Endotoxin Priming of Neutrophils Requires NADPH Oxidase-generated Oxidants and Is Regulated by the Anion Transporter ClC-3*

Received for publication, June 27, 2007, and in revised form, September 27, 2007 Published, JBC Papers in Press, October 1, 2007, DOI 10.1074/jbc.M705289200

Jessica G. Moreland‡§, A. Paige Davis‡§, James J. Matsuda‡, Jessica S. Hook§, Gail Bailey‡§, William M. Nauseef‡¶, and Fred S. Lamb‡

From the ‡Division of Critical Care, Department of Pediatrics, "The Inflammation Program, and ‡Department of Internal Medicine, University of Iowa and Veterans Affairs Medical Center, Iowa City, Iowa 52242

Several soluble mediators, including endotoxin, prime neutrophils for an enhanced respiratory burst in response to subsequent stimulation. Priming of neutrophils occurs in vitro, and primed neutrophils are found in vivo. We previously localized the anion transporter CIC-3 to polymorphonuclear leukocytes (PMN) secretory vesicles and demonstrated that it is required for normal NADPH oxidase activation in response to both particulate and soluble stimuli. We now explore the contribution of the NADPH oxidase and CIC-3 to endotoxin-mediated priming. Lipooligosaccharide (LOS) from Neisseria meningitidis enhances the respiratory burst in response to formyl-Met-Leu-Phe, an effect that was impaired in PMNs lacking functional CIC-3 and under anaerobic conditions. Mobilization of receptors to the cell surface and phosphorylation of p38 MAPK by LOS were both impaired in PMN with the NADPH oxidase chemically inhibited or genetically absent and in cells lacking functional CIC-3. Furthermore, inhibition of the NADPH oxidase or CIC-3 in otherwise unstimulated cells elicited a phenotype similar to that seen after endotoxin priming, suggesting that basal oxidant production helps to maintain cellular quiescence. In summary, NADPH oxidase activation was required for LOS-mediated priming, but basal oxidants kept unstimulated cells from becoming primed. CIC-3 contributes to both of these processes.

Polymorphonuclear leukocyte (PMN) activation occurs during ingestion of microorganisms and includes the release of preformed granular proteins, including proteolytic enzymes, and de novo generation of reactive oxygen species (ROS) by the NADPH oxidase (1). There are multiple regulatory mechanisms that modulate PMN activation so that the amplitude of the cellular response matches the intensity of the stimulus. One such mechanism is priming, whereby the functional response of PMN to an activating stimulus is amplified by previous interaction with a priming agent. Diverse agents prime PMN, including microbial components and soluble host proteins, and both the underlying mechanisms of priming and the phenotype of the primed PMN are dependent on the identity of the stimulus (for review, see Ref. 2).

Endotoxin or lipopolysaccharide, a component of the cell wall of Gram-negative bacteria and a potent inflammatory mediator, primes PMN for marked enhancement of the respiratory burst in response to formyl-Met-Leu-Phe (fMLF) (3), opsonized zymosan (4), and PMA (5). Lipopolysaccharide stimulation also primes PMN for degranulation and elastase release in response to fMLF (6) and for changes in adhesion (7). Priming occurs in vivo and primed PMNs have been demonstrated in the circulation of patients after traumatic injury (8), during the course of the acute respiratory distress syndrome (9), and in the setting of sepsis (10). In addition, low concentrations of endotoxin are present in the plasma of patients with Gram-negative bacterial infections (11). Therefore, the study of endotoxin-mediated priming is highly relevant to disease pathogenesis.

The phagocyte NADPH oxidase is a multicomponent enzyme complex that produces substantial quantities of ROS necessary for optimal microbialidal activity against certain pathogens. Patients with chronic granulomatous disease (CGD) lack a functional NADPH oxidase and are prone to frequent and serious infections (12). Oxidant signaling in PMN has also been demonstrated to be involved in the regulation of integrin activation (13), apoptosis (14), and priming by select agents (15). Impaired oxidant signaling in PMN might contribute to the complex phenotype of CGD.

We recently described the involvement of the anion transporter CIC-3 in NADPH oxidase activation of PMNs in response to both particulate and soluble stimuli (16). Concurrently, studies conducted in vascular smooth muscle cells have demonstrated impaired cytokine-initiated redox signaling in Clcn3-/- cells (17). We investigated priming in PMNs as a process similar to cytokine-induced signaling in other cell
types, as tumor necrosis factor-α is a well described priming agent. We hypothesized that NADPH oxidase-derived oxidants serve as signals during endotoxin-mediated priming and that ClC-3 participates as a regulator of NADPH oxidase function. The development of the primed phenotype in response to endotoxin, including enhancement of the respiratory burst to fMLF, up-regulation of intracellular stores of receptors, and phosphorylation of p38 MAPK, required NADPH oxidase activation and ClC-3 function. Unexpectedly, we also found that low, tonic levels of NADPH oxidase-derived ROS were required for the maintenance of the non-primed quiescent state. Our data suggest that ClC-3 is proximally involved in oxidant-dependent events in the PMN, including endotoxin priming and the maintenance of cellular quiescence.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hanks’ balanced salt solution was obtained from BioWhittaker (Walkersville, MD). Fetal bovine serum was obtained from HyClone (Logan, UT). Polyclonal antibody to ClC-3 was from Sigma. Rabbit anti-p38 MAPK and anti-phosphorylated threonine and tyrosine p38 MAPK were from Cell Signaling (Beverly, MA). Murine anti-human CD11b was purchased from Pharmingen, and rat-anti-mouse CD11b was purchased from the Developmental Hybridoma bank at the University of Iowa. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG + IgM and FITC-conjugated donkey anti-rabbit F(ab') 2 were from Jackson ImmunoResearch Laboratories (West Grove, PA). Mouse IgG 1 was purchased from Sigma, and rat IgG 2 was purchased from BIOSOURCE. Antibody to the gp91phox component of the flavocytochrome b 558 was generated by Dr. Nauseef (clone 7D5). Lipopoligosaccharide (LOS) purified from Neisseria meningitidis and lipopolysaccharide-binding protein (LBP) were generous gifts from Dr. Jerrold Weiss. Niflumic acid (NFA) was purchased from Sigma, dissolved in Me 2 SO at a concentration of 10⁻¹ M, and used at a final concentration of 10⁻³ M. Additional reagents were all obtained from Sigma.

