Methionine Sulfoxide and Proteolytic Cleavage Contribute to the Inactivation of Cathepsin G by Hypochlorous Acid: An Oxidative Mechanism for Regulation of Serine Proteinases by Myeloperoxidase

Baohai Shao1, Abderrazzaq Belaaouaj2, Christophe L.M.J. Verlinde3, Xiaoyun Fu1, Jay W. Heinecke1

1Department of Medicine and 3Department of Biological Structure, University of Washington, Seattle, WA 98195, and 2Department of Medicine, Washington University, St. Louis, MO 63110

Running title: Autolytic cleavage of HOCl-exposed cathepsin G

Address correspondence to: Jay W. Heinecke, Division of Metabolism, Endocrinology and Nutrition, Campus Box 356426, 1959 NE Pacific Street, Seattle, WA 98195; Fax: (206) 685-8346; E-mail: heinecke@u.washington.edu.

SUMMARY

Using myeloperoxidase and hydrogen peroxide, activated neutrophils produce high local concentrations of hypochlorous acid (HOCl). They also secrete cathepsin G, a serine protease implicated in cytokine release, receptor activation, and degradation of tissue proteins. Isolated cathepsin G was inactivated by HOCl but not by hydrogen peroxide in vitro. We found that activated neutrophils lost cathepsin G activity by a pathway requiring myeloperoxidase, suggesting that oxidants generated by myeloperoxidase might regulate cathepsin G activity in vivo. Tandem mass spectrometric analysis of oxidized cathepsin G revealed that loss of a peptide containing Asp108, which lies in the active site, associated quantitatively with loss of enzymatic activity. Catalytic domain peptides containing Asp108 were lost from the oxidized protein in concert with the conversion of Met110 to the sulfoxide. Release of this peptide was blocked by pretreating cathepsin G with phenylmethylsulfonyl fluoride, strongly implying that oxidation introduced proteolytic cleavage sites into cathepsin G. Model system studies demonstrated that methionine oxidation can direct the regiospecific proteolysis of peptides by cathepsin G. Thus, oxidation of Met110 may contribute to cathepsin G inactivation by at least two distinct mechanisms. One involves direct oxidation of the thioether residue adjacent to the aspartic acid in the catalytic domain. The other involves the generation of new sites that are susceptible to proteolysis by cathepsin G. These observations raise the possibility that oxidants derived from neutrophils restrain pericellular proteolysis by inactivating cathepsin G. They also suggest that methionine oxidation could render cathepsin G susceptible to autolytic cleavage. Myeloperoxidase may thus play a previously unsuspected role in regulating tissue injury by serine proteases during inflammation.

INTRODUCTION

Neutrophils play a key role in host defense by migrating to sites of infection, where they phagocyte invading microorganisms (1). After a neutrophil encloses a microbe, the resulting phagosome fuses with granules containing microbicidal and digestive enzymes to form a phagolysosome. The azurophilic granules of neutrophils are rich in serine proteases, including cathepsin G and neutrophil elastase, which play critical roles in killing bacteria (2–4).

Although such proteases are important for tissue homeostasis and host defense, an imbalance between proteases and their inhibitors is implicated in tissue damage during inflammation (5). It is therefore likely that plasma-derived inhibitors of cathepsin G, such as α1-antichymotrypsin and α1-antitrypsin, help limit proteolysis (6). However, traditional enzyme kinetics cannot fully explain the regulation of proteolysis by neutrophils. Thus, even in the presence of plasma-derived protease inhibitors, neutrophils produce evanescent “quantum bursts”...
of pericellular proteolytic activity (7). Cathepsin G could thus promote local proteolysis of a range of protein and peptide substrates, including cytokines, neutrophil chemoattractants, clotting factors, extracellular matrix, and G protein-coupled protease-activated receptors (8-10).

The substrate-binding site of cathepsin G lies in a cleft between two six-stranded β-barrel domains (11, 12). It contains the catalytic triad Asp\textsuperscript{108}-His\textsuperscript{64}-Ser\textsuperscript{201}, which forms a charge relay system that cleaves the peptide bond of proteins. The active site of a protease contains a series of subsites (S) that interact with particular residues in peptide substrates (P). The site for amidolysis is defined as the peptide bond between amino acids P\textsubscript{1} and P\textsubscript{1′} in the substrate peptide P\textsubscript{n}...P\textsubscript{2}, P\textsubscript{1}, P\textsubscript{1′}, P\textsubscript{2′}...P\textsubscript{n′} (13). The primary specificity pocket, S\textsubscript{1}, which accommodates the side chain of the P\textsubscript{1} amino acid of a peptide substrate, provides the critical interaction site for serine proteases (12). Cathepsin G has the unusual ability to recognize either large, hydrophobic amino acids (Phe, Leu, Met) or basic amino acids (Lys, Arg) in the P\textsubscript{1} site of target peptides and proteins (12, 14-16), indicating that it has both chymotrypsin- and trypsin-like specificity.

Phagocytosis of pathogens by neutrophils triggers a burst of cyanide-insensitive oxygen consumption by the phagocyte NADPH oxidase (17, 18). This membrane-associated electron transport system generates superoxide, which dismutates to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). Another major component of azurophilic granules, myeloperoxidase, can use H\textsubscript{2}O\textsubscript{2} and chloride ions to generate hypochlorous acid (HOCl), a potent cytotoxic oxidant (Eq. 1) (19, 20).

\[ \text{Cl}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O} \] (Equation 1)

Myeloperoxidase is the only known enzyme that produces HOCl in humans at plasma concentrations of halide ion (21). Recent studies with mice deficient in myeloperoxidase or cathepsin G strongly support the proposal that these enzymes are necessary for killing fungi and gram-negative bacteria (21, 22). However, reactive intermediates produced by myeloperoxidase have also been implicated in tissue damage at sites of inflammation (23). Indeed, oxidation products identical to those generated by the enzyme \textit{in vitro} have been found in proteins, lipids, and nucleic acids in inflamed human tissue (24-27), indicating that myeloperoxidase and HOCl might be pathogenic. Moreover, phagocytes store myeloperoxidase and cathepsin G in the same secretory compartment, and degranulation of these components is likely to create high local concentrations of both enzymes near the cell surface (28), where NADPH oxidase is located. Indeed, secreted cathepsin G binds tightly to the cell surface of neutrophils (2-4).

Oxidative modulation of neutrophils’ inflammatory response may be a general regulatory mechanism because HOCl can inactivate a wide range of products that are secreted by neutrophils (29-31). However, the molecular details of protein oxidation by reactive intermediates remain poorly understood.

In the current studies, we demonstrate that HOCl, a specific product of myeloperoxidase, potently inactivates cathepsin G by a pathway that involves oxidation of a specific methionine residue and autolytic cleavage. This finding raises the possibility that myeloperoxidase might restrain cathepsin G’s activity near the surface of neutrophils. If so, it might help limit—rather than promote—inadvertent proteolysis of host proteins and tissue.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cathepsin G (from human sputum, salt-free, lyophilized) was obtained from Elastin Products Co. (Owensville, MO). NaOCl, H\textsubscript{2}O\textsubscript{2}, CF\textsubscript{3}COOH, and HPLC grade CH\textsubscript{3}CN were obtained from Fisher Scientific (Pittsburgh, PA). Unless otherwise indicated, all other materials were purchased from Sigma Chemical Co. (St. Louis, MO).

**Methods**

**Oxidation Reactions.** Reactions were carried out at 37°C for 30 min in buffer A (10 mM phosphate-buffered saline, containing 138 mM NaCl and 2.7 mM KCl, pH 7.4) with 1 µM or the indicated concentrations of cathepsin G or 400 µM TIQNDIMLLQLSR (Biopeptide, San Diego, CA). Reactions were initiated by adding oxidant (HOCl or H\textsubscript{2}O\textsubscript{2}) and terminated by adding a 10-fold molar excess (relative to oxidant) of L-methionine.

