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NADPH Oxidase Activation and Assembly During Phagocytosis


Generation of superoxide (O$_{2}^-$) by the NADPH-dependent oxidase of polymorphonuclear leukocytes is an essential component of the innate immune response to invading microorganisms. To examine NADPH oxidase function during phagocytosis, we evaluated its activation and assembly following ingestion of serum-opsonized Neisseria meningitidis, serogroup B (NMB), and compared it with that elicited by serum-opsonized zymosan (OPZ). Opsonized N. meningitidis- and OPZ-dependent generation of reactive oxygen species by polymorphonuclear leukocytes peaked early and then terminated. Phosphorylation of p47$^{phox}$ coincided with peak generation of reactive oxygen species by either stimulus, consistent with a role for p47$^{phox}$ phosphorylation during NADPH oxidase activation, and correlated with phagosomal colocalization of flavocytochrome b$_{558}$ (flavocytochrome b) and p47$^{phox}$ and p67$^{phox}$ (p47$^{phox}$/p67$^{phox}$). Termination of respiratory burst activity did not reflect dephosphorylation of plasma membrane- and/or phagosome-associated p47$^{phox}$; in contrast, the specific activity of phosphorylated p47$^{phox}$ at the phagosomal membrane increased. Most significantly, termination of oxidative activity paralleled the loss of p47/p67$^{phox}$ from both NMB and OPZ phagosomes despite the continued presence of flavocytochrome b. These data suggest that 1) the onset of respiratory burst activity during phagocytosis is linked to the phosphorylation of p47$^{phox}$ and its translocation to the phagosome; and 2) termination of oxidative activity correlates with loss of p47/p67$^{phox}$ from flavocytochrome b-enriched phagosomes and additional phosphorylation of membrane-associated p47$^{phox}$. The Journal of Immunology, 1999, 163: 6732–6740.

Human phagocytic cells such as polymorphonuclear leukocytes (PMN)$^1$ are readily mobilized to sites of infection and ingest microorganisms by a process known as phagocytosis (1, 2). Ingested bacteria are killed by reactive oxygen species (ROS) derived from superoxide (O$_{2}^-$) produced by an activated, phagosome-bound NADPH-dependent oxidase. The importance of a functioning oxidase is exemplified by the recurrent bacterial and fungal infections observed in individuals with chronic granulomatous disease (CGD), a disorder in which the oxidase is nonfunctional due to a deficiency in one of its protein components (3–7). In addition to its importance in the innate immune system, the NADPH oxidase is also a prominent contributor to a variety of inflammatory disorders (8–11).

In unstimulated cells the NADPH oxidase is unassembled and inactive, with its seven protein components segregated into membrane and cytosolic locations (reviewed in Ref. 12). Once phagocytes are activated, however, oxidase components p47$^{phox}$ (13–15), p67$^{phox}$ (13, 16), and p40$^{phox}$ (17) translocate en bloc from the cytosol (18, 19) and associate with membrane-bound flavocytochrome b (4, 7, 20). A low m.w. GTPase, Rac2, translocates independently to the assembling oxidase (21–23). Presumably, the intricate assembly event involving cytosolic components regulates the onset of O$_{2}^-$ generation by limiting the potential for inadvertent generation of damaging ROS in host tissues. To date, our understanding of NADPH oxidase activation and assembly has been determined in part from studies using PMN treated with soluble stimuli such as PMA, FMLP, calcium ionophores, or analysis using the broken cell NADPH oxidase reconstitution assay (24). The applicability of conclusions derived from these studies to NADPH oxidase function during phagocytosis of microorganisms remains undetermined. Albeit more related to its role in host defense against bacteria, characterization of oxidase assembly and activation in PMN during phagocytosis is relatively limited.

To this end, we have characterized activation and assembly of the NADPH-dependent oxidase during phagocytosis of opsonized Neisseria meningitidis serogroup B (NMB), an encapsulated Gram-negative diplococcus that can cause severe septicaemia in certain individuals (reviewed in Ref. 25), and have observed phenomena not previously reported when soluble agonists such as PMA and FMLP were used. In this study we demonstrate that termination of NADPH oxidase activity parallels increased phosphorylation of membrane-associated p47$^{phox}$, an event coupled with the loss of p47/p67$^{phox}$ from phagosomes. Collectively, these data provide evidence that inactivation of the NADPH oxidase is regulated at the level of enzyme disassembly.