**Clcn 3⁻/⁻ Mice and Murine Leukocyte Isolation**—Generation of the Clcn 3⁻/⁻ mice with a C57Bl/6j-SV129 background has been previously described (18). All animals had free access to food and water. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Iowa. For isolation of murine PMNs from bone marrow, mice were euthanized, and then the femur, tibia, and iliac bones were removed and stripped of all tissue. Distal and proximal ends of each bone were transected, a 25-gauge needle was inserted into the shaft, and cold buffered Hanks’ balanced salt solution was used to flush out bone marrow cells. Cells were pelleted at 600 × g for 10 min at 4 °C then suspended in 3 ml of 45% Percoll. Percoll density gradient was made in a 15-ml tube by layering successively 2 ml each of 62, 55, and 50% Percoll on top of 3 ml of 81% Percoll. Leukocytes were then gently loaded on top of gradient. Cells were centrifuged at 1600 × g for 30 min at 10 °C, and PMNs were harvested between 81 and 62% layers and washed to remove Percoll.

**Human PMN Purification**—Human PMNs were isolated according to standard techniques from heparin anti-coagulated venous blood from healthy consenting adults in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa. PMNs were isolated using dextran sedimentation and Hypaque-Ficoll density-gradient separation followed by hypotonic lysis of erythrocytes as previously described (19).

**Measurement of NADPH Oxidase Activity Chemiluminescence**—Lucigenin-enhanced chemiluminescence (LUC-CL) assays of NADPH oxidase activity were performed in a 96-well plate using the Wallac Victor 1420 luminometer (PerkinElmer Life Sciences). 200 μl of a PMN suspension containing 2.5 × 10⁶ PMNs/ml in Hanks’ balanced salt solution with 1% human serum albumin and 0.1% dextrose was added to each well with a final concentration of lucigenin 100 μM. Cells were stimulated by the addition of LOS (10 ng/ml) in the presence of LBP (100 ng/ml) ± fMLF as specified. Chemiluminescence was quantified as relative luminescence units using a kinetic assay with readings every minute for 30–90 min.

**Reduction of Ferricytochrome c**—Extracellular O²⁻ generation was measured using the superoxide-dismutase (SOD)-inhibitable reduction of ferricytochrome c in a 96-well microplate using the SPECTRmax plus (Molecular Devices). PMN suspensions were diluted and added to the microplate as described above. Cytochrome c (100 μM) was added to the suspension just before loading in the microplate. In duplicate wells, SOD was added at a final concentration of 50 μg/ml. The maximum rate (V max) of O²⁻ generation and the total nmol O²⁻/min was calculated as the SOD-inhibitable reduction of ferricytochrome c, with readings at absorbance 550 nm every 15 s for 30 min after injection of the stimulus as specified.

**Effect of Anion Channel Inhibition on ClC-3 Current**—The short (see GenBankTM X78520) N-terminal isoform of human ClC-3 was PCR-amplified and cloned into the adenovirus shuttle plasmid pAd5 CMV. Bicistronic adenoviruses co-expressing ClC-3 (Ad-ClC-3) behind the cytomegalovirus promoter and enhanced green fluorescent protein (eGFP) behind the Rous sarcoma virus promoter were prepared and titrated by the University of Iowa Vector core. Control adenovirus expressed only eGFP (Ad-eGFP). HEK293 cells (HEK293T, adenoviral propagation-resistant) were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were infected with adenovirus in serum-free Dulbecco’s modified Eagle’s medium for 16 h before being returned to their standard serum concentration where the virus was allowed to express for 48 h before experimentation.

Chloride ion currents were measured at room temperature (22 °C) using standard whole-cell voltage-clamp techniques with an Axopatch 200B patch clamp amplifier driven by pClamp 9 software (Molecular Devices Corp., Sunnyvale, CA). Pipette resistances were 3–5 megaohms. Pipette and whole-cell capacitance and series resistance were compensated. Holding potential was −40 mV and 1-s test pulses were delivered every 3 s to test potentials from −100 to +100 mV in 20-mV increments. Currents were sampled at 5 kHz and filtered at 1 kHz. The standard bath solution contained 120 mM NaCl, 2.5 mM MgCl₂, 2.5 mM CaCl₂, 10 mM HEPES, 5.5 mM glucose, pH 7.2 with NaOH, and osmolality was titrated to 300 mosmol by osmometer (μ OSMETTE) using 1 M mannitol. Liquid junction
CIC-3 and NADPH Oxidase in Endotoxin Priming of Neutrophils

![Graphs and tables showing NADPH oxidase activity]

**FIGURE 1. NADPH oxidase activity of Clcn3<sup>+/+</sup> versus Clcn3<sup>−/−</sup> PMNs measured by LUC-CL.** A, stimulation with fMLF (100 μM) in otherwise untreated murine PMNs elicited a small increase in NADPH oxidase activity. B, after stimulation with LOS (10 ng/ml) in the presence of LBP, Clcn3<sup>+/+</sup> PMNs demonstrated significantly enhanced NADPH oxidase activity in comparison to Clcn3<sup>−/−</sup> PMNs. C and inset, fMLF (100 μM) stimulation after LOS priming elicited marked enhancement in the respiratory burst, which was greater in Clcn3<sup>−/−</sup> versus Clcn3<sup>+/+</sup> PMNs. n = 5 mice of each strain. RLU, relative luminescence units.

