Surfactant protein D (SP-D) plays diverse and important roles in innate immunity and pulmonary homeostasis. Neutrophils and myeloperoxidase (MPO) colocalized with SP-D in a murine bacterial pneumonia model of acute inflammation, suggesting that MPO-derived reactive species might alter the function of SP-D. Exposure of SP-D to the complete MPO-H2O2-halide system caused loss of SP-D-dependent aggregating activity. Hypochlorous acid (HOCl), the major oxidant generated by MPO, caused a similar loss of aggregating activity, which was accompanied by the generation of abnormal disulfide-cross-linked oligomers. A full-length SP-D mutant lacking N-terminal cysteine residues and truncation mutants lacking the N-terminal domains were resistant to the oxidant-induced alterations in disulfide bonding. Mass spectroscopy of HOCl-treated human SP-D demonstrated several modifications, but none involved key ligand binding residues. There was detectable oxidation of cysteine 15, but no HOCl-induced cysteine modifications were observed in the C-terminal lectin domain. Together, the findings localize abnormal disulfide cross-links to the N-terminal domain. MPO-deficient mice showed decreased cross-linking of SP-D and increased SP-D-dependent aggregating activity in the pneumonia model. Thus, MPO-derived oxidants can lead to modifications of SP-D structure with associated alterations in its characteristic aggregating activity.

Pulmonary surfactant protein D (SP-D) is a collagenous C-type lectin (collectin) that plays important, if not critical, roles in antimicrobial host defense, inflammatory regulation, and surfactant homeostasis (1–3). Although SP-D is primarily synthesized by respiratory epithelial cells and secreted in the alveolar spaces, it is also expressed at mucosal surfaces and other extrapulmonary sites, including the gastrointestinal and genitourinary tracts, where it could play similar roles in inflammatory and immune regulation (4).

SP-D consists of one or more trimeric subunits, each with four structurally distinct domains (5, 6). The N-terminal domains mediate the association of subunits and contain conserved cysteine residues (Cys15 and Cys20 of the mature protein) that participate in intersubunit cross-links. By contrast, the C-terminal lectin domains mediate binding to microbial cell wall glycoconjugates, such as lipopolysaccharides (LPS), organic particulate antigens, nucleic acids, and specific cellular receptors (5, 7, 8). The intervening collagenous and neck domains maintain the trimeric structure of SP-D subunits and ensure an appropriate spatial distribution of the terminal lectin domains. Although the trimerization of CRDs is necessary for high affinity binding, higher order oligomerization of trimeric subunits is required for aggregation and bridging interactions of particulate ligands and effects on surfactant metabolism.

Acute inflammation is characteristically accompanied by the recruitment and activation of neutrophils. SP-D can directly interact with neutrophils and modulate the antimicrobial functions of neutrophils in vitro (9, 10); it can also modestly enhance macrophage uptake of apoptotic neutrophils (11). SP-D-deficient mice show an exaggerated neutrophil response to viral and bacterial challenge (12, 13). Recently, we demonstrated degradation of SP-D by neutrophil serine proteases (NSPs) in vitro and in vivo (14). Notably, all three granule-associated NSPs were able to cleave at specific sites within the functionally important lectin domain, abrogating carbohydrate recognition and bacterial aggregation. Together, these observations suggest a complex interplay between neutrophils and SP-D at sites of acute inflammation in vivo.

In addition to proteases, neutrophil granules contain potent oxidant-generating enzymes, and recruited neutrophils are the major source of oxidants in the setting of acute inflammation.
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(15). Although neutrophils can generate a variety of oxidants via several distinct pathways, myeloperoxidase (MPO) is the dominant oxidant-generating system, accounting for 3–5% of total neutrophil proteins. MPO is unique in catalyzing the formation of hypochlorous acid (HOCl), a highly reactive oxidant. Although HOCl production is normally confined to phagolysosomes, it is released into the environment in the face of overwhelming challenge and/or cell death, where it can react with a variety of tissue components. The present study was undertaken to determine whether MPO and its specific product HOCl can inactivate SP-D with resultant effects on its function.

EXPERIMENTAL PROCEDURES

Reagents—MPO (lyophilized, salt-free, and highly purified by ion exchange and gel diffusion chromatography from human sputum) was purchased from Elastin Products Company, Inc. (EPC, Owensville, MO). The Reinheit Zahl value (A280 nm/A320 nm) was 0.7. The purity of MPO was confirmed by SDS-PAGE, and activity was assessed spectrophotometrically using the H2O2 and O-dianisidine method (16). HOCl (reagent grade) was prepared as previously described (17). Concentrations of HOCl were spectrophotometrically determined (E292 = 350 M−1 cm−1) (18). Sequencing grade modified trypsin was from Promega (Madison, WI), and endoproteinase Glu-C from staphylococcal serine protease Protease V8 was obtained from Roche Applied Science. Polyclonal rabbit antibodies specific for rat and human SP-D were prepared and characterized as described previously (19, 20). The rabbit anti-mouse SP-D (AB3434) and albumin were purchased from Chemicon. Antibodies to the His tag were from Novagen. Except where indicated, all other chemicals were reagent grade and purchased from Sigma. All buffers were prepared using endotoxin-free water.

Surfactant Protein D—Recombinant rat SP-D (RrSP-D), RrSP-Dser15,20, and recombinant human SP-D were expressed in CHO-K1 cells as described previously (21–23). SP-D dodecamers (tetramers of trimeric subunits) were purified by sequential maltosyl-agarose affinity and gel filtration chromatography on A-15 M-agarose. The native recombinant rat and human molecules are ultrastructurally and compositionally indistinguishable from the wild-type proteins (22, 24). Rat and human molecules are ultrastructurally and compositionally indistinguishable from the wild-type proteins (22, 24). For these experiments, the fusion tags were removed with enterokinase prior to repurification by maltosyl-agarose affinity chromatography and gel filtration (25). Crystalllographic studies have confirmed normal folding of these preparations (27).

Bacteria—For aggregation experiments, we used a well characterized microbial ligand known to be efficiently aggregated by SP-D, an unencapsulated phase variant of Klebsiella pneumoniae, K-50 cap− (14).

