

## REVIEW ARTICLE

## NADPH Oxidase: An Update

By Bernard M. Babior

**T**HE NADPH OXIDASES are a group of plasma membrane-associated enzymes found in a variety of cells of mesodermal 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:



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 neutrophils, 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<sup>PHOX</sup> (PHOX for PHagocyte OXidase), p47<sup>PHOX</sup>, p67<sup>PHOX</sup>, p22<sup>PHOX</sup> and gp91<sup>PHOX</sup>. In the resting cell, three of these five components—p40<sup>PHOX</sup>, p47<sup>PHOX</sup> and p67<sup>PHOX</sup>—exist in the cytosol as a complex. The other two components—p22<sup>PHOX</sup> and gp91<sup>PHOX</sup>—are located in the membranes of secretory vesicles\* and specific granules, where they occur as a heterodimeric flavohemoprotein known as cytochrome b<sub>558</sub>. 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<sup>PHOX</sup> becomes heavily phosphorylated and the entire cytosolic complex migrates to the membrane, where it associates with cytochrome b<sub>558</sub> to assemble 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

group(s). Activation requires the participation, not only of the core subunits, but of two low-molecular-weight guanine nucleotide-binding proteins: Rac2, which in the resting cell is located in the cytoplasm in a dimeric complex with Rho-GDI (Guanine nucleotide Dissociation Inhibitor), and Rap1A, which is located in membranes from which it can be copurified with the cytochrome. During activation, Rac2 binds guanosine triphosphate (GTP) and migrates to the membrane along with the core cytosolic complex. At the same time, cytochrome b<sub>558</sub> and Rap1A are delivered to the cell surface by fusion of the secretory vesicle membranes and later the specific granule membranes with the plasma membrane of the cell. This fusion event also releases the contents of the organelles to the exterior. When phagocytosis takes place, the plasma membrane is internalized as the wall of the phagocytic vesicle, with what was once the outer membrane surface now facing the interior of the vesicle. From this location, the enzyme pours  $O_2^-$  into the vesicle, and the rapid conversion of this  $O_2^-$  into its successor products bathes the internalized target in a lethal mixture of corrosive oxidants.

The leukocyte NADPH oxidase continues to be the object of a considerable amount of investigation by scientists who are curious as to how it works. Interest in the leukocyte oxidase has increased with the growing recognition that oxidases closely related to the leukocyte oxidase can be found in a variety of cells in which the enzymes serve a variety of purposes. The following is a review of some of the new information obtained through these recent studies.

## THE LEUKOCYTE NADPH OXIDASE

*Properties and Functions of the Oxidase Components*

**Cytochrome b<sub>558</sub>.** A question that has been under study for some time has been the exact composition of the cytochrome b<sub>558</sub> molecule: how many subunits of each kind does the cytochrome contain, and how many prosthetic groups? The most recent answers are that the cytochrome is a heterodimer containing one of each kind of subunit,<sup>1</sup> and that it contains one FAD and two hemes.<sup>2</sup> The two heme groups on the flavocytochrome are functionally distinct, with midpoint potentials at pH 7.0 of  $-225$  and  $-265$  mV.<sup>3</sup> In the enzyme-bound FAD, a corner of the benzene ring of the electron-carrying isoalloxazine group is exposed to the solvent,<sup>2</sup> suggesting that this corner is where the electron enters or leaves.

New information has appeared concerning the redox properties of cytochrome b<sub>558</sub>. The kinetics of electron flow through the flavocytochrome have been redetermined in a cell-free system containing purified cytochrome b<sub>558</sub> and recombinant cytosolic components. The turnover number† for the system was  $165 \text{ heme}^{-1} \text{ s}^{-1}$ , while the rate of oxidation of reduced heme was  $1,720 \text{ s}^{-1}$ .<sup>4</sup> Assuming that the concentration of cytochrome b<sub>558</sub> in neutrophils corresponds to  $10 \text{ pmol heme}/10^6 \text{ cells}^5$  and that all the cytochrome b<sub>558</sub> participates in  $O_2^-$

\*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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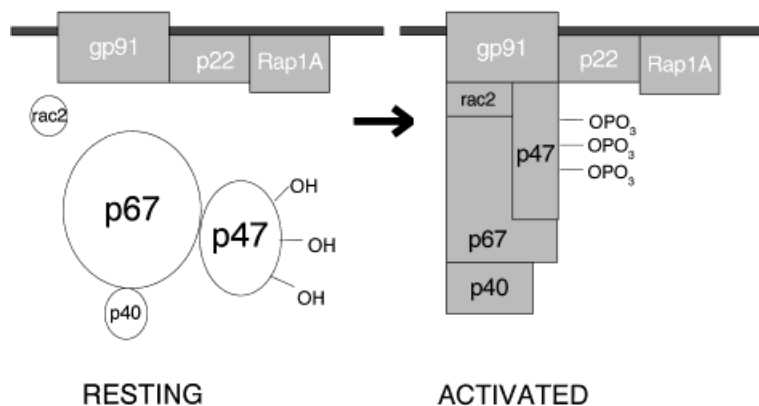
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†The turnover number is the number of molecules of product made each second by one molecule of enzyme.