Concentrations of HOCl and H\textsubscript{2}O\textsubscript{2} were determined spectrophotometrically (\(\varepsilon_{292} = 350 \text{ M}^{-1} \text{ cm}^{-1}\) and \(\varepsilon_{240} = 39.4 \text{ M}^{-1} \text{ cm}^{-1}\), respectively; ref. 32, 33).
Human Neutrophils. Human neutrophils were isolated from EDTA-anticoagulated blood by buoyant density centrifugation using Polymorph-Prep (Robbins Scientific, Sunnyvale, CA) (34). Neutrophils were washed twice at 4°C by centrifugation in buffer B (Hank’s balanced salt solution, pH 7.4; prepared without calcium chloride, magnesium chloride, magnesium sulfate, sodium bicarbonate and phenol red (Invitrogen Corporation, Grand Island, NY); 100 µM diethylenetriaminepentaacetic acid was included to inhibit metal-catalyzed reactions (35)). Neutrophils were resuspended in buffer B at 37°C and immediately used for experiments. Cathepsin G (1 µM) was exposed for 1 h to neutrophil (1 × 10⁶ cells/ml) in buffer B at 37°C. Neutrophils were activated with 200 nM phorbol 12-myristate 13-acetate (PMA). Reactions were terminated by pelleting the cells by centrifugation at 4°C. Supernatants were concentrated under vacuum and digested with trypsin for mass spectrometric analysis.

Cathepsin G Enzymatic Activity. Following the addition of L-methionine to scavenge oxidants, the activity of cathepsin G (70 nM) was assayed in buffer C (0.1 M Tris-HCl, 0.5 M NaCl, pH 7.4) using 510 µM N-succinyl-AAPF-p-nitroanilide (suc-AAPF-NA) as substrate (36). Control experiments demonstrated that L-methionine did not affect the activity of cathepsin G. suc-AAPF-NA (6.4 mM) was initially solubilized in dimethylsulfoxide. Samples were incubated in individual wells of a 96 well microtiter plate at 37°C after adding suc-AAPF-NA, and the initial rate of change in absorbance was monitored at 410 nm (SpectraMax 190, Molecular Devices, Sunnyvale, CA).

To determine cathepsin G activity in neutrophils, quiescent and activated neutrophils were sonicated, and then incubated with 510 µM suc-AAPF-NA for 60 min at 37°C in buffer C (pH 7.4). Each reaction mixture was clarified by centrifugation and the initial rate of change in absorbance was monitored at 410 nm (SpectraMax 190, Molecular Devices, Sunnyvale, CA).

High Performance Liquid Chromatography Analysis (HPLC). Synthetic peptides and cathepsin G proteolytic products were separated on a reverse-phase column (Vydac C18 MS column, 25 × 2.1 mm i.d; Grace Vydac, Hesperia, CA) at a flow rate of 0.3 ml/min using a Beckman HPLC system (Fullerton, CA). Peptides were detected by monitoring absorbance at 214 nm. Peptides were eluted using solvent A (0.06% CF₃COOH in H₂O) and solvent B (0.06% CF₃COOH in 80% CH₃CN and 20% H₂O) with a linear gradient of 10% to 60% solvent B over 60 min.

Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-ESI-MS). Cathepsin G was incubated overnight at 37°C with sequencing grade modified trypsin (Promega, Madison, WI) at a ratio of 25:1 (w/w) cathepsin G/trypsin in buffer D (1 M urea, 50 mM NH₄HCO₃). Before trypsin digestion, cathepsin G was reduced with dithiothreitol and alkylated with iodoacetamide. Digestion was halted by acidifying with CF₃COOH to a final pH of 2-3. LC-ESI mass spectrometric analyses (39) were performed in the positive ion mode with a Finnigan Mat LCQ ion trap instrument (San Jose, CA) coupled to a Waters 2690 HPLC system (Milford, MA). Peptides were separated on a reverse-phase column (Vydac C18 MS column, 25 × 2.1 mm i.d.) at a flow rate of 0.2 ml/min using solvent C (0.2% HCOOH in H₂O) and solvent D (0.2% HCOOH in 80% CH₃CN and 20% H₂O). Peptides were detected by monitoring absorbance at 214 nm. Peptides were eluted using solvent A (0.06% CF₃COOH in H₂O) and solvent B (0.06% CF₃COOH in 80% CH₃CN and 20% H₂O) with a linear gradient of 10% to 60% solvent B over 60 min.
were eluted using a linear gradient of: 10% to 40% solvent D over 50 min, and then 40% to 70% solvent D over 10 min for tryptic digests; 10% solvent D for 5 min, and then 10% to 50% solvent D over 55 min for cathepsin G proteolysis of native or oxidized cathepsin G; and 10% to 50% solvent D over 60 min for synthetic peptides. The electrospray needle was held at 4500 V. The sheath gas, nitrogen, was set at 80 units. The collision gas was helium. The temperature of the heated capillary was 220°C.

RESULTS

Cathepsin G is inactivated by HOCl but not by H₂O₂. To assess whether reactive intermediates generated by phagocytes can directly influence the proteolytic activity of cathepsin G, we exposed the enzyme to HOCl, H₂O₂ or the myeloperoxidase-H₂O₂-Cl⁻ system for 30 min at 37°C at neutral pH in a physiological buffer containing plasma concentrations of chloride, sodium, and phosphate. After adding methionine to scavenge any residual oxidant, we determined whether the enzyme could degrade suc-AAPF-NA, a synthetic peptide substrate (33). Cathepsin G exposed to increasing concentrations of HOCl progressively lost its proteolytic activity, which was inhibited by ~50% at a 10:1 mole ratio of HOCl to protein (Fig. 1A, Fig. 1B and Fig. 2A). Higher concentrations of HOCl completely inhibited proteolysis. In striking contrast, the same range of H₂O₂ concentrations did not affect cathepsin G’s proteolytic activity (Fig. 1A and Fig. 2A). Cathepsin G exposed to the complete myeloperoxidase-H₂O₂-Cl⁻ system also lost activity (Fig. 1A). Inactivation required each component of the reaction mixture: myeloperoxidase, H₂O₂, and Cl⁻ (data not shown). The IC₅₀ for HOCl and peroxide in the complete myeloperoxidase system were ~10:1 and 20:1 (mol:mol), respectively (Fig. 1A). Inactivation of cathepsin G by the myeloperoxidase system was blocked by catalase, a scavenger of H₂O₂, and azide and 3-aminotriazole, two inhibitors of heme enzymes (data not shown). Methionine, a potent scavenger of HOCl, also blocked the reaction. These results demonstrate that inactivation of cathepsin G by myeloperoxidase requires active enzyme, Cl⁻, and H₂O₂.

Oxidative inactivation of cathepsin G is quantitatively associated with loss of a tryptic digest peptide containing Asp¹⁰⁸ of the catalytic triad. To determine whether oxidation of specific amino acid residues and/or enhanced proteolysis might contribute to the oxidative inactivation of cathepsin G, we digested unmodified and oxidized enzyme with trypsin and identified the resulting peptides through LC-ESI-MS. We used high concentrations of cathepsin G (17 µM) for these experiments to facilitate MS analysis of the protein. It is important to note, however, that inactivation of cathepsin G depended only on the mole ratio of oxidant to protein (Fig. 1B and Fig. 1C). Thus, when 200 nM cathepsin G was exposed to 3 µM HOCl (a 15:1 mole ratio of oxidant to protein), the activity of the proteinase was inhibited by ~85%. These observations indicate that physiologically plausible concentrations of HOCl, but not H₂O₂, can convert cathepsin G to a catalytically inactive form.

