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Molecular Chlorine Generated by the Myeloperoxidase-Hydrogen Peroxide-Chloride System of Phagocytes Converts Low Density Lipoprotein Cholesterol into a Family of Chlorinated Sterols*

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Oxidation of low density lipoprotein (LDL) may be of critical importance in triggering the pathological events of atherosclerosis. Myeloperoxidase, a heme protein secreted by phagocytes, is a potent catalyst for LDL oxidation *in vitro*, and active enzyme is present in human atherosclerotic lesions. We have explored the possibility that reactive intermediates generated by myeloperoxidase target LDL cholesterol for oxidation. LDL exposed to the myeloperoxidase-H_2O_2-Cl^- system at acidic pH yielded a family of chlorinated sterols. The products were identified by mass spectrometry as a novel dichlorinated sterol, cholesterol α-chlorohydrin (6β-chlorocholestane-(3β,5α)-diol), cholesterol β-chlorohydrin (5α-chlorocholestane-(3β,6β)-diol), and a structurally related cholesterol chlorohydrin. Oxidation of LDL cholesterol by myeloperoxidase required H_2O_2 and Cl^-, suggesting that hypochlorous acid (HOCl) was an intermediate in the reaction. However, HOCl failed to generate chlorinated sterols under chloride-free conditions. Since HOCl is in equilibrium with molecular chlorine (Cl_2) through a reaction which requires Cl^- and H^+, this raised the possibility that Cl_2 was the actual chlorinating intermediate. Consonant with this hypothesis, HOCl oxidized LDL cholesterol in the presence of Cl^- and at acidic pH. Moreover, in the absence of Cl^- and at neutral pH, Cl_2 generated the same family of chlorinated sterols as the myeloperoxidase-H_2O_2-Cl^- system. Finally, direct addition of Cl_2 to the double bond of cholesterol accounts for dichlorinated sterol formation by myeloperoxidase. Collectively, these results indicate that Cl_2 derived from HOCl is the chlorinating intermediate in the oxidation of cholesterol by myeloperoxidase. Our observations suggest that Cl_2 generation in acidic compartments may constitute one pathway for oxidation of LDL cholesterol in the artery wall.

An elevated level of low density lipoprotein (LDL)[1] is a major risk factor for the development of atherosclerotic vascular disease (1). However, a wealth of evidence suggests that LDL must be oxidized to trigger the pathological events of atherosclerosis (2–5). A potential pathway involves myeloperoxidase, a heme protein secreted by activated phagocytes (6–8). Catalytically active myeloperoxidase is a component of human atherosclerotic tissue (9). Immunohistochemical studies co-localize myeloperoxidase with lipid-laden macrophages in vascular lesions. Moreover, patterns of immunostaining for the enzyme at different stages of atherosclerosis (9) are remarkably similar to those for protein-bound lipid oxidation products (10), suggesting that myeloperoxidase oxidizes lipoproteins *in vivo*.

Myeloperoxidase utilizes H_2O_2 as oxidizing substrate to generate a ferryl π-cation radical complex, which may be reduced to the native state by halides and other compounds (11). One substrate is L-tyrosine, which is converted to tyrosyl radical (12). Tyrosyl radical generated by myeloperoxidase initiates LDL lipid peroxidation (13) and generates *o,o′*-dityrosine cross-links in proteins (14, 15). Protein-bound dityrosine levels are markedly increased in human atherosclerotic tissue (16), suggesting that tyrosyl radical generated by myeloperoxidase may play a role in LDL oxidation *in vivo*.

