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FORUM REVIEW ARTICLE

New Insights into the Regulation of Neutrophil NADPH Oxidase Activity in the Phagosome: A Focus on the Role of Lipid and Ca^{2+} Signaling

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Abstract

Significance: Reactive oxygen species, produced by the phagosomal NADPH oxidase of neutrophils, play a significant physiological role during normal defense. Their role is not only to kill invading pathogens, but also to act as modulators of global physiological functions of phagosomes. Given the importance of NADPH oxidase in the immune system, its activity has to be decisively controlled by distinctive mechanisms to ensure appropriate regulation at the phagosome. ***Recent Advances:*** Here, we describe the signal transduction pathways that regulate phagosomal NADPH oxidase in neutrophils, with an emphasis on the role of lipid metabolism and intracellular Ca^{2+} mobilization. ***Critical Issues:*** The potential involvement of Ca^{2+}-binding S100A8 and S100A9 proteins, known to interact with the plasma membrane NADPH oxidase, is also considered. ***Future Directions:*** Recent technical progress in advanced live imaging microscopy will permit to focus more accurately on phagosomal rather than plasma membrane NADPH oxidase regulation during neutrophil phagocytosis. *Antioxid. Redox Signal*. 00, 000–000.

Introduction to the Phagosomal NADPH Oxidase

PHAGOCYTOSIS, A HALLMARK of cellular innate immunity, permits the complete enclosure of pathogens within the phagosome and their subsequent destruction through an impressive arsenal of antimicrobial proteins and products. One of the major, and best studied, host defense systems is the phagocyte NADPH oxidase. For a prolonged period, there has been limited progress in the understanding of phagocytosis in neutrophils. This condition is mostly the result of technical constraints, such as the difficulty of transfecting neutrophils. Much of the current state of knowledge is based on studies performed on macrophages that have been extrapolated to neutrophils despite the profound differences between the two cell types.

Particulate stimuli can mediate phagocytosis using different types of receptors, including FcγR (binding IgG-opsonized particles), CR (recognizing particles opsonized by complement) (28, 50), and a set of glycoconjugate receptors (interacting with bacterial lectins) (60). Engulfment and ingestion of particles are accompanied by phagosome-granule fusion before or concomitantly with the localized activation of the NADPH oxidase on the phagosomal membrane and the subsequent release of reactive oxygen species (ROS) in the lumen of the phagosome that ultimately destroy the invading microbes (5).

NADPH oxidase is a multicomponent enzyme system that is composed of two integral membrane-associated proteins and several cytosolic proteins. In the resting state, $gp91^{phox}$ (also referred to as Nox2) is associated with $p22^{phox}$ at the membrane of specific granules and, to a lesser extent, at the secretory vesicles, gelatinase granules, and the plasma membrane (102). Both proteins constitute the heterodimeric flavocytochrome b_{558} that contains, at the cytosolic side, a putative NADPH binding site, FAD, and two heme groups which are required to transfer electrons from NADPH located in the cytosol to molecular oxygen across the phagosomal membrane. Phagosomes that contain engulfed particles rapidly acquire cytochrome b_{558} that has been released by specific and gelatinase granule membrane fusion to the phagosome (58). The activity of cytochrome b_{558} is regulated by interactions between the cytosolic proteins, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$, and the small GTP-binding protein Rac1/2. On stimulation by an opsonized particle, the cytosolic subunits are recruited to the phagosomal membrane where they assemble with the cytochrome b_{558} to form the active NADPH oxidase (5, 6). Sustained phagosomal NADPH oxidase activity requires a continuous translocation of cytosolic components to the phagosome (120). The control of NADPH assembly and activation in nonphagosomal intracellular membranes is beyond the scope of the present review and has been discussed elsewhere (11, 119).

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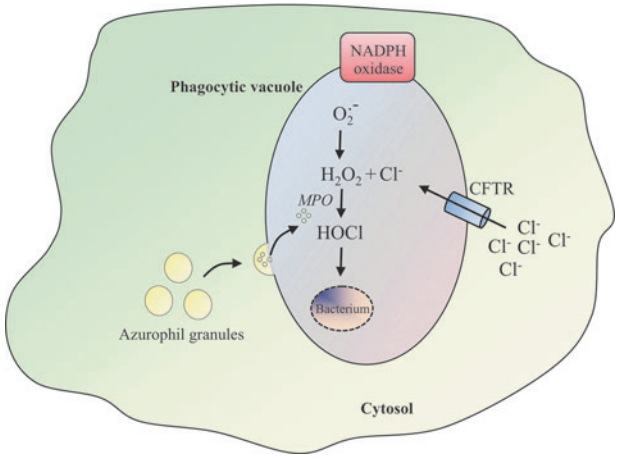

FIG. 1. Intraphagosomal reactive oxygen species (ROS) production. Initiation of phagocytosis triggers the recruitment of the cystic fibrosis transmembrane conductance regulator (CFTR) into the phagosomal membrane, allowing for an increase of chloride anion (Cl^-) transport into the phagosome for the support of HOCl generation (83). Superoxide anion ($O_2^{\cdot-}$) generated inside the neutrophil phagosome by NADPH oxidase is converted to hydrogen peroxide (H_2O_2). The reaction between H_2O_2 and Cl^- is catalyzed by myeloperoxidase (MPO), which generates hypochlorous acid (HOCl) in the proximity of the ingested microbes. MPO is released after fusion of the azurophil granules with the phagosome during phagocytosis. Dysfunction of the CFTR channel in the CF neutrophil phagolysosomal membrane is expected to limit the concentration of Cl^- in the phagosomal compartment and result in inadequate microbial killing (82).

Superoxide anion radicals ($O_2^{\cdot-}$), generated by the NADPH oxidase inside the phagosome, have a very low bactericidal potency and rapidly dismutate into hydrogen peroxide (H_2O_2). H_2O_2 is then further converted into even more toxic compounds. The reaction between H_2O_2 and chloride ions (Cl^-) generates hypochlorous acid (HOCl), which is considered the major oxidative weapon used by phagocytes. The formation of HOCl is catalyzed by myeloperoxidase (MPO), which is released after fusion of primary azurophil granules with the phagosome in proximity of the ingested pathogens (Fig. 1) (78). The physiological importance of NADPH oxidase-generated ROS in host defense is illustrated by a severe immunodeficiency called chronic granulomatous disease (CGD). The CGD immunodeficiency in which defective killing of phagocytosed pathogens caused by NADPH oxidase dysfunction results from mutations in any of the five NADPH oxidase component genes (Table 1) (12, 24, 48, 72, 109) leads to a predisposition to recurrent bacterial and fungal infections.

The role of the phagocyte NADPH oxidase is not only limited to the direct destruction of phagocytosed microbes *via* ROS production. Intraphagosomal luminal pH variation is stringently controlled by NADPH oxidase activity and drastically differs among neutrophils as compared with the well-known gradual acidification observed in macrophages (51). Indeed, luminal pH remains almost neutral for several minutes, after which the mature phagosomes eventually acidify. The acidification that is potentially established by the action of the vacuolar-ATPase delivered by fusion of neutrophil granules to the phagosomal membrane which pumps protons into the lumen of the phagosome is required for the optimal activity of proteases and hydrolases involved in pathogen killing (33).

Regulation of the phagosomal NADPH oxidase during phagocytosis is a complex and not completely understood process. The involvement of different signaling pathways is likely to vary depending on the types of receptors that are engaged (Table 2) (39, 50, 79) and the size of target particles. In this review, we consider the recent progress underlying phagosomal NADPH oxidase activation at the molecular level. We focus on the most-widely studied model of FcγR and CR3-dependent ingestion by neutrophils, which rely heavily on ROS production to mediate antimicrobial defenses within the phagosome. This review summarizes the upstream signaling pathways that lead to downstream phagosomal NADPH oxidase activation. In particular, those mechanisms by which Ca^{2+} and phospholipid metabolism might modulate NADPH oxidase activity are discussed. Since conclusions regarding the regulation of the plasma membrane NADPH oxidase may be extrapolated to its regulation at the phagosomal membrane, we also discuss recent findings that highlight not only the role of these two signaling pathways but also additional regulatory events.