For the murine pneumonia model of inflammation, we used Pseudomonas aeruginosa, H103, kindly provided by R. Hancock (University of British Columbia, Vancouver, Canada). This strain does not express a metalloproteinase previously shown to degrade SP-D (28). Overnight cultures were diluted 1:100 and grown aerobically in Luria Bertani broth (10 ml) at 37 °C to late exponential phase (3 h). Bacteria were collected by centrifugation (500 × g, 10 min), washed twice, and resuspended in 1 ml of phosphate-buffered saline (PBS) (pH 7.4). Bacteria were quantified by A600 nm (A600 nm of 1 × 109 bacteria/ml) (14).

MPO and HOCl-dependent Alterations in SP-D-mediated Aggregation—Bacterial aggregation assays were performed as previously described (14). SP-D dodecamers (2.5 μg) were incubated with the MPO system (MPO + H2O2 + NaCl), in which MPO (0.1 or 1 μM) reacts with H2O2 (100 μM) and chloride ions (NaCl at 100 mM) to generate HOCl. In parallel assays, SP-D was incubated alone or in the presence of varying concentrations of HOCl or H2O2, at 37 °C for a 20-μl reaction containing phosphate-buffered saline, pH 7.4, containing 10 mm CaCl2 (PBS/CaCl2) for designated time periods. Reactions were terminated using L-methionine as scavenger (10-fold molar excess relative to oxidant). Next, the reactions were mixed with suspensions of freshly prepared bacteria (107) in a total volume of 1 ml of PBS/CaCl2. All reactions were performed in duplicate. The A value of the bacterial suspension was recorded at designated time periods postincubation as an index for bacterial aggregation. In control assays, SP-D was either omitted or incubated with an MPO system containing heat-inactivated enzyme.

Effects on aggregation were also assessed by light or fluorescence microscopy using SYTO 59® (Molecular Probes, Inc., Eugene, OR)-stained bacteria (14). In these experiments, changes in the A values of the bacterial suspensions were recorded at 0 and 60 min. Data were analyzed with Student’s t test. Significance was accepted when p was <0.05.

Pull-down Assay—LPS-coated polystyrene beads were prepared essentially as described previously, except Escherichia coli J5 Rc-LPS was used (29, 30). Aliquots of rat SP-D dodecamer (0.4 μg) were diluted into 10 mM Hepes, 150 mM NaCl, 10 mm NaCl, pH 7.5 (Hepes-buffered saline (HBS)), in 1.5-ml microcentrifuge tubes. Samples were incubated with 1 mm HOCl in the absence or presence of a molar excess of L-methionine for 5 min at 37 °C. Samples were further incubated in the absence or presence of competing maltose (50 mm) for 30 min on ice. Freshly washed, LPS-coupled beads in Hepes-buffered saline were then added to each tube to yield ~1.44 × 108 beads/tube. The beads and protein were mixed by gentle agitation for 60 min at room temperature and then collected by microcentrifugation at 10,000 rpm for 5 min at 4 °C. Beads were washed two more times with the above buffer, but containing 0.05% Tween, and then resuspended and transferred to a new microcentrifuge tube. After one final spin, the beads were resuspended in SDS sample buffer plus DTT and boiled for 10 min at 100 °C. Bound proteins were resolved by SDS-PAGE on a 10% minigel and visualized with Coomassie Blue. Similar pull-
down assays were subsequently performed in PBS using the K-50 cap− strain of Klebsiella as the particulate ligand.

**Mass Spectrometry of Native and Oxidized SP-D**—Native SP-D was exposed to varying concentrations of HOCl as described above. Previous attempts to characterize cysteine-containing N-terminal peptides in tryptic digests of unreduced human or bovine SP-D dodecamers were unsuccessful (31). For these studies, the protein was reduced and alkylated prior to proteolysis to permit analysis of N-terminal sequences that participate in dodecamer formation.

**Proteolytic Digestion of SP-D Exposed to HOCl**—Native or HOCl-oxidized SP-D was reduced with 4 mM dithiothreitol at 70–80 °C in 50 mM ammonium bicarbonate and 10% acetonitrile for 35 min. The reduced protein was alkylated with 10 mM iodoacetamide at room temperature for 30 min. Alkylated protein was incubated overnight at 37 °C with trypsin at a ratio of 25:1 (w/w, protein/enzyme) or with endoproteinase Glu-C at a ratio of 10:1 (w/w, protein/enzyme) in 50 mM NH₄HCO₃, pH 7.8. Digestion was halted by acidifying the reaction mixture (pH 2–3) with trifluoroacetic acid. Proteolytic digests were desalted with a C18 ZipTip (Millipore) prior to MS analysis.

**Nano-liquid Chromatography-Electrospray Ionization Mass Spectrometry (Nano-LC-ESI-MS)**—Nano-LC-ESI-MS analyses of proteolytic digests of SP-D (0.75 μg of protein) were performed on a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA) coupled to a Paradigm MS4 LC system (Michrom BioResources, Inc.) (32). Peptides were separated at a flow rate of 1.0 μL/min on a Magic C18 AQ column (150 × 0.15 mm, 5 μm 200A; Michrom BioResources, Inc.), using solvent A (0.1% formic acid, 5% CH₃CN in water) and solvent B (0.1% formic acid in 90% CH₃CN). Peptides were eluted using a linear gradient of 0–35% solvent B over 75 min. A spray voltage of 1.8 kV was applied, and the heated metal capillary was maintained at 200 °C. The analyses were performed in the positive ion mode with a mass range of 200–2000 Da. MS/MS spectra were obtained using data-dependent acquisition with the following parameters (one MS survey scan followed by MS/MS scans of the nine most abundant peaks in the survey scan): isolation width, 3 Da; normalized collision energy, 35%; activation time, 30 ms; activation Q, 0.25; default charge state, 2.

**Quantification of Modified Amino Acids in SP-D Exposed to HOCl**—Peptide ion currents were used to quantify modified amino acids (33, 34). Product yield of oxidized peptides was determined with reconstructed ion chromatograms of product and precursor peptides, calculated as follows, product yield (%) = ((product ion peak area)/(precursor ion peak area + product ion peak area)) × 100. This method assumes that all precursor peptide is converted into known oxidation products and that the MS response characteristics of the product ions are similar to those of the precursor ion.

