no effect on oxidase activity in a gp91^PHOX^-deficient leukemia line; O2^- production by cells expressing these mutants was no different than O2^- production in cells expressing wild-type gp91^PHOX^~68~. This means either that this sequence is of no functional significance, or that its function can be replaced by some other element in the gp91^PHOX^ protein.

Nevertheless, this region of gp91^PHOX^ does appear to bind to p47^PHOX^, and the structure of a complex between p47^PHOX^ and the 17-mer gp91^PHOX^ 551-568 (the C-terminus of gp91^PHOX^, lacking only K569) was studied by two-dimensional nuclear magnetic resonance spectroscopy (2-D NMR).~56~ This study showed that the stretch of peptide from S55 to F564 could be clearly defined, showing an extended bend with immobilized side chains (except for H561). This is a novel approach to the study of interactions among the oxidase components.

**Phosphorylation**

Phosphorylation is an essential element in the activation of the NADPH oxidase. Phosphorylation of p47^PHOX^ during oxidase activation has been recognized for many years. More recently, the phosphorylation of p67^PHOX^ and p40^PHOX^ has been shown, and the phosphorylation of a component in the membrane has been implicated in the activation process.

The protein whose phosphorylation has been studied most thoroughly is p47^PHOX^~69~. During oxidase activation it is extensively phosphorylated, with 8 to 9 serines in the C-terminal quarter of the molecule acquiring phosphates. Of these target serines, S379 is the only one whose conversion to alanine as a sole mutation results in a major loss of oxidase activity.~69~ Phosphorylation of S379 is necessary for both the translocation of p47^PHOX^ and the activation of the oxidase. It has recently been found, however, that the mutation of both of a pair of target serines to alanine can inactivate p47^PHOX^. Serines S303 and S304 are known to be phosphorylated during oxidase activation. The mutation of this serine pair to alanines greatly decreases oxidase activity, though the phosphorylation of other serines and the translocation of the mutant p47^PHOX^ to the membrane is unaffected. Activity is normal, however, if these serines are replaced by glutamates, or, surprisingly, by lysines.~70~ The activity of p47^PHOX^ S303K,S304K mutant raises the possibility that p47^PHOX^ may in part be linked to the rest of the active oxidase by a cation bridge. Interestingly, the mutation to alanines of the MAP kinase target pair S345/S348 had no effect on the activity of the oxidase.~69,71~

A subsequence study showed another pair of serines, S359 and S370, whose phosphorylation was necessary for oxidase activation.~72~ The mutation of both these serines to alanines results not only in the loss of oxidase activity, but also in a complete failure of phosphorylation of p47^PHOX^, both recombinant and in whole cells. Replacement of those serines by glutamate or aspartate allows phosphorylation and translocation to take place, but oxidase activity is still greatly reduced. In contrast to the results obtained with S303K,S304K, the replacement of S359 and S370 with lysines led to the total loss of activity of the oxidase; p47^PHOX^-deficient cells expressing p47^PHOX^ S359K,S370K showed no more O2^- production than the untransfected p47^PHOX^-deficient cells. The conclusion drawn from these studies was that during oxidase activation, S359 and/or S370 has to be phosphorylated first; S379 then acquires a phosphate, allowing the cytosolic complex to translocate to cytochrome b558 and finally, S303 and/or S304 are phosphorylated, endowing the oxidase with full catalytic activity.

The phosphorylation of p47^PHOX^ is regulated by protein kinase A. It had been known for some time that oxidase activation is diminished in neutrophils containing elevated levels of cyclic adenosine monophosphate (cAMP) or cAMP analogs. It has now been shown that the phosphorylation of p47^PHOX^ in response to N-formylmethionylleucyl phenylalanine
NADPH oxidase is activated by kinases offers an opportunity is unknown. However, the nature of their participation is always an open question with any inhibitor, these kinase family. To the extent that these inhibitors are truly MAP kinase family, and p38, another member of the MAP acid–dependent protein kinase is postulated to account for those phosphorylations showing that only ~5% of the p67PHOX translocates to the membrane during oxidase activation. Inhibition of MAP kinase kinase by PD098059 prevents oxidase activation by either orposmonized zymosan or phorbol myristate acetate, while inhibition of p38 by SB203580 prevents oxidase activation by fMLP. MAP kinase kinase activates ERK, a member of the MAP kinase family, and p38, another member of the MAP kinase family. To the extent that these inhibitors are truly specific (always an open question with any inhibitor), these results imply that both ERK and p38 participate in the activation of the NADPH oxidase. However, the nature of their participation is unknown.