Regulation of the Phagosomal NADPH Oxidase Activity

The activation of the NADPH oxidase is tightly regulated by reversible protein–protein and protein–lipid interactions. When phagocytes encounter foreign particles, binding to cell-surface receptors triggers the engagement of multiple signal transduction pathways that are involved in the stimulation of protein kinases/phospholipases. Activation of these enzymes leads to the production of lipid second messengers and Ca^{2+} release, which ultimately progress to the formation of a functional NADPH oxidase complex on the phagosomal membrane (5). Current understanding of the phagocytic-coupled activation of the NADPH oxidase is mostly based on experiments of phagocytosis in macrophage-like cells [for

Table 1. Mutations in the NADPH Oxidase Subunit Genes Involved in Chronic Granulomatous Disease

Chromosome	*Gene*	*Protein*	*Inheritance*	*Type*	*Frequency*
Xp21.1	CYBB	$gp91^{phox}$	X-linked	$X91^{\circ}/X91^{-}/X91^{+}$	~60%
16q24	CYBA	$p22^{phox}$	autosomal recessive	$A22^{\circ}/A22^{+}$	~5%
7q11.23	NCF1	$p47^{phox}$	autosomal recessive	$A47^{\circ}$	~30%
1q25	NCF2	$p67^{phox}$	autosomal recessive	$A67^{\circ}/A67^{-}$	~5%
22q13	NCF4	$p40^{phox}$	autosomal recessive	first case described in 2009 (72)	

$^{+}$for normal subunit expression; $^{-}$for reduced subunit expression; $^{\circ}$for absence of subunit expression.

Table 2. Diversity in Receptor-Mediated Phagocytosis in Human Neutrophils

	Expression	*Ligand binding*	*Signal transduction*
Complement Receptors (CR)	CR1 (CD35) CR3 (CD11b/CD18, $\alpha_M\beta_2$ integrin)	C3b-coated particles C3b-coated particles	Outside-in signaling triggered by ligand binding ↳ $[Ca^{2+}]_c$ elevation ↳ RhoA
Immunoglobulin Receptors (FcR)	FcγRI (CD64) *(inductible by INF-γ or G-CSF)* FcγRIIA (CD32) FcγRIII (CD16) *(function cooperatively with FcγRIIA)*	Monomeric IgG (high affinity) Monomeric IgG (low affinity) Monomeric IgG (high affinity)	Tyrosine phosphorylation of ITAM motifs ↳ PLC-γ/PLD/PI3K ↳ IP_3/S1P production ↳ $[Ca^{2+}]_c$ elevation ↳ Cdc42/Rac

IP_3, inositol 1,4,5-trisphosphate; S1P, sphingosine 1-phosphate; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration.

review, (73)]. However, the idea that innovative techniques have also provided insights into the functional assembly and into the targeting at the phagosome of the NADPH oxidase on neutrophil particulate stimulation should also be considered (77).

Regulation of Phox Proteins by Phosphorylation

Phosphorylation events are key regulatory steps in NADPH oxidase assembly and activation. NADPH oxidase assembly is accompanied by phosphorylation of the cytosolic components $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$. These proteins are phosphorylated by protein kinase C (PKC) isoforms, the ERK/p38MAPK-dependent pathway, cAMP-dependent protein kinases, and protein tyrosine kinase-dependent pathway (18, 31). The current state of knowledge regarding the cytosolic phox protein phosphorylation and subsequent protein–protein interactions involved in the regulation of the NADPH oxidase assembly has been extensively obtained from neutrophils stimulated with soluble physiological agonists such as tumor necrosis factor-α or granulocyte macrophage–colony-stimulating factor (17), only rarely with particulate stimuli (13, 19), and it will be not detailed here [for reviews, see (31, 32, 42)].

Importance of cytochrome b_{558} phosphorylation

The phosphorylation of cytochrome b_{558} in neutrophils is poorly documented, although the phosphorylation of the $p22^{phox}$ subunit has been observed a long time ago (40). Later, McPhail and coworkers demonstrated that $p22^{phox}$ phosphorylation is correlated with NADPH oxidase activity, suggesting that the phosphorylation of $p22^{phox}$ regulates multicomponent enzyme activation (94). $p22^{phox}$ phosphorylation does occur on the threonine 147 residue (64), but its precise role in NADPH oxidase activation has not yet been identified. A recent publication indicated that $gp91^{phox}$ phosphorylation by PKC enhances NADPH oxidase activity as well as increases the binding to Rac2, $p47^{phox}$, and $p67^{phox}$ (90).

Role of cytosolic phox protein phosphorylation during phagocytosis

The best example of subunit phosphorylation and its physiological significance remains the well-understood intracellular signaling pathways involved in $p47^{phox}$ phosphorylation that induce conformational changes of $p47^{phox}$ (32, 53) to a state which is accessible for $p22^{phox}$ binding, allowing for NADPH oxidase activation (1). The phosphorylation of $p47^{phox}$ provides a control mechanism that modulates NADPH oxidase assembly and/or activity and, thus, prevents inadequate activation. The recent study conducted by Marcoux *et al.* demonstrated that the phosphorylation of three serine residues in the auto-inhibitory region (AIR) of $p47^{phox}$ is critical for the release of the phox homology (PX) domain of the protein and its subsequent interaction with $p22^{phox}$ at the phagosomal membrane (70). Although the PX domain of $p47^{phox}$ is only a passive requirement for phagosomal superoxide production, experiments using phosphorylation/activation-mimicking $p47^{phox}$ mutants indicated that adequate serine phosphorylation is required for $p47^{phox}$-induced, $p40^{phox}$-independent, FcγR-mediated NADPH oxidase activation (65, 117).

The recent discovery of a CGD patient expressing a mutation in the PX domain of $p40^{phox}$ has firmly established the importance of $p40^{phox}$ in oxidase activation (72). Controversies over the role of $p40^{phox}$ phosphorylation exist depending on the experimental approach. In a cell-free system, $p40^{phox}$ phosphorylated on threonine 154 was shown to inhibit oxidase activity (66). Conversely, the PKCδ-mediated phosphorylation of threonine 154 in $p40^{phox}$ (but not serine 315) as well as $p47^{phox}$ translocation to phagosomes is required for full oxidase activation in mouse neutrophils (14). The involvement of NADPH oxidase activity-mediated $p67^{phox}$ phosphorylation still remains unclear.

It is of interest to investigate whether the phosphorylation of specific phox proteins has a role in modifying the affinity of the subunits for each other or with cytochrome b_{558} and/or influencing the stability of the complex. Identification of the phosphorylated sites, determination of the phosphorylation status, and site-directed mutagenesis studies should continue to illuminate the significance of phox protein phosphorylation in the regulation of NADPH oxidase during phagocytosis.

Regulation of Phox Proteins by Lipid Metabolism

In addition to phosphorylation, accumulating evidence demonstrates that membrane lipids play a crucial role in the spatiotemporal regulation of NADPH oxidase activity.

Considerable efforts have been undertaken to understand the mechanisms that underlie the lipid-protein interactions and lipid-mediated protein-protein interactions involved in NADPH oxidase activation. It is well documented that the

production of diacylglycerol by phospholipase C (PLC) can induce the activation of PKC isoforms (68), which may mediate the phosphorylation of $p47^{phox}$ [for reviews (32, 36)]. The activation of cytosolic phospholipase A_2 ($cPLA_2$) is also required for NADPH oxidase activity, but its complete role remains relatively unclear. $cPLA_2$ has been found to translocate to the membrane fractions (45) and to catalyze arachidonic acid (AA) production in opsonized zymosan-stimulated human neutrophils (98). $cPLA_2$ translocates from the cytosol and targets the phagosome membrane during phagocytosis by mouse macrophages (41). On opsonized zymosan stimulation, substantial evidence for the essential requirement of $cPLA_2$-generated AA for the activation of neutrophil NADPH oxidase has been derived from the work of Levy's group that was based on differentiated PLB-985 cells which are deficient in $cPLA_2$ (15, 86) or PLA_2 inhibitors (16). Other groups have corroborated these results (122). It is likely that $cPLA_2$-generated AA is not required for the membrane translocation of the NADPH oxidase cytosolic factors (105). Actually, $cPLA_2$ is anchored to the plasma membrane by the assembled NADPH oxidase and releases AA (Fig. 2), promoting NADPH oxidase activity (105). It appears that the target site for AA is located within the $gp91^{phox}$ N-terminal domain where it has a role in the activation of electron transfer through the FAD reduction center of NADPH oxidase (86). Indirect evidence supplied by both cell-free systems and intact neutrophils as well as from different technical approaches (e.g., electron spin resonance, atomic force microscopy) (25, 26, 37, 81) provided an alternative hypothesis, stating that AA may affect the $gp91^{phox}$ N-term domain by inducing conformational changes in cytochrome b_{558}. In fact, several studies in cell-free systems suggest that AA promotes the translocation of $p47^{phox}$ (104) or conformational changes in both $p47^{phox}$ and $p67^{phox}$ (44, 84), thus increasing NADPH oxidase activity. However, the *in vitro* model may not reflect the entire suite of complexities of NADPH oxidase activation and, thus, probably cannot faithfully recapitulate the biology of whole neutrophils.

