Effects of *Anaplasma phagocytophila* on NADPH Oxidase Components in Human Neutrophils and HL-60 Cells

Jason Mott,1 Yasuko Rikihisa,1* and Shohko Tsunawaki2

Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio 43210-1092,1 and Department of Infectious Disease Research, The National Children’s Medical Research Center, Tokyo 154-8509, Japan2

Received 10 September 2001/Returned for modification 17 October 2001/Accepted 29 November 2001

The human granulocytic ehrlichiosis agent, *Anaplasma phagocytophila*, resides and multiplies exclusively in cytoplasmic vacuoles of granulocytes. *A. phagocytophila* rapidly inhibits the superoxide anion (O$_2^-$) generation by human neutrophils in response to various stimuli. To determine the inhibitory mechanism, the influence of *A. phagocytophila* on protein levels and localization of components of the NADPH oxidase were examined. *A. phagocytophila* decreased levels of p22$^{	ext{phox}}$, but not gp91$^{	ext{phox}}$, p47$^{	ext{phox}}$, p67$^{	ext{phox}}$, or p40$^{	ext{phox}}$ reactive with each component-specific antibody in human peripheral blood neutrophils and HL-60 cells. Double immunofluorescence labeling revealed that p47$^{	ext{phox}}$, p67$^{	ext{phox}}$, Rac2, and p22$^{	ext{phox}}$ did not colocalize with *A. phagocytophila* inclusions in neutrophils or HL-60 cells, and p22$^{	ext{phox}}$ levels were also reduced. *A. phagocytophila* did not prevent either membrane translocation of cytoplasmic p47$^{	ext{phox}}$ and p67$^{	ext{phox}}$ or phosphorylation of p47$^{	ext{phox}}$ upon stimulation by phorbol myristate acetate. The inhibitory signals for O$_2^-$ generation was independent of several signals required for *A. phagocytophila* internalization. These results suggest that rapid alteration in p22$^{	ext{phox}}$ induced by binding of *A. phagocytophila* to neutrophils is involved in the inhibition of O$_2^-$ generation. Absence of colocalization of NADPH oxidase components with the inclusion further protects *A. phagocytophila* from oxidative damage.

Human granulocytic ehrlichiosis (HGE) is an emerging tick-borne zoonosis characterized by systemic signs such as fever, chills, malaise, headache, and/or myalgia (3, 32). Laboratory tests may reveal thrombocytopenia, elevated levels of C-reactive protein, leukopenia, and elevated liver enzyme levels. Severity may range from asymptomatic infections to severe morbidity or mortality. HGE, first described in 1994 (3, 7), has become increasingly recognized in both the United States and Europe. The etiologic HGE agent, *Anaplasma phagocytophila*, is a unique obligatory intracellular bacterium that cannot survive or replicate anywhere other than in cytoplasmic inclusions in neutrophils and other granulocytes, once transmitted from ticks to mammals. Upon exposure to microbial pathogens, neutrophils are the first cells recruited to the site, where their most powerful oxygen-dependent and -independent defense systems kill invading pathogens. One of the oxygen-dependent systems generates the superoxide anion (O$_2^-$) directly from molecular oxygen by the NADPH oxidase enzyme, which is converted to more potent toxic species, such as hydrogen peroxide, hydroxyl radical, and hypochlorous acid (2).

We have previously demonstrated that *A. phagocytophila* not only does not induce O$_2^-$ generation by human peripheral blood neutrophils but also rapidly (within 30 min) deprives neutrophils of the ability to generate O$_2^-$ in response to a variety of potent stimuli, such as phorbol myristate acetate (PMA), formylmethionyl-leucyl-phenylalanine (fMLP), or *Escherichia coli* as determined by both ferricytochrome c reduction and luminol-dependent chemiluminescent assays (22).

The inhibition requires the carbohydrate rather than the protein residue of *A. phagocytophila* as well as *A. phagocytophila* and host cell contact (22). The inhibition is specific to neutrophils and does not occur with human monocytes (only a few minutes’ delay in O$_2^-$ release), and it requires at least 30 min of preincubation of *A. phagocytophila* and neutrophils, as well as host cell protein synthesis, for the complete inhibition (22). The inhibition is not caused by direct interaction of components of *A. phagocytophila* with O$_2^-$ or ferrocytochrome c (or reduced luminol). Instead, the results suggest that *A. phagocytophila* inhibits the NADPH oxidase.