**Fig 1.** Activation of the leukocyte NADPH oxidase. In the resting cell, the subunits of the oxidase are distributed between the cytosol ( $p40^{PHOX}$ ,  $p47^{PHOX}$ ,  $p67^{PHOX}$  and Rac2) and the membranes (Rap1A and cytochrome  $b_{558}$ , a  $p22^{PHOX} \cdot gp91^{PHOX}$  complex). Rac2 and Rap1A are low-molecular-weight guanine nucleotide-binding proteins that function in other processes besides oxidase activation. The other 5 proteins are unique to the NADPH oxidase. When the cell is activated,  $p47^{PHOX}$  becomes heavily phosphorylated and the cytosolic subunits migrate to the membrane, where they bind to cytochrome  $b_{558}$  to assemble the active oxidase.



production, the observed turnover number is equivalent to a rate of  $O_2^-$  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  $O_2^-$  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<sup>6</sup> (see also ref 7). Moreover, earlier studies under anaerobic 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  $O_2^-$  production by the identical systems operating in air.<sup>8,9</sup> Because a multistep reaction can go no faster than its slowest step, the rate of  $O_2^-$  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,<sup>10</sup> 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.<sup>11</sup> The oxidase is inhibited by flavin antagonists such as deaza-FAD<sup>12</sup> and diphenylene iodonium<sup>13</sup> (in contrast to the lack of effect of heme antagonists such as  $CN^-$ ,  $N_3^-$ , CO, and butyl isonitrile<sup>14</sup>). In model systems,  $O_2^-$  is produced by the reaction between oxygen and reduced flavins. Finally, recent experiments demonstrating that under special conditions purified cytochrome  $b_{558}$  alone catalyzes  $O_2^-$  production from NADPH and oxygen<sup>15,16</sup> 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.<sup>17</sup> Recently, iodonitrotetrazolium violet (INT) was added to the list of oxidants that could accept electrons from the oxidase.<sup>18,19</sup> 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.<sup>19</sup> INT reduction persisted under anaerobic conditions, indicating that the dye accepted electrons directly from the enzyme.

Chronic granulomatous disease (CGD) is an inherited disorder characterized by the failure of  $O_2^-$  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,<sup>20</sup> E309K, C537R, P339H, and  $\Delta$ K315<sup>21</sup> and  $gp91^{PHOX}$   $\Delta$ F215 or 216.<sup>22</sup> Cells containing  $gp91^{PHOX}$   $\Delta$ F215 or 216 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                 |
| $gp91^{PHOX}$   | R54S                 |
| $gp91^{PHOX}$   | E309K                |
| $gp91^{PHOX}$   | C537R                |
| $gp91^{PHOX}$   | P339H                |
| $gp91^{PHOX}$   | QKT(507-509)HIWA     |
| $gp91^{PHOX}$   | $\Delta$ F215 or 216 |
| $p22^{PHOX}$    | P156Q                |
| $p67^{PHOX}$    | $\Delta$ K58         |

residues are essential for the binding of the cofactors to the cytochrome.

#### Cytosolic Components

p47<sup>PHOX</sup> 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<sup>PHOX</sup> are unable to transfer p67<sup>PHOX</sup> from the cytosol to the membrane during activation, although p67<sup>PHOX</sup>-deficient neutrophils transfer p47<sup>PHOX</sup> in a normal fashion.<sup>23</sup> Before the cytosolic oxidase components can be transferred to the membrane, however, p47<sup>PHOX</sup> must be extensively phosphorylated. This phosphorylation is one of the characteristic events of oxidase activation. p47<sup>PHOX</sup>, however, is not absolutely indispensable for oxidase activity. Even though its deficiency results in one form of CGD, two groups have now shown that in a detergent-activated cell-free system containing purified cytochrome b<sub>558</sub> and recombinant cytosolic factors, p47<sup>PHOX</sup> can be omitted as long as the system contains high concentrations of p67<sup>PHOX</sup> and Rac.<sup>24,25</sup> The effect of p47<sup>PHOX</sup> is to tighten by nearly 100-fold the binding of each of the other cytosolic proteins to the assembled oxidase.

The function of p67<sup>PHOX</sup> has been a mystery. Unlike p47<sup>PHOX</sup>, p67<sup>PHOX</sup> is absolutely required for oxidase activity. When incubated with membranes in the detergent-activated cell-free system, a cytosol lacking p47<sup>PHOX</sup> but containing p67<sup>PHOX</sup> was able to support INT reduction.<sup>18</sup> Furthermore, p67<sup>PHOX</sup> facilitated electron transfer to the flavin of cytochrome b<sub>558</sub> in the absence of p47<sup>PHOX</sup><sup>26</sup>; when p47<sup>PHOX</sup> was also present, the system was said to allow electron transfer "to proceed beyond the flavin center to the heme in cytochrome b<sub>-245</sub>‡ 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<sup>PHOX</sup> in oxidase activity by showing that this subunit contained a catalytically essential binding site for NADPH.<sup>27</sup> However, there is evidence that an NADPH binding site also exists on cytochrome b<sub>558</sub>.<sup>28,29</sup> The relationship between these two NADPH binding sites remains to be determined, though it should be noted that cytochrome b<sub>558</sub> alone catalyzes active O<sub>2</sub><sup>-</sup> production using NADPH as the reductant,<sup>16</sup> while catalysis of NADPH dehydrogenation by p67<sup>PHOX</sup> has to date not been demonstrated.