**SDS-PAGE and Immunoblotting of HOCl-treated SP-D**—RrSP-D, recombinant human SP-D, RrSP-Dser15,20, or NCRDs were incubated alone or in the presence of the indicated concentrations of HOCl in a 20-μL reaction volume in PBS or HBS with CaCl₂ (10 mM), pH 7.4, as indicated in the figure legends. The reactions were terminated by boiling in SDS sample buffer with or without a sulfhydryl-reducing agent (β-mercaptoethanol or dithiothreitol) and resolved by SDS-PAGE (12%). Reactions products were visualized by staining with Coomassie Blue. For immunoblotting, protein gels were transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) or nitrocellulose. The membranes were sequentially incubated with rabbit polyclonal antibody to rat SP-D (1:10,000 dilution), followed by goat anti-rabbit horseradish peroxidase. Immunoreactive species were visualized by ECL (Amersham Biosciences).

**Mouse Pneumonia Model of Lung Inflammation**—WT and genetically engineered mice (C57Bl/6j, 8–10 weeks old) were maintained in the animal barrier facility with a 12-h light/dark cycle and provided with water and food ad libitum. All mouse procedures were approved by the Animal Studies Committee at our institute. MPO-deficient mice were generated and characterized as described previously (17).

P. aeruginosa H103 was passaged twice in mice before use. Mice (n = 20) were intranasally challenged with a sublethal dose of P. aeruginosa H103 bacteria or sterile PBS as described previously (14). Briefly, mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (75 mg/kg) and medotomidine hydrochloride (1 mg/kg), followed by intranasal administration of bacteria (1.5 × 10⁶ colony-forming units/mouse) in 50 μL of PBS. This sublethal dose of P. aeruginosa resulted in signs of distress, including lethargy and ruffled fur, but there was no death of either genotype within the time course of the experiments. When the ability of mice to clear bacteria was examined at designated postinfection time points, we found that the number of viable bacteria in the lungs decreased markedly and was not significantly different between MPO−/− and WT mice (data not shown). Groups of mice (n = 5) were sacrificed at designated time points (0, 6, 18, or 32 h), and their lungs were lavaged in situ using 1 mL of PBS, pH 7.4, cycled three times. Identical recoveries of lavage (700 μL/mouse) were obtained for each of the experimental groups. The total protein concentrations of the cell-free BAL fluids of both types of mice were determined by the bicinchoninic acid assay (BCA; Pierce) and were not significantly different (data not shown) (35). Cell counts from BAL fluids were immediately performed by hemocytometer and aliquots of BAL fluids were collected by cytocentrifugation for differential counting. Next, aliquots (500 μL) of the remaining BAL samples were centrifuged for 10 min at 4 °C to remove cells and assayed for MPO activity as indicated below.

In separate experiments, mice (n = 5 per experimental condition) were subjected to intranasal instillation of P. aeruginosa or sterile saline as described above, and their lungs were processed for lavage and histology 18 h postchallenge. Equivalent volumes of lavage (15 μL) supernatants were examined by zymography or by immunoblotting for MPO and SP-D. The amount of immunoreactive SP-D migrating between normal disulfide-cross-linked SP-D trimers and the top of the gel was assessed by densitometry, and the ratio of the density of the abnormal, high molecular weight species to normal SP-D trimers was calculated. Data were represented as mean ± S.D. for the indicated number of independent experiments.

**Myeloperoxidase Activity in Lavage Supernatants**—MPO activity was determined in cell-free BAL supernatants using
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modifications of previously described methods (17). Briefly, 100 μl of BAL supernatant (0, 6, 18, or 32 h) were mixed with 100 μl of O-dianisidine (1.25 mg/ml) and 100 μl of H2O2 (0.05%) in a total of 1 ml of HBSS. The enzymatic activity of MPO, which is proportional to changes in absorbance, was determined by spectrophotometry at 460 nm over a time interval of at least 6 min.

Zymography—The elastolytic activity in cell-free BAL fluids of non-infected WT mice and infected WT mice and MPO-deficient mice was assessed by zymography as previously described (35). Briefly, equal volumes of BAL supernatants (15 μl) were examined under non-reducing conditions at 4 °C on SDS-polyacrylamide gels (12%), containing 1 mg/ml elastin. Purified NE (0.1 μg) was used as a control. Following electrophoresis, gels were soaked in 2.5% Triton X-100 for 30 min, rinsed briefly, and incubated at 37 °C for 48 h in 50 mM Tris-HCl (pH 8.2), containing 5 mM CaCl2. The gels were then stained in Coomassie Blue and destained in 5% acetic acid and 10% methanol. Active NE appears as a transparent lysis band at ~30 kDa. NE activity in cell-free BAL fluids was further confirmed using conventional chromogenic peptide assays as described previously (36).

Processing of Lung Tissue for Histology and Immunohistochemistry—The lungs of control and infected mice (above) were inflated in situ with 10% buffered formalin at a constant pressure of 25 cm H2O for 15 min. The lungs were then excised, immersion-fixed with 10% buffered formalin for 24 h, dehydrated, embedded in paraffin, and cut into 5-μm sections.

Serial lung tissue sections were deparaffinized, rehydrated, stained with hematoxylin and eosin, or processed for immunohistochemistry, as described previously (14). Briefly, slides were pressure cooked for 5 min in Decloaker Solution (Biocare) to enhance antigen retrieval. Endogenous peroxidase and nonspecific binding sites were blocked by incubation with H2O2 (0.3%) and blocking reagent (Background Eraser, Biocare Medical (Walnut Creek, CA)) for 30 min. Sections were incubated with specific rabbit anti-mouse SP-D (1:750 dilution; Chemicon) and anti-MPO (1:3000 dilution) antibodies. Preimmune serum was used as a negative control. Next, sections were incubated for 20 min with biotinylated secondary antibody and labeled for 15 min with horseradish peroxidase- or alkaline phosphatase-conjugated streptavidin. Immune complexes were visualized using 3’,3-diaminobenzidine or Vulcan-Fast-Red chromogen (Biocare Medical) as substrates for horseradish peroxidase or alkaline phosphatase, respectively, and counterstained with Mayer’s hematoxylin.

Lavage Aggregation Assay—Bacterial aggregation assays were performed as described above with the following modifications. Equivalent volumes of BAL fluids (200 μl) were mixed with suspensions of freshly prepared bacteria (107) in a total volume of 250 μl of PBS/NaCl (2 mM). As controls, bacteria were incubated alone, or BALs were briefly pretreated with maltose (100 mM) prior to the addition of bacteria. All reactions were performed in duplicate. At 60 min, reactions were stained for fluorescence microscopy using SYTO 59. Micrographs of nine fields (magnification ×200) were randomly selected per condition and processed for comparative analyses using Image J software (version 1.41e, National Institutes of Health). Briefly, micrographs of bacteria alone were analyzed, and a threshold value (mean value ± S.D. in pixels) corresponding to stained bacteria was predetermined. Next, micrographs of BALs were analyzed to identify areas with equal or greater pixel values than the threshold value. The total surface of selected areas was calculated and expressed as a percentage of whole micrographs.