Role of phospholipid and $p47^{phox}$/$p40^{phox}$ interactions

Phospholipase D (PLD), which cleaves phospholipids to form phosphatidic acid (PA), is also an important modulator of NADPH oxidase activity. Regier *et al.* (95) suggested that $p22^{phox}$ phosphorylation is mediated by PLD-dependent

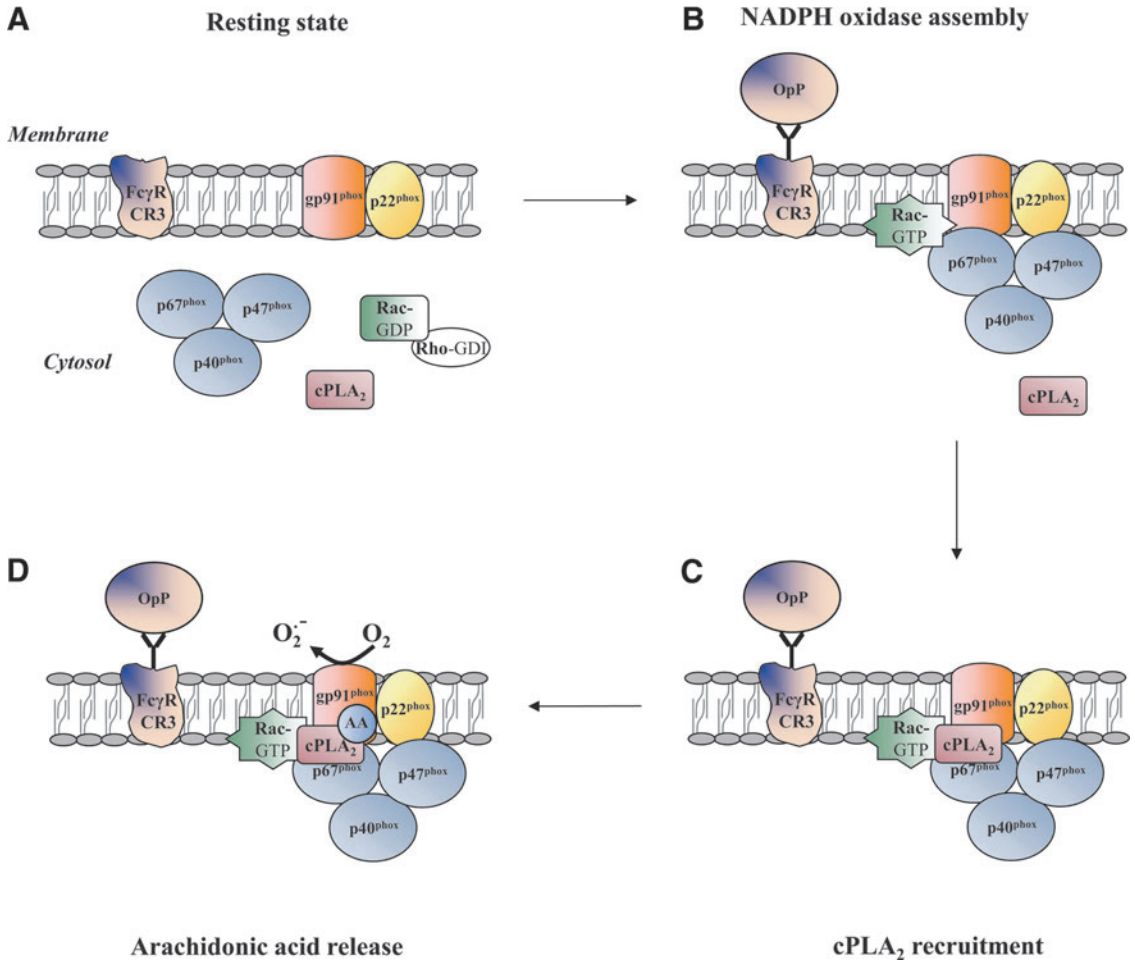

FIG. 2. Model for the role of cytosolic phospholipase A_2 ($cPLA_2$)-generated arachidonic acid (AA) in phagosomal NADPH oxidase activation [based on the review of Levy (63)]. (A) In the resting state, $cPLA_2$ is localized in the neutrophil cytosol. **(B)** On opsonized zymosan stimulation, the cytosolic subunits of NADPH oxidase are recruited to the phagosomal membrane. **(C)** $cPLA_2$ is targeted to the membrane by the assembled NADPH. **(D)** Arachidonic acid is released from $cPLA_2$ and localized within $gp91^{phox}$ N-terminal domain, where it participates in NADPH oxidase activation *via* electron transfer.

and -independent mechanisms (Fig. 3). PLD-dependent phosphorylation of $p22^{phox}$ could be activated by a not-yet-identified PA-activated protein kinase, whereas the PLD-independent mechanism is mediated by conventional PKC isoforms (94). The significance of $p22^{phox}$ phosphorylation may be the promotion of the $p22^{phox}$-$p47^{phox}$ interaction at the membrane (46).

3′-phosphoinositides (PI) are also considered pivotal regulators of NADPH oxidase activation by serving as site-specific membrane signals or by modulating cytosolic localization and/or other biological properties of effector proteins. Direct or indirect PI interactions with the subunits $p40^{phox}$ and $p47^{phox}$ regulate the NADPH oxidase assembly. Both proteins contain a lipid-binding domain, specifically the PX domain that binds PI with a broad specificity. Studies of PX domain structures by X-ray crystallography have revealed the charged binding sites for stereospecific recognition of their cognate PI (10, 57). The PX domain of $p40^{phox}$ specifically binds phosphatidylinositol 3-phosphate (PI(3)P), a lipid product of class III phosphoinositide 3-kinase (PI3K), and is required for $p40^{phox}$ accumulation at the phagosome and assembly/activation of the NADPH oxidase (Fig. 3) (111). Initial evidence regarding the importance of PI(3)P in NADPH oxidase regulation was provided *in vitro* in semi-recombinant cell-free assays and COS^{phox} systems (35, 111). Generation of the $p40^{phox}$ mutant mice expressing the $p40^{phox}$ which carries a mutation in the PX domain corroborates *in vivo* that intact binding of PI(3)P to the intact PX domain of $p40^{phox}$ is required for FcγR-mediated ROS production by professional phagocytes (9). The group of Saito (117) proposed that $p40^{phox}$ is able to acquire PI(3)P-binding capacity through conformational changes induced by H_2O_2 and functions as a carrier of the cytosolic $p47^{phox}$- $p40^{phox}$-$p67^{phox}$ complex. Binding of PI(3)P to $p40^{phox}$ PX domain has a differential impact on the downstream signaling pathways, depending on the phagocytic receptor subtypes and the type of target particles (34).

The PX domain of $p47^{phox}$ has two distinct lipid binding pockets: one with preferential affinity for phosphatidylinositol 3,4 bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol 3,4,5 trisphosphate ($PI(3,4,5)P_3$), while the other nonspecifically binds anionic phospholipids such as PA and phosphatidylserine (PS) (Fig. 3) (55, 108). The PI-dependent membrane-binding mechanisms of PX domains and their involvement in NADPH oxidase activation have not been clearly identified. However, it has recently been suggested that $p47^{phox}$ allows $p40^{phox}$ to acquire PI(3)P binding on targeted membranes that act in cooperation with $p47^{phox}$ as an adaptor of NADPH oxidase assembly on the phagosome (117).