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

The function of p40<sup>PHOX</sup> has recently been examined.<sup>31</sup> Oxidase activity can be established in K562 cells by transfecting them with plasmids that express recombinant p47<sup>PHOX</sup>, p67<sup>PHOX</sup>, and gp91<sup>PHOX</sup>.<sup>32</sup> Cells that express p40<sup>PHOX</sup> along with other recombinant oxidase components produce only about half the amount of O<sub>2</sub><sup>-</sup> generated by cells not expressing p40<sup>PHOX</sup>.

‡Cytochrome b<sub>-245</sub> is the name uniquely given to cytochrome b<sub>558</sub> by investigators from a particular laboratory.

Similarly, adding p40<sup>PHOX</sup> to a cell-free detergent-dependent oxidase activating system inhibits O<sub>2</sub><sup>-</sup> production. These results suggest that p40<sup>PHOX</sup> is an inhibitory oxidase subunit. On the other hand, interfering with the binding of p40<sup>PHOX</sup> to p67<sup>PHOX</sup> reduces by 50% the production of O<sub>2</sub><sup>-</sup> in a detergent-dependent cell-free system, a result implying that p40<sup>PHOX</sup> is a stimulatory subunit.<sup>33</sup> 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<sup>34</sup> (Fig 2). In a study of exceptional interest, Kobayashi et al<sup>35</sup> used cytochemical staining to show that O<sub>2</sub><sup>-</sup> 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<sub>2</sub><sup>-</sup>-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<sup>36</sup>), 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

Sulfhydryl groups are important for the function of the leukocyte NADPH oxidase. Recently, naturally occurring sulf-

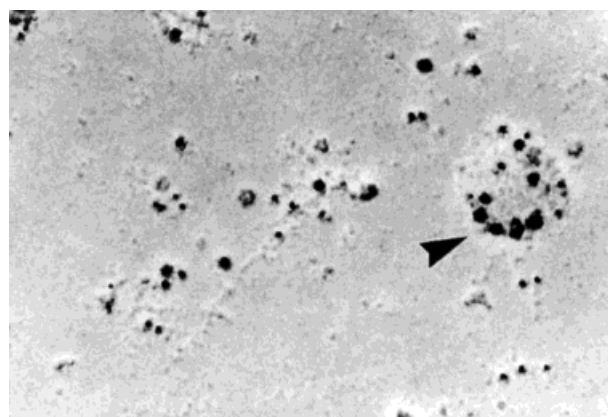


Fig 2. Localization of the oxidase to the neutrophil plasma membrane.<sup>34</sup> Normal neutrophils were activated and unroofed, and the cytoplasmic face of the plasma membrane was labeled by the immunogold technique with anti-p67<sup>PHOX</sup> (large beads) and anti-gp91<sup>PHOX</sup> (small beads). The oxidase assemblies are gathered together in small clusters. Similar results were obtained with anti-p67<sup>PHOX</sup>/anti-p47<sup>PHOX</sup> and anti-p67<sup>PHOX</sup>/anti-Rac antibody pairs. (Reprinted with permission.<sup>34</sup>)

