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Myeloperoxidase Plays Critical Roles in Killing *Klebsiella pneumoniae* and Inactivating Neutrophil Elastase: Effects on Host Defense[1]

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Activated neutrophils use myeloperoxidase (MPO) to generate an array of potent toxic oxidants. In the current studies we used genetically altered mice deficient in MPO to investigate the role of the enzyme in host defense against the Gram-negative bacterium *Klebsiella pneumoniae*, an important human pathogen. For comparison, we used mice deficient in the antimicrobial molecule, neutrophil elastase (NE). When challenged i.p., mice deficient in either MPO or NE were markedly more susceptible to bacterial infection and death. In vitro studies suggested that MPO impairs the morphology of bacteria in a distinctive way. Of importance, our in vitro studies found that MPO mediated oxidative inactivation of NE, an enzyme that has been widely implicated in the pathogenesis of various tissue-destructive diseases. This pathway of oxidative inactivation may be physiologically relevant, because activated neutrophils isolated from MPO-deficient mice exhibited increased elastase activity. Our observations provide strong evidence that MPO, like NE, is a key player in the killing of *K. pneumoniae* bacteria. They also suggest that MPO may modulate NE to protect the host from the tissue-degrading activity of this proteinase. ***The Journal of Immunology,*** **2005, 174: 1557–1565.**

The recruitment of neutrophils to injured tissues is a characteristic feature of host defense and inflammation (1). During bacterial infection, the main functions of neutrophils are killing of invading pathogens and resolution of associated inflammation. The antimicrobial importance of neutrophils is demonstrated in patients with neutropenia or disorders of neutrophil function. Such patients experience frequent life-threatening infections. Two antimicrobial systems, categorized as oxidative and nonoxidative, have been described in neutrophils. The oxidative system catalyzes the generation of toxic oxidants (2). Activated neutrophils undergo a burst of oxygen consumption, which is converted by the membrane-bound NADPH oxidase to superoxide, a precursor for various reactive oxygen species, principally hydrogen peroxide (H_2O_2). Next, myeloperoxidase (MPO)[3] uses H_2O_2 and ubiquitous chloride ions to generate hypochlorous acid (HOCl), a highly cytotoxic oxidant. The nonoxidative system involves a variety of antimicrobial peptides and proteinases, including neutrophil elastase (NE), a serine proteinase that disrupts the structural integrity of invading microbes (3).

During neutrophil development in bone marrow, both MPO and NE are synthesized at the promyelocyte stage and stored together in primary granules in active forms at high concentrations (4). MPO, a strongly basic heme-containing protein, is composed of two β heavy subunits (59 kDa) associated with two α light subunits (14 kDa) (5). At plasma concentrations of chloride ions, MPO is the main enzyme that generates HOCl. NE is a 30-kDa serine proteinase whose catalytic activity relies on the triad His^{57}-Asp^{102}-Ser^{195}, where Ser is the active residue (chymotrypsinogen numbering) (6). Although MPO and NE are neutrophil specific, they have also been found in a subset of monocytes/macrophages (7, 8).

In the setting of infections, MPO and NE are rapidly discharged into phagosomal vacuoles, where bacteria are trapped. By generating mice deficient in NE ($NE^{-/-}$ mice), we have found that the enzyme protects the host against Gram-negative, but not Gram-positive, bacteria and that neutrophils require NE for maximal intracellular killing (9). In recent years, we demonstrated that NE kills *Escherichia coli* in part by degrading the bacterium's outer membrane protein A (OmpA) (10). Genetically engineered mice deficient in MPO were susceptible to fungal, but not Gram-positive bacterial, infection (11). However, the role of MPO in host defense mechanisms against Gram-negative bacteria is less clear. It is not yet established whether neutrophil MPO and NE are both required for the killing of engulfed pathogens or whether each enzyme targets a specific microbial spectrum.

In inflamed situations, MPO and NE could leak out of the cell by a number of mechanisms (12). Many lines of evidence suggest that unchecked MPO and NE may inadvertently damage host tissue. For example, products formed only by MPO have been detected in human atherosclerotic tissue and various inflammatory conditions (13, 14). Unopposed NE creates a localized proteolytic environment where a wide variety of host molecules are degraded (15). Therefore, NE has been implicated in various tissue-destructive diseases, such as acute lung injury and cystic fibrosis (16, 17). Depending on the stimulus, MPO and NE are concomitantly released on the cell surface or extracellularly. In this regard, large amounts of MPO and NE could be detected on the cell surface of activated neutrophils (18, 19). Few studies have previously reported the oxidative damage to lysosomal enzymes of activated neutrophils (20, 21), but little is known regarding the impact of

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[3] Abbreviations used in this paper: MPO, myeloperoxidase; CG, cathepsin G; HOCl, hypochlorous acid; MMP, matrix metalloprotease; NE, neutrophil elastase; OmpA, outer membrane protein A; PR-3, proteinase 3; SP-D, surfactant protein D; WT, wild type.

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MPO-generated HOCl on NE, and its potential consequences on host defense have not been addressed.

In the present studies we show that MPO, like NE, is important for combating *Klebsiella pneumoniae* infections in mice and that MPO and NE might have unique methods of killing this pathogen in vitro. We also demonstrate that MPO oxidatively inactivates NE. These findings suggest that MPO might have a dual protective function. MPO contributes to host defense against invading Gram-negative pathogens, but is also capable of oxidatively limiting NE tissue-degrading activity.

Materials and Methods

Reagents

Purified human MPO, NE, cathepsin G (CG), proteinase 3 (PR-3), ETNA elastin, and Congo Red-conjugated elastin were obtained from Elastin Products. The activity and specificity of each enzyme were confirmed by colorimetric assay (15). MPO activity was tested using H_2O_2 and 4-aminoantipyridine as hydrogen donor and acceptor, respectively. The chromogenic peptide substrates MeO-Suc-Ala-Ala-Pro-Val-pNA, Suc-Ala-Ala-Pro-Phe-pNA, and Boc-Ala-Ala-Nva-SBzl were used against NE, CG, and PR-3, respectively.

Recombinant active human matrix metalloprotease-7 (MMP-7) and MMP-9 were provided by Drs. W. Parks and R. M. Senior (Washington University, St. Louis, MO), respectively. Recombinant rat surfactant protein D (SP-D) and its specific Ab were provided by Dr. E. Crouch (Washington University). All other chemicals were reagent grade and purchased from Sigma-Aldrich, unless otherwise stated.

Bacteria

Mouse virulent strain *K. pneumoniae* (KPA 1) was passaged twice in mice before use (9). An overnight bacterial culture diluted 1/100 was aerobically grown in Luria Bertani broth under static conditions at 37°C to late exponential phase (3 h). Cultures were centrifuged (3000 rpm, 10 min), and the bacterial pellet was washed twice with PBS (pH 7.4). The pellet was resuspended in 1 ml of PBS, and its absorbance was determined at 600 nm (1 OD $\approx 10^9$ bacteria/ml).

Mice deficient in MPO or NE

All procedures were approved by the Washington University animal studies committee. MPO-deficient ($MPO^{-/-}$) and NE-deficient ($NE^{-/-}$) mice were generated by targeted mutagenesis as previously described (9, 11). Mice were maintained in the animal barrier facility with a 12-h light, 12-h dark cycle and provided with water and food ad libitum. Unless otherwise stated, $MPO^{-/-}$ mice and their wild-type (WT) littermates and $NE^{-/-}$ mice had a 129/SvJ-C57/BL6 genetic background and were sex and age (8–10 wk) matched.