The trypsin digest of native cathepsin G contained 18 peptides that covered 70% of the native protein’s sequence. Due to poor retention on the HPLC column, we were unable to identify small peptides and single amino acid residues. When cathepsin G was exposed to HOCl (15:1, oxidant/protein, mol/mol) and digested with trypsin, LC-ESI-MS analysis revealed 8 new peaks of material (Table 1). MS/MS analysis indicated that the 8 peptides were derived from residues 35-48, 104-116, and 132-149 of cathepsin G. Five of the peptides (derived from all three regions of the protein) exhibited an increase of 16 amu, suggesting that each one had gained 1 oxygen atom (Table 1). MS/MS analysis demonstrated that methionine was oxygenated in four of these peptides (Met+16) and that tryptophan was oxygenated in the fifth (Try+16). The sixth peptide had gained 32 amu, and its methionine had been converted to a sulfone, while the seventh and eighth peptides (derived from residues 132-149) had lost 2 or 4 amu respectively. We recently showed that oxidative cross-linking of adjacent tryptophan and glycine residues creates these modifications (40, 41). In contrast, we observed only a very low level of one oxidized peptide (residues 104-116) in native cathepsin G or cathepsin G that was exposed to H₂O₂. These observations indicate that HOCl, but not H₂O₂, extensively oxidizes methionine residues in cathepsin G. It should be noted that
HOCl reacts more rapidly than H$_2$O$_2$ with thioethers (42-44).

To investigate the relationship between inactivation of cathepsin G by HOCl and the appearance of oxidation products in the enzyme, we focused on TIQNDIMLLQLSR, one of the tryptic digest peptides containing the Asp$^{108}$ of the catalytic triad (Table 1). Cathepsin G exposed to increasing concentrations of oxidant was digested with trypsin and analyzed by LC-ESI-MS. At low concentrations of HOCl (5-20:1, mol/mol, oxidant/protein), loss of the native peptide TIQNDIMLLQLSR associated quantitatively with loss of enzymatic activity (Fig. 2). In striking contrast, cathepsin G was not inactivated by the same concentrations of H$_2$O$_2$ (Fig. 2A). Moreover, there was no loss of TIQNDIMLLQLSR in cathepsin G exposed to H$_2$O$_2$ (Fig. 2B). These results implicate oxidative modification of TIQNDIMLLQLSR in the loss of enzymatic activity of cathepsin G exposed to low mole ratios of HOCl.

HOCl oxidizes specific methionine residues to generate unique proteolytic cleavage sites in cathepsin G. Because loss of the peptide TIQNDIMLLQLSR (residues 104-116) associated strongly with loss of enzymatic activity, we used reconstructed ion chromatograms (RIC; Fig. 3) and MS/MS analyses (Fig. 4) to characterize this region of cathepsin G after oxidation with HOCl and digestion with trypsin. Peptides TIQNDI(M+16)LLQLSR, TIQNDI(M+16)LL, and NDI(M+16)LL were observed in cathepsin G that had been oxidized with HOCl but not in native enzyme or cathepsin G exposed to H$_2$O$_2$ (Fig. 3B and Fig. 4). All three peptides contained methionine sulfoxide (Met+16). Importantly, two resulted from cleavage at a P$_1$ site (Leu$^{112}$) recognized by cathepsin G but not by trypsin (which recognizes Arg and Lys). The third, NDI(M+16)LL, was cleaved at Gln-Asn at the N-terminus of the peptide as well as at Leu$^{112}$ in the P$_1$ site. Since native cathepsin G has not been reported to recognize Gln in the P$_1$ site, HOCl appears to generate new proteolytic cleavage sites by oxidizing specific methionine residues in cathepsin G. Because cathepsin G can cleave proteins and peptides at P$_1$ sites that contain leucine residues and because we observed only TIQNDI(M+16)LL and NDI(M+16)LL in the oxidized protease, oxidation of Met$^{110}$ could generate a site in cathepsin G that the enzyme can cleave. Importantly, all three peptides also contained Asp$^{108}$, which lies in the enzyme’s catalytic triad, suggesting that proteolytic cleavage might help inactivate the protease.

Loss of TIQNDIMLLQLSR in cathepsin G exposed to low concentrations of HOCl occurred in parallel with the appearance of TIQNDI(M+16)LLQLSR, TIQNDI(M+16)LL, and NDI(M+16)LL (Fig. 5). It is noteworthy that the relative abundances of the oxidized peptides derived from TIQNDIMLLQLSR decreased when the mole ratio of HOCl to cathepsin G was >20 (Fig. 5). At high mole ratios of oxidant, other processes, such as random fragmentation of the peptide backbone by HOCl, may contribute to inactivation of cathepsin G and loss of precursor peptide (42). Collectively, these observations indicate that when cathepsin G is exposed to low concentrations of HOCl, oxidation of the region containing Asp$^{108}$ of the catalytic triad likely accounts for loss of enzymatic activity. The conversion of methionine 110 to its sulfoxide is likely to be an important event in this process. Moreover, we observed non-tryptic cleavage sites in cathepsin G exposed to HOCl, suggesting that oxidation generates unique autolytic or proteolytic cleavage sites in the proteinase that contribute to oxidative inactivation of the enzyme.

Cathepsin G exposed to HOCl releases an oxidized catalytic domain peptide by a pathway sensitive to inhibition by PMSF. To determine whether oxidation promotes proteolysis of intact cathepsin G, we exposed the enzyme to HOCl (15:1, oxidant/protein, mol/mol) and analyzed the reaction mixture with LC-ESI-MS (Fig. 6). Because we did not digest the protein with trypsin, any peptides detected in the reaction mixture presumably were generated by cathepsin G itself. Under these conditions, we readily detected an ion of $m/z$ 734.2, the anticipated $m/z$ of the peptide NDI(M+16)LL, which is derived from the enzyme’s catalytic site (Fig. 6B). MS/MS analysis confirmed the identity of the peptide. Formation of this peptide was directly proportional to the concentration of HOCl when the mole ratio of oxidant to protein was increased from 0 to 10 (Fig. 6D). At higher mole ratios of oxidant, the amount of peptide released from oxidized cathepsin G decreased. Treating the enzyme with PMSF, a phosphonate derivative that irreversibly
sulfonylates the serine residue at the active site of serine proteases, prior to oxidation blocked the release of the oxidized peptide (Fig. 6C). Production of oxidized peptide was directly proportional to the concentration of cathepsin G in the reaction mixture (data not shown). Moreover, the product yield of the oxidized peptide was dependent on the mole ratio of HOCl to cathepsin G but was independent of the cathepsin G concentration. These results strongly imply that proteolysis was occurring by a unimolecular reaction pathway. Thus, oxidized cathepsin G proteolytically cleaves itself, releasing NDI(M+16)LL, which contains Asp108 of the catalytic triad. The correlations between the loss of precursor peptide, the appearance of oxidized peptides, and the loss of enzymatic activity raise the possibility that proteolytic cleavage contributes to the inactivation of cathepsin G that has been exposed to HOCl. These observations further suggest that conversion of the thioether side chain of Met110 to sulfoxide generates unique autolytic cleavage sites in cathepsin G that contribute to oxidative inactivation of the enzyme.