The best characterized product of myeloperoxidase is hypochlorous acid (HOCl; Refs. 17 and 18) (Equation 1).

$$Cl^- + H_2O_2 + H^+ \rightarrow HOCl + H_2O \qquad \text{(Eq. 1)}$$

This potent cytotoxin chlorinates protein amines (19–21), converts unsaturated lipids to chlorohydrins (22, 23), oxidizes free amino acids to aldehydes (24), inactivates sulfhydryl groups (25, 26), and oxidatively bleaches heme groups and iron-sulfur centers (27). LDL exposed to HOCl at neutral pH becomes aggregated and is rapidly taken up and degraded by macrophages (28, 29). Lipoproteins with similar properties have been isolated from atherosclerotic lesions (30, 31). The unregulated uptake of modified LDL may be of critical importance in converting macrophages into foam cells (2–5). A monoclonal antibody that specifically recognizes HOCl-modified proteins reacts with epitopes within human atheroma, as well as with LDL-like particles isolated from atherosclerotic tissue (32). Thus, substantial evidence has accrued suggesting that myeloperoxidase contributes to atherogenesis by catalyzing oxidative reactions in the artery wall.

Although lipoprotein oxidation is thought to be pivotal in the development of atherosclerosis, and LDL is the major carrier of cholesterol in blood, the role of cholesterol oxidation in atherogenesis has received little attention. Oxysterols are present in human vascular lesions (33, 34) and exert potentially atherogenic effects *in vitro* (34–38); however, the reaction pathways responsible for cholesterol oxidation *in vivo* have not yet been identified. We have used a synthetic lipid bilayer model system to demonstrate that myeloperoxidase converts cholesterol to

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[1] The abbreviations used are: LDL, low density lipoprotein; GC-MS, gas chromatography-mass spectrometry; MS/MS, mass spectrometry-mass spectrometry; *m*/*z*, mass-to-charge ratio; NCI, negative-ion chemical ionization; PCI, positive-ion chemical ionization; TMS, trimethylsilyl; amu, atomic mass unit(s).

chlorinated and oxygenated sterols (23). Chlorinated sterols represent attractive candidates for monitoring phagocyte-mediated tissue damage because myeloperoxidase is the only human enzyme known to produce HOCl under physiological conditions (17, 39).

In the current study, we examine the ability of the myeloperoxidase-H_2O_2-Cl^- system of phagocytes to oxidize LDL cholesterol. We find that cholesterol is a major target for oxidation at acidic pH, yielding a family of oxidized sterols. The products were identified by mass spectrometry as a novel dichlorinated sterol, cholesterol α- and β-chlorohydrins, and a structurally related cholesterol chlorohydrin. We demonstrate further that LDL cholesterol oxidation is mediated by Cl_2, which may arise either directly from HOCl or indirectly from a chloramine intermediate.

EXPERIMENTAL PROCEDURES

Materials

Sodium hypochlorite, H_2O_2, organic solvents, and sodium phosphate were obtained from Fisher. Chelex-100 resin and catalase (bovine liver, thymol-free) were from Bio-Rad and Boehringer Mannheim, respectively. All other materials were purchased from Sigma except where indicated.

Methods

Isolation of Myeloperoxidase—Myeloperoxidase (donor: hydrogen peroxide, oxidoreductase, EC 1.11.1.7) was purified by lectin affinity and size exclusion chromatographies from human leukocytes obtained by leukopheresis (12, 40). Purified myeloperoxidase ($A_{430\ nm}/A_{280\ nm}$ ratio of 0.6) was dialyzed against water and stored in 50% glycerol at −20 °C. Enzyme concentration was determined spectrophotometrically (ϵ_{430} = 170 mM^{-1} cm^{-1}; Ref. 41).

Preparation of Chloride-free Sodium Hypochlorite—All procedures were carried out in chlorine-demand-free and chloride-free glassware (19). Chloride-free sodium hypochlorite (NaOCl) was prepared by a modification of previous methods (42). Reagent NaOCl (100 ml) mixed with 100 ml of ethyl acetate was protonated by dropwise addition of concentrated phosphoric acid (final pH ≤ 5). The organic phase containing HOCl was washed twice with H_2O, and HOCl was re-extracted into H_2O by the dropwise addition of NaOH (final pH ≥ 9). Residual ethyl acetate in the aqueous solution of chloride-free NaOCl was removed by bubbling with N_2. The concentration of NaOCl was determined spectrophotometrically (ϵ_{292} = 350 M^{-1} cm^{-1}; Ref. 43).