Intraperitoneal infection model

As previously described (9), at least 30 $MPO^{-/-}$ mice were injected i.p. with varying amounts of bacteria, and their survival was followed over time. From these experiments, the concentration of bacteria that killed ~50% of the $MPO^{-/-}$ mice was determined by interpolation for KPA 1 strain (LD_{50}, $\sim 6.5 \times 10^3$ CFU/mouse in 1 ml of PBS). Next, $MPO^{-/-}$, $NE^{-/-}$, and WT mice ($n = 12$/genotype) were i.p. infected with the LD_{50} of *K. pneumoniae*, and their survival was monitored over time.

Clearance of K. pneumoniae *bacteria in vivo*

To assess the ability of $MPO^{-/-}$ mice to clear Gram-negative bacteria, $MPO^{-/-}$ and WT mice ($n = 4$/genotype) were intratracheally infected with *K. pneumoniae* (10^5 CFU). Next, mice were killed at 4, 24, and 48 h postinfection, and their lungs were processed for histology and immunohistochemistry. Briefly, lungs were inflated with 10% formalin in PBS (22). The excised lungs were then immersion-fixed with 10% buffered formalin overnight, dehydrated, embedded in paraffin, and cut into 5-μm sections. Serial lung tissue sections were deparaffinized, rehydrated, and H&E stained. For immunostaining, the slides were immersed in citrate buffer (0.01 mol/l sodium citrate, pH 6.0) and microwave-heated to enhance Ag retrieval. Endogenous peroxidase was blocked by H_2O_2 (0.3%), followed by overnight incubation with Abs specific for MPO (dilution 1/1000) or *K. pneumoniae* OmpA (dilution 1/1000). Preimmune serum was used as the control. Ag-Ab complexes were detected with a Vectastain ABC elite kit (Vector Laboratories) according to the manufacturer's recommendations, followed by H&E counterstaining. In other experiments, mice were killed, and their lungs were aseptically removed, homogenized, serially diluted, and agar-plated to determine the number of viable bacteria.

Neutrophil influx in the absence of MPO

To determine neutrophil influx to inflamed sites in the absence of MPO, WT and $MPO^{-/-}$ mice were i.p. injected with 15% glycogen (1 ml/mouse) or *K. pneumoniae* (4×10^8/mouse) (9). At 0.5, 1, 2, 3, and 4 h postchallenge, the peritonea of mice ($n = 2$/genotype, condition, and time point) were lavaged with 5 ml of HBSS (pH 7.4). Next, the number and viability of cells in the lavage fluid were determined by hemocytometer and trypan blue exclusion, respectively. For differential counts, cells were cytospun (Cytospin 4; Thermo Shandon) and stained with a modified Wright's stain (Hema 3; Fisher Scientific).

Bactericidal activity of neutrophils in vivo

Neutrophils were glycogen-elicited to the peritoneum of $MPO^{-/-}$ and WT mice (both $n = 4$/genotype) as described above. After 4 h, the same mice were i.p. infected with *K. pneumoniae* (4×10^8 CFU). In one group of $MPO^{-/-}$ and WT mice, neutrophils were harvested 30 min postchallenge by peritoneal lavage with 5 ml of HBSS (pH 7.4) containing 100 μg/ml gentamicin and counted. Gentamicin was used to kill all extracellular bacteria (9). Next, aliquots of cells (1×10^6 neutrophils in 0.5 ml) were incubated (37°C, 5% CO_2) in duplicate. At the indicated time points, cells were gently washed three times to remove gentamicin, lysed in HBSS containing 0.1% Triton, serially diluted, and plated. In the second group of mice, neutrophils were harvested 2 h after bacterial challenge. The numbers of viable bacteria were determined after overnight incubation at 37°C. Duplicate aliquots were cytospun, stained, and compared for intracellular bacterial contents by light microscopy.

Phagocytosis function assay

The phagocytosis function of $MPO^{-/-}$ and WT neutrophils was assessed using the Vybrant phagocytosis kit (Molecular Probes) with the following modifications. Glycogen-elicited neutrophils were isolated from the peritonea of $MPO^{-/-}$ and WT mice as described above, and 200-μl cell aliquots (2.5×10^6 cells/ml DMEM containing 10% FCS) were seeded in triplicate into 96-well microplates (Microfluor II, Dynatech Laboratories) and allowed to adhere for 30 min (37°C, 5% CO_2). Nonadherent cells were gently washed off. Next, 100 μl of culture medium containing fluorescein-labeled *E. coli* (strain K-12) bioparticles (1 mg/ml) was added to each well. After 0, 30, 60, and 90 min of incubation, non-cell-associated bioparticles were gently removed. The fluorescence emitted by the remaining extracellular bioparticles was quenched by adding 100 μl of trypan blue suspension (250 μg/ml in citrate-balanced salt solution, pH 4.4). Trypan blue solution was immediately removed, and the fluorescence intensity of each well was determined with a fluorescence plate reader (Gemini XS; Molecular Devices), using 480 and 520 nm for excitation and emission, respectively.

Bactericidal activities of NE, MPO, HOCl, and H_2O_2

Freshly grown *K. pneumoniae* bacteria were incubated alone and in the presence of the indicated concentrations of the MPO system (MPO, H_2O_2, and NaCl) or NE following modifications of previously described methods (9, 23). Briefly, the reactions were incubated with bacteria in a total volume of 200 μl containing 0.155 M NaCl (pH 7.4). Under these conditions, chloride is used by the MPO system to generate HOCl (23). In other experiments, bacteria were directly incubated with the indicated concentrations of H_2O_2 or HOCl. After incubation at 37°C for various time periods, serial dilutions were plated, and the numbers of CFU were determined after overnight incubation.

Fluorescence microscopy

Bacterial viability was further assessed using two-color visualization of dead and live cells (10). Untreated and oxidant-treated bacteria were incubated with 4′,6-diamido-2-phenylindole hydrochloride (blue fluorescent live-cell stain) and SYTOX (green fluorescent dead cell stain) nucleic acid stains as recommended by the manufacturer (Molecular Probes), viewed with a Zeiss Axioshop 2 MOT Plus, and photographed.

Scanning electron microscopy of bacteria

K. pneumoniae were incubated alone and in the presence of the MPO system or NE as described above. After incubation, bacteria were seeded onto polyethyleneamine-treated coverslips (1%) and fixed in 2.5% glutaraldehyde solution as previously described (10). After ethanol dehydration, the coverslips were mounted onto aluminum stubs, coated with a 30-nm gold layer, viewed with a Hitachi S-450 scanning electron microscope, and photographed.

Enzymatic activities

In a cell-free system, MPO and NE were coincubated, and their activities were assessed. MPO (100 nM) was incubated in 100 μl of PBS (pH 7.4) alone or in the presence of the designated dose of NE for 5 min at 37°C. Next, MPO activity (Δ absorbance per minute) was determined spectrophotometrically at λ 510 nm using H_2O_2 and 4-aminoantipyridine as substrates. As controls, MPO was heat-denatured (5 min at 95°C) or replaced with NE.

NE (2 μg) was incubated in 100 μl of PBS (pH 7.4) alone and in the presence of the indicated concentration of the MPO system, H_2O_2, or HOCl for 5 min at 37°C. Next, the scavenger L-methionine (10-fold molar excess relative to oxidant) was added, and NE activity (Δ absorbance per minute) was determined spectrophotometrically at λ 410 nm using the NE-specific peptide substrate, Meo-Suc-Al-Al-Pro-Val-pNA (0.2 mM). No activity was detected when MPO, CG, or PR3 was assayed against NE peptide substrate. Additional control experiments included preincubation of NE with the serine proteinase inhibitor PMSF (1 mM) or L-methionine.