High concentrations of HOCl have been proposed to oxidatively fragment proteins by a reaction pathway that may involve formation of N-chloramides of the peptide bond, yielding a random mixture of peptides (45). To explore whether this mechanism participates in the oxidative inactivation of cathepsin G, we incubated the enzyme alone or with varying concentrations of HOCl for 5 min at 37°C in a physiological buffer at neutral pH. After we exposed cathepsin G—either native or pretreated with PMSF—to a high concentration of HOCl (100:1, mol/mol, oxidant/protein), we observed no intact protein on SDS-PAGE (data not shown). This observation suggests an oxidative mechanism that randomly cleaves cathepsin G into a mixture of lower MW proteins that would be difficult to visualize on SDS-PAGE (45). The ε amino group of lysine residues exposed to high concentrations of HOCl has been proposed to undergo deamination (46), which may also contribute to loss of staining of oxidized proteins and peptides by Coomassie blue. It is important to note that a much higher concentration of HOCl (>50:1) was required for the apparent random fragmentation of cathepsin G than for enzyme inactivation. Thus, this pathway is not likely to contribute directly to cathepsin G inactivation at low mole ratios of oxidant to enzyme (<20:1).

**Detection of autolytically released peptide NDI(M+16)LL in cathepsin G exposed to activated-neutrophils.** To determine whether oxidants generated by neutrophils might help regulate the activity of cathepsin G, we incubated human neutrophils for 1 h at 37°C in a physiological buffer (pH 7.4) supplemented with cathepsin G (1 µM). The autolytic, oxidized peptide NDI(M+16)LL was readily detected by LC-ESI-MS in the supernatant of cells activated with PMA (Fig. 7A). The peptide’s identity was confirmed using MS/MS analysis (Fig. 7B). A low level of the peptide was detected with quiescent cells, but production of the peptide increased markedly when the neutrophils were activated with PMA (Fig. 7C). Generation of the peptide was inhibited completely by methionine (a scavenger of HOCl), catalase (a scavenger of H2O2), or azide (a heme poison). Moreover, we detected the same oxidized peptide when cathepsin G was exposed to MPO-H2O2-Cl- system. These observations strongly suggest that HOCl generated by the myeloperoxidase system of activated neutrophils oxidizes cathepsin G, promoting cathepsin G autolysis and release of the oxidized peptide NDI(M+16)LL.

**Activated neutrophils isolated from myeloperoxidase-deficient mice have higher cathepsin G activity than those from wild-type mice.** To further determine whether oxidants generated by myeloperoxidase might help regulate cathepsin G in vivo, we compared cathepsin G activity in two groups of neutrophils. The first group was harvested from bone marrow of wild-type and myeloperoxidase-deficient mice; these cells were expected to be relatively quiescent. The second group consisted of neutrophils recruited into the peritoneum of wild-type and myeloperoxidase-deficient mice with glycogen and then activated by challenging each group of animals with intraperitoneal K. pneumoniae. It is important to note that this latter approach should result in phagocytosis of bacteria together with activation of NADPH oxidase and secretion of myeloperoxidase and cathepsin G into the phagolysosome. After harvesting the neutrophils, we monitored the activity of cathepsin G in supernatants of sonicated cells, using the specific peptide substrate suc-AAPF-NA (36).
The resting neutrophils derived from bone marrow of wild-type and myeloperoxidase-deficient cells had almost identical cathepsin G activity (1.40 ± 0.05 vs. 1.47 ± 0.07 units/mg protein; n = 3) (Fig. 8). In contrast, cathepsin G activity was 50% lower in neutrophils activated in vivo and then isolated from wild-type mice as in those activated in myeloperoxidase-deficient mice (0.65 ± 0.04 vs. 1.29 ± 0.07 units/mg protein; n = 3; P < 0.01, Student’s T test). Western blotting with a specific antibody demonstrated that the levels of immunoreactive cathepsin G were comparable in resting or activated wild-type and myeloperoxidase-deficient neutrophils (data not shown). These observations suggest that myeloperoxidase modulates cathepsin G activity in activated neutrophils, perhaps by oxidatively inactivating the protease. One important oxidant is likely to be HOCl. However, myeloperoxidase generates both chlorinating and nitrating intermediates in vivo (47, 48), and it is possible that a variety of oxidants contribute to the inactivation of cathepsin G in our animal studies.

Oxidation of methionine residues directs the regiospecific cleavage of synthetic peptides that mimic the catalytic domain of cathepsin G. Detection of methionine sulfoxide in the peptide released from cathepsin G after HOCl exposure raised the possibility that oxidation generates new proteolytic sites in the enzyme. To explore this hypothesis, we exposed the synthetic peptide TIQNDIMLLQLSR to HOCl (1:1, mol/mol) for 30 min at 37°C in buffer A and then determined its susceptibility to cleavage by cathepsin G. HPLC analysis revealed a single major peak of new material. LC-ESI-MS and MS/MS analyses identified this product as TIQNDI(M+16)LLQLSR. In contrast, no oxidation products were observed after H2O2 treatment, even with a 20-fold mole excess of H2O2. Thus HOCl, but not H2O2, can convert the methionine in the synthetic peptide to a sulfoxide under our experimental conditions.

Oxidation of TIQNDIMLLQLSR depended on both HOCl concentration and the length of incubation (data not shown). The reaction of the peptide with HOCl reached completion in <1 min, indicating that exposing TIQNDIMLLQLSR to low concentrations of HOCl rapidly produced a high yield of TIQNDI(M+16)LLQLSR. In contrast, the yield of Met+16 in TIQNDIMLLQLSR decreased when the concentration of HOCl was high, as did the total amount of intact and oxidized peptides detectable in the reaction mixture. This decreased yield was perhaps due to HOCl-dependent stochastic cleavage of peptide bonds (45). We observed similar results with peptides derived from TIQNDIMLLQLSR of cathepsin G that had been exposed to high HOCl concentrations (Fig. 5).

To investigate the proteolysis of TIQNDIMLLQLSR by cathepsin G, we incubated the peptide with enzyme at 37°C in a physiological buffer at neutral pH (Fig. 9). HPLC analysis revealed three major products (Fig. 9B), which MS/MS analysis identified as TIQNDIM, TIQNDML, and TIQNDMLL. These results indicate that cathepsin G cleaves peptides and proteins at Leu-Gln, Leu-Leu, and Met-Leu, as has been shown previously (15).

To determine whether oxidation can generate additional cleavage sites in the peptide, we exposed TIQNDIMLLQLSR to HOCl (1:1, mol/mol), quenched the reaction with methionine, and exposed the mixture of native and oxidized peptides to cathepsin G. HPLC analysis of the reaction mixture revealed that TIQNDIM, TIQNDML, and TIQNDMLL were derived from the native peptide. In addition, we observed two major peaks and one minor peak of new material, which MS/MS analysis identified as TIQNDI(M+16)LLQ, TIQNDI(M+16)NL, and TIQNDI(M+16)LL (Fig. 9C). In contrast, no new peaks of material were observed when the peptide was exposed to a much higher concentration of H2O2 (20:1, mol/mol). These results show that TIQNDI(M+16)LLQLSR contains two of the cleavage sites that cathepsin G recognizes in the native peptide: Leu-Gln and Leu-Leu. However, the oxidized peptide was resistant to cleavage at (Met+16)-Leu. Importantly, it had a new cleavage site—at Gln-Asn (Fig. 9C and Fig. 10). Thus, when HOCl oxidizes the synthetic peptide TIQNDIMLLQLSR, it generates the same unique proteolysis site as when it oxidizes cathepsin G.

We used reverse-phase HPLC to quantify the influence of time on product yields when native and oxidized TIQNDIMLLQLSR were incubated with cathepsin G (Fig. 9). The oxidized peptide was degraded more rapidly than the native peptide, suggesting that conversion of Met to
Met+16 increases the peptide’s susceptibility to proteolysis (Fig. 9A). With native TIQNDIMLLQLSR, the major proteolytic product after short incubation periods was TIQNDIMLL (Fig. 9B). This peak rapidly declined as TIQNDIM appeared. The level of TIQNDI remained relatively constant during the incubation. These observations suggest that TIQNDIMLLQLSR is cleaved by cathepsin G at Leu-Gln to yield TIQNDIMLL, which in turn is cleaved at Leu-Leu and then Met-Leu to produce TIQNDIML and then TIQNDIM (Fig. 11).