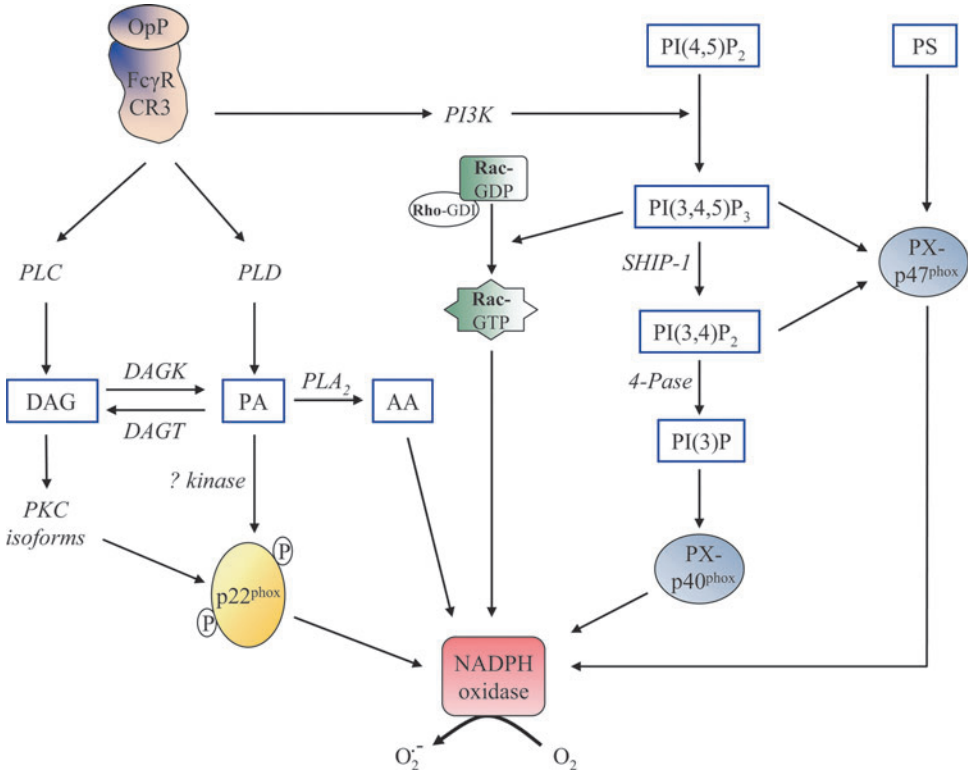

FIG. 3. Lipid metabolism. Binding of opsonized particles (OpP) to FcγR or CR3 triggers the activation of several signaling pathways. Stimulation of phosphoinositide 3-kinase (PI3K) results in a downstream inositol phospholipid signaling. It phosphorylates $PI(4,5)P_2$ to produce phosphatidylinositol 3,4,5 trisphosphate ($PI(3,4,5)P_3$), which is dephosphorylated by SHIP-1 into phosphatidylinositol 3,4 bisphosphate ($PI(3,4)P_2$), while inositol polyphosphate 4-phosphatase (4-Pase) hydrolyzed $PI(3,4)P_2$ to form PI(3)P. Products of inositol phospholipid signaling bind with domain PX of $p47^{phox}$ and $p40^{phox}$, a prerequisite for NADPH oxidase activation. In addition, $PI(3,4,5)P_3$ activates Rac proteins. In parallel, phospholipase C (PLC), Phospholipase D (PLD), and PLA_2 are also activated. The resulting products, diacylglycerol (DAG) and phosphatidic acid (PA), can activate different types of kinases, participating in the regulation of the NADPH oxidase activity. The level of DAG and PA is dependent on the activity of diacylglycerol kinase (DAGK) and transferase (DAGT). The role of AA underlying NADPH oxidase activation remains unidentified. For details, see text.

The role of the $p47^{phox}$ PX domain in phagosomal NADPH oxidase activation may be selective and may vary greatly depending on the nature of the stimulus. Mutations in the $p47^{phox}$ PX domain that result in the loss of $PI(3,4)P_2$ binding does not impair phagosomal membrane recruitment and the normal activity of NADPH oxidase during phagocytosis of a variety of particles in mouse neutrophils. In contrast, plasma membrane NAPDH oxidase activity is reduced in neutrophils expressing $p47^{phox}$ mutants (65). Thus, although the formation of the phagosomal NADPH oxidase complex is considered similar to the plasma membrane, it seems clear that the regulation of the plasma membrane NADPH oxidase activity differs in terms of signaling pathways from that of phagosomal NADPH oxidase.

Charge-dependent localization of Rac proteins

As for $p40^{phox}$ and $p47^{phox}$, the translocation of positively charged small GTPases to the membrane may involve direct interactions with anionic lipids, mainly PI, PA, and PS. Lipid metabolism regulates the net membrane charge, which is responsible for the selective recruitment of Rac proteins that rely heavily on their own inherent net-positive charge. Based on alterations of lipid metabolism during phagocytosis, a charge-shift mechanism dictates localization patterns to distinct membrane compartments of Rac1 and Rac2 (112). On stimulation, Rac proteins dissociate from RhoGDI, exchange GDP against GTP, and translocate to the membrane where they bind to $p67^{phox}$ (4). They may either act as an adaptor to ensure correct positioning of $p67^{phox}$ toward NADPH oxidase or participate in the electron-transfer reaction. The electrostatic forces between Rac1 and the plasma membrane are higher for Rac2 than for the plasma membrane. The higher electrostatic forces result in a preferential association of Rac1 with highly charged actin-rich pseudopods and primarily the localization to the plasma membrane. During phagosome maturation, membrane lipid composition is altered by the partially localized depletion of specific membrane phospholipids ($PI(4,5)P_2$, $PI(3,4,5)P_3$) and by the partial decrease in PS at the base of the phagocytic cup and phagosomal membrane (Fig. 4). This generates a membrane environment with a moderate negative charge that correlates with the preferential localization of active Rac2 with the intermediately charged phagosome membrane (69).

Contribution of lipid rafts in phagosomal NADPH oxidase activation

Lipid rafts are sphingolipid and cholesterol-enriched insoluble membrane microdomains that are associated with signaling molecules such as receptors. They have the ability to constitute platforms on cell stimulation. These lipid domains have emerged as an important factor in the regulation of NADPH oxidase activity, probably through the control of the efficacy of NADPH oxidase assembly. Indeed, Shao *et al.* (103) established that NADPH oxidase subunits localize to lipid rafts, which mediate the efficiency of NADPH oxidase coupling to FcγR. A marked negative impact on NADPH oxidase assembly is observed when lipid rafts are disrupted. Later, the work of Jin and co-workers (52) supported this conclusion by highlighting the fact that redox-signaling platforms formed by lipid rafts provide an important driving force for assembling the NADPH oxidase subunits and subsequent NADPH oxidase activation. In addition to the lipid rafts, a member of the Src family tyrosine kinases, which are responsible for the phosphorylation of FcR ITAM motifs and downstream activation of PI3K, has been identified as a regulator of NADPH oxidase recruitment to the phagosomes in rodents (56). This result opens up a new potentiality for our understanding of the mechanisms involved in the regulation of phagosomal NAPDH oxidase activity. In the future, it will be interesting to confirm that Lyn ensures the same function in human neutrophils.

Role of Ca^{2+} in the Regulation of Neutrophil NADPH Oxidase Activity

Over the years, it has become evident that an elevation of cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_c$) in the periphagosomal region participates in NADPH oxidase activation during opsonized zymosan particles-mediated phagocytosis (27, 67). Recent progress in understanding molecular mechanisms linking these two phenomena is highlighted next.