Zymography and Western blotting

The proteolytic activity of untreated and HOCl-treated NE (1, 10, 100, and 1000 μM) was determined by κ-elastin zymography (22). The reactions were incubated as described above and migrated under nonreducing conditions at 4°C in SDS-PAGE gels containing 1 mg/ml elastin. Next, gels were soaked in 2.5% Triton X-100 (twice, 15 min each time), rinsed briefly, and incubated at 37°C for 48 h in 50 mM Tris-HCl (pH 8.2) containing 5 mM $CaCl_2$. The gels were then stained with Coomassie Blue, and active NE appeared as a transparent lysis band at ~30 kDa. To examine further its proteolytic activity, NE was incubated with SP-D (1 μg), and the reactions were subjected to Western blotting (NEN) using an Ab specific for SP-D (dilution 1/10,000) (24). HOCl (1 mM) alone did not affect the apparent mobility of SP-D.

SDS-PAGE

NE was incubated alone or in the presence of HOCl, H_2O_2, or PMSF for 5 min at 37°C in a 20-μl reaction containing PBS (pH 7.4). As controls, NE was first incubated with PMSF, then HOCl was added to the reaction or HOCl was incubated with L-methionine at a 1:10 ratio before addition to NE. Next, all reactions were reduced by addition of sample buffer containing 2% β-ME, heat-denatured (5 min, 95°C), resolved by SDS-PAGE (4–16%), and stained with Coomassie blue. In parallel experiments, treatments with β-ME and heat were omitted. Similar experiments were conducted with CG, PR-3, MMP-7, and MMP-9.

NE activity in neutrophils

Resting cells were isolated from the bone marrow or blood of unchallenged $MPO^{-/-}$, $NE^{-/-}$, and WT mice (n = 4/group) and aliquoted (2×10^6) (25). In other experiments, two groups of mutant and WT mice (both n = 4/group) were i.p. injected with glycogen. After 4 h, one group of mice was killed, and peritoneal neutrophils were collected as described above and aliquoted (2×10^6). The second group of mice received an additional i.p. injection of *K. pneumoniae* (1×10^8 CFU), and 2 h later, neutrophils were collected. Next, cells from all experiments were lysed by sonication (twice, 15 s each time) on melted ice (26) or by addition of Triton (0.1% final concentration) (9). The cell lysates were incubated with NE substrate (0.2 mM) for 60 min at 37°C in a total volume of 1 ml Tris-NaCl buffer (0.1 M Tris and 1 M NaCl, pH 7.4). The reactions were then spun (30 s, 12,000 rpm), and changes in the absorbances of the supernatants were determined at λ 410 nm. Absorbances were corrected for nonspecific activity by subtracting the value of $NE^{-/-}$ neutrophil supernatants from those of $MPO^{-/-}$ and WT supernatants.

Parallel studies were performed using Congo Red-conjugated elastin (Elastin Products) as a substrate for NE (26). Briefly, lysed cells were incubated overnight with Congo Red elastin (3 mg) in 1 ml of Tris buffer (100 mM, pH 8.0). After centrifugation (1 min, 14,000 rpm), elastolytic activity was measured as the absorbance of supernatant at 495 nm. Absorbances were corrected for nonspecific activity by subtracting the value of $NE^{-/-}$ neutrophil supernatants from those of $MPO^{-/-}$ and WT supernatants.

Immunoblotting of neutrophil enzymes

WT, $MPO^{-/-}$, and $NE^{-/-}$ mice were i.p. challenged with glycogen, followed by *K. pneumoniae* as described above. Aliquots of cells were treated with acetone for protein extraction (15). Western blotting experiments were conducted using Abs specific to MPO (1/2000) and CG (1/500). MPO and CG Abs are highly specific because they do not cross-react with other neutrophil proteins.

Statistics

The Kaplan-Meier method was used to estimate the survival probabilities of mice as a function of time. The survival curves and survival rates at the end of the experiments were compared by Wilcoxon test and Fisher's exact test, respectively. All other data were analyzed with Student's t test. Significance was accepted at $p < 0.05$.

Results

MPO plays an important role in host defense against K. pneumoniae *infections*

The relative importance of MPO in combating Gram-negative bacterial infections was compared with that of NE. Groups of $MPO^{-/-}$, $NE^{-/-}$, and WT control mice were i.p. challenged with the mouse virulent pathogen *K. pneumoniae* (9). Early in the infection, both $MPO^{-/-}$ and $NE^{-/-}$ mice appeared clinically worse (ruffled fur, lethargy, and diarrhea) than WT mice. At 24 h postinfection, the mortality rates were markedly higher in mutant mice than in WT mice. Indeed, >65% of $MPO^{-/-}$ and $NE^{-/-}$ mice, but <10% of WT controls, died (Fig. 1*A*). None of the survivors died after 72 h. That MPO is required for host defense against *K. pneumoniae* was also confirmed in a pneumonia model. After lung infection, histological examination detected similar patchy infiltrates of neutrophils in $MPO^{-/-}$ and WT lungs (Fig. 1*B*). However, the inability of $MPO^{-/-}$ mice to clear bacteria as efficiently as WT mice could be clearly seen as early as 4 h and persisted at least up to 48 h (Fig. 1, *C*, *D*, and *G*). These in vivo data demonstrate that MPO, like NE, contributes significantly to the protection of mice from morbidity and mortality due to *K. pneumoniae* infections.

MPO is required for intracellular killing of K. pneumoniae *bacteria by neutrophils*

To determine the underlying basis of the vulnerability of $MPO^{-/-}$ mice to *K. pneumoniae* infection, we examined the capacity of $MPO^{-/-}$ and WT mice to clear bacteria in vivo using different approaches. After lung infection, immunostaining of tissues with a polyclonal antiserum that recognizes *K. pneumoniae* OmpA found much more immunoreactive material in $MPO^{-/-}$ lung than WT lung, particularly in the areas of neutrophil infiltrates (Fig. 1, *C* and *D*). In separate experiments we recruited neutrophils to the peritoneum of mice by i.p. injection of glycogen. Four hours later, we i.p. infected the same mice with *K. pneumoniae*. The peritonea of $MPO^{-/-}$ and WT mice had comparable numbers of neutrophils 2 h postinfection (mean ± SEM, $12.2 \pm 2.56 \times 10^6$ vs $10.7 \pm 2.06 \times 10^6$ for $MPO^{-/-}$ and WT neutrophils, respectively; $p > 0.05$). However, microscopic examination of cytospin preparations of isolated neutrophils revealed more bacteria in $MPO^{-/-}$ neutrophils than in WT neutrophils (data not shown), an observation that was supported by plating and counting the numbers of viable colonies in cell lysates ($3.1 \pm 0.7 \times 10^6$ vs $0.9 \pm 0.3 \times 10^6$ CFU/10^6 neutrophils from $MPO^{-/-}$ and WT mice, respectively; $p < 0.05$). To ensure that the observed differences are associated with a bactericidal defect in $MPO^{-/-}$ neutrophils rather than a defect in their recruitment, mice from both genotypes were i.p. challenged with either glycogen or bacteria, and neutrophil recruitment was monitored over time (0.5, 1, 2, 3, and 4 h). Analyses of differential cell counts and trypan blue exclusion found that although neutrophil influx was higher in response to glycogen than bacteria, there were no significant differences in the numbers and viability of inflammatory cells between $MPO^{-/-}$ and WT mice (data not shown). These results indicate that in the absence of MPO, neutrophils migrate normally to sites of infection, but exhibit impaired defense against bacteria.