The progress curve for proteolysis of oxidized TIQNDI(M+16)LLQLSR by cathepsin G was different (Fig. 9C) from that for the native peptide. As incubation time increased, the yield of all three cleaved oxidized peptides initially increased. The product yields were TIQNDI(M+16)LL > TIQNDI(M+16)L > NDI(M+16)LL. With prolonged incubation, the concentration of TIQNDI(M+16)LL gradually declined as the concentrations of NDI(M+16)LL and TIQNDI(M+16)L gradually increased. These observations suggest that cathepsin G can readily cleave the oxidized peptide at either Leu-Gln or Leu-Leu (Fig. 11). The oxidized peptide, but not TIQNDIMLQLSR, is also cleaved at Gln-Asn (Fig. 10 and Fig. 11). These observations indicate that cathepsin G can cleave only the oxidized peptide at Gln-Asp and that different residues in native and oxidized TIQNDIMLQLSRSs are susceptible to proteolytic cleavage by cathepsin G.

**DISCUSSION**

Cathepsin G plays a critical role in host defense, cytokine release, and receptor activation, but dysregulation of its proteolytic activity has been implicated in tissue injury (2-4, 8-10). Our observations indicate that one potential mechanism for limiting its activity involves HOCl, a specific product of myeloperoxidase. We found that isolated cathepsin G could be inactivated by HOCl but not H2O2. Moreover, when we isolated neutrophils from the peritonea of mice infected with K. pneumonia, cathepsin G activity was lower in wild-type neutrophils than in myeloperoxidase-deficient neutrophils, suggesting that oxidants derived from myeloperoxidase can regulate the activity of the protease in vivo. Tandem MS analyses of tryptic digests of cathepsin G exposed to HOCl demonstrated that enzyme inactivation associated strongly with loss of the peptide containing Met10. Importantly, the crystal structure of cathepsin G demonstrates that Met10 resides in close proximity to Asp108, a key component of the enzyme’s catalytic triad (11, 12). Moreover, loss of the peptide containing Met10 occurred in concert with the appearance of peptides containing (Met+16)10. Thus, loss of enzymatic activity apparently involved conversion of methionine to its sulfoxide in a single localized region of cathepsin G. Our observations may be physiologically relevant because we found that activated myeloperoxidase-deficient neutrophils had more cathepsin G activity than wild-type neutrophils during inflammation.

Oxidation of Met10 may contribute to cathepsin G inactivation by at least two distinct mechanisms. The first involves the introduction of unique proteolytic sites into the enzyme by alterations in local (and perhaps distant) structure. We found that low concentrations of HOCl promoted the degradation of cathepsin G, as assessed by the appearance of the peptide NDI(M+16)LL containing (Met+16)10. Importantly, this oxidized peptide also contained catalytic Asp108 (Fig. 12). Pretreatment with PMSF, a phosphonate that potently inhibits serine proteases, inhibited the appearance of this oxidized peptide. Moreover, we detected NDI(M+16)LL when cathepsin G was exposed to activated neutrophils, suggesting that HOCl generated by myeloperoxidase could play a physiological role in oxidizing the proteinase.

These findings suggest that oxidation of methionine residues generates proteolytic cleavage sites that permit cathepsin G to be inactivated. The production of NDI(M+16)LL was linearly dependent on the concentration of cathepsin G, indicating that oxidized cathepsin G autolytically digested itself, releasing the oxidized peptide. Oxidation of Met10 in concert with disruption of the charge relay system formed by Asp108-His64-Ser201 may provide a second mechanism for inactivating cathepsin G. The crystal structure of cathepsin G reveals that Met10 is in close proximity to catalytic Asp108 (11, 12). In turn, the latter residue is juxtaposed with His64, which serves as the general base that activates the hydroxyl group of Ser201 (Fig. 12). This raises the possibility that introduction of a sulfoxide into the tightly packed local environment of Asp108, His64,
and Ser\textsuperscript{201} disrupts the charge relay system that is critical for proteolysis by cathepsin G (Fig. 12). It is worth noting that oxidation of a single methionine residue also inactivates subtilisin, a bacterial serine protease (49, 50). This family of proteinases uses the Asp-His-Ser catalytic triad, although both the primary sequence and the three dimensional structure of subtilisin are completely different from that of cathepsin G (11, 49). Thus, oxidation of a specific methionine residue may disrupt the tightly packed environment of the charge relay system in both subtilisin and cathepsin G.

Model system studies indicate that, of all the amino acid side chains, the thiol and thioether groups of cysteine and methionine react most rapidly with HOCl ($K_2 = 3 \times 10^7$ M\textsuperscript{-1}s\textsuperscript{-1} and $4 \times 10^7$ M\textsuperscript{-1}s\textsuperscript{-1}, respectively; ref. 42, 44). Therefore, these two residues might be the first to be targeted when HOCl oxidizes proteins. Consistent with this proposal, our study of a synthetic peptide, TIQNDIMLLQLSR, which mimics the tryptic digest peptide from cathepsin G that contains Asp\textsuperscript{108} and Met\textsuperscript{110}, demonstrated that HOCl rapidly converts the methionine residue to the sulfoxide in near quantitative yield. In contrast, thiols react slowly with H\textsubscript{2}O\textsubscript{2} ($K_2 < 5 \times 10^1$ M\textsuperscript{-1}s\textsuperscript{-1}; ref. 43), and peroxide did not oxidize TIQNDIMLLQLSR under our experimental conditions. When this peptide was oxidized with HOCl and then incubated with cathepsin G, it was converted to NDI(M+16)LL, the same peptide that appears after HOCl oxidizes intact cathepsin G. Thus, alteration of the same residue in the peptide and the oxidized protein may confer susceptibility to cleavage by cathepsin G. Collectively, these observations indicate that oxidation of methionine residues can direct the regiospecific cleavage of peptides and proteins by cathepsin G. These findings may be of broader significance because we recently showed that neutrophil elastase, another serine protease that is abundant in neutrophils, also degrades itself as it is oxidatively inactivated by HOCl (51).

Based on these observations, we propose the following model for the oxidative regulation of cathepsin G’s proteolytic activity. When the enzyme is exposed to low concentrations of HOCl, Met\textsuperscript{110} is oxidized to methionine sulfoxide, which disrupts the interactions of the catalytic triad and generates unique autolytic cleavage sites in the intact protein at Gln\textsuperscript{106}-Asn\textsuperscript{107} and Leu\textsuperscript{112}-Gln\textsuperscript{113}. Proteolytic cleavage of this region releases NDI(M+16)LL, which contains Asp\textsuperscript{108}. Other methionine residues may also be oxidized, and these oxidations may also promote autolysis and loss of proteolytic activity. When cathepsin G is exposed to high concentrations of HOCl, however, other oxidative processes, including random fragmentation of the peptide backbone, degrade the protein (45, 46).