RESULTS

SP-D and MPO Are Co-distributed at Sites of Acute Inflammation—In conjunction with previous studies of SP-D degradation by neutrophil serine proteases, we implemented a murine model of P. aeruginosa pneumonia to study neutrophil-dependent modifications. In this model, mice were challenged with a sublethal dose of P. aeruginosa sufficient to elicit and activate neutrophils in SP-D containing regions of the lung (14).

At 18 h following instillation, the lungs showed multifocal infiltrates of neutrophils, particularly within air spaces near small airways (Fig. 1) (35). Immunohistochemical assays demonstrated strong staining for SP-D that co-distributed with the acute inflammatory infiltrates (Fig. 1B). Immunostaining of serial sections demonstrated accumulations of immunoreactive MPO at the sites of neutrophil infiltration (Fig. 1C), whereas sections of inflamed (or normal) lung incubated with control serum showed no staining (Fig. 1D). As demonstrated below, this was temporally correlated with the appearance of immunoreactive MPO in cell-free lavage, consistent with extracellular discharge of MPO. In PBS-treated animals, there was no histologically evident inflammation, and SP-D labeling was restricted to type II cells and bronchiolar cells (Fig. 1A); in addition, there was no tissue staining for MPO (data not shown), and no MPO was detected in lavage.

Although SP-D-deficient mice show slightly reduced clearance of Pseudomonas, it should be noted that these effects have only been observed at a much earlier time point and prior to maximal inflammation (37), presumably reflecting the marked redundancy of host defense mechanisms.

MPO Inhibits the Aggregating Activity of SP-D—Given the immunohistochemical findings, we examined the effects of a defined MPO oxidant-generating system on SP-D function. In particular, we exposed highly purified RrSP-D dodecamers to an “MPO system” consisting of active MPO, hydrogen peroxide, and sodium chloride, as described under “Experimental Procedures.” Aggregation of particulate ligands was selected as a sensitive read-out of SP-D binding activity, which requires both normal lectin activity and oligomeric structure. This approach avoids the need for covalent labeling of SP-D and does not require the immunologic detection of epitopes, which might be altered by oxidative modifications. Furthermore, similar assays can be used to assess SP-D activity in a complex milieu, such as murine lavage. For the in vitro aggregation experiments, we employed a well characterized, unencapsulated, strain of K. pneumoniae as the target ligand. Previous studies have demonstrated efficient, calcium-dependent, and maltose-sensitive aggregation of this organism by SP-D dodecamers (29).
As shown in Fig. 2A (top), SP-D alone caused sufficient aggregation to significantly increase sedimentation in a spectrophotometric assay. However, following exposure of SP-D to the complete MPO system in PBS, there was a time- and enzyme dose-dependent loss of the protein aggregating activity. Aggregation was unaltered when the assay was performed using a control system containing heat-inactivated MPO. The MPO system alone showed no bacterial aggregating activity (data not shown).

Because hydrogen peroxide is a component of the MPO system and can directly oxidize protein-associated thiols, we examined the effects of increasing concentrations H$_2$O$_2$ on aggregating activity (Fig. 2A, middle). Bacterial aggregation was unaltered when SP-D was incubated for up to 100 min at 37 °C with H$_2$O$_2$ as high as 10 mM, considerably higher than used with the MPO system.

![Fig. 1. SP-D and MPO co-localize in lungs of infected mice.](image)

**FIGURE 1.** SP-D and MPO co-localize in lungs of infected mice. Mice were challenged by intranasal instillation of *P. aeruginosa* as described under “Experimental Procedures.” Tissues obtained 18 h after instillation of bacteria or saline were processed for immunohistochemistry. A, SP-D immunostaining of control lung using rabbit anti-mouse SP-D. B–D, representative serial sections of acutely inflamed mouse lung reacted with anti-mouse SP-D (B), antibody to MPO (C), and preimmune serum (D). SP-D specifically co-localized with MPO at sites of neutrophil infiltration. ctrl, control.

**FIGURE 2.** “MPO system” and hypochlorous acid (HOCl), but not H$_2$O$_2$, inhibit SP-D-dependent aggregation. A, sedimentation assays of bacterial aggregation were performed after incubation of purified rat SP-D dodecamers (2.5 μg/ml) with the indicated components for 5 min at 37 °C in the presence of PBS containing 10 mM calcium. All assays used the same strain of *K. pneumoniae* as a ligand. Top, MPO system (MPO + H$_2$O$_2$ + NaCl). Open circles, SP-D alone; triangles, 100 nM MPO; open squares, 1 mM MPO. The MPO system significantly decreased bacterial aggregation (*, p < 0.035 for MPO at 1 μM). Diamonds, bacteria alone. Middle, hydrogen peroxide (H$_2$O$_2$). Open circles, SP-D alone; triangles, 1 μM H$_2$O$_2$; open squares, 10 mM H$_2$O$_2$; diamonds, bacteria alone. Bottom, hypochlorous acid. Open circles, SP-D alone; triangles, 100 μM HOCl; open squares, 1 μM HOCl; diamonds, bacteria alone. 100 μM and 1 mM HOCl significantly decreased bacterial aggregation (*, p = 0.02 for HOCl at 1 mM), but there was no detectable effect of H$_2$O$_2$ at concentrations as high as 10 mM. These findings are illustrative of three independent experiments per condition. The mean and S.D. (error bars) are shown. B, effects on bacterial aggregation were more directly visualized by fluorescence microscopy (×1000) using SYTO 59-labeled *Klebsiella*. Rat SP-D dodecamers (2.5 μg/ml) caused microscopic and macroscopic aggregation of bacteria in PBS. Aggregation was blocked by treating SP-D with 0.1 and 1 mM HOCl for 5 min at 37 °C. Note that small aggregates could be still observed in 0.1 mM treated-SP-D. There was no effect of HOCl alone. Representative light and fluorescence images and the optical density of the suspension at 60 min are indicated. The decreased absorbance reflects clearing of the suspension as aggregated bacteria sediment to the bottom of the tube. The SP-D-dependent decrease in absorbance was reproducible and significant (4 values represent the mean of three experiments; *, p = 0.015 for Klebsiella + SP-D versus Klebsiella + HOCl-treated SP-D).
Hypochlorous Acid Inhibits the Aggregating Activity of SP-D—
In the presence of hydrogen peroxide and chloride ions, MPO generates hypochlorous acid (HOCl), a potent oxidizing agent responsible for many of the oxidative effects of MPO (15). The hypochloride ion can rapidly interact with neighboring thiol, disulfide, and amino groups or modify tyrosine residues to form chlorotyrosine or dityrosine cross-links (38–41). Accordingly, we examined the effects of freshly prepared HOCl on SP-D activity. As shown in Fig. 2A (bottom), HOCl caused a time- and dose-dependent inhibition of bacterial aggregation in PBS, consistent with the effects observed for the MPO system. Inhibition was macroscopically evident after only 5 min and was even more obvious at 30 min. Incubation with 1 mM HOCl did not alter SP-D aggregating activity when incubations were performed in the presence of excess L-methionine (10-fold molar excess relative to oxidant), a scavenger of the reactive species (data not shown; see other experiments below).