Materials and Methods

Materials

2,7'-Dichlorodihydrofluorescein diacetate (DCF) and Texas Red-conjugated secondary Abs were obtained from Molecular Probes (Eugene, OR), and superoxide dismutase (SOD) was obtained from Roche Molecular Biochemicals (Indianapolis, IN). [32P]Orthophosphoric acid (10 mCi/ml) was purchased from DuPont-NEN (Boston, MA). Heat-killed, formalin-fixed, protein–A bearing Staphylococcus aureus (Pansorbin cells) were purchased from Calbiochem (San Diego, CA). RPMI 1640 was purchased...
from Life Technologies (Gaithersburg, MD). FITC-conjugated goat anti-mouse and donkey anti-rabbit IgGs were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Dr. Michio Nakamura provided the flavocytochrome b-specific mAb 7D5. Drs. Algardis J. Jesaitis, James B. Burritt, and Mark T. Quinn provided gp91-phox, and p22-phox-specific mAbs 54.1 and 44.1, respectively. All other reagents were purchased from Sigma (St. Louis, MO) unless specified otherwise.

**Neutrophil isolation**

Heparinized venous blood was obtained from healthy individuals and from an individual with X-linked CGD in accordance with a protocol approved by the institutional review board for human subjects at the University of Iowa. PMN were isolated using dextran sedimentation and Hypaque-Ficoll density-gradient separation followed by hypotonic lysis of erythrocytes as described previously (26). Purified PMN were resuspended in Dulbecco’s PBS containing 10 mM d-glucose (DPBS/g), DPBS/g containing 25 µM DCF, HBSS supplemented with 10 mM d-glucose (HBSS/g), RPMI 1640 containing 20 mM HEPES, pH 7.2 (HEPES/RPMI), or phosphate-free loading buffer (LB; see below) and kept on ice until use.

**Growth and opsonization of NMB**

For each experiment, *N. meningitidis* were plated for overnight growth at 37°C with 5.0% CO₂ on gonorrhea culture medium-based agar (Difco,Detroit, MI) and then collected into the appropriate buffer (HBSS/g, DPBS/g, LB, or HEPES/RPMI) via a glass spreading rod the following day for immediate use. Cell density was determined spectrophotometrically at 550 nm from a predetermined growth curve, and *Neisseria* were then opsonized with 100% normal human serum (NHS) for 30 min at 37°C and subsequently washed on the appropriate buffer to remove excess serum. Ospomized PMN were then resuspended in assay buffer to the indicated cell density and kept on ice until use. Zymosan were also opsonized with 100% NHS as described for NMB.

**Neutrophil assays for ROS**

To measure the intracellular generation of ROS, PMN were resuspended in DPBS/g containing 25 µM DCF to 10⁵ cells/ml and then equilibrated for 45 min at room temperature with gentle agitation. PMN (10⁵), 8.0 µg of SOD, 3 x 10⁷ NMB, 5 x 10⁵ opsonized zymosan (OPZ), or 0.2 µg of PMCA were dissolved or resuspended in DPBS/g containing 25 µM DCF, and then added to NHS-coated wells of a chilled 96-well microtiter plate. In all experiments, free NHS was removed from plates and vials following coating by two sequential washes in the appropriate assay buffer. Cold DPBS/g containing 25 µM DCF was added to each well so that the final volume was 200 µl. Exogenous DCF was added with the buffer to maximize fluorescent signal derived from NMB- or OPZ-stimulated PMN. The plate was centrifuged at 400 x g for 5 min at 4°C to synchronize phagocytosis, and generation of ROS was monitored continuously at 37°C for 90 min using a BMG FLUOstar 403 microplate spectrophotometer (BMG Lab Technologies, Durham, NC) without agitation. Alternatively, O₂⁻ generation was determined by the reduction of ferricytochrome c and measured previously with several methods (21). Briefly, PMN were adjusted to 10⁵ cells/ml in HBSS/g, and 100 µl was aliquoted to wells of a chilled 96-well microtiter plate precoated with 100% NHS for ≥1 h at 37°C. Ospomized *N. meningitidis* (3 x 10⁷) in HBSS/g were added to each well, and the plate was centrifuged at 400 x g for 5 min at 4°C to synchronize phagocytosis. Subsequently, the plate was warmed to 37°C using a microplate spectrophotometer (Bio-Rad, Hercules, CA), and the rate of O₂⁻ generation was measured by the SOD-inhibitable reduction of ferricytochrome c at 550 nm with plate agitation. Although all PMN were stimulated simultaneously with NMB, ferricytochrome c was added to individual wells at each 10-min interval. For example, the rate of O₂⁻ produced 30 min after NMB stimulation was determined by adding ferricytochrome c at 30 min and measuring O₂⁻ generation between 30 and 40 min. For comparison, O₂⁻ generation by cells stimulated with 1 µg/ml PMA was measured for the first 10 min. We also observed that after 90 min of exposure to NMB, PMN still generated O₂⁻ in response to PMA.