This suggested that the observed antibacterial defect in MPO-deficient mice could be associated with impairment of neutrophil defense functions: phagocytosis and/or bacterial killing. When

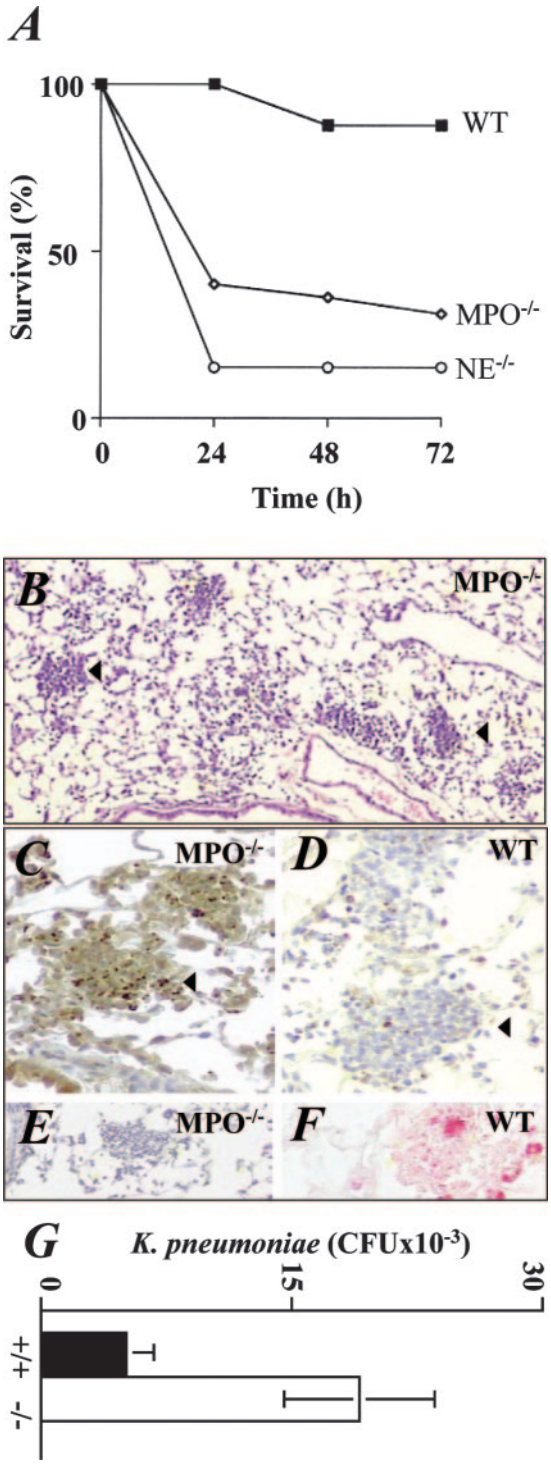

FIGURE 1. Role of MPO in host defense against *K. pneumoniae* infection. *A*, Mortality rates in $MPO^{-/-}$, $NE^{-/-}$, and WT mice ($n = 12$/genotype) in response to i.p. challenge with *K. pneumoniae* ($p < 0.01$ for $MPO^{-/-}$ and $NE^{-/-}$ vs WT mice at all three time points). *B*, Representative histology of lung tissues from $MPO^{-/-}$ mice 48 h after intratracheal infection with *K. pneumoniae* (arrowheads, neutrophil infiltrates; magnification, ×40). *C* and *D*, Representative immunostaining of lung tissue for OmpA 48 h postchallenge (brown color, peroxidase staining; magnification, ×200). *E*, No immunostaining was observed in infected $MPO^{-/-}$ lung sections using rabbit preimmune serum (peroxidase staining; magnification, ×40). *F*, Infected WT lung sections showed positive immunostaining for MPO (red color, alkaline phosphatase staining; magnification, ×40). G, Representative data of bacterial clearance from WT and $MPO^{-/-}$ lungs 4 h after challenge with *K. pneumoniae*. ■, WT mice; □, $MPO^{-/-}$ mice.

phagocytosis function was examined, the uptake of fluorescein-labeled bioparticles was comparable between $MPO^{-/-}$ and WT neutrophils (Fig. 2*A*). Next, we assessed the bactericidal activity of

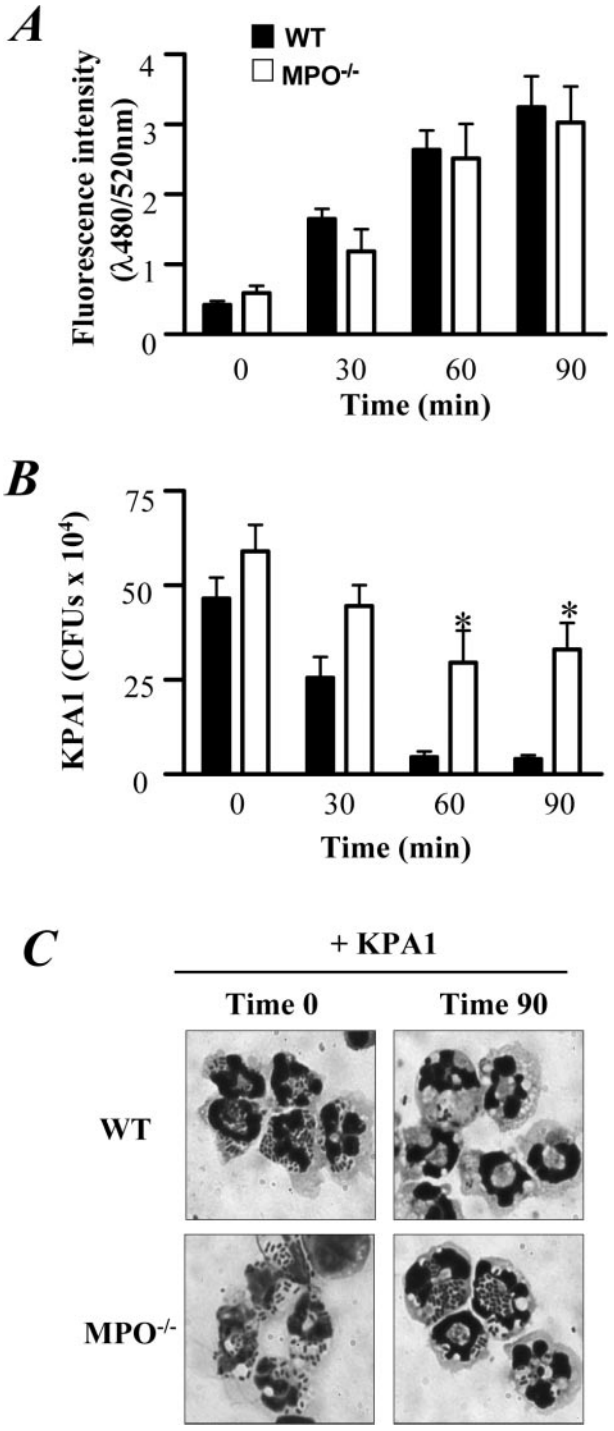

FIGURE 2. Capacity of $MPO^{-/-}$ and WT neutrophils to ingest and kill *K. pneumoniae*. *A*, Phagocytosis of fluorescent bioparticles by $MPO^{-/-}$ and WT neutrophils. *B,* Quantification of viable bacteria in isolated $MPO^{-/-}$ and WT neutrophils. After incubation for the indicated times, neutrophils (1×10^6) were washed and lysed, and the numbers of viable intracellular bacteria were determined by the plating method. *C*, Representative micrographs of cytospins from $MPO^{-/-}$ or WT neutrophils. Neutrophils were examined immediately after isolation (Time 0) or after 90 min of incubation (Time 90). Experiments were performed in triplicate; data represent the mean of at least two independent assays (bars = SEM; *, $p < 0.05$ for $MPO^{-/-}$ vs WT neutrophils).