To further confirm these findings, aggregation was directly visualized by fluorescence microscopy using labeled bacteria as described under “Experimental Procedures” (Fig. 2B). This assay is more sensitive and allows an assessment of aggregate size. Aggregation was directly correlated with absorbance as measured in parallel using a sedimentation assay. Notably, the maximal decrease in absorbance in the sedimentation assay corresponded to the presence of very large bacterial aggregates measured in parallel using a sedimentation assay. Notably, the efficient binding of SP-D following treatment with 1 mM HOCl (lanes 4 and 8). M, standards are at the left. Similar findings were obtained in four independent experiments.

Hypochlorous Acid Treatment Minimally Inhibits the Lectin Activity of SP-D at Concentrations That Inhibit Aggregation—
Defective aggregation could result from damage to the C-type lectin domains with defective binding to particulate ligands. To assess lectin activity of SP-D, we employed a pull-down assay using rough LPS-coated beads. This allows direct visualization of bound SP-D dodecamers by SDS-PAGE and protein staining in the absence of bacterial proteins. Specificity of the pull-down is indicated by inhibition of binding with the prototypical saccharide competitor, maltose. As shown in Fig. 3, 1 mM HOCl showed little decrease in maltose-sensitive binding of SP-D to LPS beads. Binding was similar when beads were incubated with 1 mM HOCl, which is sufficient to block coarse aggregation, or with the same concentration of HOCl in the presence of the L-methionine. One potential limitation is that these studies were performed in HBS to allow more precise control of the calcium concentration. However, Heps can interact with HOCl and could thereby act as a competitive inhibitor. Notably, some inhibition of binding was observed in pull-down assays using Klebsiella K50 cap− bacteria in PBS buffer (data not shown).

Hypochlorous Acid Treatment Causes Oxidative Modifications of SP-D—Thiol-containing and aromatic amino acids are the major targets for oxidation in proteins exposed to HOCl (41). To determine which residues in SP-D can be modified, we exposed the protein to HOCl, digested the modified protein with trypsin, and used LC-ESI-MS/MS to analyze the resulting peptide mixture. To ensure high coverage and to unambiguously identify the site at which each peptide was modified (34), we also used Glu-C, which cleaves bonds C-terminal to glutamic acid in ammonium bicarbonate buffer (42). Used in concert, peptides from tryptic and Glu-C digests covered ~85% of the protein’s sequence, including all of the Met, Cys, His, Trp, and Tyr residues except for Met57. To determine which residues had been oxidized, we used reconstructed ion chromatograms to detect (i) peptides that contained methionine, cysteine, histidine, tryptophan, and/or tyrosine and (ii) oxidized methionine-containing peptides (methionine sulfoxide (M+16) or methionine sulfone (M+32)), oxidized cysteine-containing peptides (sulfenic acid (C+16), sulfonic acid (C+32), or sulfonic acid (C+48)) (43), chlorinated histidine-containing peptides (chlorohistidine (H+34)), oxidized tryptophan-containing peptides (hydroxytryptophan (W+16) or dihydroxytryptophan (W+32)), and chlorinated tyrosine-containing peptides (chlorotyrosine (Y+34)). The identity of oxidized peptides was confirmed by LC-ESI-MS/MS analysis.

An example of our approach is shown in Fig. 4A, which represents the MS/MS spectrum of tryptic peptide NEAFLSM329TDSK that contains Met295. When SP-D was exposed to 10 mM HOCl, Met295 was oxidized to methionine sulfoxide (M+16; Fig. 4B) and methionine sulfone (M+32; Fig. 4C). In HOCl-oxidized protein, the y5 - y10, and the b2–b7 ions had gained 16 atomic mass units (Fig. 4B) or 32 atomic mass units (Fig. 4C), but the y2–y4 and b2–b7 ions were unmodified (Fig. 4, B and C), strongly suggesting that Met295 had been converted to methionine sulfoxide (M+16) or methionine sulfone (M+32).
FIGURE 4. MS/MS identification of methionine sulfoxide (Met\(^{295}\); M+16) and methionine sulfone (Met\(^{295}\); M+32) in SP-D exposed to HOCl. SP-D protein (0.2 mg/ml, ~5.6 μM) was exposed to 10 mM HOCl for 5 min at 37 °C in Hepes-buffered saline. After the reaction was terminated with L-methionine, SP-D was digested with trypsin, and the peptides were analyzed with LC-ESI-MS/MS. A, note that the y5–y10 and the b8–b11 ions had gained 16 atomic mass units (B; plus one oxygen) or 32 atomic mass units (C; plus two oxygens), but the y2–y4 and b2–b7 ions were unmodified, suggesting that Met\(^{295}\) in this peptide had been converted to methionine sulfoxide (M+16) or methionine sulfone (M+32).
The product yields of modified residues in SP-D exposed to 0.1, 1, or 10 mM HOCl are shown in Fig. 5. Analyses of these data suggest strongly that Met3, Met19, and Met169 were the major targets for oxidation and that the yield was nearly quantitative, even at low molar ratios of HOCl to protein. In addition, Cys15 showed low levels of oxidation. Tyr314 and His220 were susceptible to chlorination but only at high molar ratios of HOCl to protein. In contrast, the two tryptophan residues, which are buried within the core of the CRD (Trp317 and Trp340), were not susceptible to oxidation, even at the highest concentration of HOCl (data not shown). Cys15, Met169, and Cys331 were oxygenated in the native protein, suggesting that these residues were susceptible to oxidation during sample storage and/or analysis.