**Phosphorylation and immunoprecipitation of p47phox**

PMN (1-2 x 10⁶) were resuspended in 1 ml of LB (10 mM Na-HEPES, 138 mM NaCl, 2.7 mM KCl, and 7.5 mM d-glucose, pH 7.5), with 0.5 mM/ml [32P]orthophosphate and rotated at room temperature for 60 min. Following centrifugation, pellets were washed twice with LB, and 10⁵ cells of NMB were transferred to NHS-precoated glass scintillation vials containing 150 µl of LB and incubated for 10-15 min at 25°C. Subsequently, vials were iced immediately for 10 min, and 3 x 10⁶ chilled NMB or 5 x 10⁶ chilled OPZ were pipetted onto the cells. Vials were immediately centrifuged at 400 x g for 5 min at 4°C and then transferred to 37°C. At the indicated time points, cells were solubilized with an equal volume of lysis buffer (5% Triton X-100, 0.25% NP-40, 0.1 M NaF, and 10 mM NaCl, pH 7.5) for 1 h at 37°C. Opsonized ingested NMB were recovered from each time point by aspiration and scraping in a total of 2 ml of LB. Cells were centrifuged at 100 x g for 5 min to pellet only PMN and then were resuspended in 1 ml of relaxation buffer (30) containing 1 mM sodium orthovanadate. PMN were lysed by mechanical cavitation, opsonized zymosan and plasma membrane vesicles could not be satisfactorily separated, a crude plasma membrane/phagosome-enriched fraction was recovered by sequential centrifugation at 100 x g for 5 min, to remove unbroken cells, and then at 250,000 x g for 10 min. Following the 250,000 x g spin, supernatant was collected as the cytosol-derived fraction. The crude plasma membrane/phagosome-enriched fraction was washed in relaxation buffer, which included brief sonication using a low setting, and recentrifuged twice. This pellet was resuspended in RIPA lysis buffer (10 mM sodium phosphate, pH 7.2, containing 1% Triton X-100, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 200 µM sodium orthovanadate, 0.4 U/ml aprotinin, and 2 mM PMSF), precipitated with Ab specific for p47phox, and subjected to SDS-PAGE and autoradiography as described above. Unlabeled samples prepared in parallel were immunoblotted using biotinylated Ab specific for p47phox coupled with enhanced chemiluminescence detection (Super Signal West Dura, Pierce, Rockford, IL). Immunoblot signals were quantified using an IS-1000 Digital Imaging System (Innotech, San Leandro, CA). The sp. act. of the membrane-associated p47phox was calculated by determining the ratio of 32P counts obtained from the PhosphorImager per unit of p47phox protein as measured by densitometry.

Alternatively, OPZ phagosomes and a plasma membrane-enriched fraction from OPZ-stimulated cells were isolated using two separate Percoll gradients. Following nitrogen cavitation, cells were centrifuged at 300 x g for 10 min to pellet unbroken PMN, nuclei, and OPZ phagosomes. Plasma membrane-enriched fractions and a cytosol-derived fraction were isolated from the postnuclear supernatant using a Percoll step gradient as described by Borregaard et al. (30). The pellet resulting from the 300 x g spin was resuspended in relaxation buffer (30) containing 1 mM sodium orthovanadate. PMN were lysed by mechanical cavitation, OPZ phagosomes and a plasma membrane-enriched fraction were washed twice in expansion buffer containing 1 mM sodium orthovanadate and then combined. Although phagosomes and plasma membrane vesicles could be readily isolated from OPZ-stimulated PMN, these fractions were pooled for two reasons: 1) to recover any phagosomal membrane stripped from OPZ during nitrogen bomb cavitation, and 2) to mimic studies of NMB phagosomes that were isolated as crude plasma- and phagosome-enriched fractions. This way we could compare pertinent control fractions between membranes derived from OPZ- and NMB-stimulated PMN. The pooled fractions were solubilized in RIPA buffer and precipitated with Ab specific for p47phox as described above. Immunoprecipitates were processed for either autoradiography or immunoblotting.

**SOD and oxygen consumption**

O₂⁻ generation by OPZ-stimulated PMN was assayed by measuring the inactivation of SOD by superoxide radicals in vitro. The reaction mixture contained 0.15 M sodium phosphate, 0.1 M ascorbic acid, 0.1 M EGTA, 0.5 mM NBT, 0.15 M NaCl, and 0.1 MPD. The reaction was initiated by the addition of 3-4 x 10⁴ PMN in the absence or presence of indicated stimuli for 10 min at 37°C. The complexes were then centrifuged at 350 x g for 2 min, to remove unbroken cells, and the absorbance at 550 nm of the supernatants was measured. The rate of O₂⁻ formation was calculated as the percent change in absorbance per minute.
ACTIVATION OF NADPH OXIDASE DURING PHAGOCYTOSIS