The recent development of cell-free systems in which the NADPH oxidase is activated by kinases offers an opportunity to study the signal transduction pathway(s) responsible for oxidase activation in a system that is at least partly defined. In one of these systems, oxidase activation requires both adenosine triphosphate (ATP) and phosphatidic acid, the latter an anionic detergent that is able to activate the oxidase by itself, although ATP doubles O2− production in that system. A phosphatidic acid–dependent protein kinase is postulated to account for the effects of various agents on oxidase activation. The other system uses no detergents, but requires the phosphorylation of both p47PHOX and a membrane component to activate the NADPH oxidase. The identity of the phosphorylated membrane component is currently under investigation.

With kinase near, is phosphatase remote? Not likely, but studies of phosphatases in relation to the leukocyte NADPH oxidase are lagging. Three recent studies, all using whole neutrophils and inhibitors, have implied that phosphatases are involved in the deactivation of activated NADPH oxidase. Two studies showed an enhancement of oxidase activity and O2− production in fMLP-activated neutrophils treated with okadaic acid or calyculin A, both of which are inhibitors of protein phosphatases 1 and 2A. The release of p47PHOX from the cytoskeleton of activated neutrophils in response to protein kinase inhibitors was prevented by both okadaic acid and calyculin A. This interesting study suggests that the activity of the oxidase is determined by the prevailing steady-state rates of protein phosphorylation and dephosphorylation, with increases in phosphorylation activating and increases in dephosphorylation deactivating the enzyme, lending further support to inferences that could be drawn from a large number of earlier studies on protein phosphorylation and oxidase activity.

Small Guanine Nucleotide Binding Proteins

Two low-molecular-weight guanine nucleotide binding proteins (G proteins) have been implicated in the function of the leukocyte NADPH oxidase: Rac2 (in mouse macrophages, Rac1) and Rap1A. Rac2, found chiefly in the cytosol of resting neutrophils, is a member of the Rho family of G proteins, a family known principally for its function in regulating the cytoskeleton. Rap1A, located in the membranes of resting neutrophils, is in the Ras family. Like Ras, it regulates cell proliferation, acting as an antagonist of Ras-dependent transformation.

Most of the recent work on low-molecular-weight G proteins and the NADPH oxidase has been performed on Rac2, probably because as a soluble protein, Rac2 is much easier to deal with experimentally than Rap1A. These studies focused on the protein-protein interactions that developed between Rac2 and other NADPH oxidase components when the oxidase was activated in a cell-free system using anionic detergents. Incubation of neutrophil membranes with p67PHOX-deficient cytosol under activating conditions had been shown to cause p47PHOX to be transferred to the membrane, but the reciprocal experiment with p47PHOX-deficient cytosol did not lead to the translocation of p67PHOX. A similar experiment but with recombinant proteins instead of whole cytosols, the same laboratory showed that Rac2 also failed to translocate. These findings suggest that during oxidase activation, the translocation of p47PHOX precedes the translocation of both p67PHOX and Rac2. Using chimeras between Rac1, which strongly activates the leukocyte NADPH oxidase, and CDC42hs, a Rho-family G protein that barely activates the oxidase, it was shown that residues 27 and 33 were particularly important for oxidase activation. Both Rac1 and Rac2 were shown to bind to p67PHOX but not to p47PHOX. Both the effector region (residues 26-45) and the insert region (residues 125-145) of Rac1 were important for oxidase activation by this G protein, but binding to p67PHOX was only affected by mutations in the effector region.