Many lines of evidence indicate that activated phagocytic cells inflict oxidative tissue injury in humans (1, 5, 20, 23). However, the results of clinical trials of antioxidants in the prevention of human disease have generally been disappointing (52). This observation raises the possibility that oxidants such as HOCl are also involved in suppressing inflammation. Our demonstration that HOCl restrains the proteolytic activity of cathepsin G may have important implications for understanding the role of reactive intermediates in limiting tissue damage. Indeed, we have recently shown that HOCl inactivates matrilysin (MMP-7) by oxidatively cross-linking adjacent tryptophan and glycine residues in the catalytic domain of the enzyme (39, 40). This inactivation mechanism is distinct from the well-studied mechanisms involving tissue inhibitors of metalloproteinases. Our findings suggest that local, pericellular production of HOCl by phagocytes is a physiological mechanism for governing proteinase activity during inflammation. The failure of antioxidants to prevent human inflammatory diseases in clinical trials may in part reflect a beneficial regulatory effect of oxidants on proteolytic activity in inflamed tissue.

In conclusion, our studies establish a potential role for HOCl as a regulator of the proteolytic activity of cathepsin G, a serine protease implicated in host defense, cytokine release and tissue injury. The underlying mechanism involves the oxidation of a specific methionine residue, which in turn may disrupt the catalytic charge relay system and introduce proteolytic cleavage sites into the enzyme. This complex interplay between the oxidative and proteolytic systems of neutrophils raises the possibility that oxidants protect hosts from protease-mediated tissue degradation.
REFERENCES

1. Klebanoff, S. J. (1980) *Ann. Intern. Med.* 93, 480-489
2. Travis, J. (1988) *Am. J. Med.* 84, 37-42
3. Belaaouaj, A., McCarthy, R., Baumann, M., Gao, Z., Ley, T. J., Abraham, S. N., and Shapiro, S. D. (1998) *Nat. Med.* 4, 615-618
4. Reeves, E. P., Lu, H., Jacobs, H. L., Messina, C. G. M., Bolsover, S., Gabella, G., Potma, E. O., Warley, A., Roes, J., and Segal, A. W. (2002) *Nature* 416, 291-297
5. Weiss, S. J. (1989) *N. Engl. J. Med.* 320, 365-376
6. Travis, J. and Salvensen, G. S. (1983) *Annu. Rev. Biochem.* 52, 655-709
7. Campbell, E. J., Campbell, M. A., Boukedes, S. S., and Owen, C. A. (1999) *J. Clin. Invest.* 104, 337-344
8. Turkington, P. T. (1992) *Thromb. Res.* 67, 147-155
9. Molino, M., Blanchard, N., Belmonte, E., Tarver, A. P., Abrams, C., Hoxie, J. A., Cerletti C., and Brass, L. F. (1995) *J. Biol. Chem.* 270, 11168-11175
10. Adkison, A. M., Raptis, S. Z., Kelley, D. G., and Pham, C. T. (2002) *Nature* 416, 291-297
11. Hof, P., Mayr, I., Huber, R., Korzus, E., Potempa, J., Travis, J., Powers, J. C., and Bode, W. (1996) *EMBO J.* 15, 5481-5491
12. Czapinska, H. and Otlewski, J. (1999) *Eur. J. Biochem.* 260, 571-595
13. Schechter, I. and Berger, A. (1968) *Biochem. Biophys. Res. Commun.* 32, 898-902
14. Maison, C. M., Villiers, C. L., and Colomb, M. G. (1991) *J. Immunol.* 147, 921-926
15. Tanaka, T., Minematsu, Y., Reilly, C. F., Travis, J., and Powers, J. C. (1985) *Biochemistry* 24, 2040-2047
16. Bank, U., Kupper, B., Reinhold, D., Hoffmann, T., and Ansorge, S. (1999) *FEBS Letters* 461, 235-240
17. Babior, B. M., Kipnes R. S., and Curnutte, J. T. (1973) *J. Clin. Invest.* 52, 741-744
18. Klebanoff, S. J. (1975) *Semin. Hematol.* 12: 117-142
19. Harrison, J. E. and J. Schultz, J. (1976) *J. Biol. Chem.* 251, 1371-1374
20. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) *Blood* 92, 3007-3017
21. Gaut, J. P., Yeh, G. C., Tran, H. D., Byun, J., Henderson, J. P., Richter, G. M., Brennan, M. L., Lusis, A. J., Belaaouaj, A., Hotchkiss, R. S., and Heinecke, J. W. (2001) *Proc. Natl. Acad. Sci. USA* 98, 11961-11966
22. Aratani, Y., Koyama, H., Nyui, S., Suzuki, K., Kura, F., and Maeda, N. (1999) *Infect. Immun.* 67, 1828-1836
23. Heinecke, J. W. (1999) *J. Lab. Clin. Med.* 133, 321-325
24. Hazen, S. L. and Heinecke, J. W. (1997) *J. Clin. Invest.* 99, 2075-2081
25. Heller, J. I., Crowley, J. R., Hazen, S. L., Salvay, D. M., Wagner, P., Pennathur, S., and Heinecke, J. W. (2000) *J. Biol. Chem.* 275, 9957-9962
26. Henderson, J. P., Byun, J., Takeshita, J., and Heinecke, J. W. (2003) *J. Biol. Chem.* 278, 23522-23528
27. Leeuwenburgh, C., Rasmussen, J. E., Hsu, F. F., Mueller, D. M., Pennathur, S., and Heinecke, J. W. (1997) *J. Biol. Chem.* 272, 3520-3526
28. Owen, C. A., Campbell, M. A., Sannes, P. L., Boukedes, S. S., Campbell, E. J. (1995) *J Cell Biol.* 131:775-789
29. Voetman, A. A., Weening, R. S., Hamers, M. N., Meershof, L. J., Bot, A. A., Roos, D. (1981) *J. Clin. Invest.* 67, 1541-1549
30. Kobayashi, M., Tanaka, T., Usui, T. (1982) *J. Lab. Clin. Med.* 100, 896-907
31. Clark, R. A., Borregaard, N. (1985) *Blood* 65, 375-381
32. Morris, J. C. (1966) *J. Phys. Chem.* 70, 3798-3805
33. Nelson, D. P. and Kiesow, L. A. (1972) *Anal. Biochem.* 49, 474-478
34. Heinecke, J. W., Li, W., Francis, G. A., and Goldstein, J. A. (1993) *J. Clin. Invest.* 91, 2866-2872
35. Heinecke, J. W., Baker, L., Rosen, H., and Chait, A. (1986) *J. Clin. Invest.* 77, 757-761
36. DelMar, E. G., Largman, C., Brodick, J. W., and Geokas, M. C. (1979) *Anal. Biochem.* 99, 316-320
37. MacIvor, D. M., Shapiro, S. D., Pham, C. T., Belaaouaj, A., Abraham, S. N., and Ley, T. J. (1999) *Blood* **94**, 4282-4293

38. Brennan, M. L., Anderson, M. M., Shih, D. M., Qu, X. D., Wang, X. P., Mehta, A. C., Lim, L. L., Shi, W. B., Hazen, S. L., Jacob, J. S., Crowley, J. R., Heinecke, J. W., and Lusis, A. J. (2001) *J. Clin. Invest.* **107**, 419-430

39. Fu, X., Kassim, S. Y., Parks, W. C., and Heinecke, J. W. (2001) *J. Biol. Chem.* **276**, 41279-41287

40. Fu, X., Kao, J. L., Berg, C., Kassim, S. Y., Huq, N. P., d’Avigny, A., Parks, W. C., Mecham, R. P., and Heinecke, J. W. (2004) *J. Biol. Chem.* **279**, 6209-6212