Synchronized phagocytosis, staining, and fluorescence microscopy were performed as described previously (31, 32). Briefly, 10^6 PMN in 2 ml of HEPES/RPMI 1640 were allowed to adhere to glass coverslips ( precoated with 10–100% NHS for 1 h at 37°C in 35-mm dishes for 15 min at 37°C. Subsequently, cells were chilled to 4°C, 10^6 cold NMB or 5 × 10^6 OPZ was added, and dishes were immediately centrifuged at 500 × g for 2 min at 10–12°C to synchronize phagocytosis. Dishes were then incubated at 37°C for 0–90 min, at which time cells were washed with DPBS to remove unbound bacteria or OPZ and then fixed with 10% formalin for 15 min. Cells were permeabilized with acetone for 5 min at −20°C, washed, and then blocked overnight in DPBS containing 5 mg/ml BSA, 10% normal goat serum, and 0.02% sodium azide. Flavocytochrome b was detected using a combination of mAbs, 7D5, 54.1, and 44.1. P47^phox and p67^phox were detected as previously described (31, 32). Oposnized N. meningitidis was detected using mouse mAb 2C3 (a gift from Dr. Peter Rice, Boston University, Boston, MA), which reacts with outer membrane protein H8, or using rabbit polyclonal antiserum specific for NMB. Primary Abs were detected with secondary Abs conjugated to FITC or Texas Red. The specificity of staining was assessed by analysis in the absence of primary Abs.

Fluorescence was visualized using a Zeiss AxioPlan2 epifluorescence microscope (Carl Zeiss, Thornwood, NY), a Zeiss LSM 510 laser-scanning confocal microscope (Carl Zeiss) at the Veterans Administration Medical Center, or a Bio-Rad 1024 laser-scanning confocal microscope (Bio-Rad, Hercules, CA) at the University of Iowa Central Microscopy Research Facility.

Results

ROS generated by PMN stimulated with NMB

To examine the kinetics of ROS produced by PMN in response to NMB, we monitored the generation of both intracellular and extracellular ROS over a 90-min period following synchronized phagocytosis (Fig. 1A). For reference, we compared the PMN response to NMB (30:1, NMB:PMN ratio) with those to OPZ (5:1, OPZ:PMN ratio) and PMA (1 μg/ml; Fig. 1, A and B). The data obtained from these analyses are displayed as direct kinetic plots (Fig. 1A) and as maximum rates calculated from each 5-min interval over the 90-min time course (Fig. 1B). Although the PMN response to NMB was reduced compared with that elicited by OPZ, maximal rates for both occurred within 20–30 min after the assay was initiated (Fig. 1, A and B, inset). The delayed response of PMN to all three agonists appeared to be partly the result of the time necessary for the microtiter plate to warm from 4 to 37°C (±40 min), and peak activities for either OPZ or NMB occurred at 25.0–27.5°C during warming as indicated by arrows (Fig. 1B, inset). Peak activity was subsequently followed by an abrupt decline in the rate of ROS generation, which returned to near-basal levels by 70–90 min (Fig. 1B, inset). The reduction in fluorescence following peak activity was not a result of insufficient quantities of substrate (DCF), as PMA-stimulated PMN produced a response 5- and 3-fold greater than those elicited by NMB and OPZ, respectively (Fig. 1B). Although similar kinetic profiles of ROS generation were elicited by all agonists, the rate of ROS generation by PMA-stimulated PMN was still greater than the maximal rates by two to four separate trials from a representative experiment performed at least four times. Arrows indicate −25.0–27.5°C during warming of the assay. The time required to warm each type of assay (i.e., ROS, phosphorylation, and immunofluorescence assays) varied and reflected variables such as assay volume and the specific method used for warming (air vs thermoplate, for example). The inset was included to illustrate peak ROS generation with NMB and OPZ (zoomed scale). C, O_2^- generation was monitored for PMN stimulated with either NMB (filled bars) or PMA (open bars). Results are expressed as the mean ± SD of the}