LDL Isolation—Human LDL (d = 1.020–1.069 g/ml) was isolated by sequential density ultracentrifugation as described previously (44). Protein content was determined utilizing the Markwell-modified Lowry protein assay with bovine serum albumin as standard (45). LDL was rendered chloride-free by exhaustive dialysis against buffer A (65 mM sodium phosphate, 100 μM diethylenetriamine pentaacetic acid, pH 7.4).

LDL Oxidation—All incubations were performed in sealed, chlorine-demand-free and chloride-free reaction vials. [^{14}C]Cholesterol-labeled LDL was prepared by addition of tracer quantities of [^{14}C]cholesterol (DuPont NEN) in ethanol (<0.2% final concentration, v:v) to chloride-free LDL at 37 °C. Following incubation for 5 min, the LDL was subjected to size exclusion chromatography on an Econopac 10-DG column (Bio-Rad) equilibrated with H_2O. Under these conditions >99% of [^{14}C]cholesterol was incorporated into the LDL particle as determined by scintillation spectrometry and protein analysis of fractions prepared from the column. LDL was oxidized utilizing the myeloperoxidase-H_2O_2-Cl^- system, chloride-free NaOCl, or Cl_2 as described in the Fig. legends. Following a 30-min incubation at 37 °C, H_2O saturated with NaCl (0.5 ml) was added to the reaction mixture (0.5 ml), and lipid soluble products were extracted by three sequential 2-ml additions of ethyl acetate. The combined organic extracts were brought to near dryness under anhydrous N_2 and immediately analyzed by normal-phase thin layer chromatography (TLC) on silicic acid (Silica Gel 60A Linear-K with preabsorbent strips; 250 μm; Whatman) with hexane:diethyl ether (40:60, v:v) as the mobile phase. TLC plates were developed twice and reaction products subsequently quantified by phosphor imaging (Molecular Dynamics). Under the conditions employed, radioactivity was a linear function of [^{14}C]cholesterol and recovery of chlorinated sterols was ≥95%. The free cholesterol content of LDL preparations was determined by gas chromatography (46).

For experiments involving LDL oxidation with reagent molecular chlorine, Cl_2 (Aldrich) was first purged into a vented gas-tight reservoir to one atmosphere of pressure, and a known quantity of Cl_2 was then removed and added to a sealed reaction vial utilizing a gas-tight syringe rendered chlorine-demand-free. Synthesis of the monochloramine of N^{α}-acetyl-L-lysine (Sigma) was performed by dropwise addition of chloride-free NaOCl (1:1; mol:mol) to the *N*-blocked amino acid at 0 °C in 10 mM sodium phosphate (pH 7.0) with constant mixing. The solution was then warmed to 37 °C for 15 min and its monochloramine content determined spectrophotometrically (42). Preparations of monochloramine were free of excess HOCl as monitored by monochlorodimadone oxidation (17).

Preparation of Standard Cholesterol Chlorohydrins—6β-Chlorocholestane-(3β,5α)-diol (α-chlorohydrin) and 5α-chlorocholestane-(3β,6β)-diol (β-chlorohydrin) were prepared from cholesterol α-epoxide and cholesterol β-epoxide, respectively (Research Plus Inc., Bayonne, NJ), with concentrated HCl utilizing a modification of the method of Maerker *et al.* (47) as described (23).

