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Forum Review

Modulation of the Cytosolic and Phagosomal pH by the NADPH Oxidase

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ABSTRACT

Proton (equivalents) are primary participants in the control and potency of the NADPH oxidase. Both the cytosolic and intraphagosomal pH are influenced during oxidase activation, and maintenance of the optimal environment requires the coordinated action of a series of sophisticated, highly regulated H^+ transporters, including the Na^+/H^+ exchange, vacuolar H^+-ATPase, and H^+-conductive pathway(s). In addition, protons that are produced during some of the NADPH oxidase reactions then are substrates for the dismutation of superoxide, which precedes production of additional bactericidal agents. In this review, pH homeostasis is shown in conjunction with the NADPH oxidase to present an integrated picture of leukocyte physiology during the phagocytic response. Antioxid. Redox Signal. 4, 61–68.

INTRODUCTION

THE NOX PROTEINS are a family of oxidases that were grouped based on their structural and functional similarities (6, 20). The leukocyte NADPH oxidase is the best described and understood of the NOX family members and, unlike other NOX proteins, it is expressed exclusively by professional phagocytes and B lymphocytes (6). The leukocyte oxidase is a membrane-bound enzymatic complex (see Fig. 1) that catalyzes the generation of superoxide anions (O_2^-), which play a central role in the microbicidal function of neutrophils and macrophages. This remarkable enzyme mediates the one-electron transfer from NADPH to molecular oxygen giving rise to superoxide. Superoxide in turn can be converted to other active oxygen species, including H_2O_2, which is formed by dismutation. O_2^- and H_2O_2 can further react with each other, through a reaction involving a redox-active metal such as iron, to generate hydroxyl radical (·OH), a highly reactive and very toxic oxidant. In addition, H_2O_2 can be converted into hypochlorous acid (HOCl), another effective antimicrobial agent. HOCl is generated by the action of myeloperoxidase, an enzyme that is secreted into the phagosomal lumen by exocytosis of primary granules. Jointly, this collection of active oxygen intermediates contribute to the effective killing of invading microorganisms (25).

The leukocyte NADPH oxidase is composed of at least six components that are required for its proper activity *in vivo* (for reviews, see 9, 30). In the resting state, the oxidase constituents are segregated into cytosolic and membrane-bound components. Two of these,