Rap1A was first implicated in the function of the leukocyte NADPH oxidase when it was found to copurify with cytochrome b558. Functional evidence for the participation of Rap1A in oxidase activation was not developed until much later, when it was shown using a transfected Epstein-Barr virus (EBV)-transformed B-lymphocyte system that both Rap1A and Rap1A were locked in the GDP-bound and GTP-bound conformations respectively, inhibited phorbol-induced production of O2−, although the wild-type protein had no effect. The observation that O2− production was inhibited by both “locked” conformations of Rap1A raises the possibility that the GTP-bound form carries the oxidase from “state 1” to “state 2” with concomitant hydrolysis of the GTP, while the GDP-bound form brings the oxidase back to “state 1” with the concomitant exchange of GDP for GTP. Nothing is known about the nature of these hypothetical states. Another ingredient in the mixture is the finding that Rap1A can be phosphorylated...
by protein kinase A, and that the phosphorylated form binds somewhat more weakly to cytochrome b558 than the unphosphorylated form. Protein kinase A is known to suppress the activity of the NADPH oxidase, and the foregoing observations suggest that the kinase-regulated interaction between cytochrome b558 and Rap1A may be of functional significance. On the other hand, the observations concerning the phosphorylation of Rap1A were made in an in vitro system; there is no evidence to date that Rap1A is phosphorylated in whole cells under any circumstances.

A curious observation is the recent demonstration that suppressing p120 Ras-GAP biosynthesis by an antisense oligonucleotide led to enhanced O2\(^{-}\) production in EBV-transformed B lymphocytes. Because p120 Ras-GAP binds to both Ras and Rap1A, the observed effect could be a result of its interaction with one or both of these G proteins. Another effect of p120 Ras-GAP, however, is to inhibit phorbol-induced cellular events. This seems to be the likeliest explanation for the effect of the p120 Ras-GAP antisense oligonucleotide, because the increase in O2\(^{-}\) production was seen in phorbol-stimulated cells but not fMLP-stimulated cells.

The Cytoskeleton, Integrins, and Tyrosine Kinases

The role of the cytoskeleton in the function of the leukocyte NADPH oxidase has been recognized for some time. The two major pieces of evidence supporting this idea are: (1) the finding that in activated neutrophils, all the O2\(^{-}\) producing activity and portions of all the oxidase components are found in the cortical cytoskeleton; and (2) the demonstration that when neutrophils adhere to a surface, a process that involves integrins and is associated with far-reaching changes in the cytoskeleton, the time course of O2\(^{-}\) production becomes greatly altered compared with the time course of O2\(^{-}\) production in suspended cells. Recent studies have provided more details regarding the incompletely understood relationship between the cytoskeleton and the leukocyte NADPH oxidase. In adherent neutrophils, stimulation with tumor necrosis factor causes β2 integrins as well as the cytosolic oxidase components to move slowly from the Triton-soluble to the Triton-insoluble compartment, the latter generally regarded as representing the neutrophil cytoskeleton. O2\(^{-}\) is produced during this event, its time course resembling that of the migration of proteins to the cytoskeleton. A complicated series of steps involving a β2 integrin explains how antibodies and complement cooperate in the activation of the leukocyte NADPH oxidase. When the activated complement protein iC3b associates with the β2 integrin that serves as its receptor, the Ig receptor FcγII\(H\) becomes attached to the cortical cytoskeleton. The occupation of the FcγIII receptor then allows the tyrosine phosphorylation of the cytoskeletonally associated FcγII receptor to take place, triggering the signal transduction pathway that leads to the activation of the leukocyte NADPH oxidase.

The same laboratory that worked out antibody-complement cooperation has also identified the “leukocyte response integrin (LRI),” 96 This protein, which recognizes the basement membrane protein entactin, acts in association with another membrane protein, the “integrin-associated protein.” This combination of proteins activates the leukocyte NADPH oxidase when one of them—the LRI—interacts with entactin, or with the peptide KGAGDV (Fig 6). O2\(^{-}\) production activated by the LRI/integrin-associated protein (IAP) combination occurs in neutrophils deficient in CD18, the common subunit of the β2 integrins, ruling out the participation of these more traditional leukocyte integrins in the response of the neutrophil to the LRI/IAP system.