Hypochlorous Acid Modifies the Higher Order Structure of SP-D—Effects of HOCl on bacterial aggregation could also result from alterations in oligomeric structure. There were no obvious HOCl-dependent alterations in mobility when RrSP-D dodecamers were resolved by SDS-PAGE after sulphydryl reduction with dithiothreitol (Fig. 6A, left). However, treatment with 0.1 or 1 mM HOCl resulted in the appearance of abnormal disulfide-cross-linked species, as visualized by SDS-PAGE in the absence of reduction (Fig. 6A, right, asterisk). These species migrated more slowly than normal disulfide-cross-linked trimers. In some experiments, minor cross-linked species also persisted upon reduction, indicating the simultaneous formation of non-disulfide cross-links. Similar complexes were formed using PBS or HBS buffer. The HOCl-dependent generation of high molecular weight species was largely abrogated in the presence of L-methionine (+L-Met). Derivation of these species from SP-D was further confirmed by immunoblotting with anti-SP-D (Fig. 6B). Abnormal disulfide-cross-linked species (~170 and 210 kDa) were also observed for HOCl-treated nat-
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**Hypochlorous Acid Treatment Does Not Alter Normal Intrachain Disulfide Cross-linking**—As indicated in the Introduction, interchain disulfide bonds are present within the N-terminal cross-linking domains. However, four cysteine residues are also present within the C-terminal domain, where they play essential roles in maintaining the tertiary structure of the CRD. Although there was no major effect on lectin activity in the pull-down assay, we also looked for evidence of HOCl-dependent disulfide exchange using truncated molecules consisting of the trimeric neck plus CRD domains of SP-D (NCRD) (Fig. 6D). On heavily loaded gels, HOCl caused the appearance of very minor cross-linked components that migrated in the positions of dimers and trimers in the presence of reducing agent. However, there was no evidence of abnormal interchain disulfide cross-links.

**A Mutant SP-D Lacking N-terminal Cysteine Residues Shows Altered HOCl-dependent Cross-linking**—Together, the above data suggest HOCl-dependent modifications of N-terminal cysteine residues. In order to further confirm the localization of residues contributing to the abnormal interchain disulfide bonds, we examined the effects of HOCl on the mobility of RrSP-Dser15,20. This mutant recombinant rat SP-D lacks N-terminal cysteine residues and is unable to form dodecamers or other disulfide-cross-linked multimers of trimeric SP-D subunits (23). Although the mutant trimers retain lectin activity, they are unable to efficiently aggregate particulate ligands, such as influenza virus or bacteria, or correct surfactant abnormalities in SP-D-deficient mice (23, 44). As shown in Fig. 7, the major abnormal disulfide-cross-linked species was absent following exposure to 1 mM HOCl. As observed for the NCRD, very minor dithiothreitol-resistant cross-linked species were generated. No cross-linking was observed when HOCl treatment was performed in the presence of l-methionine.

**SP-D Shows MPO-dependent Alterations in Disulfide Cross-linking in the Murine Pneumonia Model**—Subsequent studies employed the model of bacterial pneumonia to elicit a neutrophil-rich inflammatory response. The time course of the acute inflammatory response to bacterial challenge was monitored by analysis of lavage. Consistent with earlier work, cytospin preparations of lavage showed a marked increase in total lavage cells by 18 h in both wild-type and MPO null mice (Fig. 8A) (14). The increase temporally correlated with the presence of neutrophils as visualized by microscopy (Fig. 1) (14). It also correlated with increased peroxidase activity (Fig. 8B), which was attributed to MPO based on immunohistochemical analysis and increases in shown). D, tagless, trimeric rat NCRDs were incubated in the absence or presence of 1 mM HOCl in Hepes-buffered saline as above. There was no evidence of HOCl-dependent disulfide cross-linking. However, HOCl generated minor, non-disulfide-cross-linked species (arrows) and caused a slight increase in dispersity of the monomer band. These modifications were blocked with l-methionine. The mobilities of the cross-linked components are consistent with NCRD dimers and trimers. M, standards are at the left. The slightly lower mobility (higher apparent mass) of proteins in the presence of DTT results from unfolding of the CRD following reduction of the normal intrachain disulfide bonds (25). The findings are representative of two independent experiments. Identical results were obtained for human NCRDs.
immunoreactive MPO (Fig. 8C, top) in the cell-free lavage supernatant of wild-type mice. In accordance with our previously published work, zymography demonstrated increased elastase activity in the lavage of infected mice, with slightly greater activity in the lavage of infected MPO/H11002/H11002 mice (Fig. 8C, middle) (14). There was also a marked increase in total protein and immunoreactive albumin (Fig. 8C, bottom) in the lavage of infected wild-type and MPO null mice. When combined with the immunolocalization data (Fig. 1C), the findings indicate neutrophil recruitment and activation with degranulation and the release of MPO in the vicinity of SP-D at sites of active pneumonia.

Given this information, our attention turned to the fate of endogenous murine SP-D and the effects of MPO deficiency on SP-D cross-linking. Preliminary studies showed that there was no difference in the level of immunoreactive SP-D in equivalent aliquots of cell-free bronchoalveolar lavage of untreated age-matched control and MPO-deficient mice (Fig. 9B). Both mouse strains showed a single major immunoreactive band migrating in the expected position of unreduced SP-D trimers (arrow, right). Essentially identical results were obtained when aliquots of cell-free lavage from PBS-treated (vehicle control) mice were examined (Fig. 9B, lanes 1 and 2, arrowhead on left).

As compared with uninfected controls, infected wild-type animals showed a much more complex pattern of immunoreactive species (Fig. 9B, lanes 3 and 4). This included higher molecular mass cross-linked forms at ~170 and 210 kDa (asterisks), components slightly smaller than natural trimers, and lower molecular weight species migrating near the expected position of reduced SP-D monomers (arrowheads on right). Essentially all of the immunoreactive protein migrated at the expected position of monomers when lavage was examined after reduction with dithiothreitol (Fig. 9B, inset). When loadings for immunoblots were normalized to protein, the SP-D signal was reduced, but there were comparable differences in the size distribution of immunoreactive species (data not shown).