The NADPH oxidase consists of a cytochrome b$_{558}$ (heterodimer of integral membrane proteins gp91$^{	ext{phox}}$ and p22$^{	ext{phox}}$) and several cytosolic components (p67$^{	ext{phox}}$, p47$^{	ext{phox}}$, and p40$^{	ext{phox}}$) (2). In resting neutrophils, the inactive oxidase components remain unassembled and segregated into membranes of secretory vesicles, specific granules, and a cytosolic complex (2). Upon activation, secretory vesicles and specific granules rapidly fuse with plasma or the phagosomal membrane, and a complex of p47$^{	ext{phox}}$, p67$^{	ext{phox}}$, and possibly p40$^{	ext{phox}}$ translocates and associates with cytochrome b$_{558}$ (2, 11). A functional NADPH oxidase enzyme is assembled at the phagosomes and/or the plasma membrane, allowing exertion of the lethal effects of O$_2^-$ and its derivatives on extracellular or ingested bacteria in close proximity. The small GTP-binding protein, Rac2, a major Rac protein expressed in neutrophils, is required for oxidase activity through direct interaction with p67$^{	ext{phox}}$ and cytochrome b$_{558}$ (14). The cytosolic component p47$^{	ext{phox}}$ becomes phosphorylated on the C-terminal eight to nine serine residues, some of which are required for unmasking the SH3 domain for binding to Pro-rich regions of p22$^{	ext{phox}}$, while phosphorylation of p67$^{	ext{phox}}$ and p40$^{	ext{phox}}$ also occurs; however,
whether it is required for NADPH oxidase activation is unknown (2, 28).

Banerjee et al. (4) reported the down-regulation of gp91phox mRNA levels in *A. phagocytophila*-infected HL-60 cells (a human promyelocytic leukemia cell line) at 5 day postinfec- tion and splenic neutrophils from mice at 2 to 8 days postinfection; however, earlier time points critical for *A. phagocytophila* survival or human peripheral blood neutrophils were not examined in the study. Furthermore, there has been no study ex- amining the effects of *A. phagocytophila* on protein levels, phosphorylation, translocation, or localization of the NADPH oxidase enzyme components. The objectives of the present study were the following: (i) to examine the influences of *A. phagocytophila* on protein levels, phosphorylation, translo- cation, and/or localization of NADPH oxidase components in human peripheral blood neutrophils and HL-60 cells, and (ii) to examine whether signals required for internalization of *A. phagocytophila* are involved in the rapid inhibition of O2− production induced by *A. phagocytophila*.

**MATERIALS AND METHODS**

*A. phagocytophila* culture. The *A. phagocytophila* HZ strain isolated from an HGE patient was cultured in the HL-60 cell line in RPMI 1640 medium as previously described (22). When approximately 75% of the HL-60 cells were infected as determined by Diff-Quik staining (Baxter Scientific Products, Obetz, Ohio), cells were suspended in Hank’s balanced salt solution (HBSS) without phenol red and sodium bicarbonate (Sigma Chemical Co., St. Louis, Mo.) and mildly sonicated on ice at predetermined conditions to release intact organisms from infected HL-60 cells (22).

Preparation of human neutrophils. Buffy coat (50–90 ml) from healthy donors was centrifuged at 1,500 × g for 5 min. Following centrifugation, the plasma was removed, and 10 ml of buffy coat was placed in a 50-ml centrifuge tube containing 10 ml of Histopaque 1077 overlaying 15 ml of Histopaque 1119 (Sigma). Following centrifugation at 2,200 × g for 25 min, the interface between Histopaque 1077 and Histopaque 1119 was collected and added to 0.85% NaCl for 5 min at room temperature to lyse any remaining red blood cells. Neutrophils were centrifuged at 1,500 × g for 5 min and washed twice with HBSS. By Diff-Quik staining, cells were determined to be >97% neutrophils. The viability of the neutrophil preparations was determined during each experiment by a trypan blue exclusion test and was found to be >99%. To consider individual human varia- tions, all experiments were independently repeated more than three times on different days using neutrophils derived from different donors and freshly pre- pared host-cell-free *A. phagocytophila*. Donor cells were never mixed, and each donor’s neutrophil assay included positive and negative controls to ensure the quality of each neutrophil and *A. phagocytophila* preparation.

Effects of various compounds on *A. phagocytophila*-induced inhibition of O2− release and internalization. H89 (10 μM), genistein (50 μM), neomycin (10 μM), monodansylcadaverine (MDC) (250 μM), or monoclonal antibody against P selectin glycoprotein ligand (PSGL-1) clone PL1 (final concentration, 2.5 μg/ml; Ancell Immunology Research Products, Bayport, Minn.) was added to 2 × 106 neutrophils in wells of a 24-well plate at 37°C for 30 min prior to addition of host-cell-free *A. phagocytophila* derived from 2 × 106 infected HL-60 cells. The O2− released in response to PMA was measured for 2 h at 37°C using the ferricytochrome c reduction assay as previously described (22).

To determine the internalization, host-cell-free *A. phagocytophila* in RPMI medium was incubated in the presence of each inhibitor with neutrophils pre- treated with each compound, as described above, in RPMI medium for 2 h at 37°C. The cells were centrifuged at 500 × g for 5 min to remove the inhibitor and host cell nonsassociated *A. phagocytophila*. Cells were further treated with 2 mg of pronase/ml in phosphate-buffered saline (PBS) for 5 min at 37°C to remove surface-bound uninternalized *A. phagocytophila*, washed twice with RPMI me- dium, and incubated at 37°C to allow internalized *A. phagocytophila* growth in the absence of any inhibitor. At 16 h, cells were cytotoxic (Cytospin; Shandon, Inc., Pittsburgh, Pa.) and Diff-Quik stained to determine the percentage of infected neutrophils. Data were compared with controls using the Student t test. P values of <0.05 were considered significant.