FIGURE 1. ROS generation by NMB and OPZ-stimulated PMN. A, PMN (10^6) were incubated for 90 min at 37°C with NMB (30:1 NMB:PMN ratio), OPZ (5:1 OPZ:PMN ratio), or PMA (1 μg/ml) following synchronized phagocytosis as indicated. PMN and opsonized N. meningitidis alone were also incubated for the 90-min period in the presence of 25 μM DCF for comparison. Reactive oxygen species generation was determined as described in Materials and Methods, and kinetic plots are from a representative experiment. B, PMN (10^6) were incubated for 90 min at 37°C with or without NMB (▲; 30:1 NMB:PMN ratio), OPZ (○; 5:1 OPZ:PMN ratio), PMA (□; 1 μg/ml), or without agonist (●) following synchronized phagocytosis, and rates of ROS generation were determined every 5–10 min as indicated. Results are expressed as the mean ± SD of
observed with NMB-stimulated PMN 50 min after peak activity (Fig. 1B). Therefore, diminishing fluorescence reflected a termination of the respiratory burst in all stimulated cells. Superoxide dismutase had little effect on ROS generated by either NMB- or OPZ-stimulated PMN in the fluorescence assays, inhibiting fluorescence by only 9.4 ± 0.7 and 13.0 ± 4.6%, respectively, but eliminated 44.2 ± 3.5% of that generated by PMA-stimulated PMN (data not shown). The inhibitory effect of SOD suggested that most of the ROS were generated within NMB- and OPZ-stimulated PMN and presumably within phagosomes, rather than at the PMN plasma membrane. The specificity of this assay to measure only NADPH oxidase-derived activity was confirmed by experiments using NMB-, OPZ-, and PMA-stimulated PMN isolated from an individual with X-linked CGD (data not shown). We also monitored the generation of O_2^- during phagocytosis over the same time period by the reduction of ferricytochrome c (Fig. 1C). Although the ferricytochrome c assay measured mainly O_2^- escaping forming phagosomes, ROS diffusing out of the cell, or that produced at the plasma membrane, the kinetics of O_2^- production were comparable to those of the DCF assay (Fig. 1C).

**Phosphorylation of p47^phox^ by NMB and OPZ**

Phosphorylation of p47^phox^ has been previously shown to correlate with activation of the respiratory burst in PMN (33–38). To investigate the mechanism of NMB- and OPZ-dependent ROS generation, we monitored the phosphorylation of p47^phox^ during synchronized phagocytosis. P47^phox^ from unstimulated PMN had little phosphate incorporated over the 90-min period studied, and correspondingly, these PMN did not generate ROS (compare Fig. 1B, filled circles, with Fig. 2, A and B, bottom panel and filled circles). By contrast, p47^phox^ precipitated from PMN stimulated with either NMB or OPZ was phosphorylated in a time-dependent manner, and this phosphorylation correlated with activation of the NADPH oxidase (compare Figs. 1 and 2). However, termination of the respiratory burst was not reflected by decreased phosphorylation of p47^phox^ using this assay (compare Figs. 1B, inset, and 2B); i.e., even after oxidase activity had terminated, the levels of phosphorylated p47^phox^ in those PMN were still increased compared with those in unstimulated cells despite similarities in oxidant generation (little or none).

Analysis of phosphorylated p47^phox^ from solubilized PMN did not resolve whether phosphorylated p47^phox^ was cytosolic or membrane associated. Therefore, to determine the subcellular distribution of phosphorylated p47^phox^, we isolated plasma membrane and/or phagosome-enriched (membrane) fractions and a cytosol-derived fraction from PMN stimulated with NMB or OPZ at time points that corresponded to the highest and lowest levels of ROS production (Fig. 3, A and B). As shown in Fig. 3, A and B, the bulk of phosphorylated p47^phox^ was recovered from the cytosolic fraction. Although only a small fraction of the total cellular pool of p47^phox^ becomes membrane associated during oxidase activation (18, 39), we determined by immunoblotting that 5.3 ± 2.2 and 7.8 ± 2.3% of the total p47^phox^ precipitated from cytosol and membrane fractions had translocated to membrane after 30 min of phagocytosis of NMB and OPZ, respectively (Fig. 3, A and B). In contrast to the coupling of phosphorylation of p47^phox^ with respiratory burst activity, termination of the respiratory burst did not reflect decreased phosphorylation of membrane-associated p47^phox^.

In fact, the sp. act. of membrane-associated p47^phox^ phosphoprotein had increased slightly by 90 min when oxidase activity was at a nadir, from 3.5 ± 0.8 and 2.7 ± 0.2 at 30 min for NMB- and OPZ-stimulated PMN, respectively, to 5.8 ± 1.8 and 3.7 ± 0.1 (Fig. 3, A and B). These data demonstrate that there was additional phosphorylation of membrane-associated p47^phox^ coincident with termination of respiratory burst activity.
FIGURE 3. Subcellular localization of phosphorylated p47phox. Following synchronized phagocytosis of NMB (A) or OPZ (B), PMN preloaded with [32P]orthophosphate were nitrogen bomb-cavitated, and cytosol and plasma and phagosomal membrane-containing fractions (membrane) were isolated and precipitated with Ab specific for p47phox as described in Materials and Methods (upper panels). In parallel, cytosol and phagosome/plasma membrane-containing fractions were precipitated with Ab specific for p47phox and then immunoblotted with biotinylated Ab specific for p47phox (lower panels). The amount of p47phox detected in the membrane and cytosol-derived fractions were quantitated as described in Materials and Methods. Translocated represents that percentage of p47phox (total detected by immunoblotting, lower panels) recovered from both the cytosol-derived and membrane-containing fractions in combination. The basal level of translocated p47phox (the percentage recovered from PMN incubated 0 min with either NMB or OPZ) was subtracted from all time points. The sp. act. is defined as the amount of phosphorylated p47phox determined via PhosphorImager per unit protein detected by immunoblotting. Results in A and B are representative of two to five separate experiments.