neutrophils. Mice were i.p. injected first with glycogen and 4 h later with *K. pneumoniae*. After 30 min, cells were collected in gentamicin-containing HBSS, washed, and incubated for varying time points. At each time point, the cells were lysed and agar-plated, and the numbers of viable bacteria were determined. Bacterial counts and analyses of cytospin micrographs indicated significant differences in the numbers of bacteria between $MPO^{-/-}$ and WT neutrophils, which became more striking as a function of time (e.g., there were 5- to 6-fold more bacteria in $MPO^{-/-}$ cell lysates than in WT lysates 90 min postincubation; Fig. 2, *B* and *C*). These data indicate that neutrophils normally engulf bacteria inside the phagolysosomes in the absence of MPO. However, the enzyme is required for intracellular killing of this Gram-negative bacterium.

Reactive intermediates produced by MPO mediate K. pneumoniae *killing*

To determine the role of MPO in neutrophil mediated-bacterial killing, we cultured *K. pneumoniae* alone or with the complete MPO system (MPO, H_2O_2, and NaCl) and assessed the viability of bacteria. MPO mediated bacterial killing in an enzyme concentration- and time-dependent fashion (Fig. 3, *A* and *B*). Under physiologically plausible conditions, MPO used H_2O_2 to convert chloride to HOCl. We found that HOCl was a much more potent toxin than its precursor H_2O_2. For example, at equal molarities (10 μM), HOCl killed 100% bacteria, whereas H_2O_2 was inefficient (Fig. 3*C*). The killing ability of the MPO system or HOCl was also confirmed by fluorescent staining to distinguish live vs dead bacteria (Fig. 3*D*). HOCl, but not H_2O_2, killed virtually all bacteria under these conditions.

To gain insight into the mechanism of MPO-mediated bacterial killing, we compared the effects of the MPO system and NE on the morphology of bacteria. These studies were performed under conditions that led to the killing of ~ 50% of bacteria by either the complete MPO system or NE (Fig. 4*A*) (9). Untreated *K. pneumoniae* exhibited typical bacillus morphology (Fig. 4*B*). When exposed to the complete MPO system, we observed that bacteria affected by HOCl shrank and became round instead of elongated, suggesting that MPO generates toxic oxidants that interfere with bacterial growth (Fig. 4*C*). In contrast, bacteria targeted by NE blebbed extensively and lost their architecture when exposed to NE, suggesting disruption of structural integrity (Fig. 4*D*), an observation similar to NE-mediated killing of *E. coli* (10). These findings suggest that MPO and NE might use different mechanisms to kill Gram-negative bacteria.

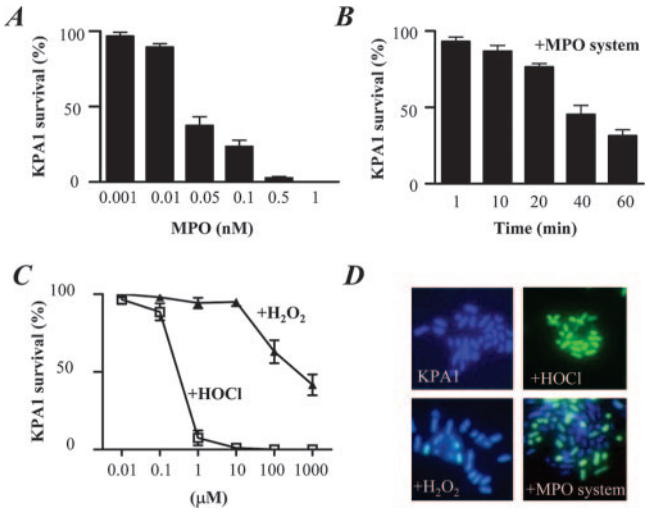

FIGURE 3. Bactericidal activity of the complete MPO system, HOCl, or H_2O_2. *A*, Effect of MPO concentration on bacterial killing. *K. pneumoniae* were incubated for 60 min in the presence of H_2O_2 (10 μM), NaCl (155 mM), and the indicated final concentration of MPO. *B*, *K. pneumoniae* were incubated with MPO (50 pM) and H_2O_2 (10 μM) for the indicated times. *C*, *K. pneumoniae* were incubated for 60 min with the indicated concentrations of oxidants. Results represent the means of three independent experiments (bars = SEM; $p < 0.05$ for differences between H_2O_2 and HOCl at concentrations ≥ 1 μM). *D*, Analysis of bacterial killing by fluorescence microscopy. *K. pneumoniae* were incubated for 60 min alone or in the presence of HOCl and H_2O_2 (10 μM each) or of the MPO system (50 pM MPO and 10 μM H_2O_2; +MPO sys; magnification, ×1000).

Reactive intermediates generated by MPO inactivate NE in vitro

MPO and NE are stored in primary neutrophil granules. To explore whether the enzymes might interact with each other after extracellular degranulation of neutrophils, we coincubated NE with the MPO system in vitro using varying concentrations (including those that were bactericidal to *K. pneumoniae*) and then assayed the activity of each enzyme. MPO did not loose its activity even upon exposure to high concentrations of NE (Fig. 5*A*). In contrast, incubation of NE with the MPO system revealed a reciprocal relation between the MPO concentration and the extent of NE inactivation, as assessed by the inability of this latter to degrade its specific

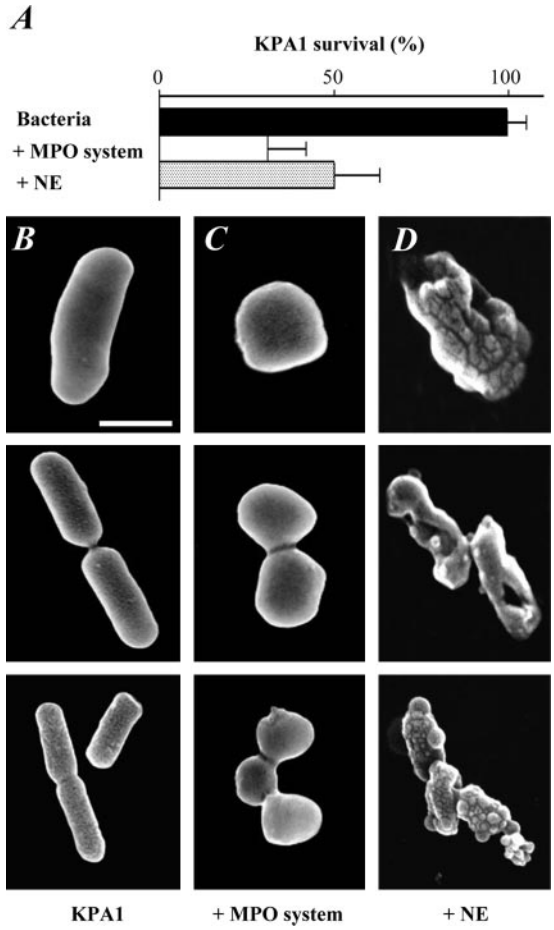

FIGURE 4. Morphology of bacteria exposed to MPO or NE. *A*, Bacterial viability in the presence of MPO system or NE. Data represent the mean of four independent experiments (bars = SEM). *B–D*, Scanning electron microscopy of untreated *K. pneumoniae* or bacteria exposed to the complete MPO system or NE. The micrographs are representative of single cells (magnification, ×40,000), doublets (magnification, ×30,000), and aggregates (magnification, ×20,000). Scale bar, 0.5 μm.