Preparation of NADPH oxidase-rich membrane fractions. The membrane fraction was prepared by the method of Clark et al. (9). Fresh human neutrophils were plated in wells of a 24-well plate at 107 cells/well in HBSS containing 2 mg of glucose per ml (HBSS). HL-60 cell lysate was added to free *A. phagocytophila* derived from sonication of 107 uninfected or infected cells to the appropriate wells and incubated for 30 min at 37°C in 5% CO2–95% air. Following incubation, PMA (0.5 μg/ml) was added to the appropriate wells and incubated for 5 min at 37°C. Reactions were stopped by adding a 10-fold excess of ice-cold HBSS containing 1 μM okadaic acid (Sigma), 1 μM phenylmethylsulfonyl fluoride (Sigma), 2 mM sodium orthovanadate (Sigma), 10 mM sodium fluoride (Sigma), and 10 μM phenylarsine oxide (Sigma). Cells were pelleted by centrifugation for 5 min at 500 × g before resuspension in 1 ml of ice-cold relaxation buffer [10 mM piperazine-N,N′-bis(ethanesulfonic acid) (pH 7.3), 3.5 mM MgCl2, 100 mM KCl, 3 mM NaCl, 200 μM phenylmethylene sulfonyl fluoride, 10 μg of leupeptin/ml, and 10 μg of pepstatin/ml]. Cells were then disrupted by sonication at power setting 2 at 20 kHz for 7 s by a W-380 ultrasonic processor (Heat Systems, Farmingdale, N.Y.) and centrifuged at 300 × g for 5 min at 4°C to pellet nuclei and unbroken cells. Supernatants were removed to new tubes and centrifuged at 4°C for 5 min at 3,000 × g. The resulting supernatants were centrifuged at 100,000 × g at 4°C for 10 min to pellet the membrane fraction. The pellet was washed twice with ice-cold relaxation buffer containing inhibitors before the final suspension in relaxation buffer. Protein concentrations were determined by a biocinchoninic acid assay (Pierce, Rockford, Ill.).

Effects of chloroquine, concanamycin A, or MG-132 on *A. phagocytophila*-induced alteration of NADPH components. Neutrophils at 5 × 106 cells/well in a 24-well plate containing 300 μM chloroquine (Sigma), 100 nM concanamycin A (Sigma), or 100 μM MG-132 (BIOCLON Research Laboratories, Plymouth Meeting, Pa.) were incubated for 1 h at 37°C followed by incubation for 1 h with host-cell-free *A. phagocytophila*. Cells were lysed in ice-cold NP-40 lysis buffer. An equal volume of 2% sodium dodecyl sulfate-polyacrylamide gel electrophore- sis (SDS-PAGE) sample buffer was added, and the lysates were boiled for 5 min prior to SDS-PAGE and Western blot analysis. We previously found that MG-132 accelerates the apoptosis of human neutrophils in vitro (33). Therefore, to confirm biological activity of MG-132 in each assay, fresh human neutrophils (107) were incubated in triplicate wells with or without 100 μM MG-132 for 16 h to evaluate the percentage of apoptotic cells under a microscope, as previously described (33). To confirm biochemical activities of lysosomal acidification inhibi- tors, human neutrophils (107) were added to a well of a 12-well plate in the presence or absence of 300 μM chloroquine or 100 nM concanamycin A for 1 h at 37°C. Acidrine orange (Sigma) at a final concentration of 2 μg/ml was added to neutrophils, and the mixture was incubated for 1 h at 37°C before cytocyto- trifugation for 2 min at 450 × g to wash away unincorporated dye. Cytosin preparations were made and the cells were mounted, and the views were displayed by epifluorescence mi- croscopy. The blots were exposed for 2 h and then developed. SDS-PAGE and Western blotting were performed as described elsewhere (24). Protein bands were imaged in blocking buffer (5% nonfat dry milk in 1× PBS) at 4°C overnight with primary antibodies (Table 1). After three rinses with TBT buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.02% Tween-20), the blots were incubated at room temperature for 2 h with peroxidase-conjugated anti-rabbit or anti-goat immunoglobulin G (IgG) (ICN pharmaceuticals, Aurora, Ohio) at 1:1,000 dilution in blocking buffer. The blots were washed three times in TBT buffer for 15 min each. Peroxidase-positive bands were detected by immersing the blots in DAB developing solution (73 mM sodium acetate [pH 6.2], 0.3% diaminobenzidine trihydrochloride [Nacalai Tesque, Inc., Kyoto, Japan], and 0.04% hydrogen peroxide) at room temperature. Washing with 0.1 M H2SO4 terminated the enzyme reaction.