Associations between particular cytoskeletal proteins and the NADPH oxidase have also been described. The human counterpart of coronin, a protein important for motility in Dictyostelium, associates with p40PHOX and accumulates around phagocytic vesicles. 97 Cofilin loses its phosphate and moves to ruffled membranes when neutrophils are stimulated with fMLP. Oxidase components also migrate to the ruffled membranes under those conditions, and O2\(^{-}\) production is stimulated with kinetics that resemble the kinetics of cofilin dephosphorylation, implying the possibility of some connection between cofilin and the

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8 The Fcγ II and Fcγ III are Ig receptors that are expressed on the surfaces of phagocytes. Occupation of the Fcγ II receptor stimulates the oxidase, but occupation of the Fcγ III receptor alone inhibits oxidase activation.
oxidase. Stimulation of the oxidase in a detergent-activated cell-free system is augmented by G actin.

Finally, some very interesting studies relating neutrophil tyrosine kinases to the activation of the NADPH oxidase have recently appeared from Berton’s laboratory. In their initial studies, this group showed that tumor necrosis factor, which activates the NADPH oxidase in adherent cells but not in suspended cells, caused the Src family tyrosine kinase p58fgr to move from the cytosol to the cytoskeleton. Neutrophils from knockout mice deficient in both p58fgr and p59/61hck, another tyrosine kinase in the Src family, were unable to produce O$_2^-$ in the course of their attachment to surfaces coated with collagen or fibronectin, though O$_2^-$ production in response to immune complexes and phorbol myristate acetate was normal. These results indicate that the trigger responsible for the adhesion-dependent activation of fgr and hck, and their subsequent actions on the oxidase, was β$_2$ integrin. Conversely, reactive oxidizing species were found to be required for the activation of the tyrosine kinases p58fgr and p53/56lyn that takes place when neutrophils adhere to surfaces, a result which indicates that at least to some extent, the activation of these kinases is a bootstrap operation with autocatalytic features.

CGD. CGD is an inherited immune deficiency in which phagocytes from affected patients are unable to manufacture O$_2^-$. All cases so far have been found to result from a deficiency of one of four oxidase-specific proteins: p47$^{PHOX}$, p67$^{PHOX}$, p22$^{PHOX}$, or gp91$^{PHOX}$. Two groups of investigators have cured CGD in vivo by genetic methods, but only in mice. Knockout mice lacking gp91$^{PHOX}$ were transplanted with their own marrow cells after transplanation with a murine stem cell virus vector that expressed the missing protein. O$_2^-$ was expressed by neutrophils from these transplanted mice, though at levels lower than seen in wild-type mice. Despite the fact that neutrophil O$_2^-$ production was only partly corrected in the transplanted mice, these mice withstood a challenge with Aspergillus fumigatus that produced pneumonia in all the untransplanted CGD mice. Similar results were obtained in p47$^{PHOX}$ knockout mice that were transplanted with stem cells transfected with retroviral vectors that expressed p47$^{PHOX}$ except that in these mice the challenge was with Burkholderia cepacia, a typical pathogen of patients with CGD, though normally its activities are confined to onions.

**NADPH OXIDASE IN NONPHAGOCYTES**

Like all other biological macromolecules and macromolecular assemblies, the leukocyte NADPH oxidase was created in the course of evolution. In considering its possible origin, it was hard to believe that an enzyme this lethal could have arisen de novo. With this in mind, it was speculated that the oxidase might have arisen through the mutation of a more ancient NADPH oxidase of much lower activity whose tissue distribution was much more widespread than that of the lethal leukocyte oxidase and whose principal function was to provide oxidants for signaling purposes. Recent findings have confirmed this speculation, showing clearly that a low-activity NADPH oxidase is present in a variety of nonphagocytic cells, most of which are derived from the embryonic mesoderm, and that this oxidase is a source of second messengers.