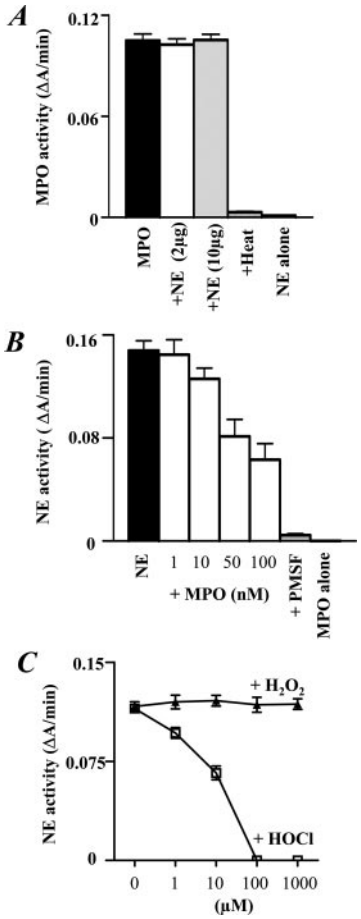

FIGURE 5. Interactions of NE and MPO. *A*, Effect of NE on MPO activity. No activity was detected with heat-inactivated MPO (+Heat) or NE alone. *B*, Effect of the complete MPO system on NE activity. NE was incubated alone or in the presence of H_2O_2 (10 μM) and the indicated concentration of MPO. *C*, Effects of varying concentrations of H_2O_2 or HOCl on the activity of NE. Results represent the mean of at least four independent experiments (bars = SEM; $p < 0.05$ for differences between H_2O_2 and HOCl at concentrations ≥ 1 μM).

peptide substrate (Fig. 5*B*). Incubation of NE with increasing concentrations of HOCl, but not H_2O_2, led to progressive loss of the enzyme's activity (Fig. 5*C*). These observations suggest that MPO uses H_2O_2 to generate HOCl, which inactivates NE in vitro.

To determine whether NE loses its ability to degrade physiologically relevant substrates after oxidation with HOCl, we exposed the enzyme to increasing concentrations of HOCl and examined its proteolytic activity against elastin and SP-D (15). HOCl-treated NE failed to cleave either protein, even though elastin is its preferred substrate (Fig. 6). Two lines of evidence indicate that oxidative modification of the substrate was not responsible for inhibition of proteolysis. First, when NE was exposed, residual oxidants were scavenged with L-methionine before addition of the substrates. Second, HOCl oxidation of substrates did not affect their degradation by NE. Thus, NE was inactivated by HOCl.

To explore potential mechanisms for inactivation of NE by HOCl, we incubated the proteinase with varying concentrations of HOCl and H_2O_2 or the broad serine proteinase inhibitor PMSF. We then determined both the enzyme's activity and its apparent m.w. by SDS-PAGE. NE exposed to either HOCl or PMSF, but not H_2O_2, lost its catalytic activity (Fig. 7*A*). As assessed by SDS-PAGE, NE protein remained intact after incubation with H_2O_2 or PMSF. In contrast, there was an apparent loss of NE protein when

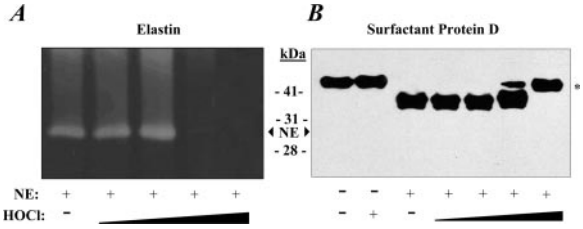

FIGURE 6. Effect of HOCl on the capacity of NE to degrade its substrates. NE was exposed to increasing concentrations of HOCl (1, 10, 100, and 1000 μM), and the ability of the proteinase to degrade its substrates was determined. *A*, κ-Elastin zymography. *B*, Western blotting of SP-D (reduced 43 kDa; *). SP-D is degraded by NE to yield an ~35-kDa fragment. Results are representative of at least three independent experiments. Molecular mass standards are shown in the *center*; arrowheads indicate the position of NE.

the proteinase was exposed to HOCl at 100 μM, and we failed to observe discrete fragments or higher apparent m.w. material (Fig. 7*B*). The presence of the oxidant scavenger L-methionine preserved both the catalytic activity and the protein integrity of NE incubated with HOCl (Fig. 7, *A* and *B*). Also, pretreating NE with PMSF prevented HOCl-mediated loss of the proteinase (Fig. 7*B*). The effect of HOCl on the activity and protein integrity of NE was dependent on the oxidant concentration, but was independent of the length of incubation (Fig. 7*C*).

MPO-generated HOCl inactivates a range of proteases implicated in inflammation in vitro

We next assessed the effects of HOCl on other proteases associated with inflammation. HOCl inhibited the proteolytic activity of both CG and PR-3, two members of the neutrophil serine proteinase family. Both enzymes lost their catalytic activity in concert with the disappearance of intact protein on SDS-PAGE, although PR-3 appeared to require higher concentrations of HOCl (data not shown and Fig. 8*A*). HOCl also inactivated two metalloproteases, MMP-7 and MMP-9, but the oxidatively inactivated proteases apparently remained intact (data not shown and Fig. 8*B*) (27). These observations indicate that a range of proteases encountered in inflamed situations is inactivated in vitro by physiologically relevant concentrations of HOCl.

MPO modulates NE activity in activated neutrophils

To determine whether MPO-mediated inactivation of NE might occur in vivo, we compared NE proteolytic activity in resting or activated neutrophils from $MPO^{-/-}$ or WT mice. Resting cells that were isolated from the bone marrow or blood of unchallenged $MPO^{-/-}$ or WT mice had comparable NE activities (data not shown and Fig. 9*A*). Activated $MPO^{-/-}$ and WT neutrophils that were isolated from the peritoneum of mice after i.p. challenge with glycogen released increased amounts of enzymatically active NE compared with resting cells (Fig. 9*A*). However, when glycogen-elicited neutrophils were exposed i.p. to *K. pneumoniae* before isolation, the levels of active NE markedly increased and became significantly higher in $MPO^{-/-}$ neutrophils than in WT neutrophils (Fig. 9). These observations suggest that MPO modulates NE activity in activated neutrophils, perhaps through oxidative inactivation.