Gas Chromatography-Mass Spectrometry—LDL cholesterol oxidation products were purified by normal phase TLC and extracted into ethyl acetate prior to derivatization reactions. Trimethylsilyl (TMS) ether derivatives of hydroxylated compounds were formed with excess *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce) in pyridine (1:1, v:v) at 60 °C for 15 min (49). Heptafluorobutyrate derivatives of hydroxylated compounds were prepared with excess heptafluorobutyric anhydride (Pierce) in acetonitrile (1:3, v:v) for 1 h at room temperature (12). Derivatized products were concentrated to dryness under anhydrous N_2 and reconstituted in heptane for analysis. Base-catalyzed dehydrohalogenation of chlorohydrins to the corresponding epoxides was performed by treating the compounds with sodium hydroxide in tetrahydrofuran (23). Mass spectra of standard cholesterol α- and β-chlorohydrins, cholesterol α- and β-epoxides, and the structurally related cholesterol chlorohydrin, Band 3, were performed as described previously (23).

Electrospray and Tandem Mass Spectrometry—Electrospray mass spectra were acquired on a Sciex API III+ triple quadrupole mass spectrometer (Sciex Inc., Thornhill, Ontario, Canada) in positive-ion mode. The TLC extracts were diluted in chloroform:methanol (1:1, v:v) containing 10 mM ammonium acetate and were infused into the mass spectrometer at a rate of 4 μl/min. The electrospray interface was maintained at 5.0 kV with respect to ion entrance of the mass spectrometer. Air was used as the nebulizing gas. Product ion mass spectrometry-mass spectrometry (MS/MS) spectra of individual chlorinated sterols were acquired by accelerating their $(M + NH_4)^+$ through a 30-V potential difference into argon gas, which was maintained at a target thickness of 2.0×10^{14} atoms/cm^2. The resulting fragment ions were mass-analyzed by scanning the third quadrupole in 0.1-amu increments from 15 amu through the mass of the precursor ion. At least 10 scans were averaged for each MS/MS spectrum. Precursor ion MS/MS spectra were acquired by scanning for $(M + NH_4)^+$ precursors that fragmented to m/z 367, a common fragment of chlorohydrins and other cholesterol species. The mass spectrometer was operated at unit mass resolution for all experiments to fully resolve individual chlorine isotopes of the chlorinated sterols.

General Procedures—All glassware was rendered chlorine-demand-free (19) and chloride-free by soaking in chloride-free NaOCl, extensive rinsing with H_2O, and pyrolysis at 500 °C overnight. Buffers were treated with Chelex-100 resin to remove transition metal ions and demonstrated to be chlorine-demand-free (defined as no detectable (<3%) consumption of 1 mM HOCl in a 10-min incubation at 37 °C within a gas-tight vial) as monitored by the oxidation of iodide to triiodide (50). Multilamellar dipalmitoyl phosphatidylcholine vesicles (cholesterol:dipalmitoyl phosphatidylcholine; 3:7, mol:mol) were prepared utilizing a modification of the method of Sepe and Clark (23, 48). H_2O_2 concentration was determine spectrophotometrically (ϵ_{240} = 39.4 M^{-1} cm^{-1}; Ref. 51).

RESULTS

Myeloperoxidase Oxidizes LDL Cholesterol to Generate a Family of Chlorinated Sterols—To determine whether LDL cholesterol might serve as a substrate for oxidation by myeloperoxidase, we incubated [^{14}C]cholesterol-labeled LDL with the complete myeloperoxidase-H_2O_2-Cl^- system. Analysis of oxidation products by normal phase TLC and autoradiography revealed that LDL cholesterol was converted to a family of products (Fig. 1). Characterization of the oxidized sterols by GC-MS (Appendix) confirmed their structures as cholesterol α-