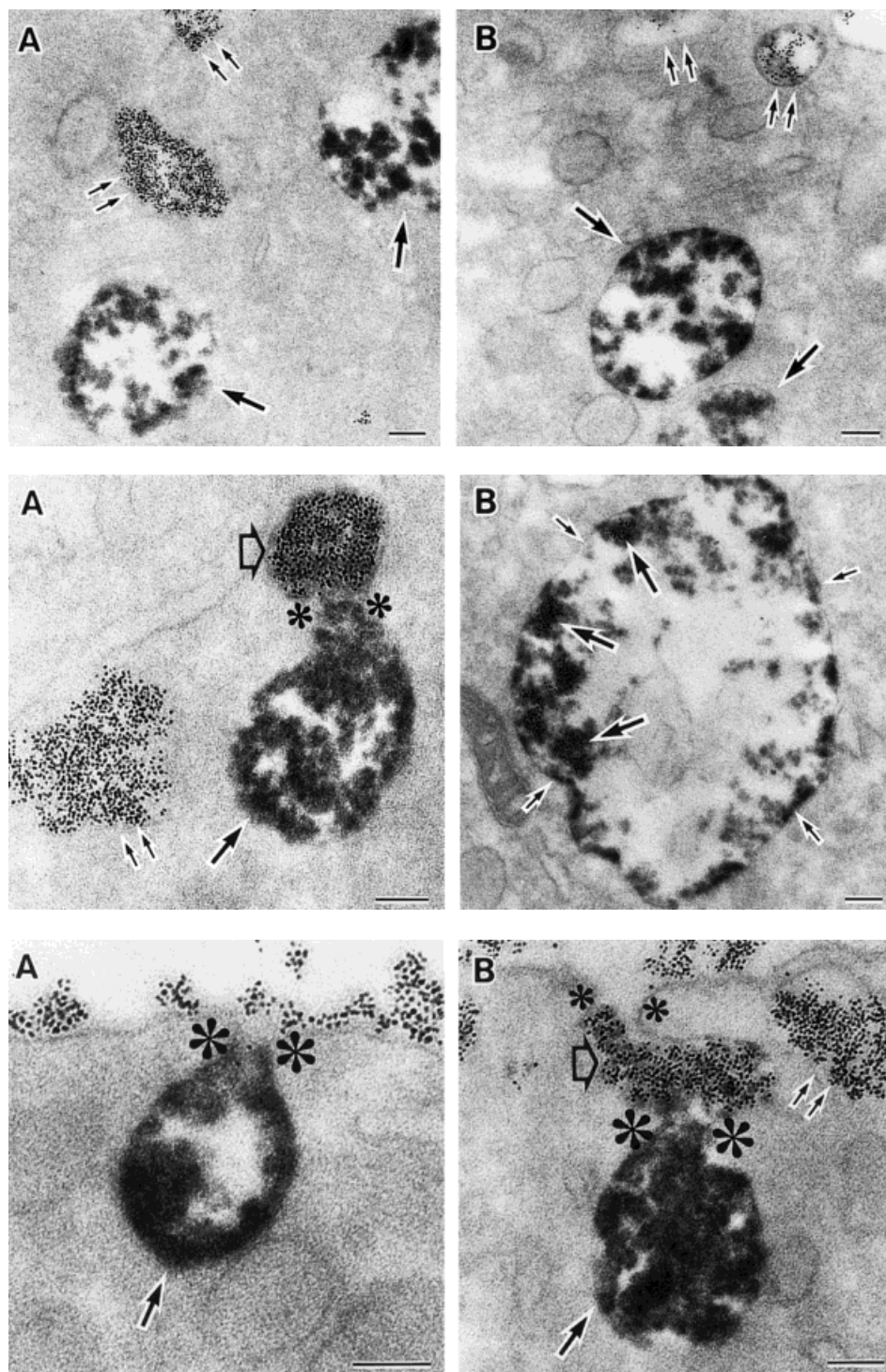


Fig 3. Sites of  $O_2^-$  production in neutrophils as a function of time after activation.<sup>35</sup> (Top)  $O_2^-$  production starts in secretory vesicles (clumped precipitate; single arrow). The membranes of these vesicles are distinct from the plasma membrane, because none of the  $O_2^-$ -forming vesicles contain the ferritin (punctate precipitate, double arrow) that was added to the extracellular medium during the incubation and then was internalized by pinocytosis. (Center) The  $O_2^-$ -forming secretory vesicles later fuse with pinocytotic vesicles to form secondary vesicles. The oxidase in the membranes of these secondary vesicles continue to deliver  $O_2^-$  into the vesicle lumina. (Bottom)  $O_2^-$ -forming secretory vesicles also fuse with the plasma membrane itself, leading to the secretion of  $O_2^-$  into the extracellular environment. (Reprinted with permission from Kobayashi T, Robinson JM, Seguchi H: Identification of intracellular sites of superoxide production in stimulated neutrophils. *J Cell Sci* 111:81, 1998, published by The Company of Biologists, Ltd, Cambridge, UK.<sup>35</sup>)

hydride blockers have been found to inhibit the oxidase. The aldehyde 4-hydroxynonenal inhibited the oxidase with an  $I_{50}$  of  $19 \mu\text{mol/L}$ .<sup>37</sup> 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 ( $\text{NO}$ ) also inhibits the oxidase, but it works by preventing the assembly of the oxidase during activation.<sup>38</sup> It has no effect on the fully active oxidase, and does not interact with either the flavin or the hemes on cytochrome  $b_{558}$ .

Nitrosothiols (RSNO) also inhibit oxidase activation, preventing translocation of the cytosolic oxidase components p47<sup>PHOX</sup> and p67<sup>PHOX</sup> through an action on the membrane.<sup>39</sup> The effect of nitrosothiols can be reversed by mercaptoethanol. It is likely that both NO<sup>•</sup> 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<sup>PHOX</sup>, thereby blocking the interaction between p47<sup>PHOX</sup> and p22<sup>PHOX</sup> that normally takes place during oxidase activation.<sup>40</sup> 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<sub>2</sub><sup>-</sup> 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<sub>2</sub><sup>-</sup> as the anion, leaving behind the proton produced in the O<sub>2</sub><sup>-</sup>-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.<sup>41-44</sup> In a recent publication, the existence of an inward current associated with the activation of the oxidase has been directly measured by patch clamping,<sup>45</sup> confirming earlier work by O.T.G. Jones and associates.<sup>41</sup> 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<sub>2</sub><sup>-</sup>] in microbial killing is unclear.” This statement is rather surprising in view of the very large body of evidence showing that this O<sub>2</sub><sup>-</sup> 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<sub>558</sub> 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.<sup>2,30,31,33,46-62</sup> 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 ~250-kD complex of undetermined stoichiometry comprising the three oxidase subunits p47<sup>PHOX</sup>, p67<sup>PHOX</sup>, and p40<sup>PHOX</sup>. Activation of the oxidase brings this cytosolic complex to the membrane, where it associates with cytochrome b<sub>558</sub>, a membrane-bound

dimer containing gp91<sup>PHOX</sup> and p22<sup>PHOX</sup> 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<sup>63</sup> 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<sup>64,65</sup> 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<sup>PHOX</sup> and the SH3 domains of both p47<sup>PHOX</sup> and p40<sup>PHOX</sup> in the resting complex. The interaction of p47<sup>PHOX</sup> with itself is thought to be responsible for the inability of resting p47<sup>PHOX</sup> to bind to cytochrome b<sub>558</sub>, the membrane-associated electron-