**RESULTS**

**Diminished LOS Priming in Murine Clcn3<sup>−/−</sup> PMNs**—To explore the contribution of CIC-3 to LOS priming, we measured the respiratory burst in Clcn3<sup>+/+</sup> and Clcn3<sup>−/−</sup> murine PMNs by fMLF using LUC-CL. fMLF is a relatively weak activator of NADPH oxidase activity in otherwise unstimulated PMNs; however, exposure to a priming agent resulted in a 5–10-fold augmentation of subsequent fMLF-stimulated ROS production. We used a highly purified LOS from N. meningitidis as the priming agent (20). Although there were minimal ROS generated in response to fMLF at 1–10 μM, 100 μM fMLF elicited similar levels of NADPH oxidase activity in both Clcn3<sup>+/+</sup> and Clcn3<sup>−/−</sup> PMNs (Fig. 1A). In Clcn3<sup>+/+</sup> PMNs, LOS exposure (10 ng/ml) elicited robust NADPH oxidase activity, whereas Clcn3<sup>−/−</sup> PMNs had less than 50% of the NADPH oxidase activity by comparison (Fig. 1B). We detected a transient but significant increase in ROS in response to fMLF 100 μM in LOS-primed murine PMNs (Fig. 1C and inset). This primed burst was 2-fold increased in Clcn3<sup>−/−</sup> versus Clcn3<sup>+/+</sup> PMNs.

**Abnormal Priming of the Respiratory Burst in PMNs after Anion Transporter Inhibition**—In view of the well defined differences between human and murine PMNs (21, 22), we utilized the anion channel inhibitor NFA to extend our findings of abnormal NADPH oxidase activity in Clcn3<sup>−/−</sup> PMNs to human PMNs. In response to fMLF (1 μM) alone, naïve PMN exhibited a small burst of NADPH oxidase activity detected in both control and NFA-treated PMNs (Fig. 2A). In response to the priming stimulus alone (LOS 10 ng/ml), ROS were gener-

potentials were minimized by using 3 mM KCl agar bridges. Pipette solutions for standard whole cell recordings contained 120 mM CsCl, 4 mM triethylammonium chloride, 5 mM EGTA, 1.187 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 5 mM Na-ATP, 0.5 mM Na-GTP, 10 mM HEPES, pH 7.2 with CsOH, osmolality was 290 mosmol, and free [Ca<sup>2+</sup>] was 55 μM (calculated using WEBMAXC). Currents were expressed as current density (pA/picofarads). GFP-positive cells were identified using a fluorescence-equipped inverted microscope (Zeiss Axiovert 25).

Analysis of Cell Surface Receptor Expression and Intracellular p38 Levels by Flow Cytometry—PMNs at a concentration of 1 × 10<sup>6</sup>/ml were analyzed using a FACScalibur flow cytometer (BD Biosciences). For surface expression of flavocytochrome b<sub>558</sub> and CD11b, PMNs were incubated in Hanks’ balanced salt solution alone or in the presence of LOS/LBP in the presence or absence of NFA or diphenyleneiodonium (DPI) as specified. After incubation, PMNs were centrifuged and resuspended in blocking buffer containing phosphate-buffered saline with 2% nonfat dry milk and 1% normal goat serum for 20 min on ice. Primary antibodies were added after blocking; mouse IgG<sub>2a</sub> control, anti CD11b, or anti-gp91<sub>phox</sub>, all at final concentrations of 10 μg/ml and incubated for 1 h on ice. Cells were centrifuged and resuspended in fluorescein isothiocyanate-conjugated goat anti-mouse at 1:1000 dilution and incubated for 30 min on ice. Cells were resuspended in buffer containing 5 μg/ml pro-p56l for analysis. Analysis of murine PMN p38 levels were performed on Clcn3<sup>+/+</sup> and Clcn3<sup>−/−</sup> PMNs that were exposed to LOS/LBP as described and then fixed in 2% formaldehyde for 10 min and placed on ice for 1 min followed by centrifugation and permeabilization with 90% methanol on ice for 30 min. Samples were analyzed for levels of anti-total p38 and anti-phospho-p38 using the manufacturer’s recommended dilutions.

Analysis of p38 MAPK Phosphorylation—PMNs (2 × 10<sup>6</sup>) were treated with purified LOS/LBP for the specified time points. Some PMNs were treated with inhibitors (DPI, N-acetelycysteine (N-Ac), rotenone, NFA) at the specified concentration before incubation with LOS. After incubation, cells were centrifuged and lysed in PMN lysis buffer (100 mM Tris, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 2% leupeptin/peptatin A) for 45 min at 4 °C with tumbling. Lysates were centrifuged at 14,000 rpm for 7 min and removed to fresh tubes, and samples were heated to 103 °C for 3 min before analysis by SDS-PAGE. To quantify total p38 MAPK, blots were stripped and reprobed with a phosphorylation state-independent anti-p38 MAPK antibody.

**Protein Electrophoresis and Immunoblotting**—Samples were resolved in an 11% gel by SDS-PAGE and then transferred to nitrocellulose. Immunoblots were processed using polyclonal antibody specific for phospho-p38 or total p38 MAPK, blots were stripped and reprobed with a phosphorylation state-independent anti-p38 MAPK antibody.

---

JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 282 • NUMBER 47 • NOVEMBER 23, 2007

---

33960
ClC-3 and NADPH Oxidase in Endotoxin Priming of Neutrophils

extracellular and intracellular function. To gain a better understanding of PMN NADPH oxidase activity, the oxidase assembly and function were studied. Using the SOD-inhibitable reduction of ferricytochrome c, inhibition of lucigenin, and cannot be used quantitatively, we examined the effect of NFA treatment on LOS priming of NADPH oxidase activity using the SOD-inhibitable reduction of ferricytochrome c. Extracellular superoxide generation in response to LOS alone was very low. Control PMNs primed for 30 min with LOS and then stimulated with fMLF generated 1.38 ± 0.09 nmol O$_2^-$/10$^6$ PMN/10 min versus 0.15 ± 0.007 nmol O$_2^-$/10$^6$ PMN/10 min in NFA-treated PMNs. Taken together with our previous findings of decreased NADPH oxidase activation in NFA-treated PMNs after opsonized zymosan or PMA stimulation (16), these data suggested a role for anion transport and ClC-3 during LOS priming.