An NADPH oxidase similar to the one found in phagocytes has been reported to occur in all three layers of the aorta. O$_2^-$ was generated in endothelial cell sonicates to which NADPH oxidase was added. In addition, mRNA for the four specific oxidase subunits and protein for the two cytosolic subunits were found, but no heme protein was detected by spectroscopy. Human aortic smooth-muscle cells produced O$_2^-$ in response to platelet-derived growth factor (PDGF). This O$_2^-$ production was inhibitable by diphenylene iodonium, suggesting that a phagocyte-like NADPH oxidase was responsible for its production. In these cells, the activation of NF-κB by PDGF was dependent on O$_2^-$, but its activation by interleukin-1β was not. In fibro-
blasts from aortic adventitia, O$_2^-$ production occurred constitutively.$^{100}$ Its rate of production was increased by angiotensin II$^{100,111}$ but not by norepinephrine.$^{111}$ It was postulated that the O$_2^-$ generated by the aorta was functioning as a blood pressure regulator by consuming nitric oxide, a well-known hypotensive agent with which it reacts at a diffusion-limited rate.$^{110,111}$

In joint tissues, O$_2^-$ production has been detected in synovocytes (both type A and type B)$^{106}$ and in chondrocytes.$^{112}$ In synovocytes, O$_2^-$ production is induced by phorbol myristate acetate, suggesting that it is generated by an NADPH oxidase. In chondrocytes, O$_2^-$ production is elicited by a calcium ionophore, but not by phorbol myristate acetate. In these cells O$_2^-$ production is inhibited by diphenylene iodonium, and mRNA for p22$^{PHOX}$, p40$^{PHOX}$, and p47$^{PHOX}$ (or their homologs) was detected by reverse transcriptase-polymerase chain reaction, relatively strong evidence that O$_2^-$ from chondrocytes is produced by an NADPH oxidase like that in phagocytes.

There is evidence that NADPH oxidases serve as components of oxygen sensors in various tissues. Erythropoietin production in some hepatoma lines appears to be regulated by O$_2^-$, and p22$^{PHOX}$, the α-subunit of cytochrome b$_{558}$, has been detected immunologically in renal peritubular fibroblasts and in Ito cells of the liver, both of which may be sources of erythropoietin.$^{113}$ In the lung, pulmonary neuroepithelial bodies have been proposed as airway oxygen sensors.$^{114}$ The cells of these organs contain mRNAs corresponding to a subunit of H$_2$O$_2$-regulated potassium channel as well as messages for p22$^{PHOX}$ and gp91$^{PHOX}$, the two membrane-associated oxidase subunits, or their homologs. H$_2$O$_2$ production by the neuroepithelial body cells was constitutive, but was stimulated by phorbol myristate acetate. In these cells O$_2^-$ or H$_2$O$_2$ production by diphenylene iodonium (Inhibition of O$_2^-$ or H$_2$O$_2$ production by diphenylene iodonium is generally regarded as presumptive evidence for an NADPH oxidase.) Furthermore, the K$^+$ current in these cells increased when the cells were exposed to H$_2$O$_2$. These results suggest that the cells contain an NADPH oxidase whose output is a function of the ambient oxygen tension and whose dismuted product (ie, H$_2$O$_2$) regulates the flow of current through the K$^+$ channel, the latter representing the signal by which the oxygen tension is communicated to the rest of the organism.

Plants contain an NADPH oxidase that they use for host defense. Cells from suspension cultures of Arabidopsis thaliana produced O$_2^-$ when activated with phorbol myristate acetate. Harpin, a bacterial elicitor protein,$^{115}$ had the same effect, eliciting the production of O$_2^-$ from the cultured cells. The Arabidopsis cells were found to contain proteins that were recognized by antibodies against human p47$^{PHOX}$ and p67$^{PHOX}$, and membranes from the plant produced O$_2^-$ when incubated with human neutrophil cytosol under activating conditions.

Finally, a b-type cytochrome homologous to human gp91$^{PHOX}$ was discovered in yeast.$^{116}$ This protein is an iron reductase that contains a low-potential heme and sequences homologous to flavin-binding sequences in other proteins. Binding of the heme to the apoprotein requires the presence of four essential histidine residues, because the conversion of any of these essential histidines to alanine resulted in a protein with no heme spectrum.$^{117}$ This protein, the product of the yeast FRE1 gene, participates in iron uptake by the yeast.

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NADPH Oxidase: An Update

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