Abnormal cross-linked components were decreased in BAL from infected MPO-deficient mice (lanes 5 and 6), as compared with infected wild-type mice. This was initially confirmed by comparing the total density of immunoreactive components migrating more slowly than unreduced wild-type trimers for the various experimental conditions.
MPO Alters SP-D Aggregating Activity in the Setting of Acute Inflammation—We next sought to determine whether MPO can alter the activity of SP-D in the context of the pneumonia model. Lavage was assayed for aggregating activity, again using unencapsulated *K. pneumoniae* as a convenient multivalent ligand. Previous studies have shown that SP-D is a major agglutinin of Gram-negative bacteria in rodent lavage and that SP-D is the major component in rodent lavage that binds to malosyl-agarose (45, 46). Preliminary experiments performed in conjunction with other studies showed that unconcentrated C57Bl/6J WT murine lavage causes malose-sensitive aggregation of beads stably coated with rough Gram-negative LPS and that aggregation is blocked with anti-SP-D but not an unrelated antibody (30). As shown in Fig. 10, lavage from WT or MPO−/− control mice showed efficient aggregation of *K. pneumoniae* (PBS-WT and PBS-MPO−/−, black bar) with the appearance of coarse bacterial aggregates. Aggregation was greatly decreased in the infected WT mice (white bar). However, aggregation was significantly greater in the infected MPO-deficient animals (MPO−/−, gray bar, *p* < 0.005) and approached the level observed for wild-type uninfected mice (PBS-WT and PBS-MPO−/−, black bar). Very large bacterial aggregates, as observed using wild-type lavage, were conspicuously absent. As expected for an SP-D-mediated effect, the aggregating activity was almost completely inhibited with maltose (hatched bar).

DISCUSSION

We have demonstrated that neutrophil myeloperoxidase and its specific reactive oxidant product, hypochlorous acid, can cause modifications and abnormal disulfide cross-linking of SP-D with loss of its distinctive aggregating activity in vitro and in the setting of acute inflammation in vivo.

Previous studies have demonstrated oxidative modifications of lung collectins (30, 47–50). However, the present studies are the first to demonstrate modification of SP-D by a MPO-generated oxidant and the first to implicate neutrophil-dependent covalent modifications in a loss of SP-D activity in vivo. A 5-min exposure to HOCl, in the concentration range of 0.1–1 mM, was...
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The in vitro conditions of HOCl exposure, which sometimes included Hepes, could have altered the dose response or specific pattern of oxidative modifications. Regardless, all of our assays showed similar qualitative effects on SP-D oligomeric structure and aggregating activity, and a variety of HOCl competitors and secondary reactive species, such as chloramines, are undoubtedly present at sites of neutrophil activation in vivo.

HOCl-dependent Modifications of SP-D—Mass spectroscopic analysis demonstrated several alterations in human SP-D structure following incubation with HOCl. However, none of these modifications involved residues previously implicated in ligand binding or known to be important for CRD structure. Methionine was the most sensitive to oxidation, but none of the oxidized methionines have known structural or functional roles. Oxidation of Met\(^{19}\) is interesting, given its occurrence in a normally hydrophobic sequence in proximity Cys\(^{20}\), but this residue is replaced by leucine in rat SP-D, which showed similar changes in cross-linking. The partial, HOCl-dependent oxidation of Met\(^{295}\) is also of potential interest. This residue participates in a non-canonical “WIGL” motif, FLSM. Met\(^{295}\) resides at the base of a depression, well posterior and lateral to the known binding surface of the CRD. The WIGL motif is highly conserved in many C-type lectins and may contribute to the maintenance of CRD structure (55). The distinctive variant motif is conserved in all SP-Ds, but its role is unknown.

The chlorinated tyrosine residues, Tyr\(^{228}\) and Tyr\(^{314}\), have been previously shown to be sites of nitration and/or cross-linking following exposure of human SP-D NCRDs to peroxynitrite (30). However, site-directed mutagenesis of these residues did not significantly alter mannan binding activity. Chlorination of His\(^{320}\) was quite selective, but this residue is replaced by arginine in rat SP-D and other members of the SP-D family.

There was detectable oxidation of Cys\(^{15}\) with the formation of sulfenic acid (Fig. 5). However, there was no evidence for HOCl-dependent oxidation of Cys\(^{20}\) or any of the cysteines within the C-terminal lectin domain. The finding of some oxidized Cys\(^{15}\) and Cys\(^{331}\) in our untreated preparations of SP-D was consistent with the recent report of free cysteine in rat SP-D dodecamers (50). Because there is little precedent for the disruption of disulfide bonds by HOCl, HOCl-dependent oxidation of Cys\(^{15}\) is also consistent with the presence of some free cysteine in the N-terminal peptide domain of the human SP-D dodecamers.

Localization of Abnormal Disulfide Cross-links—Interchain disulfide bonds, which are confined to the N-terminal peptide of native SP-D, are required to maintain the dodecameric structure of SP-D, whereas intrachain disulfide bonds are required to maintain a functional conformation of the C-terminal lectin domains (5). The HOCl- or MPO-dependent generation of sulfhydryl reagent-sensitive species of greater than \(\sim\)170 kDa is consistent with cross-linking of additional SP-D chains to the normal disulfide-cross-linked trimeric subunit. Because trimeric NCRDs showed no evidence of abnormal disulfide cross-linking following treatment with HOCl, we can exclude interchange among the C-terminal cysteines and reasonably conclude that N-terminal interchain disulfide bonds are preferentially susceptible to HOCl-dependent modification. This