The Anion Channel Inhibitor Niflumic Acid Inhibits ClC-3 Current—Although we previously suggested that NFA inhibits ClC-3 function (16), we recognized that electrophysiological evidence that NFA blocked the ClC-3 current was required. Using a heterologous adenoviral ClC-3 expression system, we assessed the effects of NFA on ClC-3 currents in HEK293 cells. The basic biophysical properties of the currents induced by ClC-3 overexpression were very similar to those previously published for ClC-4 and ClC-5 (23) and consistent with ClC-3 functioning as a chloride proton exchanger at physiologic pH (24, 25). ClC-3 currents were sharply outwardly rectifying, strongly favoring Cl$^-$ movement from the extracellular to the intracellular compartment (Fig. 3, A and B). $^3$ NFA (1 mM) significantly inhibited this current (Fig. 3C) with a mean percent inhibition of 57 ± 4.2% (mean ± S.E., n = 7) at a test potential of +80 mV. Thus, NFA clearly inhibited ClC-3 current and can be used to assess the contribution of ClC-3 currents to PMN function.

Oxidants Generated in Response to LOS Priming Are Both Extracellular and Intracellular—According to current understanding of PMN NADPH oxidase activity, the oxidase assem-

$^3$ J. J. Matsuda, M. S. Filali, K. A. Volk, M. M. Collins, J. G. Moreland, and F. S. Lamb, submitted for publication.

FIGURE 2. NADPH oxidase activity of control versus NFA-treated PMNs as measured by LUC-CL. A, stimulation with fMLF (1 μM) in otherwise untreated human PMNs elicited a small burst of NADPH oxidase activity that was increased in control versus NFA (1 μM) PMNs. B, after stimulation with LOS (10 ng/ml) in the presence of LBP, PMNs demonstrated a delayed generation of ROS that was increased in control PMNs versus NFA-treated PMNs. C, fMLF (1 μM) stimulation after LOS priming elicited a marked enhancement in the respiratory burst in control PMNs but no enhancement of NADPH oxidase activity in NFA-treated PMNs. n = 7 experiments, triplicate wells. RLU, relative luminescence units.

FIGURE 3. Effect of niflumic acid on ClC-3 current. A, adenoviral overexpression of ClC-3 in HEK293 cells produces a time-dependent, sharply outwardly rectifying anion current. Representative current tracings from eGFP expressing (left) and ClC-3 expressing (right) cells (holding potential = -40 mV, test potential = -100 to +100 mV in 20-mV increments). B, the current-voltage plots for eGFP expressing (open circles, n = 6) and ClC-3 expressing cells (closed circles, n = 11). C, NFA (1 mM) significantly inhibits the current induced by ClC-3 expression, p < 0.05. Representative tracings from a ClC-3 infected HEK293 cells before and after application of 1 mM NFA, n = 7. RLU, relative luminescence units. pF, picofarads.
bles at the plasma membrane in response to soluble agonists and generates extracellular ROS but assembles at the phagosome membrane to generate intraphagosomal ROS after a particulate stimulus. However, several studies suggest that the oxidase can assemble on vesicular or granular membranes and generate ROS into an intracellular, non-phagosomal compartment (26–28). Our data with LUC-CL suggested that ROS were generated during the priming response to LOS, but minimal extracellular superoxide was measured by ferri-cytochrome c. To locate the ROS produced during LOS priming, we investigated the effect of added extracellular SOD on the recovery of ROS produced directly in response to LOS. Extracellular SOD significantly inhibited NADPH oxidase activity stimulated by LOS as detected by LUC-CL, and the membrane-permeable PEG-SOD reduced detectable ROS levels below background (Fig. 4A). After LOS priming in the presence of extracellular SOD or PEG-SOD, cells were centrifuged and washed to remove SOD and then stimulated with fMLF. The primed fMLF burst in PMN that were LOS-primed in the presence of extracellular SOD was identical to control PMN but was completely inhibited in cells primed in the presence of PEG-SOD (Fig. 4B). Cells treated with PEG-SOD and then washed had normal responses to PMA, indicating that there was adequate removal of the permeable dismutase (data not shown). These data suggest that intracellular ROS generated during priming are required for the development of the primed burst.

We reasoned that if priming requires ROS, cells primed in an anaerobic environment would not demonstrate amplification in the fMLF-stimulated respiratory burst. To test this hypothesis, we primed cells with LOS in an anaerobic chamber and then removed the cells to room air immediately before stimulation with fMLF. PMN primed in the anaerobic chamber had near complete inhibition of the fMLF-induced burst in comparison to PMN primed in room air (Fig. 4C). PMA-stimulated NADPH oxidase activity was identical to control cells in PMN removed from the anaerobic chamber after 30 min, suggesting that the conditions had not permanently altered the ability of the NADPH oxidase to function (data not shown). Oxygen concentration in the chamber was estimated to be <1% based on PMA-stimulated superoxide generation as measured by the reduction of ferri-cytochrome c as compared with previous studies performed under anaerobic conditions (29). Taken together, these data strongly support the hypothesis that endotoxin-elicited priming of the respiratory burst is an oxygen-dependent process and requires intracellular generation of ROS.