Source of Ca^{2+}-mediated phagosomal NADPH oxidase activity

There is no consensus on the source of Ca^{2+}, and the question of whether phagosomal oxidase activity requires Ca^{2+} release from intracellular stores or Ca^{2+} influx remains unanswered. Changes in Ca^{2+} are global and not restricted to the phagosomal region but are temporally correlated with NADPH oxidase activity (22). Hallett and co-workers research (22) has given rise to speculation that extracellular Ca^{2+} entry is a rapid event which contributes to $[Ca^{2+}]_c$ elevation during C3bi-opsonized zymosan particles-mediated phagocytosis. The fact that knockdown by the specific siRNA of Ca^{2+} release-activated Ca^{2+} modulator 1 (Orai1), an essential pore subunit of Ca^{2+} channels, decreases ROS production on FcγR activation tends to confirm that phagosomal NADPH oxidase activity is dependent on extracellular Ca^{2+} entry (110). However, Ca^{2+} influx *per se* does not exert a sufficient signal that ensures an optimal NADPH oxidase activation during phagocytosis but instead acts in synergy with other events. Given the importance of phospholipids in phagosomal NADPH oxidase activity, it is reasonable to assume that inositol and Ca^{2+} signaling pathways are interconnected. Observations in B cells that SHIP-1 plasma membrane recruitment on FcγR activation decreases the $PI(3,4,5)P_3$ level and block Ca^{2+} signals suggest that $PI(3,4,5)P_3$ is a critical regulator of Ca^{2+} signaling (99). Further, restricting $PI(3,4,5)P_3$ formation by LY294002, a PI3K inhibitor, has been shown to impair both global Ca^{2+} signal and ROS production triggered by CR3 activation in neutrophils (23). The exact mechanisms linking $PI(3,4,5)P_3$ formation to Ca^{2+} signals are not yet known. However, this phospholipid may initiate Tec kinase activation, resulting in tyrosine phosphorylation of PLCγ (99) and leading to the formation of inositol 1,4,5-trisphosphate (IP_3), the second messenger responsible for Ca^{2+} release from intracellular stores *via* the opening of IP_3 receptors during FcγR-mediated phagocytosis (110). Depletion of intracellular Ca^{2+} stores induces activation of the intraluminal Ca^{2+} sensor STIM1 (stromal interaction molecule 1), which, in turn, interacts with Orai1 to allow extracellular Ca^{2+} entry (Fig. 5). This mechanism is known as store-operated Ca^{2+} entry and has been well detailed in research published over the past 20 years (89).

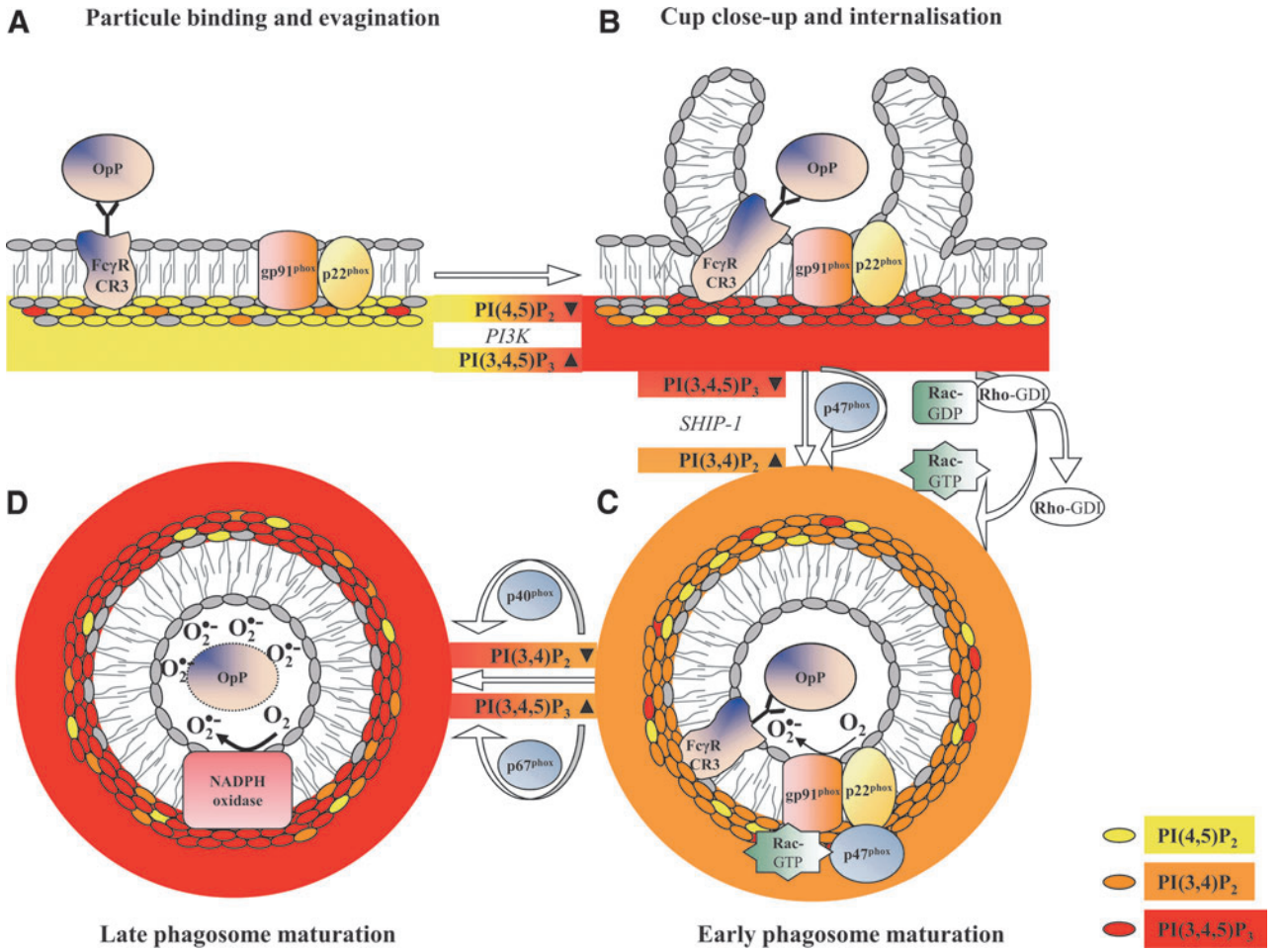

FIG. 4. Chronological phospholipid-dependent localization of NADPH oxidase subunit. (A) On particle binding, $PI(4,5)P_2$ transiently accumulates at the site of particle engagement and at the tips of the pseudopods, extending around the phagocytic cup. **(B)** The concentration of $PI(4,5)P_2$ decreases rapidly on internalization, and early phagosomes are instead enriched in $PI(3,4,5)P_3$, generated from $PI(4,5)P_2$ by PI3K. The disruption of an isoform of inositol hexakisphosphate kinase augments downstream $PI(3,4,5)P_3$ signaling in phagocytes (88). This increase of $PI(3,4,5)P_3$ triggers Rac2 activation (dissociation from Rho-GDI). **(C)** The membrane-associated SHIP-1, which dephosphorylate $PI(3,4,5)P_3$ yielding $PI(3,4)P_2$, enhances the early ROS production by transiently increasing $PI(3,4)P_2$ on the phagosomal membrane (54). Localized alteration of the membrane 3′PI composition affects the recruitment of $p47^{phox}$ (55, 99) and Rac. **(D)** Late phagosomes are depleted of $PI(4,5)P_2$ and predominantly contain $PI(3,4,5)P_3$.

The role of IP_3 is the subject of much controversy. On the one hand, its role might be dependent on individual FcγR and CR3 for IgG subclasses and complement activity, respectively. On the other hand, as observed in platelets, $PI(3,4,5)P_3$ may sustain NADPH oxidase activity during neutrophil phagocytosis without an increase in PLC activity but through another lipid-based signaling pathway (85). CR3 could preferentially activate the PLD-dependent cascade, triggering the depletion of Ca^{2+} from intracellular stores *via* sphingosine-1 phosphate and subsequent STIM1/Orai1-mediated extracellular Ca^{2+} entry (Fig. 5) (79).

In addition to Orai1, Hv1 voltage-gated proton channels could regulate phagosomal Ca^{2+} turnover by preventing the depolarization generated by NADPH oxidase activity, therefore, enhancing the driving force for extracellular Ca^{2+} entry and sustaining NADPH oxidase activity. Initially, proton channels responsible for H^+ efflux were described as a part of the $gp91^{phox}$ subunit (46). Later, convincing evidence demonstrated that channels nonrelated to $gp91^{phox}$ constitute voltage-gated proton channels (21). In this sense, Hv1 channels also preserve physiological membrane electroneutrality by compensating the charge generated by the electrogenic transfer of electrons from NADPH to $O_2^{\cdot -}$ in the cytosol and hydrolysis of NADPH [for review, see (33)] (Fig. 6).