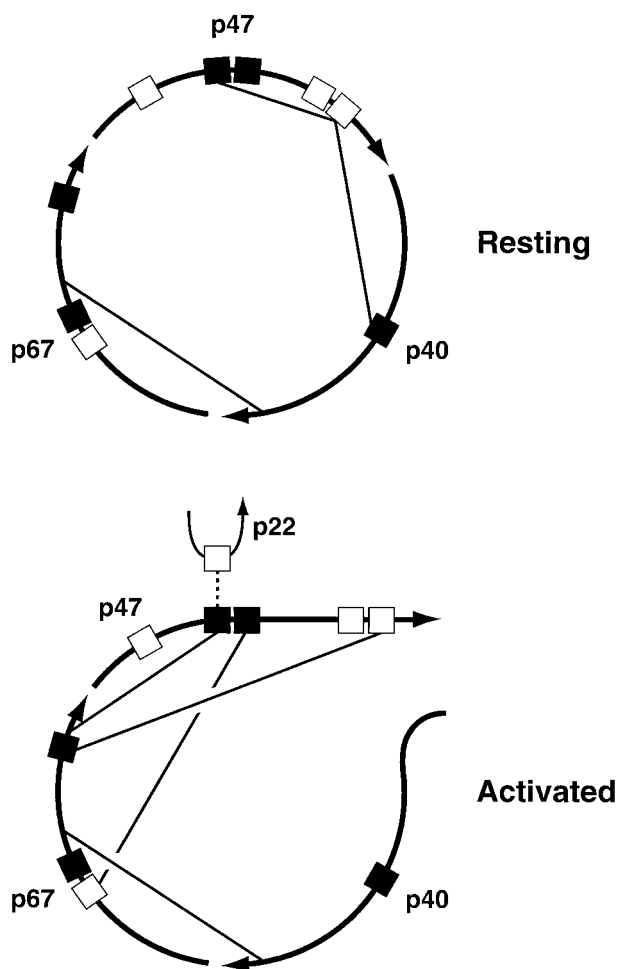


Fig 4. Proposed protein-protein interactions among the cytosolic components of the leukocyte NADPH oxidase in the resting and activated states. For an explanation of the figure, see text. (■), SH3 domains; (□), proline-rich SH3 binding domains.

carrying component of the oxidase. During activation, however, the C-terminal proline-rich domain of p47<sup>PHOX</sup> is occupied by the C-terminal SH3 domain of p67<sup>PHOX</sup>, an interaction that is required for translocation. This presumably means that this proline-rich domain separates from the p47<sup>PHOX</sup> SH3 domain that it occupies in the resting state, and perhaps from the SH3 domain of p40<sup>PHOX</sup> as well. Another interaction required for translocation is the one between the C-terminal SH3 domain of p47<sup>PHOX</sup> and the N-terminal proline-rich domain of p67<sup>PHOX</sup>; 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<sup>PHOX</sup>, freed from its interaction with the distal p47<sup>PHOX</sup> proline-rich domain, binds to the C-terminal portion of p67<sup>PHOX</sup>. But in the active oxidase, this p47<sup>PHOX</sup> SH3 domain has to interact with p22<sup>PHOX</sup>. 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<sup>PHOX</sup> in which the C-terminal portion of p67<sup>PHOX</sup> is swapped for the proline-rich domain of p22<sup>PHOX</sup>. 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<sup>PHOX</sup>, translocation of p47<sup>PHOX</sup> and the other cytosolic oxidase components to the membrane, and then final phosphorylation of p47<sup>PHOX</sup> 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<sup>PHOX</sup>, are reasonably well defined, interacting domains between gp91<sup>PHOX</sup> and the remainder of the oxidase subunits are uncertain. Phage display libraries have identified certain p47<sup>PHOX</sup> peptides that recognize gp91<sup>PHOX</sup>,<sup>66</sup> and the examination of hydrophobic domains<sup>67</sup> has identified certain peptides in gp91<sup>PHOX</sup> 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 electroporated neutrophils. However, peptide inhibition studies can be deceptive. Illustrating this is work carried out on a peptide from the C-terminus of gp91<sup>PHOX</sup>. For a number of years, an interaction involving seven residues near the C-terminus of gp91<sup>PHOX</sup> (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<sup>PHOX</sup>-deficient leukemia line; O<sub>2</sub><sup>-</sup> production by cells expressing these mutants was no different than O<sub>2</sub><sup>-</sup> production in cells expressing wild-type gp91<sup>PHOX</sup>.<sup>68</sup> 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<sup>PHOX</sup> protein.

Nevertheless, this region of gp91<sup>PHOX</sup> does appear to bind to p47<sup>PHOX</sup>, and the structure of a complex between p47<sup>PHOX</sup> and the 17-mer gp91<sup>PHOX</sup> 551-568 (the C-terminus of gp91<sup>PHOX</sup>,

lacking only K569) was studied by two-dimensional nuclear magnetic resonance spectroscopy (2-D NMR).<sup>56</sup> This study showed that the stretch of peptide from S555 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<sup>PHOX</sup> during oxidase activation has been recognized for many years. More recently, the phosphorylation of p67<sup>PHOX</sup> and p40<sup>PHOX</sup> 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<sup>PHOX</sup>. 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.<sup>69</sup> Phosphorylation of S379 is necessary for both the translocation of p47<sup>PHOX</sup> 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<sup>PHOX</sup>. 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<sup>PHOX</sup> to the membrane is unaffected. Activity is normal, however, if these serines are replaced by glutamates, or, surprisingly, by lysines.<sup>70</sup> The activity of p47<sup>PHOX</sup> S303K,S304K mutant raises the possibility that p47<sup>PHOX</sup> 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.<sup>69,71</sup>