We next determined whether the observed differences in neutrophil serine proteinase activity between activated $MPO^{-/-}$ and WT neutrophils were associated with differences in the levels of immunoreactive serine proteinases. Lysates derived from resting or activated neutrophils, isolated from $MPO^{-/-}$, $NE^{-/-}$, or WT mice, were subjected to Western blotting using Abs specific for

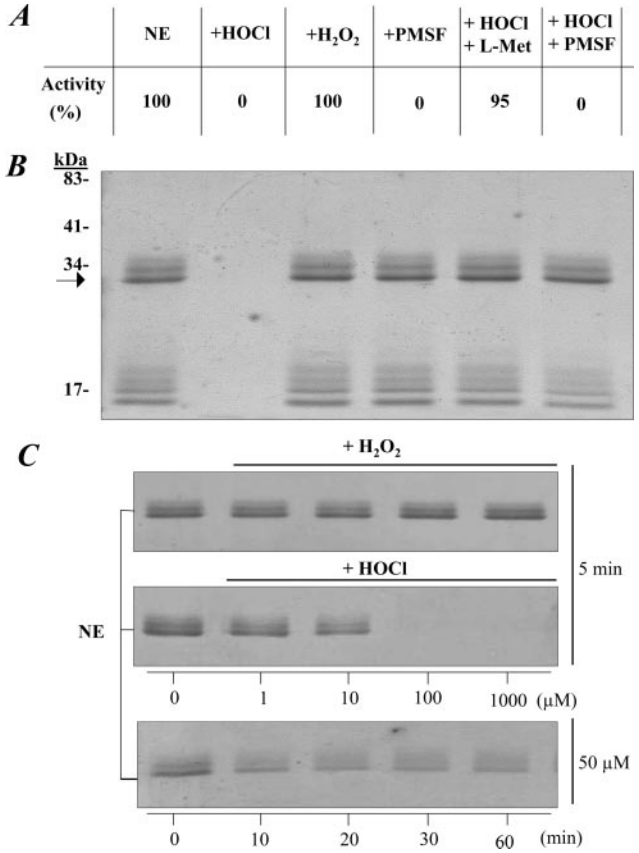

FIGURE 7. Effects of HOCl on the activity and protein integrity of NE. *A*, The effect of HOCl or H_2O_2 (100 μM each) on NE activity was assessed in the presence and the absence of PMSF or L-methionine (L-Met; 1 mM each). *B*, Protein integrity was assessed by SDS-PAGE and Coomassie blue staining. Molecular mass standards are shown on the *left*; the arrow indicates the position of NE. *C*, Effects of increasing concentrations of H_2O_2 and HOCl and time of incubation on NE protein. Results are representative of at least three independent experiments.

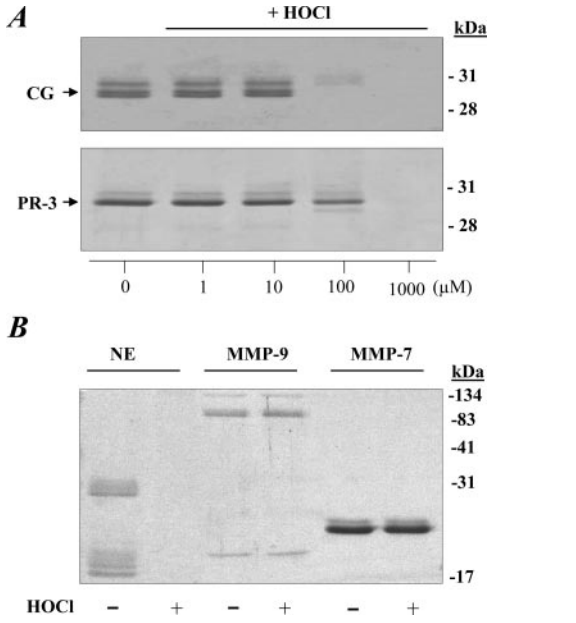

FIGURE 8. Oxidation of proteinases by HOCl. *A*, CG or PR-3 (2 μg each) were exposed to the indicated concentrations of HOCl. Protein integrity was assessed by SDS-PAGE and Coomassie blue staining. *B*, NE, MMP-9, and MMP-7 (2 μg each) were exposed to HOCl (100 μM), then subjected to SDS-PAGE and Coomassie blue staining. Results are representative of at least three independent experiments. Molecular mass standards are shown on the *right*.

MPO and CG. MPO protein levels were comparable in resting or activated WT and $NE^{-/-}$ neutrophils; these data were confirmed by MPO activity assay using the enzyme's chromogenic substrate (Fig. 10*A* and data not shown). Also, CG protein levels were similar in WT, $NE^{-/-}$, and $MPO^{-/-}$ neutrophils (Fig. 10*B*). These observations suggest that there were no major differences in the composition of granule proteins in mutant mice.

Discussion

In the current studies we demonstrate that MPO-deficient mice, although not immunocompromised, cannot resist overwhelming *K. pneumoniae* infections. Because $MPO^{-/-}$ mice are also susceptible to *Candida albicans*, but not to the Gram-positive *Staphylococcus aureus*, this may suggest that the enzyme is active against certain pathogens (11, 28). In support of this possibility, we previously reported that mice deficient in NE are sensitive to Gram-negative, but not Gram-positive, bacteria (9). Our in vivo and in vitro studies suggest that MPO, like NE, is critical for host immunity because neutrophils require the enzyme for maximal intracellular killing of bacteria. This finding is significant, because MPO-deficient patients who are not immunocompromised for other reasons do not appear to be susceptible to frequent bacterial or fungal infections (29). This could reflect the fact that human neutrophils express potent antimicrobial molecules, such as defensins and bactericidal/permeability-increasing proteins that compensate for MPO deficiency (30–32). Because mice lack these antimicrobial molecules, they may rely more on MPO for resistance to Gram-negative bacterial infection, but the increased vulnerability of MPO-deficient mice to infection also suggests the importance of this enzyme in host defense against certain pathogens, and that mice and humans use different spectra of antimicrobial weapons to combat invading pathogens.

Our morphological studies suggest that NE and MPO attack bacteria in different ways. Whereas NE kills by proteolysis (10), MPO probably oxidizes or halogenates various bacterial molecules. Indeed, high levels of chlorotyrosine, a specific product of MPO, are found in bacteria that have been phagocytosed by neutrophils (33, 34). The highly reactive nature of MPO-derived oxidants suggests that the bacterial wall might be the major target, which could potentially affect pathways associated with bacterial growth, such as energy production and DNA synthesis (7, 23, 35). It is also possible that MPO contributes to the efficient killing of neutrophils by acting in an orchestrated fashion in conjunction with other antimicrobial molecules. For example, NE degrades outer membrane proteins of bacteria (10), which may allow MPO to diffuse intracellularly and generate reactive intermediates to target molecules essential for bacterial survival. Alternatively, MPO oxidation of bacterial membrane proteins may enhance their degradation by NE (36, 37).