Monitoring NADPH oxidase assembly during phagocytosis of opsonized NMB

Recovering sufficient phosphorylated p47phox from these assays required enormous quantities of NMB phagosomes, and we routinely used 100 NMB/PMN. Therefore, only early phagocytic events were synchronized, as PMN were continually ingesting NMB at up to 90 min after activation (data not shown). Despite this technical complication, the results shown in Fig. 3 suggested that less p47phox was membrane associated at 90 min after stimulation compared with that at 30 min (3.8 ± 2.7 vs 5.3 ± 2.2%, respectively, for NMB, and 2.9 ± 1.2 vs 7.8 ± 2.3%, respectively, for OPZ). To examine this event more precisely we monitored phagosomal association of p47phox/p67phox following synchronized phagocytosis using immunofluorescence microscopy (32). This assay allows precise control of phagocytosis using low NMB and OPZ to PMN ratios, ∼10:1 and 5:1, respectively. Opsonized N. meningitidis and OPZ phagosomes were enriched for flavocytochrome b immediately upon ingestion as judged using mAbs specific for gp91phox and p22phox (Fig. 4, A and B). Although NMB were difficult to identify at early time points due to the large number of flavocytochrome b-laden granules, it was obvious that by 60 min these phagosomes, and also those containing OPZ, remained enriched for flavocytochrome b (Fig. 4, A and B). We then monitored the NMB phagosomal association of p47phox and p67phox as an indicator of NADPH oxidase assembly (12, 18, 32, 40). Immediately after centrifugation at 10°C, the majority of PMN-bound NMB were not stained using Abs specific for p47phox and p67phox, suggesting that NMB phagosomes had not yet formed, and the oxidase was unassembled (Fig. 5, A and B). Subsequently, phagosomal association of p47phox/p67phox occurred rapidly as NMB were ingested, and translocation peaked at 10–15 min (e.g., by 10 min 62.4 ± 4.0 and 68.9 ± 10.3% of the NMB associated with PMN were p47phox/p67phox positive, respectively; Figs. 5, A and B, and 6). As with peak ROS-generating activity, peak phagosomal association of p47phox/p67phox with NMB phagosomes occurred at 26–27.5°C (indicated with an arrow in Fig. 6), indicating a correlation between translocation (assembly) and induction of the respiratory burst by NMB (compare peaks in Figs. 1B, inset, and 6). Although a large percentage of NMB phagosomes remained p47phox/p67phox positive at 30 min (∼42.6 ± 10.1 and 46.4 ± 5.2%, respectively), by 90 min this was reduced significantly to 12.2 ± 3.9 and 13.7 ± 3.7% (p = 0.0006 and 0.005 for 10 vs 90 min; Figs. 5, A and B, and 6).

To determine whether this phenomenon was characteristic of phagocytosis and not peculiar to NMB, we also monitored NADPH oxidase assembly at OPZ phagosomes (Fig. 5, C and D). As we have shown previously (32), p47phox and p67phox are recruited to forming OPZ phagosomes rapidly upon initiation of ingestion, with peak association of p47phox and p67phox with OPZ phagosomes between 5 and 15 min (68.5 ± 1.4 to 92.3 ± 2.5%; Figs. 5, C and D, and 6). As we observed with NMB phagosomes, the quantity of p47phox/p67phox-positive OPZ phagosomes decreased significantly over time, and by 90 min, there was little p47phox/p67phox association with OPZ phagosomes (Figs. 5, C and D, and 6). Thus, dissociation of p47phox/p67phox from PMN phagosomal membranes appeared to be a general phenomenon of late phagocytosis. Furthermore, the point at which phagosomal association of p47phox/p67phox either peaked or was at a nadir corresponded to the highest and lowest levels of ROS generation, respectively (compare Figs. 1 and 6). These results were consistent with our immunoblotting data using the phosphorylation assay, although kinetics in those assays were skewed due to the large particulate load (compare 10 and 60 min in Fig. 6 with 30 and 90 min in Fig. 3, A and B). Analysis by immunofluorescence microscopy was more selective in that it was restricted to localization of the assembled oxidase on phagosomes, whereas data from the phosphorylation assays reflected the sum of reactivity at the plasma membrane and phagosomal membrane.
stained with Abs specific for flavocytochrome b specific for NMB as described in localized phagocytosis. In

determined using indirect immunofluorescence and confocal microscopy in secondary Ab) and rabbit Ab specific for p67 phox granules.