A subsequent study showed another pair of serines, S359 and S370, whose phosphorylation was necessary for oxidase activation.<sup>72</sup> 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<sup>PHOX</sup>, 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<sup>PHOX</sup>-deficient cells expressing p47<sup>PHOX</sup> S359K,S370K showed no more O<sub>2</sub><sup>-</sup> production than the untransfected p47<sup>PHOX</sup>-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 b<sub>558</sub>; and finally, S303 and/or S304 are phosphorylated, endowing the oxidase with full catalytic activity.

The phosphorylation of p47<sup>PHOX</sup> 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<sup>PHOX</sup> in response to N-formylmethionylleucyl phenylalanine

(fMLP) is inhibited by cAMP agonists, and that this effect is prevented by an inhibitor of protein kinase A. In contrast, cAMP agonists had no effect on p47<sup>PHOX</sup> phosphorylation induced by phorbol myristate acetate, an activator of protein kinase C.<sup>73</sup>

Recently, both p40<sup>PHOX</sup> and p67<sup>PHOX</sup> were found to take up phosphate when neutrophils are stimulated.<sup>74,75</sup> It was shown that p40<sup>PHOX</sup> is phosphorylated in the resting cell, takes up additional phosphate when the NADPH oxidase is activated, and loses the additional phosphate when the oxidase is deactivated. Both p40<sup>PHOX</sup> and p67<sup>PHOX</sup> acquire phosphate when the cells are activated with either fMLP or phorbol; the latter suggests that protein kinase C participates in the phosphorylation of both oxidase components. On the basis of in vitro experiments, however, it was claimed that the phosphorylation of p40<sup>PHOX</sup> and p47<sup>PHOX</sup> involve distinct signal transduction pathways. In regard to the phosphorylation of p67<sup>PHOX</sup>, an earlier report<sup>76</sup> claimed that two 67K proteins are phosphorylated in activated neutrophils, but that neither one is p67<sup>PHOX</sup>; their results, however, do not rule out the possibility that a small amount of p67<sup>PHOX</sup> is phosphorylated during oxidase activation, a qualification that becomes important in the light of observations showing that only ~5% of the p67<sup>PHOX</sup> translocates to the membrane during oxidase activation.<sup>77,78</sup> Inhibition of MAP kinase kinase by PD098059 prevents oxidase activation by either opsonized zymosan or phorbol myristate acetate,<sup>79</sup> while inhibition of p38 by SB203580 prevents oxidase activation by fMLP.<sup>80</sup> 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<sup>81,82</sup> 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 O<sub>2</sub><sup>-</sup> production in that system. A phosphatidic acid-dependent protein kinase is postulated to account for those findings. The other system uses no detergents, but requires the phosphorylation of both p47<sup>PHOX</sup> 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 O<sub>2</sub><sup>-</sup> production in fMLP-activated neutrophils treated with okadaic acid<sup>83</sup> or calyculin A,<sup>84</sup> both of which are inhibitors of protein phosphatases 1 and 2A. The release of p47<sup>PHOX</sup> from the cytoskeleton of activated neutrophils in response to protein kinase inhibitors was prevented by both okadaic acid and calyculin A.<sup>85</sup> 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.<sup>86</sup>

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 p67<sup>PHOX</sup>-deficient cytosol under activating conditions had been shown to cause p47<sup>PHOX</sup> to be transferred to the membrane, but the reciprocal experiment with p47<sup>PHOX</sup>-deficient cytosol did not lead to the translocation of p67<sup>PHOX</sup>.<sup>87</sup> In a similar experiment but with recombinant proteins instead of whole cytosols, the same laboratory showed that Rac2 also failed to translocate.<sup>61</sup> These findings suggest that during oxidase activation, the translocation of p47<sup>PHOX</sup> precedes the translocation of both p67<sup>PHOX</sup> 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.<sup>88</sup> Both Rac1 and Rac2 were shown to bind to p67<sup>PHOX</sup> but not to p47<sup>PHOX</sup>. 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 p67<sup>PHOX</sup> was only affected by mutations in the effector region.<sup>2</sup>

Rap1A was first implicated in the function of the leukocyte NADPH oxidase when it was found to copurify with cytochrome b<sub>558</sub>. 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<sup>S17N</sup> and Rap1A<sup>Q63E</sup>, which are locked in the GDP-bound and GTP-bound conformations respectively, inhibited phorbol-induced production of O<sub>2</sub><sup>-</sup>, although the wild-type protein had no effect.<sup>89</sup> The observation that O<sub>2</sub><sup>-</sup> 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  $b_{558}$  than the unphosphorylated form.<sup>90</sup> 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  $b_{558}$  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<sup>Ras-GAP</sup> biosynthesis by an antisense oligonucleotide led to enhanced  $O_2^-$  production in EBV-transformed B lymphocytes.<sup>91</sup> Because p120<sup>Ras-GAP</sup> 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<sup>Ras-GAP</sup>, however, is to inhibit phorbol-induced cellular events.<sup>92</sup> This seems to be the likeliest explanation for the effect of the p120<sup>Ras-GAP</sup> antisense oligonucleotide, because the increase in  $O_2^-$  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  $O_2^-$  producing activity and portions of all the oxidase components are found in the cortical cytoskeleton<sup>77,93</sup>; 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  $O_2^-$  production becomes greatly altered compared with the time course of  $O_2^-$  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  $\beta_2$  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.<sup>94</sup>  $O_2^-$  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  $\beta_2$  integrin explains how antibodies and complement cooperate in the activation of the leukocyte NADPH oxidase.<sup>95</sup> When the activated complement protein iC3b associates with the  $\beta_2$  integrin that serves as its receptor, the Ig receptor Fc $\gamma$ II $\beta$  becomes attached to the cortical cytoskeleton. The occupation of the Fc $\gamma$ III receptor then allows the tyrosine phosphorylation of the cytoskeletonally associated Fc $\gamma$ II receptor to take place, triggering the signal transduction pathway that leads to the activation of the leukocyte NADPH oxidase (Fig 5).