Impaired Mobilization of the Flavocytochrome b_{558} and CD11b in NFA-treated and Clcn3^{-/-} PMNs—To examine the impact of CIC-3 on LOS priming, we examined up-regulation of cell surface expression of gp91^{phox}, a subunit of the flavocytochrome b_{558} (cyt b_{558}), as a manifestation of priming (30) and of CD11b/CD18. PMNs incubated with LOS for 30 min increased surface expression of cyt b_{558} by 128 ± 40.3%, whereas NFA-treated PMNs showed virtually no increase after LOS (Fig. 5A). CD11b expression was similarly affected (Fig. 5B). Interestingly, NFA treatment of PMNs in the absence of other stimulation elicited a 2.2-fold increase above basal cyt b_{558} levels and a 2.9-fold increase in CD11b expression (Fig. 5, C and D). These data raised the issue of whether the failure of LOS to elicit up-regulation of cyt b_{558} and CD11b in NFA-treated cells was secondary to the fact that intracellular stores were fully mobilized by the NFA alone. However, it appears that the “priming” effect of LOS and NFA are additive (see Fig. 9).

To determine whether the effects of NFA on receptor expression resulted from inhibition of CIC-3, we compared CD11b expression in Clcn3^{+/+} and Clcn3^{-/-} murine PMNs. In
CIC-3 and NADPH Oxidase in Endotoxin Priming of Neutrophils

Cln3\(^{+/+}\) PMNs, LOS priming induced a 288% increase in surface CD11b expression (Fig. 6, A and C). Cln3\(^{-/-}\) PMNs also demonstrated an increase in CD11b after LOS; however, it was significantly reduced from that seen in the wild-type PMNs (Fig. 6, B and C). In addition, similar to NFA-treated human PMNs, basal surface levels of CD11b were 1.5-fold increased in Cln3\(^{-/-}\) PMNs as compared with Cln3\(^{+/+}\) (Fig. 6D). Considered in combination, the human and murine data suggest a specific role, direct or indirect, for the anion transporter CIC-3 during LOS priming.

NADPH Oxidase-derived ROS Are Required for Mobilization of cyt b\(_{558}\) and CD11b in Response to LOS—Although previous reports have described low level oxidant generation in response to priming stimuli (31), it is not clear if these oxidants are causally related to acquisition of the primed phenotype. Our data using the anaerobic chamber strongly suggested that development of priming required an oxygen-dependent process. To further investigate the role of ROS in the development of the primed phenotype, we studied the requirement for NADPH oxidase-derived ROS in mobilization of intracellular stores of cyt b\(_{558}\) and CD11b/CD18. DPI (50 \(\mu\)M), an inhibitor of flavoproteins, was used to inhibit the NADPH oxidase. This concentration of DPI was chosen after preliminary studies demonstrated >99% inhibition of superoxide generation in response to PMA as measured by reduction of ferricytochrome c, whereas 10 \(\mu\)M DPI inhibited ~95% of superoxide, consistent with previously published data (data not shown) (32). DPI completely inhibited the LOS-mediated enhancement of surface cyt b\(_{558}\) expression (Fig. 7A). Surface CD11b expression was also investigated in PMNs from two patients with X-linked CGD, whose PMNs lack expression of the cytochrome b\(_{558}\) and, thus, have no NADPH oxidase function. The CGD-PMNs failed to augment levels of CD11b after 30 min of incubation with LOS, and DPI pretreatment significantly reduced the up-regulation by LOS (Fig. 7B). These data suggest that NADPH oxidase-derived ROS are required for receptor mobilization after priming with LOS. In addition, similar to NFA-treated and Cln3\(^{-/-}\) PMNs, DPI alone elicited up-regulation of both surface cyt b\(_{558}\) levels and surface CD11b levels (Fig. 7, C and D), suggesting that in the absence of basal ROS, cells acquire features of the primed phenotype. The CGD-PMNs also had higher basal levels of CD11b on the cell surface as compared with our control population.

p38 MAPK Phosphorylation in Response to LOS—To assess the potential role of NADPH oxidase-derived oxidants in intra-
cellular signaling pathways relevant to LOS priming, we examined p38 MAPK, a required signaling intermediate in endotoxin-mediated priming (33) and a kinase that phosphorylates both p47phox and p67phox (34, 35), events necessary for assembly and activation of the NADPH oxidase complex. Control PMNs maintained in buffer displayed relatively low levels of p38 phosphorylation over 1–20 min (Fig. 8, top panel) with some increase in phosphorylation after 60 min (data not shown). In response to LOS, there was rapid phosphorylation of p38 in control PMNs. PMNs treated with NFA, DPI (not shown), or N-Ac all displayed significantly enhanced levels of phosphorylated p38 in the absence of any other treatment and did not further increase p38 phosphorylation in response to LOS (Fig. 8). Of note, CGD PMNs displayed a pattern of phosphorylation that was identical to that seen in the presence of DPI or N-Ac, with high basal levels of p38 MAPK phosphorylation and no detectable augmentation in response to LOS (Fig. 8, bottom panel). The mitochondrial inhibitor rotenone (100 μM) had no effect on basal or LOS stimulated phosphorylation (data not shown).

To investigate specifically the involvement of ClC-3 in p38 MAPK phosphorylation, we analyzed phosphorylated p38 levels in fixed, permeabilized Clcn3+/+ and Clcn3−/− PMNs using flow cytometry. In unstimulated cells there was only one population of cells identified by staining for phosphorylated p38 MAPK. The relative mean fluorescence intensity of phospho-p38 was not different in unstimulated cells between Clcn3+/+ and Clcn3−/− PMNs. After LOS priming, two distinct populations of fluorescent PMNs were apparent with one population demonstrating lower fluorescence than resting cells and a second population with “high” phospho-p38 fluorescence (Fig. 9, A and B). In wild-type PMNs there was significant enhancement (1.5-fold) in the overall mean fluorescence (all cells) after LOS treatment for 10 min (Fig. 9C), with the majority (56%) of PMNs displaying high levels of phospho-p38 fluorescence (Fig. 9, A and D). In ClC-3-deficient PMNs there was enhanced phosphorylation in ~27% of cells (Fig. 9B and D), but phosphorylation was diminished as compared with wild-type PMNs (Fig. 9C). Total p38 levels were not different in the two cell populations (data not shown). Taken together, these data suggest a specific role for both ClC-3 and NADPH oxidase generated ROS in the activation of p38 MAPK in response to LOS at priming doses. Furthermore, these data suggest that basal ROS levels may contribute to the maintenance of the resting or quiescent PMN by limiting the phosphorylation of p38 MAPK.