FIGURE 4. Flavocytochrome b is enriched at forming phagosomes. Flavocytochrome b enrichment on NMB (A) or OPZ (B) phagosomes was determined using indirect immunofluorescence and confocal microscopy in combination with Abs specific for flavocytochrome b following synchronized phagocytosis. In A NMB were detected with a rabbit polyclonal Ab specific for NMB as described in Materials and Methods. In B cells were stained with Abs specific for flavocytochrome b (Texas Red-conjugated secondary Ab) and rabbit Ab specific for p67phox (FITC-conjugated secondary Ab) and were visualized with a confocal laser-scanning microscope as described in Materials and Methods. Stained cells are representative of at least four independent experiments. Arrows indicate the position of OPZ (at 0 min) or representative phagosomes visualized in single confocal sections. Arrowheads indicate the position of flavocytochrome b-enriched granules.

Taken together, these data suggest that termination of the respiratory burst reflects dissociation of p47/67phox from phagosomal membranes.

Discussion

The NADPH-dependent oxidase has been the focus of intense study because of its importance in host defense and the increasing interest in and/or awareness of a role for $O_2^{-}$ in cell-cell signaling (41, 42) and in the pathogenesis of inflammatory disorders such as arthritis (43) and ischemia/reperfusion injury (8–11). Much of our understanding of NADPH oxidase activation and assembly has been derived from in vitro assays using PMA or other soluble stimuli as agonists (18, 33, 34, 40, 44–48) and analysis using broken cell NADPH oxidase reconstitution assays (24, 49–56). However, characterization of this important system in PMN during phagocytosis has been limited.

We examined the kinetics of ROS generation, phosphorylation of p47phox, and translocation of p47/67phox in response to NMB and OPZ to understand activation, assembly, and subsequent termination of NADPH oxidase activity during phagocytosis. Using NMB- or OPZ-stimulated PMN, we observed that respiratory burst activity peaked following phagocytosis and subsequently declined. These kinetics were also observed in PMA-stimulated PMN, although the magnitude of the response was much greater. The ability of PMA-stimulated PMN to generate significantly more ROS than either OPZ- or NMB-stimulated PMN demonstrates that the decline in the generation of ROS following peak respiratory burst activity during phagocytosis of NMB or OPZ reflected neither a limitation of available substrate nor the inability of PMN to produce ROS, but rather the termination of NADPH oxidase activity. A general mechanism for termination of NADPH oxidase activity has not been previously identified, although it may reflect receptor internalization in the case of FMLP-stimulated PMN (57, 58). Our data suggest that termination of the respiratory burst correlates with the removal of p47/67phox from phagosomes and/or the plasma membrane.

Initial phosphorylation of p47phox coincided with the generation of ROS by NMB and OPZ-stimulated PMN (compare peaks in Figs. 1B and 2B). Moreover, initial p47phox phosphorylation kinet-

ics using either NMB or OPZ-stimulated PMN closely mirrored the kinetics of oxidase assembly at phagosomes for the first 10–15 min (compare Figs. 2B and 6). Thus, our findings with NMB or OPZ-stimulated PMN agree with previous reports suggesting that NADPH oxidase activation and assembly are linked to the phosphorylation of p47phox (33, 38, 44, 46, 47, 53, 59–61). Recent biochemical evidence suggests that domains of p47phox necessary for flavocytochrome b and/or p67phox binding during NADPH ox-

idase activation are sequestered when the enzyme is inactive (54, 60, 62, 63), and in vitro studies demonstrate that p47phox changes conformation when phosphorylated and functionally competent (53, 54, 60). During phagocytosis, phosphorylation of p47phox may be a permissive event, necessary only to expose domains and/or impart conformational change to allow association with p67phox and subsequently with flavocytochrome b. Precise quantitation of the subcellular distribution of phosphorylated p47phox is difficult, because translocated p47phox may undergo additional modification, such as dephosphorylation and/or hyperphosphorylation, which would change the sp. act. of membrane-associated species relative to those remaining in or having just returned to the cytosolic compartment. However, it was possible to compare the sp. act. of translocated p47phox phosphoprotein at different times during phagocy-

tosis (Fig. 3, A and B). If dephosphorylation were a mechanism for releasing membrane-associated p47phox, it was not apparent in our assays, because no decrease in p47phox phosphorylation was observed in NMB- or OPZ-stimulated PMN by 90 min, a time by which ROS generation was minimal.