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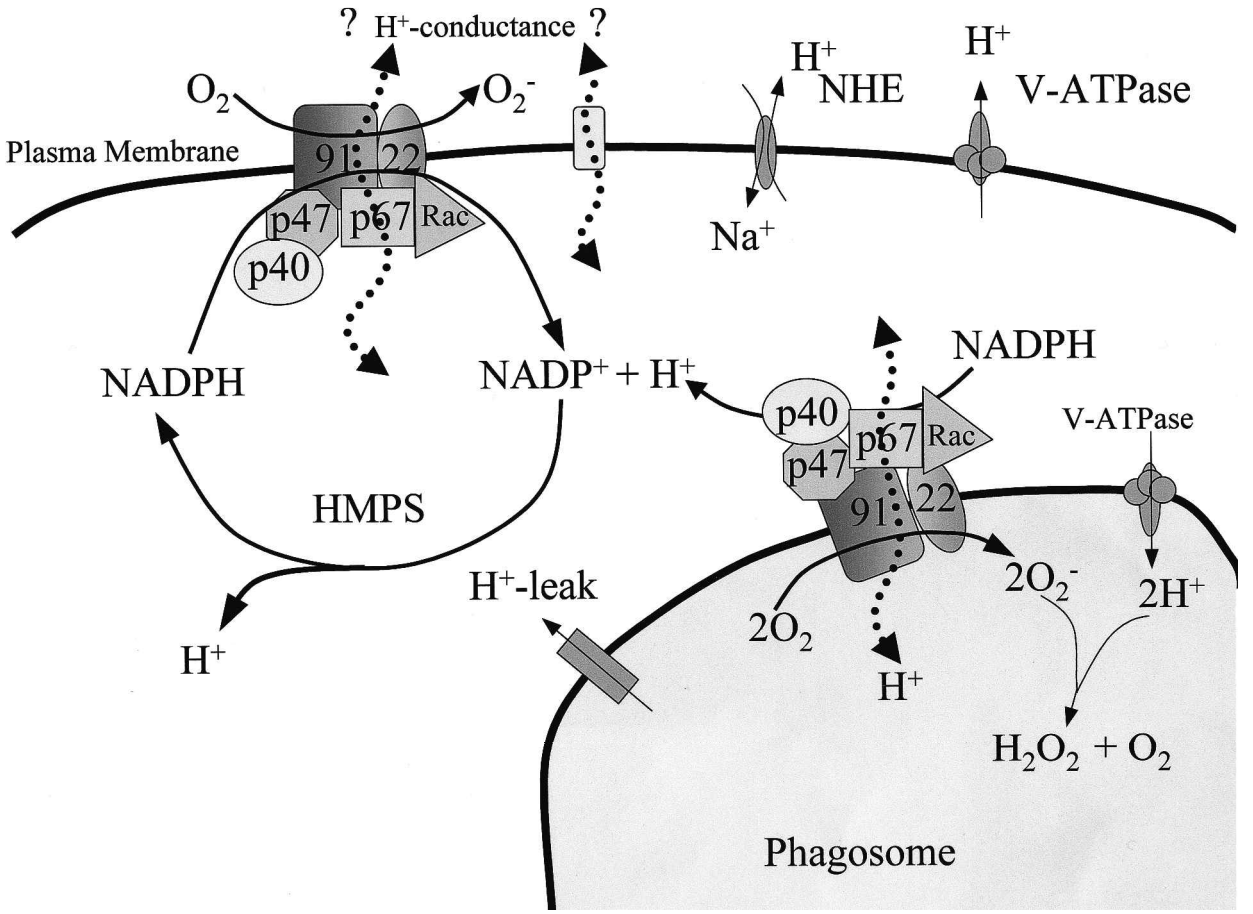

FIG. 1. Model of the regulatory mechanisms controlling cytosolic and phagosomal pH. gp91^{phox} and p22^{phox} are integral membrane proteins that in resting cells are present in the plasmalemma (10%), as well as in endomembrane compartments. There are four additional components—p40^{phox}, p47^{phox}, p67^{phox}, and Rac2—that reside in the cytosol. For the enzyme to become operational, the cytosolic components must translocate to the membrane and interact with the membrane-bound flavocytochrome. Upon NADPH oxidase activation, O_2^- is generated extracellularly (or in the phagosomal lumen), whereas H^+ is released into the cytosol during NADPH oxidation to $NADP^+$ and during resynthesis of NADPH by the hexose monophosphate shunt (HMPS). Excess cytosolic H^+ is extruded by the Na^+/H^+ exchanger (NHE), by active H^+ pumps (V-ATPase), and via a H^+ conductance that may involve gp91^{phox}. Inside the phagosome, H^+ are thought to be consumed by the dismutation of superoxide to generate H_2O_2. The V-ATPase is very active in the phagosomal membrane, and the specific H^+ conductance or nonspecific H^+ leak pathways may also function.

gp91^{phox} and p22^{phox}, are integral membrane proteins that in resting cells are present in the plasmalemma (10%), as well as in endomembrane compartments (90%), specifically secretory vesicles and secondary granules. This heterodimeric transmembrane unit, which forms the flavocytochrome b_{558}, functions as a docking site for the soluble components of the enzyme. There are four additional components—p40^{phox}, p47^{phox}, p67^{phox}, and Rac2—that reside in the cytosol. For the enzyme to become operational, the cytosolic components must translocate to the membrane and interact with the membrane-bound flavocytochrome. *In vitro* experiments suggest that NADPH oxidase activity can be reconstituted using only the membrane-bound p67^{phox} and Rac1/2. The presence of p47^{phox}, although not essential for activity, seemingly stabilizes the complex. The precise role of p40^{phox}, which is the least studied of the components, remains ambiguous as it was reported to either inhibit or stimulate the oxidase (7, 31). Another membrane factor, Rap1A, is thought to help in docking granules containing flavocytochrome b_{558} to the plasma or phagosomal membranes during activation (23). Several

studies suggest that activation of the enzyme can occur in endomembrane vesicles prior to fusion with the target membrane (15, 19).