At this point, it is interesting to emphasize that a feedback mechanism could exist between Ca^{2+} homeostasis and NADPH oxidase activity at the phagosomal level. Indeed, it has been shown that extensive membrane depolarization dependent on ROS generation results in a marked decrease of the driving force for Ca^{2+} influx (91, 115). TRPM2, a nonselective and ROS sensitive channel cation found to be expressed in lysosomes (61), might be a putative ROS target linking the redox state of the membrane to Ca^{2+} homeostasis (114, 121). Moreover, TRPM2 possesses the ability to increase $[Ca^{2+}]_c$ and the bactericidal activity of neutrophils (49). A model is proposed in Figure 6 that depicts the feedback mechanism for the inactivation of ROS production.

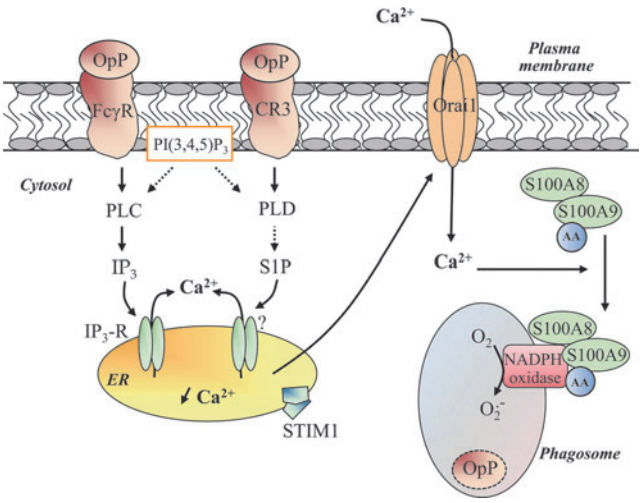

FIG. 5. Proposed model for Ca^{2+}-dependent phagosomal NADPH oxidase activity. IgG or complement fragments-opsonized particles are recognized by neutrophils through its FcγR or CR3. Phagocytic engagement of these both receptors activate PI3K, leading to the formation of $PI(3,4,5)P_3$. This phospholipid participates in the activation of PLCγ and PLD-mediated downstream signaling pathways. The generation of IP_3 by PLCγ triggers Ca^{2+} release from intracellular stores (endoplasmic reticulum, ER) *via* the opening of IP_3 receptors (IP_3-R). PLD is involved in the process of sphingosine kinase-produced sphingosine 1-phosphate (S1P), leading to the depletion of intracellular Ca^{2+} stores. The emptying of intracellular Ca^{2+} stores induces the activation of the Ca^{2+} sensor STIM1, which, in turn, interacts with Orai1 at the plasma membrane where it activates Ca^{2+} channels Orai1 and extracellular Ca^{2+} entry. The resulting $[Ca^{2+}]_c$ elevation mediates the recruitment of the cytosolic S100A8/A9 complex to the phagosomal membrane. The translocation of S100A8/A9 allows the transfer of S100A9-binding AA to cytochrome b_{558}, favoring the conformational change of cytochrome b_{558} and triggering intraphagosomal NADPH oxidase activation.

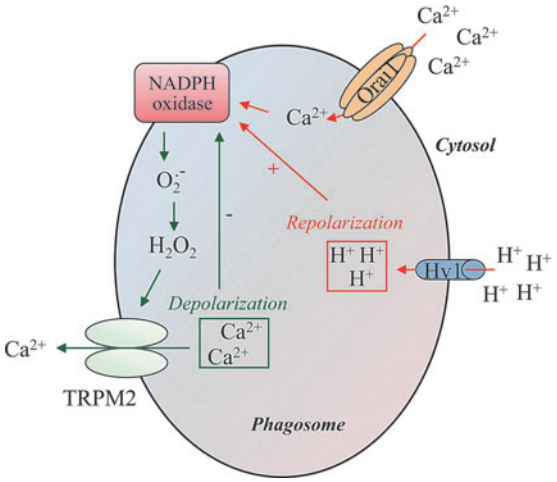

FIG. 6. Proposed model for the feed-back mechanism between redox state and phagosomal NADPH oxidase activity. Hv1 voltage-gated proton channels prevent the depolarization due to NADPH oxidase activity and promote $[Ca^{2+}]_c$ elevation, probably through Orai1-mediated Ca^{2+} entry (in red). The intraphagosomal Ca^{2+} mobilization sustains NADPH oxidase-produced ROS. H_2O_2 activates TRPM2 channels, which, in turn, trigger membrane depolarization and damp NADPH oxidase activity (in green).

There is a close cooperation between the NADPH oxidase and the Hv1 proton channels to adjust intraphagosomal and cytosolic pH variations. Hv1 proton channels are present in specific granules and accumulated in phagosomes along with the oxidase during FcγR-dependent phagocytosis (80). It has been recently shown that NADPH oxidase-dependent bacterial killing was significantly reduced in Hv1-deficient mice neutrophils *in vitro* (87, 93). During phagocytosis-mediated NADPH activation, the consumption of NADPH produces H^+ that acidifies the cytosol (74). The acidification and the electrogenic translocation of electrons into the phagosome are perfectly compensated by Hv1 proton channels, as they are activated by intracellular acidification and depolarizing voltages (75). Surprisingly, the intraphagosomal pH remains near neutral for several minutes, possibly due to several mechanisms such as the net consumption of luminal H^+ during the dismutation of $O_2^{\cdot -}$ to hydrogen superoxide, a large passive proton leak to the cytosol, preventing accumulation in the lumen and a reduced insertion of V-ATPases in the phagosome membrane, resulting in lower rates of H^+ efflux (Fig. 7) (33, 51).

S100A8/A9: the link between Ca^{2+} and NADPH oxidase

Over the last decade, the phagocyte-specific Ca^{2+}-binding S100A8 and S100A9 proteins have been proposed as essential regulators of the plasma membrane NADPH oxidase activity. These proteins are abundantly expressed in the cytosol of neutrophils and are able to form Ca^{2+}-dependent heterocomplexes, with heterotetramers being a probable prerequisite for their biological activities in myeloid cells (62). S100A8 and S100A9 have been proposed as essential regulators that exert their role through interactions with NADPH oxidase subunits (8).

The addition of Ca^{2+}-loaded S100A8/A9 to the reconstituted assembled NADPH oxidase complex prepared with neutrophil cytochrome b_{558} and B lymphocyte cytosol is able to increase the constitutive activity of cytochrome b_{558} in the absence of any stimulus. Initially, S100A8/A9 has been designated as a positive allosteric effector of NADPH oxidase activity that interacts preferentially with $p67^{phox}$ and increases its affinity for cytochrome b_{558}. However, the increased affinity of $p67^{phox}$ for cytochrome b_{558} as inferred by S100A8/S100A9 was confirmed by Berthier and co-workers (8) in a semi-recombinant cell-free system, whereas S100A8/S100A9 appear to also interact directly with cytochrome b_{558}. Observations conducted on structural conformation changes by atomic force microscopy underlie the fact that the S100A8/A9 complex is able to enhance or induce a transition from an inactive to an active conformation state of cytochrome b_{558}. Preincubation of S100A8/S100A9 in the absence of Ca^{2+} led to an interaction with cytochrome b_{558} but not to a conformational change, allowing ROS production. Thus, the relevant role for S100A8/A9 in NADPH oxidase activity is dependent on Ca^{2+} (7) and is probably mediated *via* S100A8/A9 translocation to the membrane where NADPH oxidase is activated (97, 100, 101). An elevation of $[Ca^{2+}]_c$ is necessary