The same laboratory that worked out antibody-complement

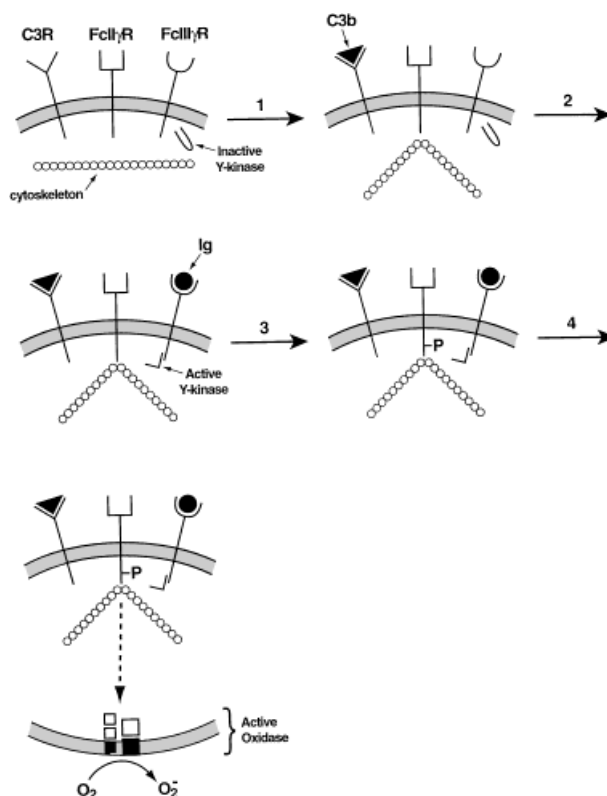


Fig 5. Mechanism of collaboration of the C3 receptor (a  $\beta_2$  integrin) and the Fc $\gamma$ III receptor in the activation of the leukocyte NADPH oxidase.<sup>95</sup> In the resting membrane, the C3, FcII $\gamma$ , and FcIII $\gamma$  receptors are empty. (1) Occupancy of the C3 receptor causes the FcII $\gamma$  receptor to bind to the cytoskeleton. (2) Occupancy of the FcIII $\gamma$  receptor activates a tyrosine kinase. (3) The activated tyrosine kinase phosphorylates the cytoplasmic portion of the FcII $\gamma$  receptor, which was rendered susceptible to phosphorylation through its interaction with the cytoskeleton. (4) The phosphorylated FcII $\gamma$  receptor activates the oxidase via a multistep signal transduction pathway.

cooperation has also identified the “leukocyte response integrin (LRI).”<sup>96</sup> 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).  $O_2^-$  production activated by the LRI/integrin-associated protein (IAP) combination occurs in neutrophils deficient in CD18, the common subunit of the  $\beta_2$  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 p40<sup>PHOX</sup> and accumulates around phagocytic vesicles.<sup>97</sup> *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  $O_2^-$  production is stimulated with kinetics that resemble the kinetics of cofilin dephosphorylation, implying the possibility of some connection between cofilin and the

§The Fc $\gamma$ II and Fc $\gamma$ III are Ig receptors that are expressed on the surfaces of phagocytes. Occupation of the Fc $\gamma$ II receptor stimulates the oxidase, but occupation of the Fc $\gamma$ III receptor alone inhibits oxidase activation.



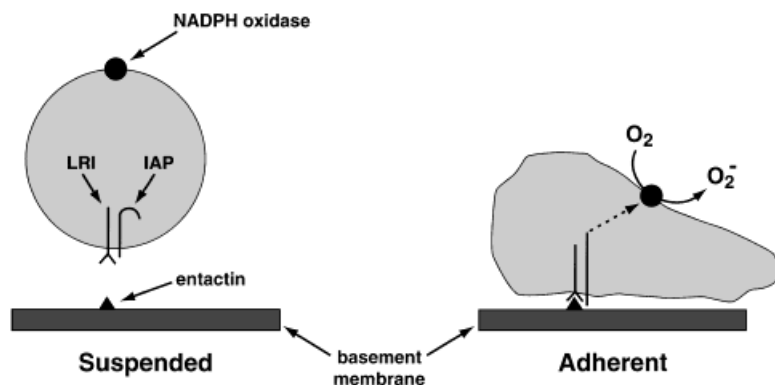


Fig 6. Provisional mechanism for the activation of the NADPH oxidase by the LRI. The LRI and the IAP are thought to form a complex in the leukocyte plasma membrane. When leukocytes are in suspension, the signal transduction pathway between this complex and the NADPH oxidase is blocked. The blockade is lifted by the following combination of events: (1) the binding of LRI to entactin, a basement membrane protein; (2) adhesion of the phagocyte to a surface (in suspended cells, NADPH oxidase is not activated by the binding of LRI to entactin); and (3) possibly a conformational change in IAP. These events somehow activate tyrosine kinase C, which then activates the oxidase. Tyrosine phosphorylation does not participate in oxidase activation by LRI/IAP.<sup>96</sup>

oxidase.<sup>98</sup> Stimulation of the oxidase in a detergent-activated cell-free system is augmented by G actin.<sup>99</sup>