Phosphorylation and immunoprecipitation of p47phox. The assay was performed as described by DeLeo et al. (11). Neutrophils at a concentration of 3.2 × 107 cells/ml in loading buffer (10 mM Na-HEPES, 138 mM NaCl, 2.7 mM KCl, and 7.5 mM t-glucose [pH 7.5]) containing 0.4 mM [32P]orthophosphoric acid (NEN Life Science Products, Inc., Boston, Mass.) were incubated for 1 h at 25°C. [32P]-labeled neutrophils (0.8 × 107 cells/ml) in loading buffer containing 1 mM MgCl2 and 0.5 mM CaCl2 were incubated in the presence of either HL-60 cell lysate or host-cell-free *A. phagocytophila* derived from 0.8 × 107 uninfected or infected cells for 30 min at 25°C as stimulated by addition of PMA (0.8 μg/ml) and (ii) 10 μM *A. phagocytophila* for 5 min at 37°C before termination of 10 volumes of ice-cold loading buffer supplemented with a phosphatase inhibitor mixture (5 mM EDTA, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 6.25 μM okadaic acid, and 1 mM p-nitrophenol phosphate). The neutrophils were lysed in ice-cold NP-40 lysis buffer (150 mM NaCl, 1.2% [wt/vol] Nonidet
TABLE 1. Primary antibodies used to label A. phagocytophila, NADPH oxidase components, or α-tubulin in human peripheral blood neutrophils and HL-60 cells

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Origin</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. phagocytophila (BDS strain) gp91phox (C-15)</td>
<td>Horse</td>
<td>1:100 (serum)</td>
<td>J. E. Madigan, University of California, Davis</td>
</tr>
<tr>
<td>gp91phox C-terminal aa 536–555</td>
<td>Goat</td>
<td>1:1,000 (serum)</td>
<td>Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.</td>
</tr>
<tr>
<td>p22phox C-terminal aa 177–195</td>
<td>Rabbit</td>
<td>1:100, 1:1,000 (purified goat IgG in PBS)</td>
<td>S. Tsunawaki, National Children’s Medical Research Center, Tokyo, Japan</td>
</tr>
<tr>
<td>p47phox C-terminal aa 376–390</td>
<td>Rabbit</td>
<td>1:100, 1:1,000 (serum)</td>
<td>S. Tsunawaki</td>
</tr>
<tr>
<td>p40phox N-terminal aa 1–15</td>
<td>Rabbit</td>
<td>1:1,000 (serum)</td>
<td>S. Tsunawaki</td>
</tr>
<tr>
<td>p67phox C-terminal aa 317–469</td>
<td>Mouse</td>
<td>1:100, 1:1,000 (IgG2b in PBS)</td>
<td>Transduction Laboratories, Lexington, Ky.</td>
</tr>
<tr>
<td>Rac2 (C-11)</td>
<td>Mouse</td>
<td>1:1,000 (purified rabbit IgG in PBS)</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Mouse</td>
<td>1:1,000 (ascitic fluid in PBS)</td>
<td>Cedarlane, Hornby, Ontario, Canada</td>
</tr>
</tbody>
</table>

* Dilution used for double immunofluorescence labeling.
* Dilution used for Western immunoblotting.

RESULTS

Effects of A. phagocytophila on the levels of NADPH oxidase components. The p22phox level but not levels of gp91phox, p47phox or p67phox in human peripheral blood neutrophils decreased within 30 min after addition of A. phagocytophila and in HL-60 cells at 7 days postinfection, as revealed by microscopic examination of Diff Quik-stained neutrophils and previously described (33), in contrast with untreated neutrophils (43.0% of cells had apoptotic nuclei). The result suggests that A. phagocytophila-induced change in p22phox is independent of proteasome activity. Lysosomal acidification inhibitors, chloroquine (a membrane-permeative lysosomotropic reagent that becomes protonated in an increasing vacuolar pH) (23) and concanamycin A (a highly sensitive and specific inhibitor of vacuolar-type H+ -ATPase) (12) did with or without A. phagocytophila in the neutrophil (data not shown). MG-132 used in this assay was confirmed to be biologically active, since it induced significant apoptosis in neutrophils after 16 h of treatment: 99.6% cells had apoptotic nuclei (condensed nuclei which lost all lobular connections) as determined by microscopic examination of Diff Quik-stained neutrophils as previously described (33), in contrast with untreated neutrophils (43.0% of cells had apoptotic nuclei). The result suggests that A. phagocytophila-induced change in p22phox is independent of proteasome activity. Lysosomal acidification inhibitors, chloroquine (a membrane-permeative lysosomotropic reagent that becomes protonated in an increasing vacuolar pH) (23) and concanamycin A (a highly sensitive and specific inhibitor of vacuolar-type H+ -ATPase) (12) did

FIG. 1. Analysis of cellular levels of NADPH oxidase components following exposure to A. phagocytophila. HL-60 culture cells or human neutrophils were infected with A. phagocytophila (HGE) for 7 days or 30 min, respectively. Cell lysates (25 μg of infected and uninfected cells were subjected to SDS-PAGE and Western immunoblotting and probed with antibodies against gp91phox (rabbit), p67phox, p47phox, p40phox, p22phox, and α-tubulin. Results shown are representative of three independent experiments.