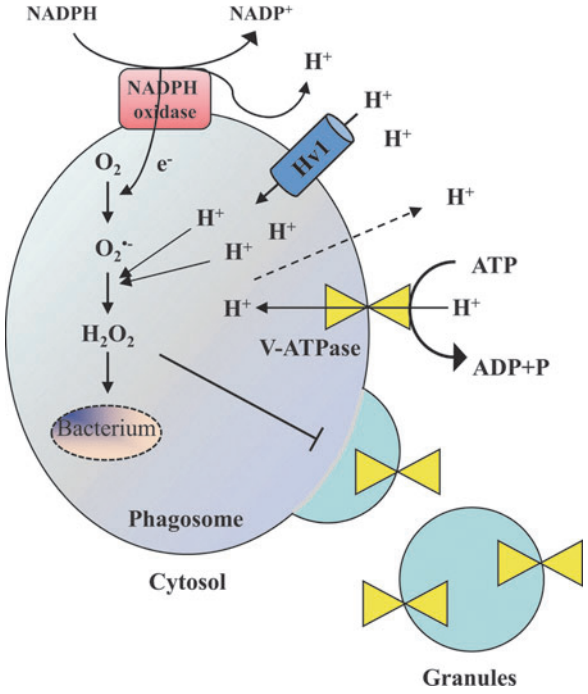

FIG. 7. Correlation between NADPH oxidase and Hv1 activities. Phagocytosis-triggered NADPH oxidase activation leads to a flux of electrons that depolarizes the membrane and an accumulation of protons, both events activating the Hv1 proton channels. Hv1 promotes proton influx into the phagosomal lumen, counteracting cytosol acidification and favoring sustained NADPH oxidase activity. Intraphagosomal accumulation of protons during early phagosome maturation is prevented by three mechanisms: (i) Consumption of protons for the production of ROS species, (ii) ROS-dependent passive leak of protons to the cytosol (51), and (iii) reduced fusion of V-ATPases-containing granules to the phagosome membrane, resulting in low rates of H^+ flux.

for S100A8/A9 redistribution to the plasma membrane; thus, intracellular Ca^{2+} store depletion appears to be substantially responsible for this phenomenon (100).

From these observations, it is logical to question whether S100A8/A9 could also constitute the link between $[Ca^{2+}]_c$ elevation and phagosomal NADPH oxidase activity. Different research groups have provided indirect evidence for such a speculation. Kumar *et al.* (59) showed that phagocytosis was associated with a rapid reduction of cytoplasmic immunostaining of the S100A8 homodimeric form, and a Ca^{2+} dependence of S100A8/A9 translocation in cytoskeletal structures was reported (97). Direct evidence that S100A8/A9 constitute the molecular switch between $[Ca^{2+}]_c$ elevation and phagosomal NADPH oxidase activity has been provided by a recent work of Steinckwich *et al.* (110). These authors established that S100A8/A9 siRNA knockdown decreases intraphagosomal ROS production. Further, on FcγR-induced $[Ca^{2+}]_c$ elevation, endogenous S100A8/A9 is recruited to the phagosomal membrane at the beginning of the formation of the phagocytic cup and persists throughout the zymosan internalization process. Therefore, extracellular Ca^{2+} entry-mediated S100A8/A9 phagosomal membrane recruitment serves as a determinant for the NADPH oxidase activity. *Via* a mechanism similar to that proposed at the plasma membrane level (8, 81), S100A8/S100A9 could deliver AA to the phagosomal membrane (Fig. 5), increasing its local concentration and favoring the conformational change of cytochrome b_{558}. This hypothesis requires confirmation, but it may provide a foundation for further investigation of the link between S100A8/A9 and phagosomal NADPH oxidase. In the near future, it will also be of interest to (i) identify phagosomal NADPH oxidase subunits involved in direct interactions with S100A8/A9 and (ii) characterize the additional signaling pathways leading to S100A8/A9 phagosomal membrane recruitment with an emphasis on phospholipid and phosphorylation signaling cascades.

The pro-inflammatory role of S100A8/A9 is not restricted to the intracellular level. Neutrophils are known to secrete S100A8/A9 that are involved in autocrine/paracrine regulatory mechanisms underlining the inflammatory process (30, 92). In a variety of cell types, this dual action is mediated through the engagement of Receptor for Advanced Glycation End (RAGE) products signaling (47). This signaling contributes to the activation of p38 MAPK and the downstream effector NF-κB (29). S100A8 and S100A9 have recently been identified as target genes of NF-κB (76), which regulate their expression, thus allowing them to fulfill their intracellular roles (Fig. 8). In parallel, exogenous S100A9 may have the ability to induce NADPH oxidase activation, although it mainly stimulates neutrophil microbicidal activity by promoting phagocytosis (106). Further, other RAGE ligands have been associated with a modulation of bacterial destruction by neutrophils and activation of NADPH oxidase. Definitely, the prototypical RAGE

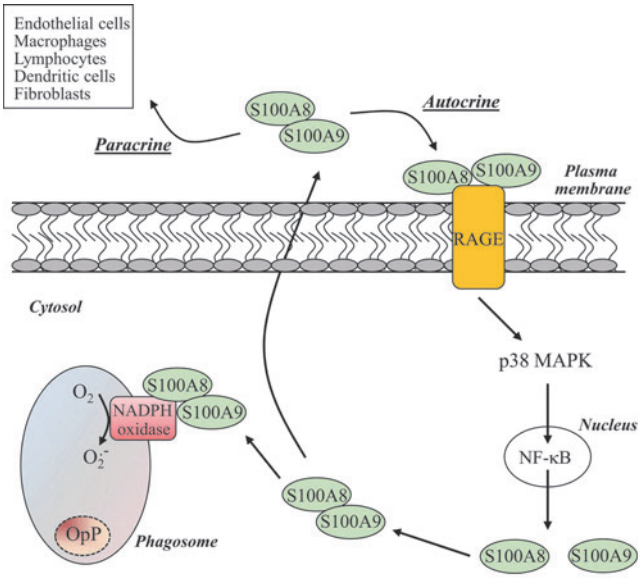

FIG. 8. Possible feed-forward signaling mediated by S100A8/A9 during phagocytosis. Receptor for Advanced Glycation End (RAGE) ligation by S100A8/A9, on the neutrophil surface, leads to the activation of the transcription factor NF-(B, which, in turn, induces the production of S100A8 and S100A9. The S100A8/A9 complex is recruited to the phagosomal membrane to regulate NADPH oxidase activation or is secreted in the extracellular environment. Extracellular S100A8/A9 amplifies the pro-inflammatory response by either activation of neutrophils (autocrine mode of action) or other inflammatory cell types (paracrine mode of action).

ligand AGE has been found to increase bacterial killing by neutrophils and activation of NADPH oxidase, likely through the phosphorylation of the $p40^{phox}$ subunit. In contrast, high mobility group box 1 diminished the ability of neutrophils to destroy pathogens and NADPH oxidase activation (113).

In light of these results, an intriguing possibility arises that S100A8/A9 secreted by neutrophils on opsonized zymosan stimulation bind to RAGE and regulate neutrophil phagosomal NADPH oxidase activity (Fig. 8). Kumar *et al.* (59) have hypothesized that the rapid loss of cytoplasmic immunoreactivity for homodimeric S100A8 was the consequence of S100A8 release by active secretion on opsonized zymosan stimulation. Using the same line of evidence, Guignard *et al.* (43) showed that S100A8 and S100A9 were recruited to the plasma, underlining the eventual and subsequent S100A8/A9 secretion after opsonized zymosan. However, these authors have provided no clear indication for a secretion of S100A8 and S100A9 proteins. While the release of S100A8 and S100A9 upon soluble stimuli is well established, to our knowledge, no direct evidence for the release of neutrophil S100A8 and S100A9 in response to particulate stimuli has yet been convincingly provided. Further investigations are, thus, compulsory. Understanding the S100A8/A9-RAGE-NADPH oxidase axis, in an autocrine/paracrine fashion, potentially offers some interesting perspectives for providing a more comprehensive mechanism for the regulation of the phagosomal NADPH oxidase.