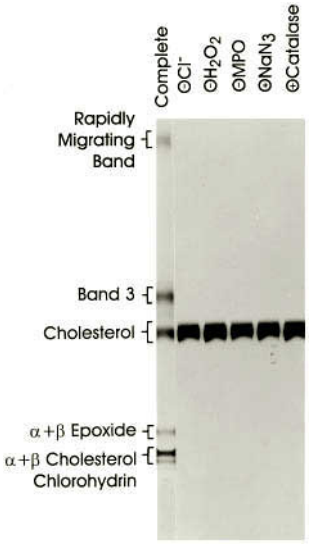


FIG. 1. **Normal-phase TLC analysis of LDL cholesterol oxidation products generated by myeloperoxidase.** The complete myeloperoxidase-H_2O_2-Cl^- system consisted of 20 nM myeloperoxidase (*MPO*), 250 μM H_2O_2, 100 mM NaCl, and [^{14}C]cholesterol-labeled LDL (512 μg of protein/ml) in buffer A (50 mM sodium phosphate, 100 μM diethylenetriamine pentaacetic acid, pH 4.5). Reaction mixtures were incubated for 30 min at 37 °C in sealed reaction vials, extracted with ethyl acetate, analyzed by normal phase TLC, and [^{14}C]sterols visualized by autoradiography as described under "Methods." Where indicated, NaN_3 (1 mM) and catalase (20 μg/ml) were included. Identity of individual reaction products was confirmed by mass spectrometric analysis as described under "Results."

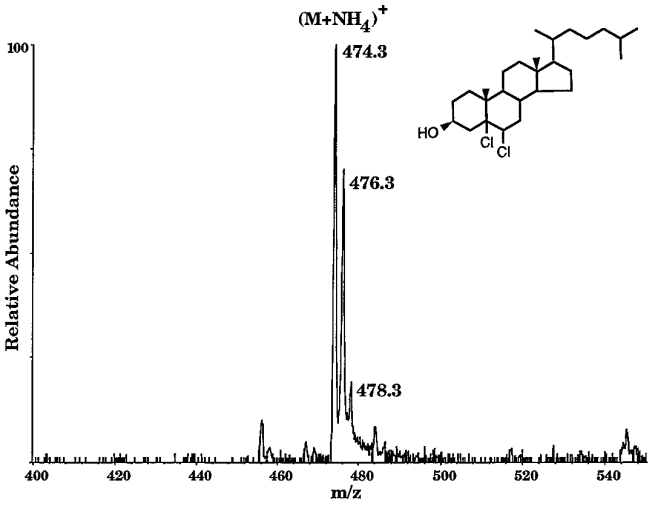


FIG. 2. **Positive-ion electrospray mass spectrum of the dichlorinated sterol(s) in the Rapidly Migrating Band.** Cholesterol oxidation products generated by the myeloperoxidase-H_2O_2-Cl^- system were separated by normal phase TLC as described under "Methods." The Rapidly Migrating Band (Fig. 1) was extracted into ethyl acetate, ammoniated with ammonium acetate, and analyzed by electrospray MS as described under "Methods." The predicted m/z of an ammoniated, dichlorinated compound derived from cholesterol (*inset*) is 474 $(M + NH_4)^+$. The mass spectrum demonstrates the isotopic cluster expected for a dichlorinated sterol, with ions of relative intensity of 10:6:1 at M^+, $M^+ + 2$, and $M^+ + 4$, respectively.

and β-chlorohydrins (6β-chlorocholestane-3β,5α-diol and 5α-chlorocholestane-3β,6β-diol, respectively), cholesterol α- and β-epoxides (cholesterol 5α,6α-epoxide and cholesterol 5β,6β-epoxide, respectively), and a previously identified cholesterol chlorohydrin termed "Band 3," which is structurally related to the α- and β-chlorohydrins (23). A novel dichlorinated sterol designated "Rapidly Migrating Band" (Fig. 1) was also identified (see below).

The principal products of the myeloperoxidase-catalyzed reaction were the α- and β-cholesterol chlorohydrins and Band 3, which collectively constituted ~80% of the oxidized sterols. Generation of the chlorinated and oxygenated sterols demonstrated an absolute requirement for the presence of Cl^-, H_2O_2, and enzyme and was inhibited by the peroxide scavenger catalase. Furthermore, addition of either sodium azide or sodium cyanide, two heme protein poisons, inhibited chlorinated sterol generation, consistent with a peroxidase-dependent mechanism of generation (Fig. 1). When [^{14}C]cholesterol-labeled LDL was treated with reagent HOCl in the presence of Cl^-, the product yield was essentially identical to that generated by myeloperoxidase, strongly implicating HOCl in the reaction pathway.

Myeloperoxidase Generates a Novel Dichlorinated Cholesterol Oxidation Product—To determine whether the LDL cholesterol oxidation product designated "Rapidly Migrating Band" (Fig. 1) was chlorinated, the compound was subjected to electrospray mass spectrometric analysis. The positive ion mass spectrum of the ammoniated compound was that expected for a dichlorinated derivative of cholesterol (Fig. 2, *inset*) and contained a single major ion at m/z 474 $(M + NH_4)^+$. The mass spectrum (Fig. 2) also demonstrated the isotopic cluster expected for a dichlorinated sterol, with ions at m/z 474 ($[M + NH_4]^+$ for $^{35}Cl_2$), 476 ($[M + NH_4]^+$ for $^{35}Cl^{37}Cl$) and 478 ($[M + NH_4]^+$ for $^{37}Cl_2$).

Product ion scans of both the $^{35}Cl_2$- and $^{37}Cl_2$-containing precursor ions revealed fragmentation patterns consistent with the loss of two molecules of HCl, confirming the presence of two chlorine atoms per sterol nucleus in the Rapidly Migrating Band. Product ions observed in the mass spectrum of the precursor ion of m/z 474 ($[M + NH_4]^+$ for $^{35}Cl_2$) included m/z 456 ($[M + NH_4]^+ - H_2O$), 420 ($[M + NH_4]^+ - H_2O - H^{35}Cl$), 403 ($[M + NH_4]^+ - H_2O - H^{35}Cl - NH_3$) and 367 ($[M + NH_4]^+ - H_2O - 2H^{35}Cl - NH_3$). Product ions observed in the mass spectrum of the precursor ion of m/z 478 ($[M + NH_4]^+$ for $^{37}Cl_2$) included m/z 460 ($[M + NH_4]^+ - H_2O$), 422 ($[M + NH_4]^+ - H_2O - H^{37}Cl$), 405 ($[M + NH_4]^+ - H_2O - H^{37}Cl - NH_3$) and 367 ($[M + NH_4]^+ - H_2O - 2H^{37}Cl - NH_3$). These results indicate that the Rapidly Migrating Band is a novel dichlorinated sterol, and raise the possibility that the formation of the compound involves Cl_2, which undergoes an addition reaction with the C5–C6 double bond of cholesterol.

Reaction Conditions for the Oxidation of LDL Cholesterol by Myeloperoxidase—The reaction requirements for oxidation of LDL cholesterol by the myeloperoxidase-H_2O_2-Cl^- system are illustrated in Fig. 3. Near-maximal chlorinated sterol production was seen at physiological Cl^- concentrations (100 mM) and in a pH range similar to that ultimately achieved in the phagolysosome (52, 53). Under the conditions employed for these studies, up to 50% of the H_2O_2 in the reaction mixture was used by myeloperoxidase to generate chlorinated sterols from LDL cholesterol.

LDL Cholesterol Oxidation Is Executed by Chlorine Gas—The chlorinating intermediate generated by myeloperoxidase is generally thought to be HOCl or its conjugate base hypochlorite (ClO^-; Refs. 19 and 43). However, HOCl is also in equilibrium with Cl_2 via a reaction that requires Cl^- and H^+ (54) (Equation 2).

$$HOCl + H^+ + Cl^- = Cl_2 + H_2O \qquad \text{(Eq. 2)}$$

This observation, coupled with identification of the novel dichlorinated sterol, suggested that Cl_2 might serve as the oxidizing intermediate in LDL cholesterol oxidation by the myeloperoxidase-H_2O_2-Cl^- system. To determine whether HOCl or Cl_2 was the chlorinating intermediate in cholesterol oxida-