41. Fu, X., Kassim, S. Y., Parks, W. C., and Heinecke, J. W. (2003) *J. Biol. Chem.* **278**, 28403-28409

42. Pattison, D. I., and Davies M. J. (2001) *Chem. Res. Toxicol.* **14**, 1453-1464

43. Winterbourn, C. C., Metodiewa, D. (1999) *Free Radical Bio. Med.* **27**, 322-328.

44. Winterbourn, C. C. (1985) *Biochim Biophys Acta.* **840**, 204-210.

45. Hawkins, C. L. and Davies, M. J. (1998) *Biochem. J.* **332**, 617-625

46. Hazell, L. J., van den Berg, J. J., and Stocker, R. (1994) *Biochem. J.* **302** (Pt 1), 297-304

47. Hazen S. L., Hsu, F. F., Crowley, J. R., Mueller, D. M., Heinecke, J. W. (1996) *J. Clin. Invest.* **98**, 1283-1289

48. Gaut, J. P., Byun, J., Tran, H. D., Lauber, W. M., Carroll, J. A., Hotchkiss, R. S., Belaaouaj, A., and Heinecke, J. W. (2002) *J. Clin. Invest.* **109**, 1311-1319

49. Bott, R., Ultsch, M., Kossiakoff, A., Graycar, T., Katz, B., and Power, S. (1988) *J. Biol. Chem.* **263**, 7895-906

50. DePaz, R.A., Barnett, C. C., Dale, D. A., Carpenter, J. F., Gaertner, A. L., and Randolph, T. W. (2000) *Arch. Biochem. Biophys.* **384**, 123-132

51. Hirche, T. O., Gaut, J. P., Heinecke, J. W., and Belaaouaj, A. (2005) *J. Immunol.* **174**, 1557-1565

52. Vivekananthan, D. P., Penn, M. S., Sapp, S. K., Hsu, A., and Topol, E. J. (2003) *Lancet* **361**, 2017-2023

**ACKNOWLEDGEMENTS**

This work was supported by grants from the National Institutes of Health (AG021191, DK02456, P50 HL073996, P01 HL030086, P01 HL60886, HL030086, HL075381), the National Institute of Environmental Health Sciences (P30 ES007033), and the Donald W. Reynolds Foundation.

The abbreviations used are: ESI, electrospray ionization; HPLC, high performance liquid chromatography analysis; HOCl, hypochlorous acid; H$_2$O$_2$, hydrogen peroxide; LC, liquid chromatography; MS, mass spectrometry; m/z, mass to charge ratio; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; RIC, reconstructed ion chromatogram; S, protease subsite; S$_1$, primary specificity pocket; P, peptide substrate subsite; suc-AAPF-NA, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide.
FIGURE LEGENDS

Figure 1. Proteolytic activity of cathepsin G exposed to HOCl, H₂O₂ or the myeloperoxidase-H₂O₂-Cl⁻ system. (A) Cathepsin G (1 µM) was incubated with HOCl, H₂O₂ or myeloperoxidase (MPO)-H₂O₂-Cl⁻ at the indicated mole ratio of oxidant to protein. (B) Cathepsin G (1 µM or 17 µM) was incubated with HOCl at the indicated mole ratio. (C) The indicated concentration of cathepsin G was incubated with HOCl (15:1, mol/mol). All reactions were carried out for 30 min at 37°C in buffer A (10 mM phosphate buffered saline, pH 7.4). Reactions were initiated by adding oxidant (HOCl or H₂O₂) and terminated by adding a 10-fold mole excess (relative to oxidant) of L-methionine. Control experiments demonstrated that L-methionine did not affect enzyme activity. The activity of cathepsin G was assessed using suc-AAPF-NA as the substrate. Results represent the means and standard deviations of triplicate determinations. Similar results were observed in 3 independent experiments.

Figure 2. Proteolytic activity (A) and abundance of peptide TIQNDIMLLQLSR (B) of cathepsin G exposed to HOCl or H₂O₂. Cathepsin G (17 µM) was incubated with HOCl or H₂O₂ at the indicated mole ratio and its enzymatic activity was assessed as described in the legend to Fig. 1. (A) Enzymatic activity of cathepsin G. (B) TIQNDIMLLQLSR in a tryptic digest of reduced, alkylated cathepsin G was quantified by LC-ESI-MS. Results represent the means of duplicate determinations from three independent experiments.

Figure 3. LC-ESI-MS analysis of cathepsin G exposed to HOCl. Cathepsin G was incubated for 30 min at 37°C in buffer A alone or buffer A supplemented with HOCl (15:1, mol/mol, oxidant/protein). Reactions were initiated by adding oxidant and terminated by adding a 10-fold mole excess of L-methionine. Cathepsin G was then reduced, alkylated and digested with trypsin, and the tryptic digest was analyzed with LC-ESI-MS. Reconstructed ion chromatograms (RIC) of tryptic products of (A) native cathepsin G and (B) HOCl-exposed cathepsin G. Peak 1, ions of m/z 1544.7 and m/z 773.1; peak 2, ions of m/z 734.2; peak 3, ions of m/z 1560.9 and m/z 781.1; peak 4, ions of m/z 1076.4 and m/z 538.8.

Figure 4. MS and MS/MS analysis of cathepsin G exposed to HOCl. Cathepsin G was incubated in buffer A alone or buffer A supplemented with HOCl (15:1, mol/mol, oxidant/protein), digested with trypsin, and analyzed by LC-ESI-MS as described in the legend to Fig. 2. (A) MS and MS/MS analysis of the ion of m/z 773.1 (peak 1). (B) MS and MS/MS analysis of the ion of m/z 781.1 (peak 2). (C) MS and MS/MS analysis of the ion of m/z 1076.4 (peak 4). (D) MS and MS/MS analysis of the ion of m/z 734.2 (peak 2). The ions of m/z 773.1 and 781.1 are doubly charged, and the ions of m/z 734.2 and 1076.4 are singly charged. Note that the mass of the methionine residue in three of the peptides (peaks 3, 4 and 2; m/z 781.1, 1076.4, and 734.2, respectively) has increased by 16 amu (plus one oxygen). Moreover, two of these peptides are cleaved at sites that are not recognized by trypsin (Leu-Gln, TIQNDI(M+16)LL, m/z 1076.4; Gln-Asn, NDI(M+16)LL, m/z 734.2).

Figure 5. Quantification of peptide TIQNDIMLLQLSR and its oxidized products in a tryptic digest of cathepsin G exposed to HOCl. Cathepsin G was incubated with HOCl at the indicated mole ratio for 30 min at 37°C in buffer A. Reactions were initiated by adding oxidant and terminated by adding L-methionine. The enzymatic activity of native or oxidized cathepsin G was assessed using suc-AAPF-NA as the substrate (Fig. 2A). In parallel studies, cathepsin G was reduced, alkylated and digested with trypsin, and the tryptic digest was analyzed with LC-ESI-MS. The ion currents of peptides of m/z 773.1 (TIQNDI(M+16)LLQLSR), m/z 781.1 (TIQNDI(M+16)LLQLSLR), m/z 1076.4 (TIQNDI(M+16)LL), and m/z 734.2 (NDI(M+16)LL) were monitored by LC-ESI-MS. Peptides were quantified relative to the ion current of the precursor peptide TIQNDI(M+16)LLQLSR of control cathepsin G incubated in buffer A alone.
Figure 6. Detection of autolytically released peptide NDI(M+16)LL in cathepsin G exposed to HOCl. Native (B) or PMSF-treated (C) cathepsin G was exposed to HOCl (15:1, mol/mol, oxidant/protein) for 60 min at 37°C in buffer A. In parallel studies, cathepsin G alone was incubated in buffer A (A). The reaction mixture was then analyzed directly by LC-ESI-MS with selected reaction monitoring of ions of m/z 734.2. Note that under these conditions only peptides derived from the proteolytic activity of cathepsin G itself will be detected. (D) HOCl-dependence for relative abundance of peptide NDI(M+16)LL in cathepsin G exposed to HOCl.