The leukocyte oxidase effects the transfer of electrons from NADPH to molecular oxygen. In the process, $NADP^+$ and H^+ are liberated in the cytosol, whereas the reduced oxygen species are believed to form extracellularly and/or within the phagosome (Fig. 1), implying net transfer of electrical charge across the membrane. Indeed, numerous reports have documented the occurrence of a marked depolarization of the plasma membrane potential (15, 33). Using a novel procedure that estimates the rates of Mn^{2+} influx, we estimated that the membrane potential can reach +58 mV, indicative of a net change of >100 mV from the resting potential (15).

The importance of the leukocyte oxidase to the innate immune response is best illustrated by the clinical disorder resulting from its impairment, called chronic granulomatous disease (CGD). Patients suffering from CGD have impaired oxidase activity owing to the defective expression of one of the oxidase components. This results in increased susceptibility to recurrent bacterial and fungal infections (22). In the laboratory, this phenotype can be mimicked pharmacologically by treatment of phagocytes with diphenyleneiodonium (DPI), which binds to and blocks the NADPH oxidase. The depolarization noted upon activation of leukocytes is absent in CGD cells, as well as in DPI-treated neutrophils, confirming the involvement of the oxidase in the potential change.

THE NADPH OXIDASE AND CYTOSOLIC pH HOMEOSTASIS

Proton generation

As the turnover of the active NADPH oxidase is large (up to 0.3 nmol of $O_2^-/10^6$ cells/min) and because protons are involved at several stages of the reaction, pH is predicted to be an important factor in the activation and maintenance of the respiratory burst. Based on the stoichiometry of the reaction catalyzed by the enzyme (Eq. 1), the generation of O_2^- is expected to release net acid equivalents into the cytoplasm:

$$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+ \quad (1)$$

In addition, protons are also generated by related pathways. To sustain the oxidase reaction, NADPH must be continuously replenished. This is accomplished by the hexose monophosphate shunt. Regeneration of NADPH from $NADP^+$ by this pathway entails the oxidation of glucose 6-phosphate, which in turn releases additional H^+ (Eq. 2)

$$\text{Glucose 6-phosphate} + 12NADP^+ \rightarrow 12NADPH + P_i + 12H^+ + 6CO_2 \quad (2)$$

Lastly, the CO_2 produced by oxidation of hexose can then become hydrated, and the resulting H_2CO_3 will in turn dissociate, liberating further H^+. Because CO_2 can diffuse through most membranes, formation and dissociation of H_2CO_3 will occur both inside and outside the cell, and the reaction will thus contribute to the acidification of both the cytosol and/or the extracellular medium.

Based on the amount of superoxide produced during the respiratory burst, and taking into account the measured buffering capacity of the cytosol, it was calculated that the protons generated when the oxidase is activated for as little as 5 min would reduce the intracellular pH by as much as 0.33–0.66 pH units (11). It was further predicted that such a drop in pH would be absent in CGD patients or in DPI-treated cells.

Proton consumption

While protons are being generated in the cytosol of activated leukocytes, net proton consumption is likely to take place within the phagosome as a result of metabolism of oxidase products. In particular, the dismutation of superoxide (Eq. 3) will consume one proton per superoxide generated.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

Therefore, when considered in isolation, the activation of the oxidase tends to acidify

the cytosol and alkalinize the phagosomal lumen. The resulting large transmembrane pH gradient, together with the change in membrane potential, would give rise to an electrochemical gradient that is thermodynamically unfavorable to the continued operation of the oxidase, leading to its autoinhibition. Does such an inhibition in fact occur?

Observed pH changes during leukocyte activation

Contrary to the predicted massive cytosolic acidification, direct measurements using fluorescent dyes revealed that the cytosol of activated neutrophils undergoes a biphasic pH change: a small initial acidification followed within 1–2 min by a substantial sustained alkalinization (12). These observations prompted additional studies to define the underlying molecular mechanisms. These experiments unmasked the existence of at least three separate processes utilized by the cells to compensate for the excess metabolic acid generated by the oxidase: (a) Na^+/H^+ exchange (NHE), (b) the vacuolar (V-type) H^+-ATPase (V-ATPase), and (c) a proton-conductive pathway (Fig. 1).

NHE is the most robust of these pathways. It is a ubiquitous, electroneutral exchange system that, in the case of leukocytes, is not only activated by the excess protons generated by the oxidase, but also directly stimulated by the same agonists that trigger the respiratory burst. Inhibition of NHE by omission of Na^+ or by addition of inhibitors like amiloride precludes the alkalinization of the cytosol and in turn unmasks a sizable acidification, as anticipated from the metabolic generation of protons by the oxidase (12). The magnitude of the acidification is, however, lower than predicted on a theoretical basis, suggesting that other pH regulatory mechanisms may contribute to the observations.

The next H^+ extrusion mechanism to be identified was the H^+ pump or V-ATPase. Such ATPases are generally associated with organellar acidification, rather than with cytosolic pH homeostasis. However, in the unique case of leukocytes, a large number of endomembrane vesicles and granules are delivered via exocytosis to the plasma and phagosomal membranes, thereby delivering multiple copies of the V-ATPase. Active H^+ pumping across the plasma/phagosomal membrane not only tends to acidify the phagosomal lumen (see below), but also contributes to the net extrusion of H^+ from the cytosol, minimizing the metabolic acidification. Thus, dual inhibition of the NHE and of the V-ATPases results in a more profound acidification of the cytosol when the NADPH oxidase is stimulated (29).

That the NADPH oxidase and its attendant reactions are indeed responsible for the acidification was shown in two different ways. First, inhibition of the respiratory burst with DPI virtually eliminated the large acidification recorded when the pH regulatory pathways were inactivated (26). Secondly, the acidification was similarly absent in cells from CGD patients (12). In both instances, when no inhibitors were present, the secondary alkalinization attributed to stimulation of NHE appeared faster and was larger. It is also interesting that the hexose monophosphate shunt (Fig. 1) alone can produce cytosolic acidification. This was demonstrated in cells where the NADPH oxidase was not activated, but the shunt was stimulated by exposure to exogenous permeant oxidizing agents, such as methylene blue or phenazine methosulfate. In cells suspended in Na^+-free medium (from either normal or CGD donors), these oxidants induced a gradual cytoplasmic acidification (11), suggesting that at least part of the acidification observed in stimulated neutrophils is due to the conversion of $NADP^+$ back to NADPH.

H^+ conductance and the regulation of cytosolic pH

An additional pathway has been postulated to offer an efficient mechanism of H^+ extrusion from the cytosol and to provide charge compensation for the electrogenic activity of the NADPH oxidase. This pathway is a passive conductance, possibly mediated by a H^+ channel. A specific H^+ conductance was initially described in giant molluscan neurons and later experimentally characterized in human neutrophils, microglial cells, and osteoclasts (for review, see 21). The electrophys-

iological signature of the "channel" holds remarkable similarities across species. In all cases, the conductance is inactive at or below the resting membrane potential, but becomes detectable after a threshold voltage is achieved. The steady-state current–voltage curves of the H^+ conductance show marked outward rectification, consistent with a role in H^+ extrusion from cells (18). The activation of the conductance is sensitive to the concentration of H^+ in both the cytosolic and extracellular spaces, as demonstrated initially by Byerly and colleagues (4). As the intracellular pH is lowered, the threshold voltage for activation of the conductance is shifted to more negative potentials. The opposite was noted when the external pH was lowered. In this case, the voltage dependence was shifted toward more positive potentials. Because of these properties, it is likely that H^+ binds both a cytosolic site and an extracellular site on the transporter to modify its activation properties. The conductance in all systems is inhibited by heavy metal ions, particularly Zn^{2+} and Cd^{2+} (3, 4, 8, 34). This inhibition is rapid and occurs at micromolar concentrations, and is readily reversible by washing out the metals. The block is apparently mediated by shifting the threshold activation voltage to more positive potentials, decreasing the maximal conductance and slowing its rate of activation (3, 4). Arachidonic acid has the opposite effect on the H^+ conductance, namely activation of proton fluxes by accelerating the rate of activation of the conductance, accomplished by shifting the threshold of activation to more negative voltages.

The molecular identity of the H^+-conductive pathway remains controversial. The $gp91^{phox}$ subunit of the NADPH oxidase has been postulated to function as the channel. In support of this notion, it is argued that both the NADPH oxidase and H^+ conductance are activated by similar agonists, including chemoattractants and phorbol esters. Moreover, both develop in parallel during differentiation of HL-60 cells into granulocytes. More convincingly, heterologous expression of $gp91^{phox}$ in Chinese hamster ovary cells is correlated with the appearance of a H^+-conductive pathway (14).

On the other hand, many cells that do not express $gp91^{phox}$ or other components of the phagocyte NADPH oxidase display robust H^+ currents with properties virtually indistinguishable from those of leukocytes (16, 17, 27, 28). Also, CGD monocytes lacking $gp91^{phox}$ have clearly detectable H^+ currents. The activation of the conductance in CGD cells is somewhat altered, which suggests that the oxidase may be a modulator of the conductance, rather than the conductive pathway itself.

As mentioned in the Introduction, $gp91^{phox}$ is but one of a family of related proteins called NOX. Some members of the NOX family have been shown to generate superoxide radicals in a variety of cells, although with much lower efficiencies than the phagocyte NADPH oxidase. A splice variant of NOX1, NOX-1S, has been shown to induce the appearance of voltage-gated H^+ currents when transfected into HEK293 cells (24). This new information suggests that the H^+ conductance observed in nonphagocytic cells that do not express NADPH oxidase might originate from one or more NOX homologues of $gp91^{phox}$. Based on these findings, Krause and colleagues (1, 2, 24) have speculated that phagocytes may have two types of proton channels: $gp91^{phox}$ and another member of the NOX family. They argue that the resting proton conductance, analogous to the one observed in cells expressing NOX-1S or in X91 CGD patients, activates slowly and deactivates rapidly, allowing proton extrusion and repolarization. In favor of this concept, NOX-1S mRNA has been shown to be expressed in HL-60 cells. In addition, Krause and colleagues (1, 24) suggest that the agonist (phorbol ester or arachidonate)-activated conductance, which is absent in CGD patients, is mediated by $gp91^{phox}$. The latter activates rapidly, inactivates slowly, and can mediate both H^+ influx and efflux.

Regardless of the precise molecular nature of the pathway, it is clear that conductive H^+ efflux occurring during the course of activation of the oxidase would not only alleviate the cytosolic acidification, but would also counteract the depolarization, favoring the continued formation of superoxide.

THE NADPH OXIDASE AND PHAGOSOMAL pH (pH_p) HOMEOSTASIS

During the course of the response to infection, the major site of activation of the leukocyte NADPH oxidase is the phagosome. The membrane, of nascent phagosomes displays a composition similar to that of the plasma membrane, and the phagosomal contents are initially like the extracellular milieu. As such, the phagosome is unsuitable to prevent the growth of pathogens. To acquire their microbicidal properties, phagosomes need to mature through a series of fusion/fission reactions with cellular endomembranes, which lead to the remodeling of the phagocytic membrane and to changes in the composition of the phagosomal lumen. One of the essential initial events is the recruitment of additional NADPH oxidases by fusion with granules and vesicles, as detailed above.