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**FIGURE 10.** MPO alters SP-D aggregating activity in the setting of acute inflammation. The aggregating activity of cell-free BAL was assessed by fluorescence microscopy (×200) as described above. Control experiments included bacteria alone or BAL treated with maltose before the addition of bacteria. Upper panels, representative micrographs of SYTO 59-labeled bacteria incubated with BAL from control mice (PBS-WT and PBS-MPO\(^{−/−}\)). Middle panels, representative micrographs of SYTO 59 labeled bacteria incubated with BAL from infected WT and MPO\(^{−/−}\) mice (infected WT and infected MPO\(^{−/−}\)). Left lower panel, the activity of BAL from infected MPO\(^{−/−}\) mice was examined in the presence of maltose (+Mal), the prototypical SP-D competitor. Right lower panel, the surface area of bacterial clumps formed under the above conditions was determined as described under “Experimental Procedures.” SP-D-dependent formation of bacterial clumps by BAL was reproducible. Infected MPO-deficient mice showed significantly greater bacterial aggregating activity than infected wild-type mice (gray and white bars, *; \(p < 0.05\)), which was inhibited by maltose (+Mal). Error bars, S.D. Of note, there was no significant difference in the aggregating activity of non-infected WT and MPO\(^{−/−}\) mice (black bar) (data not shown). These findings are illustrative of four independent experiments.
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Conclusion is strongly supported by our findings with RecSP-Dser15,20, which lacks N-terminal cysteine residues. Disulfide interchange among disulfide-cross-linked subunits is also consistent with the apparent absence of increased dimers or monomeric forms following HOCl treatment of SP-D dodecamers in vitro.

Oxidation of Cys15 to sulfenic acid provides a potential mechanism for the N-terminal disulfide exchange because sulfenic acid can condense with cysteine to form disulfide bonds. Efforts are in progress to further characterize the abnormal cross-links; however, this will be a challenging task because both N-terminal cysteines normally participate in interchain bonds. It is likely that the native molecules contain both symmetrical (Cys15-Cys15, Cys20-Cys20) and asymmetrical bonds (Cys15-Cys20), as demonstrated for the trimeric bovine serum homolog, CL-43 (56).

MPO Contributes to Abnormal Disulfide Cross-linking in Vivo—Although we cannot yet prove that HOCl directly mediates the cross-linking at sites of acute inflammation in vivo, no other oxidizing agent has been shown to cause the formation of abnormal disulfide cross-links in SP-D. In addition, the major oxidant formed by the MPO in the presence of physiological chloride concentrations is HOCl (15). Our findings are also consistent with known effects of HOCl on protein structure. HOCl can modify several types of amino acid side chains. However, methionine and cysteine are particularly susceptible at physiological pH, and HOCl-dependent oxidation of cysteine can lead to the formation of interchain disulfide bonds (41, 57, 58). It should be noted that we also observed the generation of minor, non-disulfide-cross-linked species in vitro. At present, the mechanism is uncertain, but it probably involves oxidative modification of other amino acids.

Potential Mechanisms of Defective Aggregation—The mechanism(s) by which oxidative modifications lead to defective aggregation require further elucidation. Although we detected modifications in the lectin domain and there was a detectable reduction in bacterial binding, it is unclear whether this is sufficient to account for the marked inhibition of aggregating activity. Significantly, none of the observed modifications involve residues known to be required for lectin activity.

Disulfide-stabilized multimers of trimeric subunits are required for particle aggregation (59, 60), and the efficiency of aggregation is influenced by modifications of the collagen domain that alter the spatial distribution of lectin domains (61). Theoretically, cross-linking of no more than 2 of 12 chains (17%) would be needed to cross-link the trimeric subunits of a dodecamer, and cross-linking of no more than 2 of 24 chains (8%) would be required to cross-link dodecameric molecules. Either type of interchain cross-linking could alter the spatial organization of the lectin domains, thereby altering the efficiency of bridging interactions required for microbial aggregation. Modest levels of peroxynitrite-dependent, non-disulfide cross-linking caused a similar loss of aggregating activity without detectably inhibiting lectin activity (30). It is important to emphasize that aggregation is not an “all or none” phenomenon and that sedimentation assays are insensitive to the formation of small bacterial aggregates. Studies with other multivalent lectins have shown that lectin-ligand aggregates can form highly organized cross-linked lattices and that the structure and size of the lattice depends on the protein valency and structure of the complexity of the ligand. We speculate that abnormal cross-linking of even a small subpopulation of SP-D chains can alter lattice formation and interfere with the formation of large bacterial aggregates.

Potential Consequences of Alterations in Aggregating Activity—There is great redundancy in defense and immunoregulatory mechanisms. Thus, none of the defense activities of SP-D is known, or can even be expected, to be unique to SP-D. However, maltose-sensitive bacterial aggregation provides a remarkably specific assay of SP-D activity. Aggregation contributes to the efficient neutralization of specific pathogens, particularly respiratory viruses, such as influenza A virus (62), and oligomers of trimeric subunits are also necessary to fully rescue the surfactant abnormalities and structural changes in SP-D null mice (44). Gardai et al. (7) reported that SP-D can inhibit macrophage activation via its lectin activity but further hypothesized that ligand-dependent aggregation of SP-D dodecamers can activate macrophages via enhanced recognition of the collagen domain by calreticulin/CD91. As indicated previously, S-nitrosylation of the N-terminal peptide is accompanied by enhanced macrophage chemotaxis and activation but also results in disruption of normal molecular assembly (50). In addition, abnormally assembled molecules with normal lectin activity could theoretically inhibit the activities of native molecules. Thus, oxidant-mediated changes in oligomeric structure could have complex and potentially disparate effects on lung inflammation, microbial clearance, and surfactant function. These are important areas for future investigation.

Potential Interactions between Neutrophil-derived Oxidants and Proteases—As shown in Fig. 9, the lavage of infected animals showed disulfide-cross-linked fragments that migrated more rapidly than intact SP-D on immunoblots. Although oxidants could potentially cleave peptide bonds, neutrophil serine proteases, including elastase, can liberate similar fragments both in vitro and in vivo (14). Thus, the combined findings suggest that SP-D is undergoing both proteolytic and oxidative modifications at sites of acute inflammation. Oxidative modifications could theoretically enhance or inhibit specific proteolytic degradation, and patterns of oxidation might be influenced by conformational alterations associated with proteolytic cleavage. Studies are under way to generate mice doubly deficient in MPO and NE to determine the relative contribution of these molecules in neutrophil-mediated inactivation of SP-D. These studies will be further supported by in vitro experiments using neutrophil-deficient in their capacity to express serine protease activities and/or MPO-derived oxidants.

In conclusion, neutrophil inactivation of SP-D is anticipated in any neutrophil-rich inflammatory microenvironment with an unchecked MPO-generating oxidant system. Based on the totality of available information, we infer that MPO-generated oxidants interfere with SP-D function, which may in turn contribute to defects in host defense, innate immunity, and surfactant homeostasis.
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