P-40, 1% Triton X-100, 2.5% [wt/vol] glycerol, 1 mM EGTA, 5 mM MgCl2, and 50 mM Tris-HCl [pH 7.4] supplemented with the phosphatase inhibitor cocktail and incubated with 5 μl of anti-p47phox rabbit polyclonal antibody for 1 h at 4°C. To each sample, 20 μl of protein G-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) in NP-40 lysis buffer was added and incubated for 1 h at 4°C with constant gentle rotation. Beads were boiled in SDS-PAGE sample buffer for 5 min, and solubilized proteins were subjected to SDS-PAGE on 10% polyacrylamide gels. Gels were autoradiographed with Hyperfilm-enhanced chemiluminescence film (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Double immunofluorescence labeling. Neutrophils in RPMI 1640 medium at a concentration of 5 x 10⁶/ml and host-cell-free A. phagocytophila from an equal amount of infected HL-60 cells were added to each well of a six-well plate in a total volume of 2 ml and incubated for 16 h at 37°C. Uninfected or A. phagocytophila-infected HL-60 cells or neutrophils with phosphatase inhibitor cocktail and incubated with 5 μl of anti-p47phox rabbit polyclonal antibody for 1 h at 4°C. To each sample, 20 μl of protein G-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) in NP-40 lysis buffer was added and incubated for 1 h at 4°C with constant gentle rotation. Beads were boiled in SDS-PAGE sample buffer for 5 min, and solubilized proteins were subjected to SDS-PAGE on 10% polyacrylamide gels. Gels were autoradiographed with Hyperfilm-enhanced chemiluminescence film (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

RESULTS

Effects of A. phagocytophila on the levels of NADPH oxidase components. The p22phox level but not levels of gp91phox, p47phox or p67phox in human peripheral blood neutrophils decreased within 30 min after addition of A. phagocytophila and in HL-60 cells at 7 days postinfection, as revealed by Western blot analysis (Fig. 1). The housekeeping protein α-tubulin was used as a control for neutrophil and HL-60 cells protein input across the lanes. Antibody to p22phox was specific to its C terminus (Table 1), suggesting modification of C-terminal p22phox or loss of this protein.

MG-132, a peptide-aldehyde and potent reversible inhibitor of the chymotryptic-like activity of the proteasome (16), did not have any effect on levels of p47phox, p22phox, and p67phox with or without A. phagocytophila in the neutrophil (data not shown). MG-132 used in this assay was confirmed to be biologically active, since it induced significant apoptosis in neutrophils after 16 h of treatment: 99.6% cells had apoptotic nuclei (condensed nuclei which lost all lobular connections) as determined by microscopic examination of Diff Quik-stained neutrophils as previously described (33), in contrast with untreated neutrophils (43.0% of cells had apoptotic nuclei). The result suggests that A. phagocytophila-induced change in p22phox is independent of proteasome activity. Lysosomal acidification inhibitors, chloroquine (a membrane-permeative lysosomotropic reagent that becomes protonated in an increasing vacuolar pH) (23) and concanamycin A (a highly sensitive and specific inhibitor of vacuolar-type H+ -ATPase) (12) did
not prevent the reduction of levels of whole-cell p22\textsuperscript{phox} following incubation with host-cell-free A. phagocytophila. On the contrary, levels of p47\textsuperscript{phox} and p67\textsuperscript{phox} were greater in A. phagocytophila-infected neutrophils than in uninfected neutrophils in the presence of inhibitors (Fig. 2). Control neutrophils incubated with concanamycin A or chloroquine showed decreased accumulation of the acridine orange vital stain, indicating the effectiveness of both acidification inhibitors (data not shown). These results suggest that A. phagocytophila-induced loss of the protein recognizable with antibody to p22\textsuperscript{phox} does not occur through trafficking of p22\textsuperscript{phox} to lysosomes and subsequent digestion by lysosomal enzymes. The results also suggest that the A. phagocytophila either enhanced synthesis of p47\textsuperscript{phox} and p67\textsuperscript{phox} or slowed down their degradation in the presence of lysosomal acidification inhibitors (Fig. 2).

Double immunofluorescence labeling of infected HL-60 cells and neutrophils. Overall intensity of the fluorescence labeling of p47\textsuperscript{phox}, p67\textsuperscript{phox}, and Rac2, which appeared as white dots, did not change between uninfected and infected HL-60 cells or neutrophils, whereas the intensity of p22\textsuperscript{phox} labeling, which also appeared as white dots throughout the cells, was reduced in infected cells from that in uninfected HL-60 cells and neutrophils (Fig. 3, data of neutrophils not shown). p47\textsuperscript{phox}, p67\textsuperscript{phox}, p40\textsuperscript{phox}, and p22\textsuperscript{phox} did not colocalize with A. phagocytophila inclusions (Fig. 3), and the PMA treatment did not induce colocalization of these components (data not shown). The results of immunofluorescence labeling, along with Western blot results (Fig. 1 and 2), indicate that A. phagocytophila reduced the levels of the protein recognizable with antibody to p22\textsuperscript{phox} in the host cell and the loss was not reversed by adding PMA. The results also suggest that cytoplasmic inclusions occupied by A. phagocytophila are able to exclude most of the NADPH oxidase enzyme components.

Effects of A. phagocytophila on translocation of p47\textsuperscript{phox} and p67\textsuperscript{phox} to the neutrophil membrane in response to PMA. To determine whether A. phagocytophila inhibits the translocation of cytosolic complex p47\textsuperscript{phox} and p67\textsuperscript{phox} to the membrane, the neutrophil membrane fraction was isolated following incubation with or without host-cell-free A. phagocytophila and PMA stimulation. A. phagocytophila did not induce translocation of p47\textsuperscript{phox}, p67\textsuperscript{phox}, and p40\textsuperscript{phox} to the membrane fraction (Fig. 4). Following stimulation of neutrophils by PMA, levels of p47\textsuperscript{phox} and p67\textsuperscript{phox} in the membrane fraction increased in both control neutrophils and neutrophils preincubated with host-cell-free A. phagocytophila (Fig. 4). However, membrane fractions from neutrophils preincubated with host-cell-free A. phagocytophila consistently showed decreased levels of p22\textsuperscript{phox} with or without PMA stimulation compared with uninfected unstimulated neutrophils (Fig. 4). gp91\textsuperscript{phox} levels in the membrane fraction did not decrease with infection. These results indicate that A. phagocytophila did not inhibit membrane translocation of cytosolic components in response to PMA.

Phosphorylation of p47\textsuperscript{phox} in the presence of A. phagocytophila. To examine whether the phosphorylation of p47\textsuperscript{phox} is inhibited in the presence of A. phagocytophila, A. phagocytophila and p47\textsuperscript{phox} for 30 min prior to stimulation with PMA. The p47\textsuperscript{phox} was then immunoprecipitated and autoradiographed. A. phagocytophila did not induce p47\textsuperscript{phox} phosphorylation in neutrophils (Fig. 5). In contrast, neutrophils stimulated with PMA showed increased levels of p47\textsuperscript{phox} phosphorylation in both infected and uninfected neutrophils (Fig. 5). This result indicates that A. phagocytophila does not inhibit the phosphorylation of p47\textsuperscript{phox} in response to PMA.

Signals required for internalization of A. phagocytophila are not required for inhibition of O\textsuperscript{2-} release by A. phagocytophila. Previous results in our laboratory (33) have shown that internalization of A. phagocytophila in human neutrophils was inhibited by the transglutaminase inhibitor, MDC (18). In the present study, H89, a cell-permeative, selective protein kinase A (PKA) inhibitor (8), neomycin, phospholipase-C (PLC) inhibitor (5), and genistein, a PTK inhibitor (1), were found to inhibit internalization of A. phagocytophila bacteria into neutrophils (Table 2). Herron et al. (13) reported that monoclonal antibody PL1 directed against PSGL-1 significantly prevented A. phagocytophila binding and infection in HL-60 cells. None of these inhibitors or the antibody abrogated A. phagocytophila inhibition of O\textsuperscript{2-} generation in response to PMA (Table 3). Altogether, these results indicate that A. phagocytophila inhibition of the NADPH oxidase is independent of signals required for internalization of A. phagocytophila.

DISCUSSION

Our previous study showed active inhibition of O\textsuperscript{2-} generation in response to divergent stimuli (PMA, FMLP, and E. coli) by A. phagocytophila, suggesting that the site of inhibition is a converged step downstream to divergent signaling pathways induced by all these stimuli (22). The present results indicate reduction in p22\textsuperscript{phox} protein levels in neutrophils within 30 min and in HL-60 cells within 7 days after incubation with A. phagocytophila by both Western blotting and immunofluorescence labeling, suggesting that this reduction is responsible for the inhibition of O\textsuperscript{2-} generation by A. phagocytophila.
Protein levels of gp91phox and other components, as well as membrane translocation of p47phox or p67phox and phosphorylation of p47phox in response to PMA, were not reduced by A. phagocytophila infection. It remains to be studied why the inhibition of O₂⁻/H₂O₂ generation by A. phagocytophila is 80 to 100% (22), while the reduction of p22phox was partial, if the reduction of p22phox is solely responsible for the inhibition. According to the 35S-methionine pulse-chase experiment, both p22phox and gp91phox are produced in excess and their rapid degradation is evident within 1 h (10). Therefore, there seems to be a pool of biologically inactive but antibody-reactive p22phox, which may not be affected by A. phagocytophila. Both gp91phox and p22phox were still visible by Western immunoblot analysis when NADPH enzyme activity was reduced by 95% compared with controls by the treatment with an inhibitor of heme synthesis (34), suggesting that a complete loss of antibody-reactive p22phox is not required for ~95% inhibition of superoxide generation. In our previous study we demonstrated that the host protein synthesis is required for the complete inhibition of O₂⁻ generation by A. phagocytophila (22); preliminary data of members of our group (M. Lin and Y. Rikihisa, unpublished data) revealed that cycloheximide prevents p22phox reduction, supporting our hypothesis that the reduction of p22phox is responsible for the inhibition.

The p22phox antibody used in the present study targets C-terminal amino acids (aa) 177 to 195, which includes aa 181 to 188 exposed to the cytoplasmic side (6) but does not include the Pro-rich sequence of aa 149 to 162 that is critical for

![FIG. 3. Double immunofluorescence labeling of the NADPH oxidase enzyme components p22phox, p47phox, p67phox, and Rac2, which appear as white dots in uninfected (A) or A. phagocytophila-infected (B) HL-60 cells. Note reduced amounts of p22phox in infected cells compared to those in uninfected cells. An arrow in the panel showing p67phox labeling in uninfected HL-60 cells (A) indicates an area of intense fluorescent labeling near the Golgi apparatus. Arrows in the infected HL-60 cells (B) indicate the locations of A. phagocytophila (HGE) morulae. Magnification, ×750. Results are representative of three independent labeling experiments.](http://iai.asm.org)
FIG. 4. Analysis of neutrophil membrane-associated NADPH oxidase components after incubation with *A. phagocytophila* and PMA stimulation. Human neutrophils were incubated in the presence or absence of host-cell-free *A. phagocytophila* (HGE) for 30 min prior to stimulation with PMA (5 min with 0.5 μg of PMA/ml). Cells were lysed, and membrane fractions were obtained by high-speed centrifugation. Membrane fractions (25 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane prior to being probed with antibodies against gp91phox, p67phox, p47phox, and p22phox. Western blot results are representative of more than three independent experiments, each performed with neutrophils derived from different donors and freshly prepared host-cell-free *A. phagocytophila*.

**p22phox** interaction with the SH3 domains of p47phox to translocate to the membrane (17, 28). We don’t know whether the whole p22phox molecule was lost or modified by *A. phagocytophila* so that the C-terminal cytoplasmic domain was no longer recognizable with this antibody. Since membrane translocation of p47phox or p67phox in response to PMA was not impaired by *A. phagocytophila* infection, the p47phox binding site on p22phox appears still to be available. Thus, p22phox may be modified rather than completely lost by *A. phagocytophila* infection. The fact that *A. phagocytophila*-induced inhibition was reversible upon its removal from the surface of neutrophils (22) further supports the possibility of p22phox modification. Proteolysis through proteasomes is suggested to be involved in part in the normal turnover process of gp91phox and p22phox in human neutrophils (10). However, neither neutrophil proteasomes nor lysosomes alone appear to be responsible for the decreased p22phox antigen levels. Either modification or degradation must have made the proper assembly of NADPH oxidase subunits impossible, resulting in no or minimum enzyme activity. The reason for apparent discrepancy between Banerjee et al. (4) and the present studies is unknown. Since we found a reduc-

| Treatment | % Infected cells
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.0 ± 1.7</td>
</tr>
<tr>
<td>MDC</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Genistein</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>Neomycin</td>
<td>0.3 ± 0.6</td>
</tr>
<tr>
<td>H89</td>
<td>0.7 ± 1.2</td>
</tr>
</tbody>
</table>

*Neutrophils preincubated for 30 min with inhibitors were further incubated with host cell-free *A. phagocytophila* in the presence of each inhibitor for 2 h at 37°C. The cells were treated with 2 μg of pronase/ml to remove unincorporated *A. phagocytophila* and cultured in the absence of inhibitors for 16 h at 37°C, and infection rates were determined.

Means ± standard deviations (n = 3). Results are representative of three independent experiments.

TABLE 2. Effects of MDC, genistein, neomycin, and H89 treatment on *A. phagocytophila* internalization in human peripheral blood neutrophils

| Treatment | Reduction of ferricytochrome c (nmol of O₂⁻/10⁶ neutrophils)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>Medium control</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>PMA</td>
<td>21.1 ± 3.4*</td>
</tr>
<tr>
<td>MDC</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>MDC + PMA</td>
<td>18.7 ± 0.3*</td>
</tr>
<tr>
<td>MDC + <em>A. phagocytophila</em> + PMA</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>Neomycin</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>Neomycin + PMA</td>
<td>14.7 ± 0.2*</td>
</tr>
<tr>
<td>Neomycin + <em>A. phagocytophila</em> + PMA</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>Genistein</td>
<td>3.7 ± 2.2</td>
</tr>
<tr>
<td>Genistein + PMA</td>
<td>15.4 ± 1.2*</td>
</tr>
<tr>
<td>Genistein + <em>A. phagocytophila</em> + PMA</td>
<td>4 ± 3.1</td>
</tr>
<tr>
<td>H89</td>
<td>3.8 ± 1.4</td>
</tr>
<tr>
<td>H89 + PMA</td>
<td>16.4 ± 0.3*</td>
</tr>
<tr>
<td>H89 + <em>A. phagocytophila</em> + PMA</td>
<td>2.4 ± 0.1</td>
</tr>
</tbody>
</table>

*Neutrophils pretreated with or without reagents (250 μM MDC, 10 μM neomycin, 50 μM genistein, 10 μM H89, and 2.5 pg of anti-PSGL-1) for 30 min prior to addition of *A. phagocytophila* and PMA stimulation. Reagents were present throughout assays.

Mean ± standard deviation (n = 3). Results are representative of more than three independent experiments. *P < 0.01 compared to neutrophil-alone (no PMA) control (Student’s t test).