Another critical component acquired by the phagosomal membrane during maturation is the V-ATPase, which is generally thought to mediate phagosomal acidification (Fig. 1). Such acidification is felt to be required for optimal microbicidal activity, potentiating the effects of lytic enzymes, cationic peptides, etc. Accordingly, in macrophages the pH of the phagosomal lumen has been repeatedly reported to be acidic, ranging between 5.0 and 6.0 (13). It can be demonstrated that the V-ATPase is responsible for the observed acidification, because specific inhibitors of this H^+ pump, such as bafilomycin or the concanamycins, rapidly and completely dissipate the pH gradient.

In view of the consistent reports that phagosomes are very acidic in macrophages, it is striking that in the other major phagocytic cell type, namely neutrophils, no such acidification of pH_p was detected. Several reports found that in neutrophils pH_p undergoes a biphasic change: an alkalinization occurs during the first few minutes, which is then followed by a variable, slowly developing secondary acidification (5, 32). It is unclear whether the apparent discrepancy between macrophages and neutrophils resulted from differences in the methodology used or whether pH_p is truly regulated differently in these two cell types.

The source of the initial alkalosis seen by some in neutrophils is not fully understood. Segal *et al.* (32) proposed that it results from the metabolic consumption of protons within the phagosome through the dismutation of superoxide, as described above in Eq. 3. The ongoing consumption of protons by this process would offset the acidifying effects of the V-ATPase. As the NADPH oxidase activity eventually decreases, the V-ATPase predominates and the phagosome acidifies, although at a rate and to an extent lower than those seen in macrophages, which have much less oxidase activity. Consistent with this notion, in neutrophils from patients lacking active NADPH oxidase, pH_p acidifies at rates comparable to those seen in macrophages (13, 32).

The contribution of other H^+-transporting pathways to the regulation of pH_p has not been systematically considered. In particular, the possible role of the H^+ conductance has been overlooked. Although the conductance was originally detected on the plasma membrane, there is every reason to believe that it exists also in phagosomes, not only because their membrane originates from the plasma membrane during internalization, but because phagosomes are uniquely enriched in $gp91^{phox}$. If present and active in phagosomes, this "channel" could facilitate leakage of H^+ equivalents out of the phagosomes and could explain the failure of neutrophil phagosomes to acidify fully. This interpretation would apply only if the conductance is bidirectional and not outward-rectifying (1, 2, 24). If only the rectifying species is active, the conductance would serve to neutralize the electrical gradient, but instead of dissipating the pH gradient, it would contribute to luminal acidification.

The failure of phagosomes to acidify in the case of neutrophils may also be due to an unrelated "leak" pathway. The highly reactive milieu of the lumen could conceivably render the phagosomal membrane more permeable to small ions (including H^+), dissipating the pH gradient. In fact, the oxidative environment may even impair the activity of the V-ATPase, or the fusion of the granules containing the V-ATPase with the phagosome. In support of the former, the V-ATPase is known

to be susceptible to inactivation by oxidation of some of its sulfhydryl moieties (10). Regarding impairment of fusion by products of the oxidase, Segal and his collaborators (32) observed a two- to fourfold increase in the amount of granular contents delivered to the phagosomes of CGD patients.

One or more of these proposed mechanisms may account for the failure of phagosomes to acidify in neutrophils and would resolve the apparent inconsistencies reported between macrophages and neutrophils.

CONCLUDING REMARKS

Proton (equivalents) are central players in the control and effectiveness of the NADPH oxidase. Not only are they intrinsic products of some of the reaction steps and essential substrates for the dismutation of superoxide, but they also play a role in the electrical neutralization required for continued production of reactive oxygen intermediates. As a consequence of the activity of the oxidase, both the cytosolic and intraphagosomal pH are challenged, and maintenance of the optimal conditions requires the coordinated action of a series of sophisticated, highly regulated H^+ transporters, including the NHE, V-ATPase, and H^+-conductive pathway(s). Thus, pH homeostasis must always be considered in conjunction with the NADPH oxidase to produce an integrated picture of leukocyte physiology during the phagocytic response.

ABBREVIATIONS

CGD, chronic granulomatous disease; DPI, diphenyleneiodonium; NHE, Na^+/H^+ exchange; pH_p, phagosomal pH; V-ATPase, vacuolar H^+-ATPase.

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