NFA- and N-Ac-treated PMNs Are Primed for an Enhanced Respiratory Burst—The use of NFA as an inhibitor of ClC-3 or DPI to inhibit NADPH oxidase function elicited a phenotype similar to that of LOS-primed PMNs with respect to enhanced cell surface receptor expression and phosphorylation of p38 MAPK. However, despite these phenotypic changes, NADPH oxidase activity was not primed. Although the DPI-treated cells would have NADPH oxidase function irreversibly inhibited, the NFA-treated PMN can generate oxidants in response to certain stimuli (16), albeit at reduced levels. To eliminate the potential...
tially confounding influence of the continued presence of NFA, we measured LUC-CL in NFA-treated PMNs that were centrifuged and washed after the priming period to remove NFA. Control PMNs were similarly centrifuged and washed to control for effects of this handling procedure on NADPH oxidase activity. Washed NFA-treated PMNs displayed marked enhancement of the LOS-primed respiratory burst in response to subsequent stimulation with fMLF, with peak levels almost twice those seen in control-washed primed PMNs (Fig. 10A). PMNs treated with NFA alone for 30 min (no LOS) followed by washing had an 11-fold enhancement in the respiratory burst in response to fMLF (Fig. 10B), thus mirroring the increased surface expression of cyt b558 and CD11b of the NFA-treated PMN that was consistent with the primed phenotype. Notably, the peak fMLF-stimulated NADPH oxidase activity in the washed NFA-treated PMNs was 2-fold greater after LOS priming (Fig. 10A) in comparison to PMNs treated with NFA alone (Fig. 10B), suggesting that the LOS priming effect is additive to the priming effect of NFA. To assess whether the ability of NFA to elicit priming of the respiratory burst is mediated by inhibition of basal oxidant production, we treated PMNs with N-Ac for 30 min followed by centrifugation and washing. Similar to NFA, N-Ac treatment (5 mM) primed PMNs for an enhanced respiratory burst in response to fMLF as measured by LUC-CL (Fig. 10C). PMNs that were not washed lacked any detectable ROS, indicating that 5 mM N-Ac was an effective concentration in our experimental system (data not shown). Thus, inhibition of oxidant production by NFA or N-Ac resulted in a PMN phenotype that functionally resembled the primed state. Considered together, these data support our hypotheses that basal oxidants are required to maintain the cell in a resting state and that CIC-3 is involved in the regulation of this process.

**DISCUSSION**

Neutrophil priming by a variety of soluble mediators, including endotoxin, occurs both in vitro and in vivo and allows PMNs to attain a state of enhanced readiness to respond to subsequent stimuli without release of their full complement of toxic products in response to the priming stimulation. Mechanisms underlying PMN priming by endotoxin include mobilization of intracellular stores of the flavocytochrome b558 to the plasma membrane and enhanced phosphorylation of one of the cytosolic subunits of the oxidase, p47^phox^ (30), up-regulation of other receptors to the cell surface (13), and involvement of MAPK signaling pathways (33).

The current investigation focuses on a novel role for the NADPH oxidase in neutrophil priming and provides evidence for two distinct findings. First, our data suggest that NADPH oxidase-derived ROS function in intracellular signaling processes that are required not only during endotoxin-mediated...
priming but as part of a continuum of signaling events that maintains the resting versus primed versus activated state of the cell. Second, our data suggest that the anion transporter ClC-3 is required for normal intracellular NADPH oxidase function both during LOS priming and in the maintenance of the resting state. Although the current paradigm of priming suggests that there is incomplete assembly and activation of the oxidase by the priming agent, we and other investigators (31) have demonstrated that ROS are produced during priming. Here we provide evidence that these oxidants were required for achieving the primed state; the primed PMN phenotype in response to LOS did not occur in cells lacking NADPH oxidase generated oxidants (i.e. DPI-treated or CGD PMNs).

Although ROS have been unequivocally demonstrated to function in signal transduction in a number of cell types (36), the source and spatial localization of oxidant species production, the identity of ROS that function as second messengers, and the specific protein modifications elicited by the oxidants remain under intense investigation. In PMNs, both integrin activation (13) and apoptosis (14) are regulated by oxidant signaling, with specific involvement of NADPH oxidase-derived ROS during apoptosis (37). Redox regulation of p38 MAPK is critical during PMN priming by tumor necrosis factor-α (15) and during lipopolysaccharide priming (33). Both p38 and extracellular signal-regulated kinase 2 can phosphorylate p47phox and p67phox in vitro (34, 35), and the specific serine on p47phox that is phosphorylated by these kinases was recently identified (38). Blockade of this phosphorylation event inhibited priming of PMNs by tumor necrosis factor-α and granulocyte-macrophage colony-stimulating factor. In the current study we demonstrate NADPH oxidase-dependent phosphorylation of p38 MAPK in response to LOS priming. In the setting of reduced or absent intracellular ROS, basal phosphorylation of p38 MAPK was markedly enhanced. Augmentation of basal levels of p38 MAPK phosphorylation in response to inhibition of NADPH oxidase function has previously been reported in endothelial cells (39). These data suggest that basal levels of NADPH oxidase generated ROS play a role in maintaining p38 MAPK in the dephosphorylated state. This might occur either via tonic stimulation of a critical phosphatase or inhibition of an upstream kinase and is currently an area of investigation in our laboratory.