Figure 7. Detection of autolytically released peptide NDI(M+16)LL in cathepsin G exposed to activated neutrophil. Cathepsin G (1 μM) was exposed to neutrophil (PMN, 10^6 cells/ml) for 60 min at 37°C in buffer B (Hank’s balanced salt solution, pH 7.4). Where indicated, 200 nM PMA, 5 mM methionine, 50 μg/ml catalase, and/or 5 mM azide was included in the buffer. At the end of the incubation, cells were pelleted by centrifugation, and the supernatant was concentrated and digested by trypsin and then subjected to analysis by LC-ESI-MS and MS/MS. (A) Detection of NDI(M+16)LL using RIC (ions of m/z 734.2). (B) MS/MS analysis of NDI(M+16)LL. (C) Product yield of NDI(M+16)LL.

Figure 8. Cathepsin G activity in resting and activated neutrophils isolated from wild type and myeloperoxidase-deficient mice. Resting neutrophils were isolated from the bone marrow of myeloperoxidase-deficient and wild type mice (37). To prepare activated cells, neutrophils were recruited into the peritoneum of mice using glycogen. After 4 h, the mice were infected intraperitoneally with K. pneumoniae (4 x 10^8 CFUs), and the cells were isolated 1 h latter (37). Cathepsin G activity in cell lysates were assessed using suc-AAPF-NA as the substrate. Results represent the means and SDs of three independent experiments.

Figure 9. Proteolysis of native or HOCl-exposed peptide TIQNDIMLLQLSR by cathepsin G. TIQNDIMLLQLSR (400 μM) was incubated for 30 min at 37°C in buffer A alone (B) or buffer A supplemented with HOCl (1:1 mole ratio) (C). Following the addition of L-methionine, native or oxidized peptide (400 μM) was incubated with cathepsin G (2 μM) for 60 min at 37°C in buffer A. (A) Consumption of native or oxidized peptide by cathepsin G proteolysis; (B) Product yields of cleavage products from cathepsin G proteolysis of native peptide; (C) Product yields of cleavage products from cathepsin G proteolysis of oxidized peptide. The reaction mixture was analyzed by reverse-phase HPLC. Peaks of material were identified by LC-ESI-MS and MS/MS analysis.

Figure 10. Detection of NDI(M+16)LL in HOCl-exposed peptide incubated with cathepsin G. TIQNDIMLLQLSR (400 μM) was incubated for 30 min at 37°C in (A,B) buffer A alone or (C,D) buffer A supplemented with HOCl (1:1 mole ratio). Following the addition of L-methionine, native or oxidized peptide (400 μM) was incubated (A,C) alone or (B,D) with cathepsin G (2 μM) for 60 min at 37°C in buffer A. The reaction mixture was then analyzed directly by LC-ESI-MS/MS with selected reaction monitoring of ions of m/z 718.2 (NDIMLL) or m/z 734.2 (NDI(M+16)LL).

Figure 11. Proposed reaction pathways for cleavage of native and oxidized TIQNDIMLLQLSR by cathepsin G. Model system studies (Fig. 9 and Fig. 10) indicate that the oxidized peptide, but not the native peptide, is cleaved at Gln-Asn by cathepsin G. The same cleavage site is apparent in cathepsin G exposed to HOCl. These observations indicate that different residues in native and oxidized TIQNDIMLLQLSR are susceptible to cleavage by cathepsin G. They also suggest that oxidation of methionine residues generates proteolytic cleavage sites that permit cathepsin G to be inactivated.
Figure 12. Met$^{110}$ and the catalytic triad of cathepsin G. Met$^{110}$ lies next to Asp$^{108}$ in the crystal structure of cathepsin G (11). The relative positions of the residues in the catalytic triad (Asp$^{108}$-His$^{64}$-Ser$^{201}$; green) must be maintained for the enzyme to retain proteolytic activity. Thus, converting the methionine residue to a sulfoxide may disturb the tightly packed local environment of Asp$^{108}$, His$^{64}$, and Ser$^{201}$, facilitating autolysis of NDI(M+16)LL (yellow) and disrupting the enzyme’s charge relay system.

Table I. Detection of modified peptides in cathepsin G exposed to HOCl.

| No. | Position | Sequence of fragment | Predicted $m/z$ ([M+H]$^+$) | Observed $m/z$ (charge state) | Modification |
|-----|----------|----------------------|-----------------------------|-----------------------------|--------------|
| 1   | 35-48    | *MAYLQIQPAGQSR       | 1549.8                      | 783.5 (+2), 1565.7 (+1)     | Met$^{15}$+16 |
| 2   | 104-116  | TIQNDIMLLQLSR        | 1544.8                      | 781.2 (+2), 1561.5 (+1)     | Met$^{110}$+16 |
| 3   | 104-116  | TIQNDIMLLQLSR        | 1544.8                      | 789.1 (+2), 1576.6 (+1)     | Met$^{110}$+32 |
| 4   | 104-112  | TIQNDIMLL*           | 1060.6                      | 538.9 (+2), 1076.5 (+1)     | Met$^{110}$+16 |
| 5   | 107-112  | *NDIMLL*             | 718.4                       | 367.6 (+2), 734.3 (+1)      | Met$^{110}$+16 |
| 6   | 132-149  | AQEGLRPGLCTVAGWGR    | 1929.0                      | 649.2 (+3), 973.3 (+2)      | Trp$^{147}$+16 |
| 7   | 132-149  | AQEGLRPGLCTVAGWGR    | 1929.0                      | 643.4 (+3), 964.5 (+2)      | M-2          |
| 8   | 132-149  | AQEGLRPGLCTVAGWGR    | 1929.0                      | 642.6 (+3), 963.4 (+2)      | M-4          |

* Peptide cleavage at a P$_1$ site not recognized by trypsin (Lys, Arg).
Figure 1

A. Variable Oxidant/Cath G Ratio

B. Variable HOCl/Cath G Ratio

C. Fixed HOCl/Cath G Ratio
Figure 2

A. Proteolytic Activity

B. Peptide TIQNDIMLLQLSR

Activity (% Control)

Peptide (% Control)

Oxidant/Cath G (mol/mol)
Figure 3

A. Cath G (control)

Peak 1 (m/z 1544.7)

B. HOCl-exposed Cath G

Peak 1 (m/z 1544.7)

Peak 3 (m/z 1560.9)

Peak 2 (m/z 734.2)

Peak 4 (m/z 1076.4)
Figure 5

A. TIQNDI(M+16)LLQLSR (m/z 1560.9)

B. TIQNDI(M+16)LL (m/z 1076.5)

C. NDI(M+16)LL (m/z 734.3)
Figure 6

A. Cath G (control)

B. Cath G + HOCl

C. Cath G + PMSF + HOCl

D. NDI(M+16)LL

Relative Abundance

Minutes

Relative Abundance

HOCI/Cath G (mol/mol)
Figure 8

![Bar chart showing Cath G Activity (units/mg) for WT and MPO -/- mice in Resting and Activated states.](image-url)
Figure 10

A. Peptide
m/z 718.2
NDIMLL

B. Peptide + Cath G
m/z 718.2
NDIMLL

C. Peptide + HOCl
m/z 734.2
NDI(M+16)LL

D. Peptide + HOCl + Cath G
m/z 734.2
NDI(M+16)LL

Relative Abundance (%)

Minutes

100

0

100

0

100

0

20

40
Methionine sulfoxide and proteolytic cleavage contribute to the inactivation of cathepsin G by hypochlorous acid: An oxidative mechanism for regulation of serine proteinases by myeloperoxidase

Baohai Shao, Abderrazzaq Belaaouaj, Christophe L. M. J. Verlinde, Xiaoyun Fu and Jay W. Heinecke

*J. Biol. Chem.* published online June 20, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M504040200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts