Generation of Intramolecular and Intermolecular Sulfenamides, Sulfinamides, and Sulfonamides by Hypochlorous Acid: A Potential Pathway for Oxidative Cross-Linking of Low-Density Lipoprotein by Myeloperoxidase†

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ABSTRACT: Oxidized low-density lipoprotein (LDL) is implicated in atherogenesis, and human atherosclerotic lesions contain LDL oxidized by myeloperoxidase, a heme protein secreted by activated phagocytes. Using hydrogen peroxide (H_2O_2), myeloperoxidase generates hypochlorous acid (HOCl), a powerful oxidant. We now demonstrate that HOCl produces sulfenamides, sulfinamides, and sulfonamides in model peptides, which suggests a potential mechanism for LDL oxidation and cross-linking. When we exposed the synthetic peptide PFKCG to HOCl, the peptide's thiol residue reacted rapidly, generating a near-quantitative yield of products. Tandem mass spectrometric analysis identified the products as the sulfenamide, sulfinamide, and sulfonamide, all formed by intramolecular cross-linking of the peptide's thiol and lysine residues. An intramolecular sulfinamide was also observed after the peptide PFRCG was exposed to HOCl, indicating that the guanidine group of arginine can also form a sulfur−nitrogen cross-link. The synthetic peptide PFVCG, which contains a free thiol residue but lacks nucleophilic amino acid side chains, formed an intermolecular sulfonamide when exposed to HOCl. Tandem mass spectrometric analysis of the dimer revealed that the free N-terminal amino group of one PFVCG molecule cross-linked with the thiol residue of another. This peptide also formed intermolecular sulfonamide cross-links with N^{α}-acetyllysine after exposure to HOCl, demonstrating that the ϵ-amino group of a lysine residue can undergo a similar reaction. Moreover, human neutrophils used the myeloperoxidase−H_2O_2 system to generate sulfinamides in model peptides containing lysine or arginine residues. Collectively, our observations raise the possibility that HOCl generated by myeloperoxidase contributes to intramolecular and intermolecular protein cross-linking in the artery wall. Myeloperoxidase might also use this mechanism to form sulfur−nitrogen cross-links in other inflammatory conditions.

Phagocytic white blood cells use the heme enzyme myeloperoxidase to kill invading pathogens (*1*−*4*). Myeloperoxidase reacts with hydrogen peroxide (H_2O_2)[1] to form a ferryl π-cation radical complex, which is reduced back to the native state when it oxidizes halides and other substrates (*5*, *6*). The best-characterized product of the myeloperoxidase−H_2O_2−chloride system is hypochlorous acid (HOCl), a potent cyotoxin (eq 1). The critical role of

$$Cl^- + H_2O_2 + H^+ \rightarrow HOCl + H_2O \qquad (1)$$

myeloperoxidase in host defense mechanisms is indicated by the finding that mice deficient in myeloperoxidase exhibit increased mortality when subjected to fungal (*7*) and bacterial infection (*8*).

Reactive intermediates produced by phagocytes might also injure normal tissue under pathological conditions. For example, they might generate oxidized low-density lipoprotein (LDL), which is thought to play a pivotal role in atherogenesis (*9*). Recent studies demonstrate that active myeloperoxidase is a component of human atherosclerotic lesions (*10*). Moreover, LDL isolated from such lesions contains high levels of 3-chlorotyrosine (*11*) and *p*-hydroxyphenylethanolamine (*12*), specific protein and lipid oxidation products of HOCl. Because myeloperoxidase is the only enzyme known to generate HOCl in humans, this observation strongly implies that it contributes to oxidative damage in the artery wall.

HOCl cross-links proteins in vitro (*13*, *14*), raising the possibility that it might similarly damage biomolecules in the artery wall. One target might be LDL because aggregated

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[1] Abbreviations: H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; LC, liquid chromatography; LDL, low-density lipoprotein; MPO, myeloperoxidase; MS, mass spectrometry; MS/MS, tandem mass spectrometry; *m*/*z*, mass to charge ratio; TIC, total ion chromatogram; RIC, reconstructed ion chromatogram; RSNHR, sulfenamide; RSONHR, sulfinamide; RSO_2NHR, sulfonamide; S−N, sulfur−nitrogen.

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lipoproteins promote macrophage foam cell formation, a key early event in atherogenesis (*15*−*18*). The molecular mechanisms of aggregation are poorly understood, but one potential pathway involves oxidative protein cross-linking (*19*−*21*). LDL exposed to HOCl in vitro loses reactive amino groups and undergoes aggregation and cross-linking (*22*, *23*). LDL aggregated by HOCl also rapidly converts cultured macrophages into lipid-laden foam cells (*22*, *23*). LDL isolated from human atherosclerotic lesions is aggregated, exhibits protein cross-links, and promotes the unregulated accumulation of lipid by macrophages, suggesting that lipoprotein aggregation could represent one pathway for foam cell formation in vivo (*24*, *25*).

HOCl reacts rapidly and nearly quantitatively with thiols, thioethers, and amino groups (*26*−*28*), suggesting that myeloperoxidase might target these moieties. Recent studies demonstrate that HOCl also oxidizes the thiol residue of glutathione in cultured endothelial cells (*29*). One of the major oxidation products has no reactive amino group, and its molecular mass is 30 amu greater than that of the parent molecule. On the basis of these observations, Winterbourn and colleagues (*29*, *30*) have proposed that HOCl oxidizes the thiol group of glutathione (RSH) to an intramolecular sulfonamide (RSO_2NHR) (eq 2).

$$3HOCl + RSH + RNH_2 \rightarrow RSO_2NHR + H_2O + 3HCl \quad (2)$$

In the current studies, we used synthetic peptides and tandem mass spectrometric analysis to investigate this proposed reaction pathway. Our observations indicate that HOCl generates intramolecular sulfenamides, sulfinamides, and sulfonamides and intermolecular sulfonamides from protein thiol and amino groups and that the guanidine group of arginine can also form sulfinamide cross-links. A major product of the myeloperoxidase system and activated human neutrophils is the sulfinamide. Our observations suggest a potential mechanism for generating oxidative cross-links in LDL and other lipoproteins and proteins in atherosclerosis and other inflammatory diseases (*10*−*14*, *19*−*25*).

EXPERIMENTAL PROCEDURES

Materials. Sodium hypochlorite (NaOCl), H_2O_2, and HPLC-grade CH_3CN were obtained from Fisher Scientific (Pittsburgh, PA). The Protein Nucleic Acid Chemistry Laboratory (Washington University) provided peptides. Myeloperoxidase (A_{430}/A_{280} ratio >0.8) was purified from HL-60 cells by sequential lectin-affinity, ion-exchange, and size-exclusion chromatography as described previously (*31*, *32*). Myeloperoxidase concentration was determined spectrophotometrically ($\epsilon_{430} = 0.18\ M^{-1}\ cm^{-1}$) (*33*). Unless otherwise indicated, all other materials were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of Human Neutrophils. Neutrophils were isolated from EDTA-anticoagulated blood by buoyant density centrifugation using Polymorph-Prep (Nycomed, Sunnyvale, CA) (*31*). Neutrophils were washed twice by centrifugation with buffer A [Hank's balanced salt solution, pH 7.4; magnesium, calcium, phenol, and bicarbonate free (Life Technologies, Inc.); 100 μM diethylenetriaminepentaacetic acid was included to inhibit metal-catalyzed reactions (*34*)]. Residual red blood cells were removed by hypotonic lysis at 4 °C. Neutrophils were pelleted by centrifugation, resuspended in buffer A, and immediately used for experiments. Neutrophils (7×10^5 cells/mL) were activated with 200 nM phorbol myristate acetate. Reactions were terminated by pelleting cells by centrifugation.

Oxidation Reactions. Reactions were carried out for 30 min at 37 °C in buffer A (pH 7.4) containing 100 μM peptide. They were initiated by addition of oxidant (or neutrophils) and terminated by addition of a 5−10-fold molar excess (relative to oxidant) of L-methionine. Concentrations of HOCl and H_2O_2 were determined spectrophotometrically ($\epsilon_{292} = 350\ M^{-1}\ cm^{-1}$ and $\epsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$, respectively) (*35*, *36*).

HPLC Analysis of Peptides. Peptides were separated at a flow rate of 1 mL/min on a reverse-phase column (Ultrasphere, C18, 5 μm, 4.6 × 250 mm; Beckman) using a Beckman HPLC system (Fullerton, CA), using solvent A (0.2% formic acid in H_2O) and solvent B (0.2% formic acid in 20% H_2O and 80% CH_3CN) and UV detection at 258 nm. The peptides were eluted using a linear gradient of 10%−35% solvent B over 18 min, followed by isocratic separation using 35% solvent B over 5 min.

Liquid Chromatography (LC)−*Electrospray Ionization Mass Spectrometry (MS)*. Electrospray ionization MS analyses were performed in the positive ion mode with a Finnigan Mat LCQ ion trap mass spectrometer (San Jose, CA) coupled to a Waters 2690 HPLC system (Milford, MA). Peptides were separated at a flow rate of 0.2 mL/min on a reverse-phase column (Ultrasphere C18, 5 μm, 2.0 × 250 mm; Beckman), using solvents A and B. Peptides were eluted using a linear gradient of 10%−40% solvent B over 20 min. The electrospray needle was held at 4500 V. Nitrogen, the sheath gas, was set at 80 units. The collision gas was helium. The collision energy for MS/MS was 25%−30%. The temperature of the heated capillary was 220 °C.

RESULTS

HOCl Converts the Cysteine and Lysine Residues of PFKCG to an Intramolecular Sulfenamide, Sulfinamide, and Sulfonamide. To investigate potential mechanisms for oxidative protein cross-linking by myeloperoxidase, we used the synthetic peptide PFKCG, which contains adjacent cysteine and lysine residues. The peptide was exposed to HOCl (2:1 mol/mol) for 30 min at 37 °C in buffer (pH 7.4) containing plasma concentrations (110 mM) of chloride. Analysis of the reaction mixture by LC with monitoring of the total ion chromatogram (Figure 1A; TIC) revealed four major peaks of reaction products (peaks 1−4). LC−MS analysis of the unmodified peptide detected major ions at *m*/*z* 551.3 and 276.3 (Figure 1B1), the predicted molecular masses of the singly and doubly charged peptides ($[peptide + H]^{1+}$ and $[peptide + 2H]^{2+}$).

The sequence of the unmodified peptide was confirmed using MS/MS and MS analysis, which selects a precursor ion for low-energy gas-phase collisional activation and subjects the resulting product ions to MS. Under these conditions, peptide ions fragment primarily at the amide bond, yielding a ladder of sequence ions. Retention of charge on the N-terminal portion of the peptide ion yields a b-type ion; retention on the C-terminal portion of the peptide ion yields a y-type ion. Subtracting the masses of adjacent ions

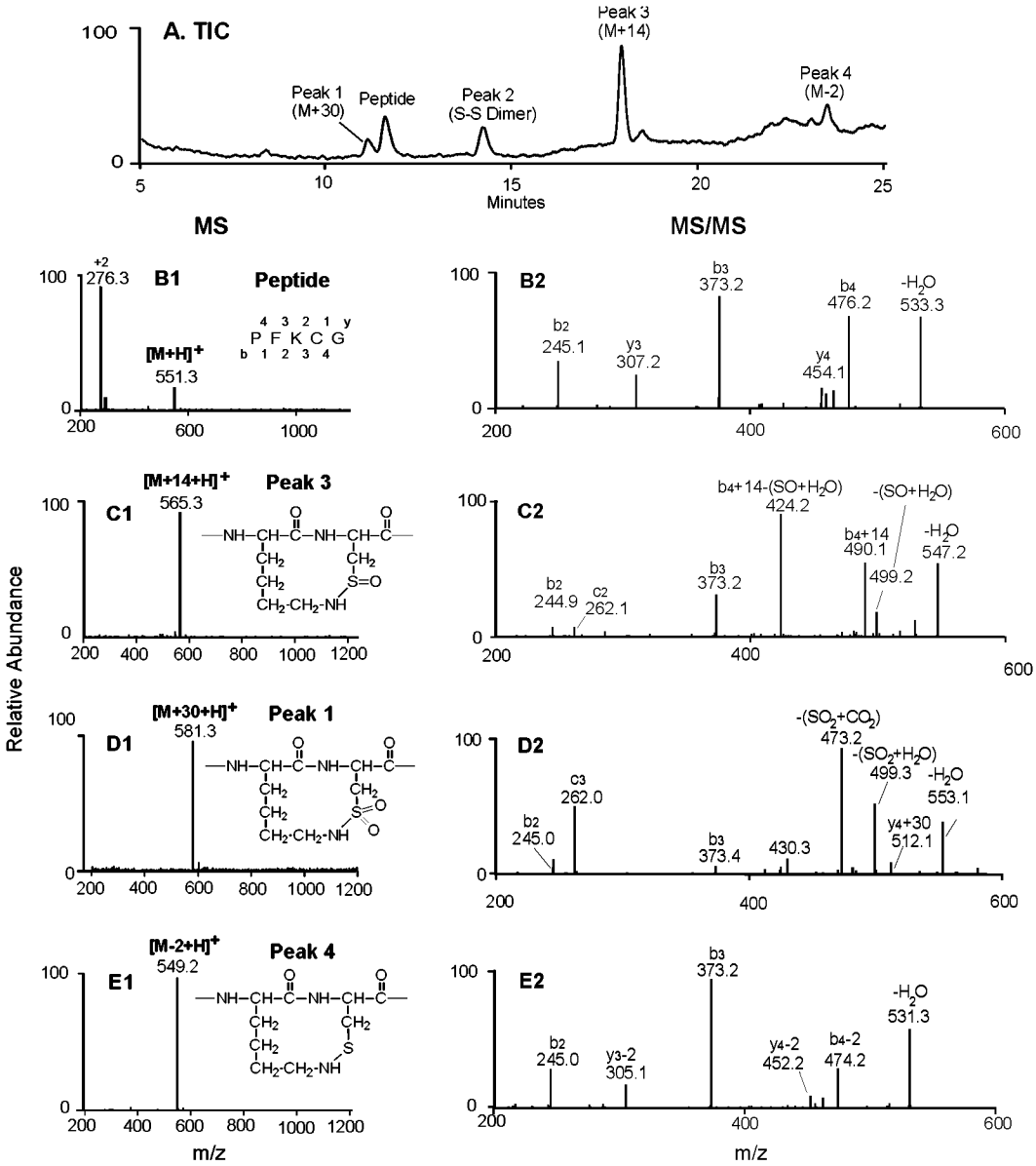

FIGURE 1: LC−MS and MS/MS analysis of PFKCG exposed to HOCl. Peptide (100 μM) was incubated for 30 min at 37 °C in buffer A (Hank's balanced salt solution, pH 7.4, containing 100 μM diethylenetriaminepentaacetic acid; magnesium, calcium, phenol, and bicarbonate free). Reactions were initiated by addition of 50 μM HOCl and terminated by addition of 500 μM L-methionine. The reaction mixture was analyzed by reverse-phase HPLC with a positive ion electrospray ionization MS detector. (A) Total ion chromatogram (TIC), (B1−E1) mass spectrometric (MS) analysis, and (B2−E2) tandem MS (MS/MS) analysis.

reveals the amino acid sequence. MS/MS analysis of the *m*/*z* 551.3 ion ([peptide + H]$^{1+}$) revealed a series of b-type ions (b_2, *m*/*z* 245.1; b_3, *m*/*z* 373.2; b_4, *m*/*z* 476.2) and y-type ions (y_3, *m*/*z* 307.2; y_4, *m*/*z* 454.1) consistent with the sequence of the original peptide (Figure 1B2).

Peak 3 represented the major product in the reaction mixture. LC−MS analysis of this material demonstrated a major ion at *m*/*z* 565.3 (Figure 1C1), the predicted mass of the singly charged peptide plus 1 oxygen and minus 2 hydrogens ([peptide + 16 amu − 2H + 1H]$^{1+}$). MS/MS analysis revealed ions of *m*/*z* 244.9 and 373.2 (b_2 and b_3) derived from the ion of *m*/*z* 565.3 (Figure 1C2), consistent with the sequence PFK in the parent peptide. MS/MS analysis of the *m*/*z* 565.3 ion demonstrated an ion of *m*/*z* 490.1, suggesting that the cysteine residue had gained 1 oxygen and lost 2 hydrogens ([b_4 + 16 amu − 2H + 1H]$^{1+}$). Consistent with this proposal, ions were seen at *m*/*z* 424.2 and 499.9, suggesting loss of (SO + H_2O) from the b_4 ion and the protonated peptide, respectively. When the ion of *m*/*z* 424.2 was subjected to MS analysis, it produced a major ion at *m*/*z* 188.2, which indicates loss of PF from the precursor ion ([KC + 16 − 2H − SO − H_2O + H]$^{+}$) and suggests that residues KC were modified in the peptide. These observations strongly suggest that HOCl oxidizes the cysteine residue of PFKCG to form an intramolecular sulfinamide cross-link (RSONHR; [M + 14 + H]$^{1+}$) and that the cross-link involves the amino group of the lysine residue.

LC−MS analysis of peak 1 material (Figure 1D1) revealed a major ion at *m*/*z* 581.3, the predicted mass of the singly charged cysteine peptide plus 2 oxygens and minus 2 hydrogens ([peptide + 32 amu − 2H + H]$^{1+}$). Consistent with this interpretation, MS/MS analysis of the *m*/*z* 581.3 ion (Figure 1D2) demonstrated ions of *m*/*z* 473.2 and 499.3 exhibiting a loss of 108 amu (SO_2 + CO_2) and 82 amu (SO_2 + H_2O), respectively. An ion at *m*/*z* 512.1 suggested that

the modification involved residues FKCG ([y_4 + 32 amu − 2H + H]$^{1+}$). MS of the *m*/*z* 473.2 ion showed an ion at *m*/*z* 229.2, indicating that the oxidative modification involved residues KCG ([KCG + 30 − SO_2 − CO_2 + H]$^{1+}$). These observations strongly suggest that HOCl converts the thiol residue of the cysteine peptide to an intramolecular sulfonamide (RSO_2 NHR; [M + 30 + H]$^{1+}$) and that the cross-link involves the amino group of the lysine residue.

LC−MS analysis of peak 4 material (Figure 1E1) revealed a major ion at *m*/*z* 549.2, the predicted mass of the singly charged cysteine peptide minus 2 hydrogens ([peptide − 2H + H]$^{1+}$). MS/MS analysis of the *m*/*z* 549.2 ion (Figure 1E2) demonstrated ions consistent with loss of 2 amu from b_4, y_4, and y_3 (*m*/*z* 474.2, 452.2, and 305.2), strongly suggesting that the peptide's thiol group had been oxidized to an intramolecular sulfenamide (RSNHR; [M − 2H + H]$^{1+}$). Collectively, these observations indicate that HOCl generates a sulfenamide, sulfinamide, and sulfonamide when it creates intramolecular cross-links from the thiol residue of the PFKCG peptide.

HOCl Converts PFKCG to a Disulfide Cross-Linked Dimer. We next characterized the material in peak 2. This was one of the four products that appeared after PFKCG was exposed to HOCl, but it was the only product seen after the peptide was exposed to H_2O_2. MS analysis of peak 2 (Figure 1A) material revealed major ions at *m*/*z* 1099.7, 550.3, and 367.4 (data not shown), the predicted masses of the singly charged, doubly charged, and triply charged dimer (RSSR) containing a disulfide cross-link ([peptide + peptide − 2H + *n*H)$^{n+}$]. The isotopic pattern of the ions confirmed these charges. LC−MS/MS of peak 2 material demonstrated an ion of *m*/*z* 583.2 (data not shown), which is consistent with the gain of SH by the peptide (peptide + SH + H)$^+$. These observations indicate that HOCl and H_2O_2 cross-link the PFKCG peptide into a dimer containing a disulfide bond.

Peptide PFKCG Reacts Rapidly with HOCl, Generating a Near-Quantitative Yield of Product. We used HPLC to quantify the progress curve and product yields for oxidation of peptide PFKCG by HOCl. All reactions were carried out at 37 °C in buffer (pH 7.4) containing plasma concentrations of chloride. The peptide reacted rapidly with HOCl, and the reaction was essentially complete at the earliest time point examined (2 min). The major oxidation products were the disulfide cross-linked dimer and the sulfinamide (Figure 2). When the mole ratio of HOCl to peptide was 1:1, conversion of PFKCG to oxidation products was nearly quantitative (Figure 2). As the ratio of HOCl to peptide increased, so did the yield of the sulfinamide and the sulfonamide (Figure 2). In contrast, the yield of the disulfide and the sulfenamide decreased, suggesting that these compounds are precursors of the sulfinamide and sulfonamide. Collectively, these observations indicate that HOCl reacts rapidly with the thiol group of PFKCG to produce a high yield of sulfur−nitrogen (S−N) cross-linked products.

The Intramolecular Sulfinamide Is the Major Product When the Myeloperoxidase−H_2O_2−Chloride System Oxidizes PFKCG. To determine whether HOCl generated by myeloperoxidase can also oxidize PFKCG, we exposed the peptide to the complete myeloperoxidase system (enzyme + H_2O_2 + chloride) for 30 min at 37 °C in buffer (pH 7.4) containing plasma concentrations of chloride. Peptide and peroxide were present at a 1:1 mole ratio. HPLC analysis revealed that the

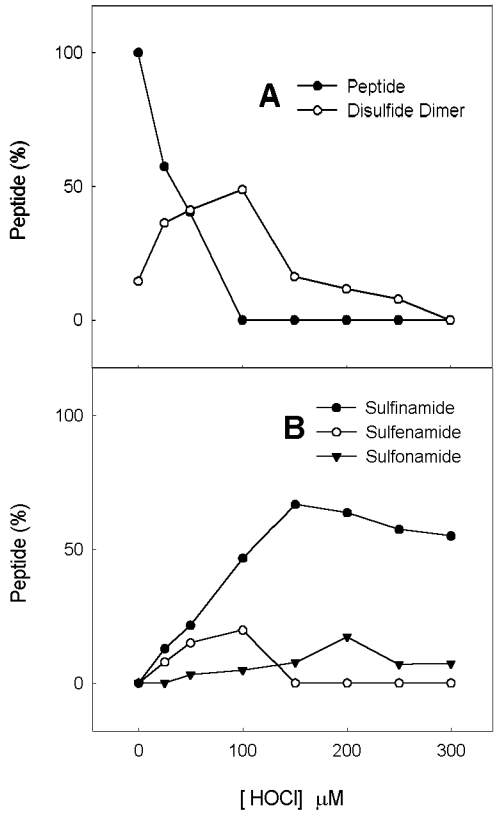

FIGURE 2: Product yield of products in PFKCG exposed to HOCl. PFKCG peptide (100 μM) was incubated in buffer A supplemented with the indicated concentration of HOCl as described in the legend to Figure 1. Peptides in the reaction mixture were quantified by reverse-phase HPLC.

complete myeloperoxidase system oxidized the peptide in near-quantitative yield, producing approximately equal amounts of the disulfide dimer and the intramolecular sulfinamide (Figure 3; complete). A 30 min exposure to an equimolar amount of H_2O_2 (Figure 3, complete − MPO) converted about half of the peptide to the disulfide-linked dimer, which was the only product. HOCl alone converted the peptide into products that had the same retention times as the sulfenamide, sulfinamide, and sulfonamide (Figure 3, HOCl). Collectively, these observations indicate that HOCl reacts much more readily than H_2O_2 with the peptide's thiol group and that continuous, slow production of HOCl by the myeloperoxidase−H_2O_2−chloride system favors formation of an intramolecular sulfinamide.

The Lysine Residue of PFKCG Contributes to the Intramolecular Sulfinamide and Sulfonamide Cross-Links. The sulfenamide, sulfinamide, and sulfonamide cross-links in PFKCG exposed to HOCl might involve the ϵ-amino group of the lysine residue and/or the free amino group at the peptide's N-terminus. Three lines of evidence strongly favor the first possibility. First, the major ion observed in the full-scan mass spectrum of unmodified PFKCG was doubly charged (*m*/*z* 276.3; Figure 1B1), presumably reflecting protonation of both the N-terminal amino group and the ϵ-amino group of the lysine residue. In contrast, only the singly charged ion was observed in the full-scan mass spectra of the materials from peaks 1, 3, and 4 (Figure 1C1−E1). These observations suggest that one of the modified peptide's amino groups had been altered to a form that was not protonated under the mildly acidic conditions used for LC−

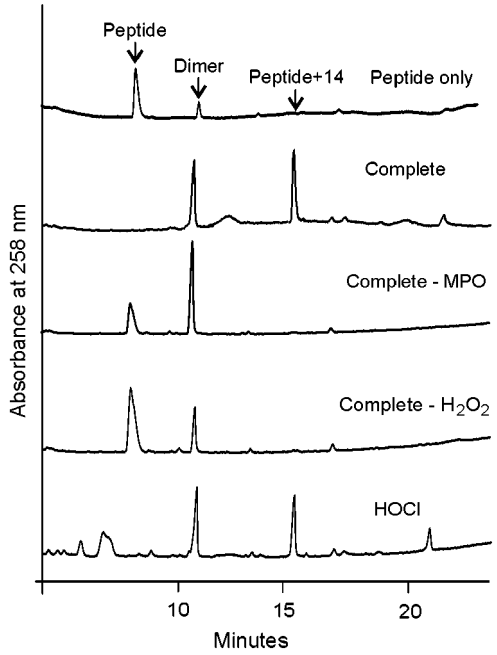

FIGURE 3: HPLC analysis of PFKCG exposed to H_2O_2 plus myeloperoxidase, H_2O_2 alone, or HOCl. PFKCG peptide (100 μM) was incubated in buffer A supplemented with 100 μM H_2O_2 and 2.5 nM myeloperoxidase (complete), 100 μM H_2O_2 alone (complete − MPO), 2.5 nM myeloperoxidase alone (complete − H_2O_2), or 100 μM HOCl. Reactions were initiated by addition of oxidant and terminated after 30 min incubation at 37 °C by addition of 500 μM L-methionine. Peptides in the reaction mixture were quantified by reverse-phase HPLC.

MS. Second, we used LC−MS/MS/MS to demonstrate that the modification of the peptide involved KC (see above). Third, we observed y_4 ions that had gained 30 amu (for the sulfonamide; Figure 1D2) or lost 2 amu (for the sulfenamide; Figure 1E2). These ions had lost the free amino group on the N-terminal proline (inset, Figure 1 B1), but they exhibited the anticipated molecular masses of the sulfinamide and sulfenamide, respectively. In concert, these observations provide strong evidence that HOCl forms an intramolecular cross-link in PFKCG by a reaction involving the peptide's lysine residue.

HOCl Generates Intermolecular Sulfonamide Cross-Links in Peptides. To obtain further evidence that lysine participates in the cross-linking reaction, we prepared the synthetic peptide PFVCG, which contains valine instead of lysine. LC−MS analysis of this peptide detected a major ion at *m*/*z* 522.2 (Figure 4A1), the predicted molecular mass of the singly charged peptide ([peptide + H]$^{1+}$). Only the singly charged ion was observed in the full-scan mass spectrum, which is consistent with the presence of a single basic residue in the peptide. MS/MS analysis of the *m*/*z* 522.2 ion derived from the unmodified peptide (Figure 4A2) revealed a series of b-type ions (b_2, *m*/*z* 245.1; b_3, *m*/*z* 344.0; b_4, *m*/*z* 447.1) consistent with the sequence PFVCG.

When this peptide was exposed to HOCl (1:1 mol/mol) for 30 min at 37 °C in buffer (pH 7.4) containing plasma concentrations of chloride, monitoring of the reconstructed ion chromatogram (RCI) of the reaction mixture demonstrated loss of the peptide (Figure 4A, *m*/*z* 522), indicating that the peptide had reacted with the oxidant. With PFVCG exposed to HOCl, we observed a peak of material that eluted later than unmodified peptide on HPLC. LC−MS analysis with selected ion monitoring of this material detected a major ion at *m*/*z* 1073.6 (Figure 4B), the predicted molecular mass of the singly charged, sulfonamide cross-linked dimer [peptide + peptide + 30 + H]$^{1+}$. Because we detected only the singly charged species (Figure 4B1), we strongly suspect that the free amino group of one of two PFVCG molecules in the dimer contributed to the cross-link.

MS/MS analysis of the ion of *m*/*z* 1073.6 revealed ions of *m*/*z* 586.1 and 552.1, suggesting collision-induced fragmentation of the cross-link between the two peptide molecules with retention of SO_2 or 2 oxygens by PFVCG (Figure 4 B2). A series of ions (-P, -PF, -PFV, b_3) were also consistent with the proposed structure of the intermolecular sulfonamide cross-link (Figure 4B2). Collectively, these observations strongly suggest that exposing PFVCG to HOCl induces intermolecular cross-linking that involves a sulfonamide bond between the cysteine residue of one peptide molecule and the free amino terminus of the other (RSO_2HNR).

To further explore this possibility, we incubated PFVCG with N^{α}-acetyllysine and HOCl. The nucleophilic α-amino group of lysine was acetylated to prevent it from reacting and to mimic the structure of a protein-bound lysyl residue. LC−MS analysis of the reaction mixture detected a major ion at *m*/*z* 740.3 (Figure 4C), the predicted molecular mass of the singly charged sulfonamide adduct of N^{α}-acetyllysine and PFVCG ([peptide + N^{α}-acetyllysine + 30 + H]$^{1+}$). Only the singly charged species was detected in the full-scan mass spectrum of this material (Figure 4C1), suggesting that the free terminal N^{ϵ}-amino group of N^{α}-acetyllysine was involved in the cross-link. Moreover, material of this *m*/*z* was detected only in reaction mixtures containing PFVCG, N^{α}-acetyllysine, and HOCl (Figure 4C). MS/MS analysis of the *m*/*z* 740.3 ion (Figure 4 C2) revealed an ion of *m*/*z* 551.1, consistent with loss of N^{α}-acetyllysine from the adduct. Collectively, these observations indicate that HOCl can create intermolecular sulfonamide cross-links in peptides.

HOCl Also Generates Intramolecular Sulfinamide Cross-Links with Arginine. The ability of HOCl to generate intramolecular and intermolecular sulfonamide cross-links with the ϵ-amino group of lysine raised the possibility that other nucleophilic side chains of amino acids might undergo a similar reaction. To test this hypothesis, we prepared the synthetic peptide PFRCG, which contains arginine instead of lysine. LC−MS analysis of this peptide detected major ions at *m*/*z* 579.3 and 290.3 (Figure 5A), the predicted molecular masses of the singly and doubly charged peptides ([peptide + H]$^{1+}$ and [peptide + 2H]$^{2+}$). The major ion in the full-scan mass spectrum was doubly charged, consistent with the presence of two basic groups in the peptide. MS/MS analysis of the *m*/*z* 579.3 ion (Figure 5B) revealed a series of b-type ions (b_2, *m*/*z* 244.9; b_3, *m*/*z* 401.2; b_4, *m*/*z* 504.3) consistent with the sequence PFRCG.

When this peptide was exposed to a 1:1 molar ratio of HOCl for 30 min at 37 °C in buffer (pH 7.4) containing plasma concentrations of chloride, LC−MS monitoring of the reaction mixture demonstrated the appearance of material of *m*/*z* 593.3 (Figure 5C). This material was singly charged, suggesting loss of the basic arginine residue, and it had the predicted molecular weight of peptide that had formed an intramolecular sulfinamide cross-link. MS/MS analysis of the ion of *m*/*z* 593.3 showed ions consistent with loss of $H_2NCHNSOH$ and SO from the HOCl-modified peptide

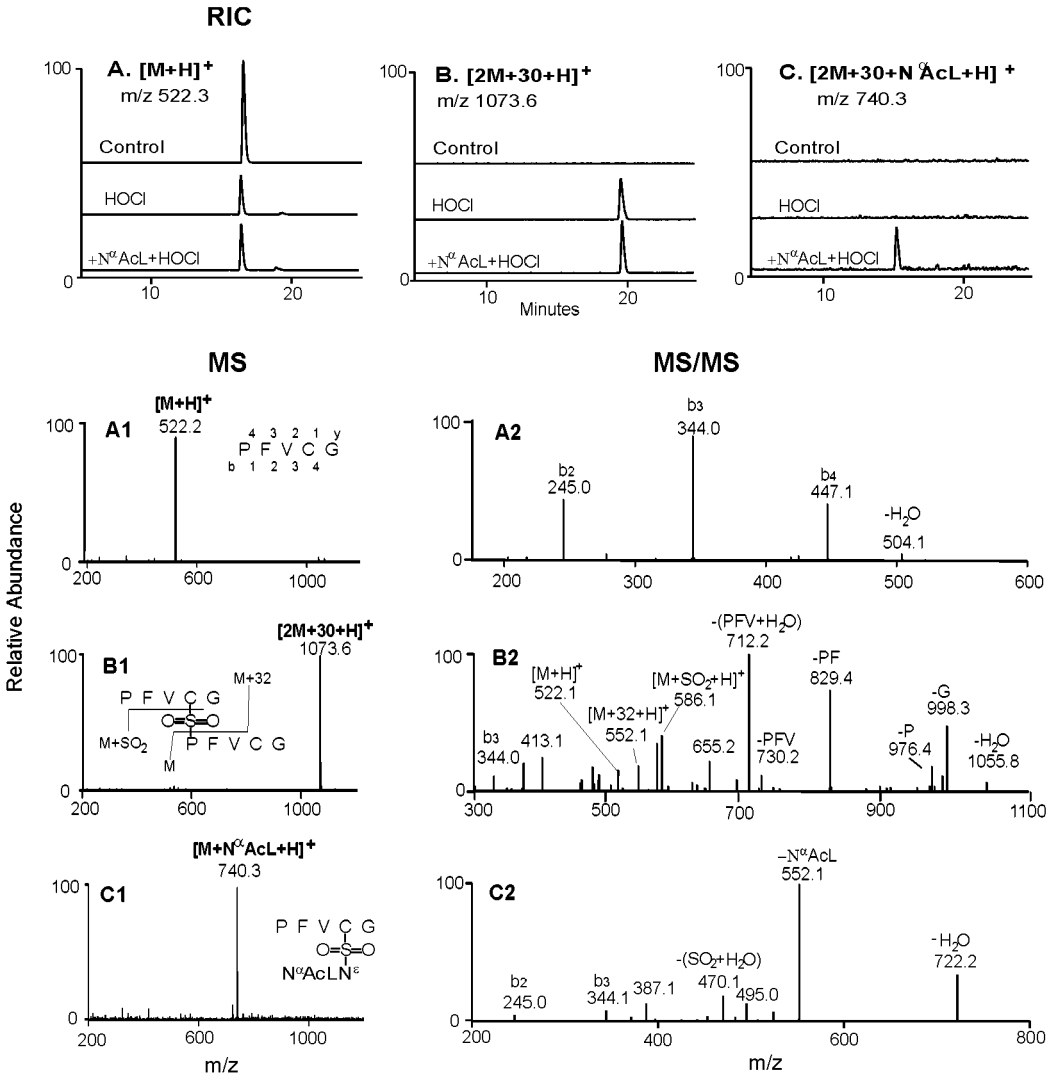

FIGURE 4: LC−MS and MS/MS analysis of PFVCG exposed to HOCl. Peptide (100 μM) was incubated for 30 min at 37 °C in buffer A alone (control) or with 100 μM HOCl. Where indicated, 100 μM N^{α}-acetyllysine (N^{α}AcL) was included in the reaction mixture. The reaction mixture was analyzed by reverse-phase HPLC with electrospray ionization MS or MS/MS. RIC, reconstructed ion chromatogram.

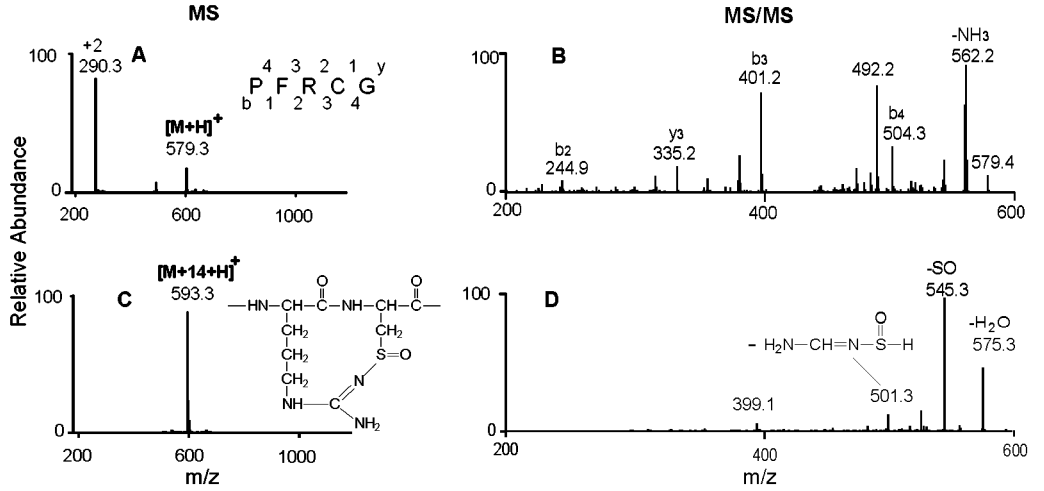

FIGURE 5: LC−MS and MS/MS analysis of PFRCG exposed to HOCl. Peptide (100 μM) was incubated for 30 min at 37 °C in buffer A containing 100 μM HOCl. The reaction mixture was analyzed by reverse-phase HPLC with electrospray ionization MS (A, C) or by MS/MS (B, D).

(Figure 5D). These observations indicate that HOCl converts the arginine and thiol residues of PFRCG to an intramolecular sulfinamide.

Human Neutrophils Can Use the Myeloperoxidase−H_2O_2 System To Generate Sulfinamide Cross-Links. To determine whether oxidants generated by neutrophils might also gener-

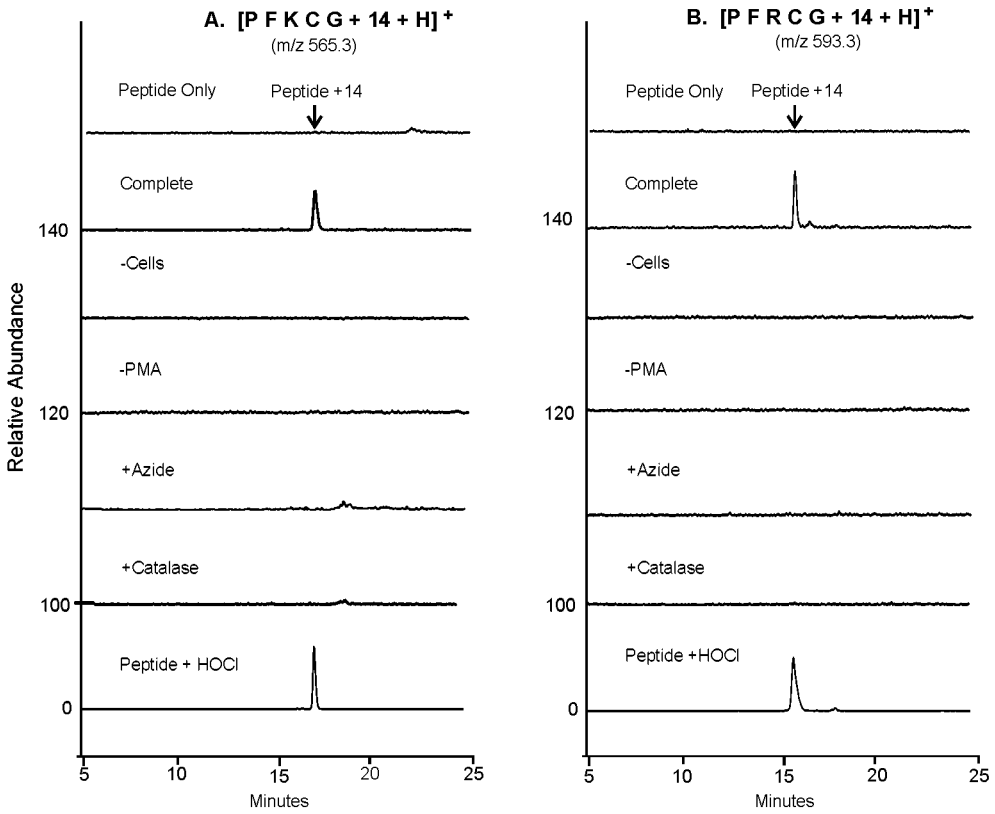

FIGURE 6: Reconstructed ion chromatograms of PFKCG or PFRCG exposed to activated human neutrophils. Neutrophils (7×10^5/mL) in buffer A containing 100 μM peptide were stimulated with 200 nM phorbol myristate acetate (PMA) and incubated for 60 min at 37 °C (Complete). Reaction conditions were varied as indicated. Formation of intramolecular sulfonamide cross-links was monitored by LC−MS as the appearance of material with a *m*/*z* of 565.3 (PFKCG) or a *m*/*z* of 593.3 (PFRCG). Azide, 1 mM; catalase, 10 μg/mL.

ate sulfinamide cross-links, we activated human neutrophils with phorbol myristate acetate at 37 °C in Hank's balanced salt solution supplemented with 100 μM DTPA and 100 μM peptide PFKCG or PFRCG. LC−MS analysis with selected ion monitoring revealed the formation of sulfinamides derived from lysine (Figure 6A) or arginine (Figure 6B) in the medium of stimulated cells. MS/MS analysis confirmed that the oxidation products were sulfinamides. Generation of sulfinamide required cellular activation and was inhibited by the peroxide scavenger catalase and the heme poison sodium azide (Figure 6). Reagent HOCl mimicked the reaction. These observations indicate that HOCl generated by the myeloperoxidase system of activated human neutrophils can generate sulfinamide cross-links in model peptides.

DISCUSSION

We showed that HOCl reacts with the thiol group of the cysteine residue in synthetic peptides to form intramolecular sulfenamide (RSNHR), sulfinamide (RSONHR), and sulfonamide (RSO_2NHR) cross-links. When HOCl was generated continuously by the myeloperoxidase−H_2O_2−chloride system or by activated neutrophils, the major peptide oxidation product was the sulfinamide. HOCl could convert either lysine or arginine residues into intramolecular sulfinamides, and it also produced intermolecular sulfonamides. Collectively, these observations indicate that cysteine residues in peptides form intramolecular and intermolecular S−N cross-links with lysine and arginine residues when they are exposed to HOCl. Similar reactions might promote the oxidative protein cross-linking that contributes to atherosclerosis and other inflammatory diseases.

On the basis of thiol chemistry and the reactions of HOCl (*26*, *29*, *30*, *37*−*39*), we propose the following pathway for thiol oxidation and the formation of intramolecular and intermolecular cross-links (Figure 7). Initially, HOCl reacts with a thiol residue to form a sulfenyl chloride (RSCl). This species then undergoes nucleophilic attack. The oxidation product is a disulfide (RSSR) if the nucleophile is a thiol. Nucleophilic attack by water yields sulfenic acid (RSOH). Nucleophilic attack by amino groups or guanidine groups forms sulfenamide (S−N) cross-links. The sulfur group can subsequently be oxygenated to yield sulfinamides, sulfonamides, and other thiol oxidation products.

In the pathway described in the current studies, HOCl would damage lipoproteins by generating sulfinamide cross-links with lysine and arginine residues. This pathway predominated when we exposed the peptides PFKCG or PFRCG to a continuous, low concentration of HOCl generated by the myeloperoxidase system or activated neutrophils, and it yielded an intramolecular cross-link. In contrast, the peptide PFVCG, which lacks an internal nucleophilic amino acid side chain, formed intermolecular sulfonamide cross-links when oxidized with HOCl. In future studies, it will be important to identify the factors that determine which of the S−N cross-links are the predominant products in model proteins.

Our observations suggest that oxidation of thiol residues by HOCl might represent a previously unsuspected pathway for lipoprotein cross-linking in atherosclerosis. Cross-linking mechanisms already proposed include a pathway in which HOCl generates Schiff base adducts via lysine chloramines (*22*, *23*) and a pathway in which myeloperoxidase generates

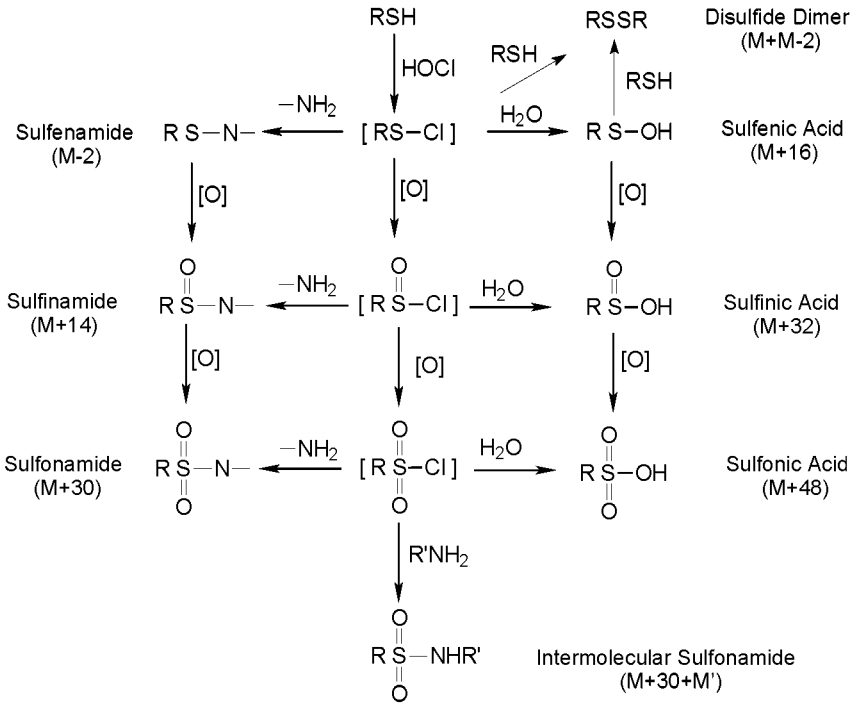

FIGURE 7: Proposed reaction pathways for S−N cross-linking and thiol oxidation by HOCl.

o,o′-dityrosine cross-links via tyrosyl radical (*20*). Schiff base formation by HOCl has been proposed to involve the initial chlorination of the ϵ- amino group of lysine, followed by deamination and generation of a reactive aldehyde. The aldehyde would then undergo nucleophilic attack by another lysine ϵ-amino group to form the Schiff base. However, in vitro studies indicate that α-amino acids require the presence of both a carboxylic acid and a free amino group on the α carbon to form a reactive carbonyl from the chloramine (*40*, *41*). In contrast, the chlorinated ϵ-amino group of lysine appears to be stable. These observations suggest that a reaction pathway that involves Schiff base formation by aldehydes derived from chlorinated ϵ-amino groups of lysine residues is unlikely to be physiologically relevant to protein cross-linking.

Cross-linking by tyrosyl radical could be physiologically relevant because lipoproteins isolated from human atherosclerotic lesions contain markedly elevated levels of *o,o′*-dityrosine (*42*, *43*). However, protein cross-linking by tyrosyl radical is a relatively inefficient reaction that generates a low yield of *o,o′*-dityrosine residues in model proteins and LDL (*31*, *44*). Indeed, quantitative analysis of LDL modified by a tyrosyl radical in vitro and LDL isolated from atherosclerotic lesions demonstrates that *o,o′*-dityrosine is present at levels of ∼1−10 cross-links per 10000 tyrosine residues (*43*). Thus, *o,o′*-dityrosine cross-links are unlikely to account for all of the protein cross-linking observed in LDL isolated from atherosclerotic tissue (*24*, *25*).

In striking contrast, we found that the thiol group of the model peptides reacted with HOCl in near quantitative yield: the sulfenamide, sulfinamide, and sulfonamide accounted for approximately half of the oxidant in the reaction mixture. It is noteworthy that thiol residues in LDL are highly reactive with HOCl (*22*, *23*) and that they are converted to a form that is resistant to reduction with disulfide reducing agents (*22*, *23*). These observations suggest that formation of S−N cross-links might represent a previously unsuspected, quantitatively significant pathway for protein cross-linking by HOCl. To assess the potential physiological significance of this reaction, it will be important to develop methods to quantify S−N cross-links in model proteins and tissues.

It is likely that protein-bound thiols and nucleophilic groups would form intramolecular and intermolecular sulfinamides and sulfonamides if suitably juxtaposed in vivo. Indeed, we have proposed that proteins must achieve specific orientations before they can be cross-linked by HOCl (*45*). Thus, when single-stranded DNA binding protein (SSB) alone or BSA alone is incubated with HOCl, only SSB forms a covalent protein−protein complex (*45*). In solution, SSB is predominantly a homotetramer, whereas BSA is a monomer, strongly suggesting that protein−protein cross-linking by HOCl requires intimate interaction between participating proteins.

In summary, our studies with model peptides indicate that HOCl can form sulfenamide, sulfinamide, and sulfonamide cross-links from thiol groups and lysine or arginine residues. Our observations raise the possibility that HOCl generated by myeloperoxidase contributes to intramolecular and intermolecular cross-linking of artery wall proteins, thus playing an important role in atherosclerosis (*10*−*14*, *19*−*25*). It is also possible that myeloperoxidase could use this mechanism to form oxidative S−N cross-linked proteins in other inflammatory conditions.

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