Basal oxidant signaling has not been described in PMNs, and the existing literature suggests that there is no fully assembled NADPH oxidase in resting PMNs. The evidence supporting this contention includes a failure to demonstrate association of p47phox or p67phox with light membrane fractions by immunoblotting (40, 41). However, basal oxidant signaling is well described in a number of other cell types (42–44), and the phagocyte NADPH oxidase (gp91phox) is the source of ROS in some of these cells. The possibility that a small percentage of the total phagocyte NADPH oxidase is assembled and generates low level intracellular oxidants under basal conditions to serve a signaling function has not been ruled out. Another possibility is that the flavocytochrome b558 alone might move very low levels of electrons involved in signaling. Both of these potential explanations for our data require further testing.

The current investigation also explored a role for the anion transporter ClC-3 in PMN priming by endotoxin. ClC-3 protein is expressed in human PMNs and is required for normal PMN NADPH oxidase activity (16). The biophysical nature of ClC-3 has been a subject of controversy, with several studies suggesting that it was a cell surface channel regulated by 1) swelling (45), 2) calcium-calmodulin-dependent protein kinase II (46), or 3) the cystic fibrosis transmembrane regulator (47). Most recently, ClC-3 has been suggested to be a chloride/proton antiporter rather than an ion channel, based on the structural similarity of ClC-3 to subfamily members ClC-4 and ClC-5, which clearly function as electrogenic Cl−/H+ exchangers that display a steep voltage dependence and are activated only at positive voltages (24, 25). Our recent characterization of ClC-3 currents in HEK293 cells suggests that ClC-3 currents are very similar to those of the other subfamily members (4).

The focus on a potential role for ClC-3 during priming stemmed from our observations in Clcn3−/− vascular smooth muscle cells demonstrating abnormal oxidant-dependent signaling in response to tumor necrosis factor-α and interleukin-1β (17, 48). We found that the LOS priming of FMLF-stimulated NADPH oxidase activity was 100% inhibited in NFA-treated PMNs and significantly inhibited in Clcn3−/− PMNs. The failure of Clcn3−/− PMNs to display complete inhibition of the primed FMLF burst may reflect compensatory changes occurring in the knock-out mice. In addition, the conditions and phenotypes for priming are clearly species-specific, as previous investigations have suggested that murine cells have reduced capacity for priming (49).

Similarities between ClC-3-deficient PMNs and oxidant-deficient PMNs suggested to us that the anion transporter was involved in regulation of NADPH oxidase function during basal oxidant signaling and during LOS priming. We speculate that ClC-3 functions in charge neutralization during intracellular ROS generation into a vesicular/endosomal compartment. If ClC-3 were acting as a Cl−/H+ antiporter in an intracellular vesicular compartment, the membrane depolarization produced by the respiratory burst would strongly favor Cl−/H+ exchange with Cl− exiting and H+ entering the compartment. The NADPH oxidase, acting to transfer electrons, generates a rapid depolarization, and proton movement is required to balance this charge imbalance (50, 51). It should be noted that a potential charge-neutralizing function of ClC-3 in an intracellular vesicular compartment does not imply that ClC-3 has a similar function at the plasma membrane. Our previous observations that NFA-treated and ClC-3-deficient PMNs have normal extracellular ROS production in response to PMA (16) would suggest that an NFA-insensitive pathway, likely a proton channel, fills this role. In addition, electrophysiological analysis of ClC-3 current in PMNs or another myeloid cell type is required to confirm that our findings in HEK cells are directly applicable to the role of ClC-3 current in PMN. An alternative hypothesis to explain our findings would be that ClC-3 has a role in assembly of the NADPH oxidase on vesicular membranes. Both of these potential explanations are currently being explored.

In summary, the current investigation demonstrates that NADPH oxidase-derived ROS are critically involved in intra-
cellular signaling both during endotoxin priming of neutrophils and in the maintenance of the resting or non-primed state. We would anticipate that patients with CGD would have impaired priming responses to endotoxin, a deficiency that may contribute to the incidence or severity of overwhelming sepsis in these patients. In addition, the anion transporter CIC-3 also appeared to contribute to LOS-mediated priming and cellular quiescence. We speculate that CIC-3 functions in charge neutralization for the NADPH oxidase under certain conditions, specifically when oxidants are generated into an intracellular vesicular compartment. This hypothesis is currently under investigation as well as studies to define the intracellular localization of oxidant generation during priming by endotoxin.