Finally, some very interesting studies relating neutrophil tyrosine kinases to the activation of the NADPH oxidase have recently appeared from Berton's laboratory.<sup>94,100,101</sup> 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  $\beta_2$  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 4 oxidase-specific proteins: p47<sup>PHOX</sup>, p67<sup>PHOX</sup>, p22<sup>PHOX</sup>, or gp91<sup>PHOX</sup>. Virtually all the recent work on the oxidase in CGD has been directed toward developing a genetic cure of the disease.

B lymphocytes and hematopoietic progenitors from patients with various forms of CGD have been cured by transfection with vectors expressing the oxidase component missing from the patients' cells. Various retroviral vectors have been used to correct CGD in cells from patients with the X-linked form of the disease, who are missing gp91<sup>PHOX</sup>. In the earliest study, the respiratory burst was restored in 15% of transfected human myeloid cells, with the active cells producing  $O_2^-$  at about 60% of the normal rate.<sup>102</sup> Using a different vector, the same group transfected a CGD myeloid line as well as marrow cells from a knockout mouse with gp91<sup>PHOX</sup> deficiency.<sup>103</sup> Results with the new vector were greatly superior to results obtained previously, with oxidase activity being largely to completely restored in both human and mouse cells. CD34<sup>+</sup> cells from a p67<sup>PHOX</sup>-deficient patient were cultured after transfection with a replication-defective amphotropic retrovirus that expressed p67<sup>PHOX</sup>.

$O_2^-$  production was restored in neutrophils arising from the transfected cells.<sup>104</sup>

Two groups of investigators have cured CGD in vivo by genetic methods, but only in mice. Knockout mice lacking gp91<sup>PHOX</sup> were transplanted with their own marrow cells after transfection with a murine stem cell virus vector that expressed the missing protein.<sup>105</sup>  $O_2^-$  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<sup>PHOX</sup> knockouts that were transplanted with stem cells transfected with retroviral vectors that expressed p47<sup>PHOX</sup>,<sup>106</sup> 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.<sup>107</sup> Recent findings have confirmed this speculation, showing clearly that a low-activity NADPH oxidase is present in a variety of nonphagocyte 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 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.<sup>108</sup> 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- $\kappa$ B by PDGF was dependent on  $O_2^-$ , but its activation by interleukin-1 $\beta$  was not.<sup>109</sup> In fibro-

blasts from aortic adventitia,  $O_2^-$  production occurred constitutively.<sup>110</sup> Its rate of production was increased by angiotensin II<sup>110,111</sup> but not by norepinephrine.<sup>111</sup> 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.<sup>110,111</sup>

In joint tissues,  $O_2^-$  production has been detected in synovio-cytes (both type A and type B)<sup>106</sup> and in chondrocytes.<sup>112</sup> In synovio-cytes,  $O_2^-$  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<sup>PHOX</sup>, p40<sup>PHOX</sup>, and p47<sup>PHOX</sup> (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<sup>PHOX</sup>, the  $\alpha$ -subunit of cytochrome b<sub>558</sub>, has been detected immunologically in renal peritubular fibroblasts and in Ito cells of the liver, both of which may be sources of erythropoietin.<sup>113</sup> In the lung, pulmonary neuroepithelial bodies have been proposed as airway oxygen sensors.<sup>114</sup> The cells of these organs contain mRNAs corresponding to a subunit of a H<sub>2</sub>O<sub>2</sub>-regulated potassium channel as well as messages for p22<sup>PHOX</sup> and gp91<sup>PHOX</sup>, the two membrane-associated oxidase subunits, or their homologs. H<sub>2</sub>O<sub>2</sub> production by the neuroepithelial body cells was constitutive, but was stimulated by phorbol myristate acetate and inhibited by diphenylene iodonium. (Inhibition of  $O_2^-$  or H<sub>2</sub>O<sub>2</sub> production by diphenylene iodonium is generally regarded as presumptive evidence for an NADPH oxidase.) Furthermore, the K<sup>+</sup> current in these cells increased when the cells were exposed to H<sub>2</sub>O<sub>2</sub>. These results suggest that the cells contain an NADPH oxidase whose output is a function of the ambient oxygen tension and whose dismutated product (ie, H<sub>2</sub>O<sub>2</sub>) regulates the flow of current through the K<sup>+</sup> 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,<sup>115</sup> 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<sup>PHOX</sup> and p67<sup>PHOX</sup>, 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<sup>PHOX</sup> was discovered in yeast.<sup>116</sup> 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.<sup>117</sup> This protein, the product of the yeast FRE1 gene, participates in iron uptake by the yeast.

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