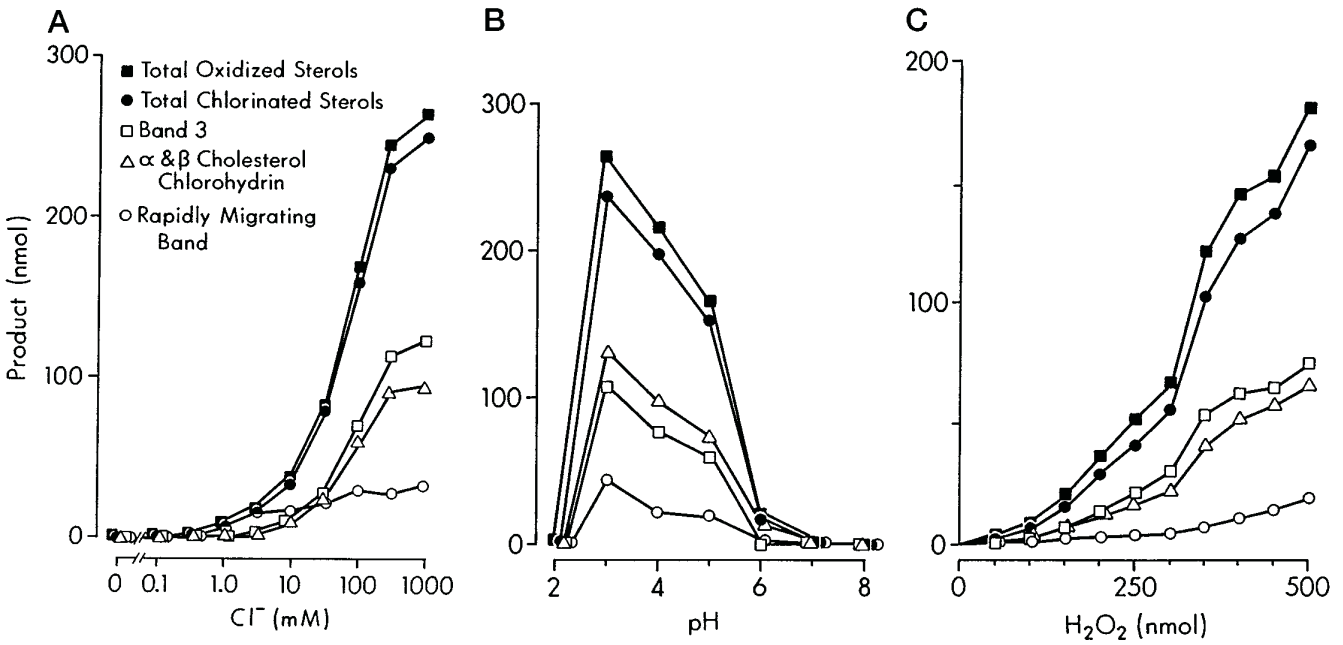


FIG. 3. **Reaction requirements for the oxidation of LDL cholesterol by myeloperoxidase.** Standard conditions: [^{14}C]cholesterol-labeled LDL (512 μg of protein/ml) incubated for 30 min at 37 °C within sealed reaction vials with 20 nM myeloperoxidase (*MPO*), 500 μM H_2O_2, and 100 mM NaCl in buffer A (pH 4.5). Standard conditions were varied by assaying the reaction mixture with the indicated Cl^- concentration (as the sodium salt) (*A*), pH (*B*), or H_2O_2 concentration (*C*). Reactions products were extracted into ethyl acetate, resolved by normal phase TLC, and individual [^{14}C]cholesterol oxidation products quantified by phosphorimaging as described under "Methods."

tion, we first examined the Cl^- and H^+ dependence of the reaction using HOCl and [^{14}C]cholesterol-labeled LDL. According to Equation 2, no Cl_2 should be generated from HOCl in the absence of Cl^-. Remarkably, [^{14}C]cholesterol failed to undergo oxidation when LDL was incubated with HOCl in the absence of Cl^- (Fig. 4, *right panel*). In striking contrast, [^{14}C]cholesterol-labeled LDL exposed to HOCl in the presence of Cl^- yielded large quantities of chlorinated radiolabeled sterols (Fig. 4, *left panel*). Moreover, the reaction was optimal under acidic conditions. The acidic pH optimum and Cl^- dependence of LDL cholesterol oxidation by HOCl is consistent with a requirement for the equilibrium-driven formation of Cl_2 as the halogenating intermediate (Equation 2).