Monitoring of phagosomal ROS production and Ca^{2+} mobilization

To understand the relationship between intracellular Ca^{2+} mobilization and NADPH oxidase activity, quantitative concomitant measurement of phagosomal NADPH oxidase activity and $[Ca^{2+}]_c$ changes with sufficient resolution is not only necessary but also a challenge. One of the issues is the difficulty of distinguishing the contribution of $[Ca^{2+}]_c$ elevation for the phagosomal oxidative event from earlier phagocytic events (chemotaxis, phagocytic cup formation, engulfment, and phagosome closure). Commonly used techniques for following intraphagosomal ROS production are provided in Table 3 [for a more detailed description, see (38, 96, 116, 123)]. An original technique was developed by Dewitt and co-workers (22) in which C3bi-opsonized particles labeled with an oxidant-sensitive probe (2′,7′-dichlorodihydrofluorescein [$DCFH_2$]) were presented to neutrophils at a defined time and location using micromanipulation. Later, Steinckwich *et al.* (110), using fluorescence light microscopy, which allows for the detection of intraphagosomal ROS production, measured simultaneously $[Ca^{2+}]_c$ elucidating the relationship between phagosomal NADPH oxidase activity and changes in $[Ca^{2+}]_c$. Classical indicators used to monitor $[Ca^{2+}]_c$ changes remain fluorescent dyes such as fluo-4 or fura-2, but genetically encoded probes are being developed to follow Ca^{2+} amplitude and kinetics at specific subcellular locations and improve Ca^{2+} imaging resolution [for review, see (20)].

To overcome the lack of sensitivity, and problems with auto-oxidation and quenching observed with the fluorescein derivative $DCFH_2$, research has been devoted to the development of a specific probe to improve the detection and measurement of intracellular H_2O_2 production. Genetically encoded probes, which can be targeted at specific subcellular locations (96), represent potentially useful tools for monitoring H_2O_2 production within the phagosomal compartment.

Table 3. Techniques Used to Monitor Intraphagosomal Reactive Oxygen Species Production

Probes	*Specificity*	*Comments*
NBT (colorimetry)	Various oxidants	+ Detection in individual phagosome – Qualitative – Low-time resolution
DHR-123 (fluorometry)	Various oxidants	+ Direct measurement – Difficulty to follow oxidant production – Dependent on MPO activity
$DCFH_2$ (fluorometry)	Various oxidants	+ Covalent attachment to particles + No diffusion into different compartments + Monitoring of amplitude and kinetics – Dependent on MPO activity
Luminol (luminometry)	$O_2^{\cdot -}$ (in the presence of peroxidase)	+ Exclusive detection of intracellular $O_2^{\cdot -}$ production (with SOD and catalase addition) + Highly sensitive – Not directly quantitative
SNAP-tag	H_2O_2	+ Subcellular resolution + Deprotection chemistry + Targeted to specific compartments – Irreversible fluorescent probes – No monitoring of H_2O_2 production – Low sensitivity
HyPer	H_2O_2	+ Reversible fluorescent probes + Targeted to various subcellular compartments

$DCFH_2$, 2,7-dichlorodihydrofluorescein; DHR-123, dihydrorhodamine 123; HyPer, genetically encoded fluorescent indicator; MPO, myeloperoxidase; NBT, nitroblue tetrazolium; SNAP-tag, organelle-targetable fluorescent probes; + and – indicate advantages and inconveniences of each technique.

Hypothetical Involvement of *N*-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor-Mediated Exocytosis in Phagosomal NADPH Oxidase Activity

One point of regulation could occur at the level of proteins collectively termed soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNAREs). SNAREs are key regulators in all fusion events occurring in the exocytosis pathway, which lead to protein trafficking between intracellular compartments. SNAREs comprise a series of proteins that can be divided into vesicular (v-SNAREs), including VAMP family members and target (t-SNAREs) including syntaxins and SNAP-23/25 family members. These proteins mediate the formation of extremely stable complexes between adjacent membranes, bringing the membranes into close apposition (107).

Several authors have identified a number of SNAREs in neutrophils, including the t-SNARE SNAP-23. SNARE proteins and their regulators have been involved in the process of cytosolic secretory granule fusion and exocytosis of their contents (71). SNAP-23 has been described as being a mediator of specific granule secretion (71) and recently, Uriarte and co-workers (118) demonstrated that the SNAP-23-mediated neutrophil granule exocytosis contributes to ROS production during phagocytosis. Thus, the assumption can be made that SNAP-23 participates in the appropriate localization of cytochrome b_{558} by specific and gelatinase-granule release in phagosomes. Further studies are needed to determine the mechanisms by which SNARE proteins are precisely involved in the regulation of the phagosomal NADPH oxidase activity.

Conclusion

Phagosomal ROS production is critical for efficient host innate defense against infections through direct microbial killing. In addition, ROS control spatiotemporal phagosomal proteolytic processes, through modification of the luminal redox environment of phagosomes, which are also required to mediate pathogen destruction. It is becoming increasingly evident that variations in the types of engaged receptors trigger different downstream signaling cascades. The challenge will be to define the contribution of the distinct molecular mechanisms involved in phagosomal NADPH oxidase activation during individual receptor-mediated phagocytosis. Ca^{2+} signals control many pathways, and growing evidence shows that $[Ca^{2+}]_c$ elevation exerts an effect on NADPH oxidase activity through interconnections with other regulatory pathways, including lipid metabolism. However, despite many decades of intensive research regarding the vast numbers of pathways and molecules that are involved in phagocytosis, the regulation of the phagosomal NADPH oxidase remains poorly understood.

Some bacteria have developed highly sophisticated mechanisms compromising the bactericidal function of phagosomal ROS production and contributing to long-lasting infections and the progression of pathological states. For example, Allen *et al.* (2) proposed a model in which *Helicobacter pylori* disrupt NADPH oxidase assembly by preventing granule mobilization to the phagosome. This inefficient recruitment triggers a privation of $gp91^{phox}$ and $p22^{phox}$ on the phagosome and impairs the retention of $p47^{phox}$ and $p67^{phox}$. Consequently, superoxide anions do not accumulate inside the phagosome and are released into the extracellular space. This mechanism allows these nonopsonized bacteria to evade phagocytic destruction and promote damage to the gastric mucosa (2). A large set of other bacteria is also able to inhibit ROS production in neutrophils through distinct mechanisms, including superoxide scavenging or impairment of cytochrome b_{558} accumulation on phagosomes [for review, see (3)]. To understand such strategies of bacterial resistance, we need to improve our understanding of the mechanisms underlying the regulation of the phagosomal NADPH oxidase activity. With this goal in mind, it is imperative to define how signaling pathways are integrated at the molecular level to regulate phagosomal NADPH oxidase activity. Research on NADPH oxidase at the plasma membrane level of neutrophils could be used to learn more about phagosomal ROS production, but caution should be exercised in extrapolating these results, as the involvement of individual pathways might vary greatly between both enzyme localizations. Recent advances in the field of computational biology coupled with traditional techniques open up new possibilities for yielding insights into the regulation of the phagosomal NADPH oxidase activity.

Acknowledgments

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Abbreviations Used

$[Ca^{2+}]_c$ elevation = cytosolic free Ca^{2+} concentration
AA = arachidonic acid
CFTR = transmembrane conductance regulator
CGD = chronic granulomatous disease
Cl^- = chloride anion
$cPLA_2$ = cytosolic phospholipase A_2
DAG = diacylglycerol
DAGK = diacylglycerol kinase
DAGT = diacylglycerol transferase
$DCFH_2$ = 2′,7′-dichlorodihydrofluorescein
H_2O_2 = hydrogen peroxide
HOCl = hypochlorous acid
IP_3 = inositol 1,4,5 trisphosphate
MPO = myeloperoxidase
$O_2^{\cdot -}$ = superoxide anion
OpP = opsonized particles
PA = phosphatidic acid
PI = 3′ phosphoinositides
PI3K = phosphoinositide 3-kinase

Abbreviations Used (Cont.)

PI(3)P = phosphatidylinositol 3-phosphate
$PI(3,4)P_2$ = phosphatidylinositol 3,4 bisphosphate
$PI(3,4,5)P_3$ = phosphatidylinositol 3,4,5 trisphosphate
PKC = protein kinase C
PLC = phospholipase C
PLD = phospholipase D
PS = phosphatidylserine
PX domain = phox homology domain
RAGE = Receptor for Advanced Glycation End
ROS = reactive oxygen species
S1P = sphingosine 1-phosphate
SNARE = *N*-ethylmaleimide-sensitive factor attachment protein receptor