TABLE 3. Inhibition of O₂⁻ release by human peripheral blood neutrophils in response to PMA by *A. phagocytophila* in the presence of MDC, neomycin, genistein, H89, and anti-PSGL-1

| Treatment | Reduction of ferricytochrome c (nmol of O₂⁻/10⁶ neutrophils)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>Medium control</td>
<td>0.4 ± 0.6</td>
</tr>
<tr>
<td>PMA</td>
<td>8.5 ± 0.6*</td>
</tr>
<tr>
<td><em>A. phagocytophila</em></td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td><em>A. phagocytophila</em> + PMA</td>
<td>1.9 ± 1.0</td>
</tr>
<tr>
<td>PL1 + PMA</td>
<td>6.3 ± 0.6*</td>
</tr>
<tr>
<td><em>A. phagocytophila</em> + PL1 + PMA</td>
<td>1.8 ± 0.5</td>
</tr>
</tbody>
</table>

*Neutrophils pretreated with or without reagents (250 μM MDC, 10 μM neomycin, 50 μM genistein, 10 μM H89, and 2.5 pg of anti-PSGL-1) for 30 min prior to addition of *A. phagocytophila* and PMA stimulation. Reagents were present throughout assays.

Mean ± standard deviation (n = 3). Results are representative of more than three independent experiments. *P < 0.01 compared to neutrophil-alone (no PMA) control (Student’s t test).
tion in p22phox antigen levels but not in gp91phox antigen levels using both human peripheral blood neutrophils at 30 min postinfection and HL-60 cells at 7 days postinfection, differences in host cell types or in postinfection time points of examination do not seem to be the reason. Since there are several posttranscriptional and -translational modifications on p22phox and gp91phox, the discrepancy may be due to different detection targets: mRNA in the study by Banerjee et al. (4) versus antigenically recognizable proteins in the present paper.

The replicative inclusion of A. phagocytophila that excludes the NADPH components partially resembles the compartment occupied by Salmonella spp. In the case of Salmonella enterica serovar Typhimurium, the localization of the NADPH oxidase components to the salmonella phagosome in murine macrophages was prevented by a cluster of genes encoded by the salmonella pathogenicity island 2; however, the overall production of O$_2^-$ in response to salmonella was not inhibited (30).

The inhibition of O$_2^-$ generation induced by A. phagocytophila does not seem to involve any previously reported mechanisms. It has been suggested that elevated levels of cAMP and activation of PKA are required for the inhibition by NADPH oxidase enzyme (2, 25). However, PKA is not responsible for dephosphorylation and inactivation of the human granulocytic Ehrlichia species as the etiologic agent of human disease. J Clin Microbiol. 32:589–595.

It has been suggested that elevated levels of cAMP and activation of PKA lead to activation of a downstream phosphatase responsible for dephosphorylation and inactivation of the NADPH oxidase enzyme (2, 25). However, PKA is not required for the inhibition by A. phagocytophila of production of O$_2^-$ by neutrophils. Protein-tyrosine phosphatase activity of Yersinia enterocolitica and Coxella burnetti is responsible for inhibition of the fMLP-stimulated O$_2^-$ generation (19, 31). However, since the PTK inhibitor genistein had no influence on PMA-induced O$_2^-$ release, as shown in the present study and by Mocsai et al. (20), and A. phagocytophila can inhibit PMA-induced O$_2^-$ release, a protein-tyrosine phosphatase is not the inhibitory mechanism of O$_2^-$ release by A. phagocytophila. Interleukin 13 (IL-13) inhibition of O$_2^-$ production by human monocytes following PMA stimulation is mediated by intracellular Ca$^{2+}$ mobilization induced by PLC activation (26, 27). Pseudomonas aeruginosa hemolytic PLC suppresses PMAbut not fMLP-stimulated NADPH oxidase activity in human neutrophils (29). The O$_2^-$ inhibition by A. phagocytophila did not require the PLC activity. Legionella pneumophila was also shown to inhibit the PMA-induced O$_2^-$ generation in human monocytes by down-modulation of PKC isozymes α and β (15). A. phagocytophila did not interfere with the phosphorylation of 47phox in response to PMA. Altogether, lack of involvement of PTK, PTK, or PLC in the inhibition of O$_2^-$ production induced by A. phagocytophila represents a novel mechanism.

Our previous study using Transwell showed that A. phagocytophila binding is required (22). Although we found that several signals are required for A. phagocytophila internalization, for the inhibition of O$_2^-$ production binding alone is sufficient, and signals required for internalization of A. phagocytophila are not involved in the inhibition. In the present study we found that the previously described antibody to PSGL-1 (14) did not have any effect the inhibition of O$_2^-$ production. A. phagocytophila did not induce fusion of secretory vesicles containing cytochrome b$_558$ with the plasma membrane. Therefore, engagement of A. phagocytophila to an undetermined neutrophil-specific receptor on the plasma membrane induces the synthesis of a host protein which facilitates generalized (not limited to the plasma membrane) reduction of p22phox in the neutrophils, leading to the inhibition of O$_2^-$ production.

**ACKNOWLEDGMENT**

This work was supported by grant R01 AI30010 from the National Institutes of Health.

**REFERENCES**