REFERENCES
1. Babior, B. M. (1999) Blood 93, 1464–1476
2. Condilffe, A. M., Kitchen, E., and Chilvers, E. R. (1998) Clin. Sci. (Lond.) 94, 461–471
3. Aida, Y., and Pabst, M. J. (1990) J. Immunol. 145, 3017–3025
4. Fittschen, C., Sandhaus, R. A., Worthen, G. S., and Henson, P. M. (1988) J. Leukocyte Biol. 43, 547–556
5. Forehand, J. R., Bomalski, J. S., and Johnston, R. B., Jr. (1991) Exp. Med. Biol. 297, 65–73
6. Fitztschen, C., Sandhaus, R. A., Worthen, G. S., and Henson, P. M. (1988) J. Leukocyte Biol. 43, 547–556
7. Condliffe, A. M., Chilvers, E. R., Haslett, C., and Dransfield, I. (1996) Immunology 89, 105–111
8. Ogura, H., Tanaka, H., Koh, T., Hashiguchi, N., Kuwagata, Y., Hosotsubo, H., Shimazu, T., and Sugimoto, H. (1999) J. Trauma 47, 774–783
9. Chollet-Martin, S., Montravers, P., Gibert, C., Elbim, C., Desmots, J. M., Fagon, J. Y., and Gougerot-Pocidalo, M. A. (1992) Am. Rev. Respir. Dis. 146, 990–996
10. Bass, D. A., Olbrantz, P., Szejda, P., Seeds, M. C., and McCall, C. E. (1986) J. Biol. Chem. 261, 439–446
11. Venet, C., Zeni, F., Viallon, A., Ross, A., Pain, P., Gery, P., Page, D., Viallon, A., Ross, A., Pain, P., Gery, P., Page, D., Vermeesch, R., Bertrand, M., Rancon, F., and Bertrand, J. C. (2000) Intensive Care Med. 26, 538–544
12. Segal, B. H., Leto, T. L., Gallin, J. I., Malech, H. L., and Holland, S. M. (2000) Medicine (Baltimore) 79, 170–200
13. Blouin, M., Halwachs-Mecarelli, L., and Rieu, P. (1999) Eur. J. Immunol. 29, 3419–3431
14. Watson, R. W. (2002) Antioxid. Redox Signal. 4, 97–104
15. Boussouina, M., Blouin, E., Halwachs-Mecarelli, L., Lesavre, P., and Rieu, P. (2004) J. Immunol. 173, 1313–1320
16. Moreland, J. G., Davis, A. P., Bailey, M. C., Nehrke, K., Williamson, R. A., and Lamb, F. S. (2002) J. Biol. Chem. 278, 12277–12288
17. Miller, F. J., Jr., Filali, M., Huss, G. J., Stanic, B., Chamseddine, A., Barna, T. J., and Lamb, F. S. (2007) Circ. Res. 101, 663–671
18. Dickerson, L. W., Bonthius, D. J., Schutte, B. C., Yang, B., Barna, T. J., Bailey, M. C., Nehrke, K., Williamson, R. A., and Lamb, F. S. (2002) Brain Res. 958, 227–250
19. Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 97, 77–89
20. Giardina, P. C., Gioaninni, T., Buscher, B. A., Zaleski, A., Zheng, D. S., Stoll, L., Teghamnet, A., Apicella, M. A., and Weiss, J. (2001) J. Biol. Chem. 276, 5883–5891
21. Nauseef, W. M. (2001) J. Clin. Investig. 107, 401–403
22. Mestas, J., and Hughes, C. C. (2004) J. Immunol. 172, 2731–2738
23. Friedrich, T., Breiderhoff, T., and Jentsch, T. J. (1999) J. Biol. Chem. 274, 896–902
24. Scheel, O., Zdebik, A. A., Lourdel, S., and Jentsch, T. J. (2005) Nature 436, 424–427
25. Picollo, A., and Pusch, M. (2005) Nature 436, 420–423
26. Kobayashi, T., Robinson, J. M., and Seguchi, H. (1998) J. Cell Sci. 111, 81–91
27. Brown, G. E., Stewart, M. Q., Liu, H., Ha, V. L., and Yaffe, M. B. (2003) Mol. Cell 11, 35–47
28. Ambruso, D. R., Cusack, N., and Thurman, G. (2004) Mol. Genet. Metab. 81, 313–321
29. Gabig, T. G., Bearman, S. I., and Babier, B. M. (1979) Blood 53, 1133–1139
30. DeLeo, F., Renee, J., McCormick, S., Nakamura, M., Apicella, M., Weiss, J., and Nauseef, W. (1998) J. Clin. Investig. 101, 455–463
31. Yaffe, M. B., Xu, J., Burke, P. A., Forse, R. A., and Brown, G. E. (1999) J. Clin. Investig. 103, 1075–1082
32. Dickerson, L. W., Bonthius, D. J., Schutte, B. C., Yang, B., Barna, T. J., Bailey, M. C., Nehrke, K., Williamson, R. A., and Lamb, F. S. (2002) Brain Res. 958, 227–250
33. Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 97, 77–89
34. Giardina, P. C., Gioannini, T., Buscher, B. A., Zaleski, A., Zheng, D. S., Stoll, L., Teghamnet, A., Apicella, M. A., and Weiss, J. (2001) J. Biol. Chem. 276, 5883–5891
35. Nauseef, W. M. (2001) J. Clin. Investig. 107, 401–403
36. Mestas, J., and Hughes, C. C. (2004) J. Immunol. 172, 2731–2738
37. Friedrich, T., Breiderhoff, T., and Jentsch, T. J. (1999) J. Biol. Chem. 274, 896–902
38. Scheel, O., Zdebik, A. A., Lourdel, S., and Jentsch, T. J. (2005) Nature 436, 424–427
39. Picollo, A., and Pusch, M. (2005) Nature 436, 420–423
40. Kobayashi, T., Robinson, J. M., and Seguchi, H. (1998) J. Cell Sci. 111, 81–91
41. Brown, G. E., Stewart, M. Q., Liu, H., Ha, V. L., and Yaffe, M. B. (2003) Mol. Cell 11, 35–47
42. Ambruso, D. R., Cusack, N., and Thurman, G. (2004) Mol. Genet. Metab. 81, 313–321
43. Gabig, T. G., Bearman, S. I., and Babier, B. M. (1979) Blood 53, 1133–1139
44. DeLeo, F., Renee, J., McCormick, S., Nakamura, M., Apicella, M., Weiss, J., and Nauseef, W. (1998) J. Clin. Investig. 101, 455–463
45. Yaffe, M. B., Xu, J., Burke, P. A., Forse, R. A., and Brown, G. E. (1999) J. Clin. Investig. 103, 1075–1082
46. Dickerson, L. W., Bonthius, D. J., Schutte, B. C., Yang, B., Barna, T. J., Bailey, M. C., Nehrke, K., Williamson, R. A., and Lamb, F. S. (2002) Brain Res. 958, 227–250
47. Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 97, 77–89
48. Giardina, P. C., Gioannini, T., Buscher, B. A., Zaleski, A., Zheng, D. S., Stoll, L., Teghamnet, A., Apicella, M. A., and Weiss, J. (2001) J. Biol. Chem. 276, 5883–5891