When we investigated the potential interaction between MPO and NE, we found that the complete MPO system (MPO-H_2O_2-Cl^-) or its specific product, HOCl, but not H_2O_2, potently inhibited the proteolytic activity of NE in vitro. In contrast, NE did not alter MPO activity despite its potent catalytic activity against a wide range of other substrates. Our data show that in the presence of low HOCl concentrations, NE lost most of its activity, but appeared largely intact on SDS-PAGE, suggesting that HOCl oxidation modified specific amino acids or altered the enzyme's substrate affinity and/or conformation. High concentrations of HOCl

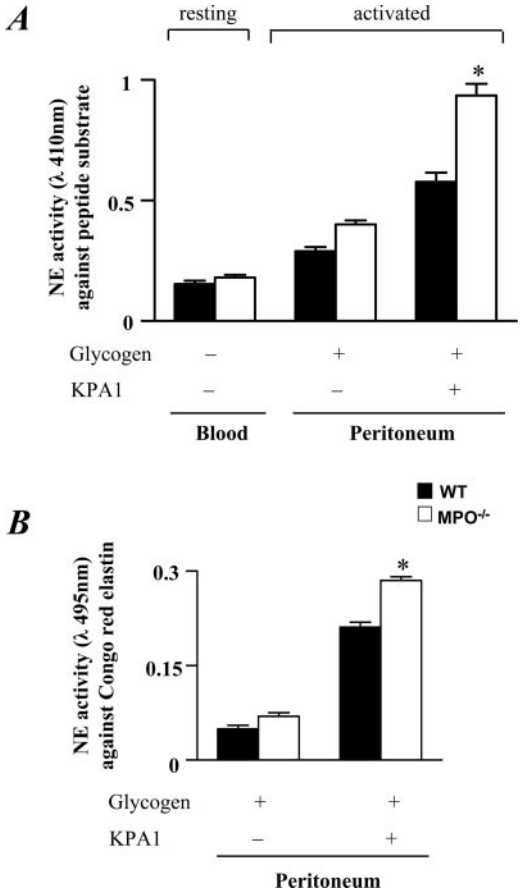

FIGURE 9. NE activities in WT and $MPO^{-/-}$ neutrophils. *A*, Resting and activated cells (2×10^6/reaction) were lysed and assayed for NE activity using its specific peptide substrate. *B*, NE elastolytic activity in peritoneal cell lysates. Analyses were performed in triplicate and are representative of at least three independent experiments (bars = SEM; *, $p < 0.05$ for $MPO^{-/-}$ neutrophils vs WT neutrophils). Values are corrected for the proteolytic activity observed in cell lysates isolated from $NE^{-/-}$ mice.

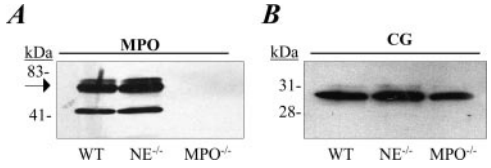

FIGURE 10. Immunoblot analysis of MPO and CG levels in WT, $NE^{-/-}$, and $MPO^{-/-}$ neutrophils. Activated neutrophils were subjected to Western blotting using Abs specific for MPO (*A*) and CG (*B*). MPO Ab detects only the H chain (~59 kDa; arrow) and an autocatalytic cleavage product (~45 kDa), but not the L chain of MPO (5). Results are representative of two independent experiments; molecular mass standards are shown on the *left*.

resulted in the complete loss of NE protein on SDS-PAGE, raising the possibility of either extensive protein degradation and/or loss of staining of oxidized proteins and peptides by Coomassie blue. Oxidative fragmentation of proteins in the presence of high concentrations of HOCl could occur through decarboxylation of amino acids/formation of *N*-chloramides, resulting in a random mixture of peptides (38, 39). Deamination of the ϵ-amino group of lysine residues exposed to high concentrations of HOCl might contribute to the loss of staining of polypeptides as well (40). However, when the proteinase's active site was blocked with PMSF, we readily detected intact inactive protein after treatment with HOCl, suggesting that oxidation might render NE susceptible to autodegradation.

HOCl inactivated other neutrophil granule enzymes, including MMP-9 and NE homologues, CG and PR-3. However, the impact of HOCl on the apparent integrity of each protein was variable. For example, HOCl inactivated MMP-9, but the protein remained intact, as assessed by SDS-PAGE, even when it was exposed to high concentrations of the oxidant. In this regard, we recently showed that low concentrations of HOCl activate MMP-7 by oxygenating the thiol residue in the pro domain of the latent enzyme (41). Higher concentrations of HOCl inactivated MMP-7 by site-specific oxidation of adjacent tryptophan and glycine residues (27). Collectively, these observations indicate that HOCl oxidatively inactivates proteases through multiple mechanisms. In future studies, it will be important to elucidate the biochemical mechanism(s) for oxidative inactivation of neutrophil serine proteinases by MPO.

We found that resting cells isolated from $MPO^{-/-}$ and WT neutrophils had similar levels of enzymatically active NE. However, upon activation in vivo, NE levels increased and became more pronounced in $MPO^{-/-}$ neutrophils compared with WT neutrophils. Our in vitro studies suggest that this difference in the activity of NE is likely to reflect in part inactivation of the serine proteinase by MPO-derived oxidants. In support of our observations, previous studies have demonstrated that granule enzymes are oxidatively damaged in activated neutrophils (20, 21, 42).

Our data show that the neutrophil serine proteinases NE, CG, and PR-3 are susceptible to oxidative inactivation by HOCl and the complete MPO system in vitro. More importantly, NE activity was enhanced in $MPO^{-/-}$ neutrophils. A body of evidence has shown that activated neutrophils can mobilize their primary granule enzymes to the cell surface (19). MPO might then use H_2O_2 catalyzed by NADPH oxidase to generate HOCl and inactivate NE as well as other proteinases secreted in the surroundings. It has been reported that 5×10^6 activated neutrophils generate ~100 μM HOCl within 2 h (43). Considering the large number of neutrophils that accumulate in inflamed tissues (e.g., lungs of patients with acute respiratory distress syndrome), the concentrations of HOCl that we used in our studies are of physiologic relevance. Taken together, these observations suggest that MPO might be a physiologically relevant regulator of proteinase activity in vivo. Studies are underway to investigate the in vivo significance of these findings.

Reactive intermediates derived from the NADPH oxidase of phagocytes have been implicated in the killing of bacterial and fungal pathogens. However, recent studies by Segal et al. (44) have cast doubts on this proposal. They suggest instead that NADPH oxidase acts by increasing the concentration of potassium in the phagocytic vacuole, which allows neutrophil proteinases, including NE and CG, to detach from the granule matrix and kill ingested microbes (44). Moreover, they proposed that MPO protects proteinases from oxidative inactivation by converting H_2O_2 to HOCl. In contrast, we provide compelling evidence that susceptibility to infection with the Gram-negative *K. pneumoniae* is enhanced in $MPO^{-/-}$ mice. Because MPO's only known enzymatic activity is to generate reactive intermediates, this observation strongly suggests that oxidants derived from NADPH oxidase contribute significantly to killing invading pathogens. We also found that MPO-deficient neutrophils exhibit enhanced NE activity, and that MPO-generated HOCl, but not its precursor H_2O_2, markedly inactivated the proteolytic activity of neutrophil serine proteinases. These data strongly argue against the proposal that MPO protects proteinases from oxidative inactivation by converting H_2O_2 into HOCl (44, 45). It is important to note that extracellular NE might damage tissue at sites of inflammation. Therefore, our findings led us

to speculate that the oxidative system could protect the host in different situations. It uses reactive intermediates derived from NADPH oxidase to turn on the proteolytic system that digests and kills bacteria. When secreted in the pericellular space, MPO could generate HOCl to inactivate NE and other proteinases, which may play a role in protecting tissues from unregulated proteolysis. Other reports have also shown that MPO could mediate inactivation of the physiologic inhibitors of neutrophil serine proteinases (46–48). These observations suggest that oxidants might have the capacity to both inhibit and promote proteolysis, and that their impact on pericellular proteolysis in vivo will be complex during inflammation.

In conclusion, our studies establish that MPO plays a critical role in protecting mice against overwhelming Gram-negative bacterial infection. These observations strongly support the proposal that neutrophils use reactive oxygen intermediates to kill bacteria. We also demonstrate that MPO-generated HOCl, but not its precursor H_2O_2, is a potent inhibitor of the proteolytic activity of NE and other enzymes, raising the possibility that oxidants might contribute to protection of the host from proteinase-mediated tissue degradation.

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