Based on these findings, one can conclude that termination of the respiratory burst is not coincident with dephosphorylation of membrane-associated p47phox. In contrast, the data suggest that additional phosphorylation of membrane-associated p47phox or a change in specific phosphorylated residues could mediate its re-

lease to the cytosol and concomitant termination of oxidase activity. The results shown in Fig. 3, viz., increased p47phox phosphorylation over time concurrent with decreased membrane-associated p47phox immunoreactivity, support the interpretation that hyperphosphorylation of membrane-associated p47phox precedes its disso-

ciation from the membrane. Thus, our data suggest that addi-

tional phosphorylation of membrane-associated p47phox correlates with the loss of p47/67phox from phagosomes during termination of the respiratory burst. Studies to identify and characterize specific phosphorylated species of p47phox from cytosol and phagosomes are necessary to prove this hypothesis and are ongoing.

Translocation of p47/67phox to the plasmalemmal and/or phagosomal membrane is necessary for assembly of the NADPH oxidase.
and initiation of oxidase activity, although features of oxidase termination are undefined. Our results show that association of p47/67phox with NMB- and OPZ-containing phagosomes coincided with the onset of respiratory burst activity (compare Figs. 1B, inset, and 6) and with the phosphorylation of p47phox (compare Figs. 2B and 6). In addition, this correlation held as well during termination of the respiratory burst, as little p47/67phox were phagosome associated when burst activity was minimal; e.g., 68.7 ± 10.3% of NMB phagosomes were p67phox positive at 10 min compared with 13.7 ± 3.7% at 90 min (Figs. 5 and 6). These data indicate that termination of the respiratory burst is coupled to the loss of p47/67phox from the membrane-associated oxidase by either dissociation or limited proteolysis. Phagosomes remained enriched for flavocytochrome b 90 min after PMN stimulation, suggesting that termination of the respiratory burst and release of p47/67phox from phagosomes are not related to selective partitioning of flavocytochrome b from the phagosomal membrane. It should also be noted that colocalization of p47/67phox with flavocytochrome b was restricted to the phagosomal membrane; hence, the cytosolic complex did not colocalize with flavocytochrome b-laden granules during activation (Fig. 4B). Therefore, p47/67phox were targeted specifically to flavocytochrome b docking sites at phagosomes containing NMB or OPZ. The mechanism for specific targeting is unknown, but our previous studies (32) suggest that flavocytochrome b is insufficient for such targeting.

Many clinically important sequelae of inflammation reflect unbridled PMN activation, supporting the need to understand better the determinants of the termination of cell activation as well as the

![Figure 5](http://www.jimmunol.org/Downloadedfrom/A6738ACTIVATIONOFNADPHOXIDASEDURINGPHAGOCYTOSIS)

**FIGURE 5.** Assembly of the NADPH oxidase at phagosomal membranes. Following synchronized phagocytosis of NMB (A and B), PMN were fixed, permeabilized, and double stained with pAb specific for p67phox (A) or p47phox (B) and mAb specific for NMB H8 protein at the indicated times. Following synchronized phagocytosis of OPZ (C and D), PMN were fixed, permeabilized, and stained for p67phox (C) or p47phox (D). Opsonized zymosan phagosomes was visualized by phase-contrast light microscopy. Stained cells are representative of at least three or four independent experiments. Arrows in A and B indicate representative phagosomes visualized in single confocal sections or by indirect immunofluorescence (C and D).

![Figure 6](http://www.jimmunol.org/Downloadedfrom/A6738ACTIVATIONOFNADPHOXIDASEDURINGPHAGOCYTOSIS)

**FIGURE 6.** Kinetics of NADPH oxidase assembly at phagosomal membranes. Opsonized *N. meningitidis* phagosomes positive for p67phox (△) and p47phox (●) and OPZ phagosomes positively stained for p67phox (○) and p47phox (◇) were quantitated using confocal laser-scanning microscopy or indirect immunofluorescence microscopy, respectively. The arrow indicates ~25.0–27.5°C during warming of the assay. Results are expressed as a percentage of the total number of phagosomes that stained positively using Abs specific for p47/67phox and are the mean ± SD of two to four separate experiments.
features important in its initiation. Although this study extends our understanding of the features of oxidase assembly and activation during phagocytosis, many questions remain unanswered. Our data implicate loss of p47<sub>phox</sub> from the phagosome as well as additional phosphorylation of plasma membrane/phagosome-associated p47<sub>phox</sub> as features of oxidase termination. Additional studies are necessary to identify the exact mechanism(s) for release of p47<sub>phox</sub> from phagosomal membranes and for termination of NADPH oxidase activity during phagocytosis.

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