To investigate further whether Cl_2 or HOCl served as the reactive intermediate in cholesterol oxidation, we compared the ability of equimolar amounts of Cl_2 and chloride-free HOCl to chlorinate LDL cholesterol (Fig. 5). At neutral pH and under chloride-free conditions, incubation with Cl_2, but not an equimolar amount of HOCl, resulted in oxidation and chlorination of LDL cholesterol. Collectively, these results suggest that Cl_2-derived from myeloperoxidase-generated HOCl, and not HOCl itself, serves as the chlorinating intermediate in LDL cholesterol oxidation.

Reaction Pathways for Chlorination of LDL Cholesterol by Cl_2—Cl_2 may be generated by Cl^--dependent reactions either from HOCl directly (Equation 2) or from an HOCl-generated chloramine (55, 56). To investigate the relative contribution of each of these pathways, we first compared the yield of total and individual chlorinated sterols when equimolar quantities of either HOCl or N^{α}-acetyl-L-lysine monochloramine (a model for protein-bound chloramines) were incubated with [^{14}C]cholesterol-labeled LDL under acidic conditions (Fig. 6). Both HOCl and the monochloramine required Cl^- to chlorinate LDL cholesterol, indicating that Cl_2 was likely to be the oxidizing intermediate (Fig. 6). Under the conditions examined, the total yield of chlorinated sterols was 10-fold greater with HOCl compared with N^{α}-acetyl-L-lysine monochloramine.

To investigate further the relative contributions of HOCl and

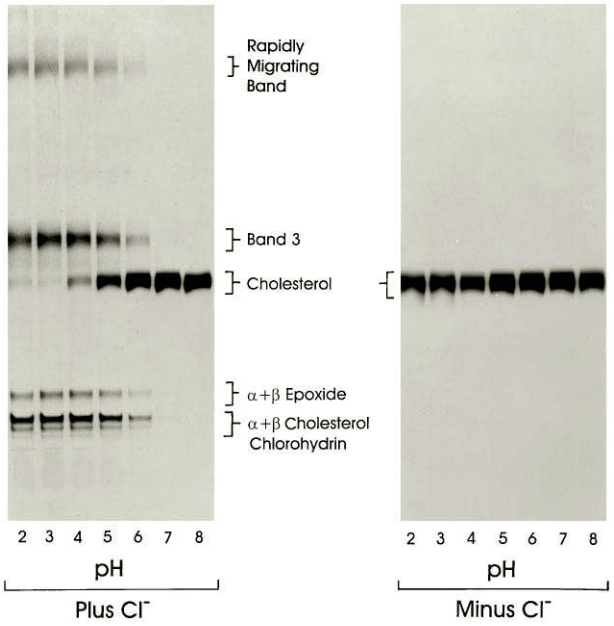


FIG. 4. **Oxidation of LDL cholesterol by HOCl in the presence and absence of chloride.** [^{14}C]Cholesterol-labeled LDL (512 μg of protein/ml) and HOCl (500 μM; chloride-free) were incubated in sodium phosphate buffer (50 mM) at the indicated pH in the presence (*left panel*) or absence (*right panel*) of NaCl (100 mM) within sealed reaction vials for 30 min at 37 °C. Reaction products were subsequently extracted into ethyl acetate, resolved by normal phase TLC, and visualized by autoradiography as described under "Methods." Identity of each reaction product was confirmed by mass spectrometric analysis.

N^{α}-acetyl-L-lysine monochloramine in LDL cholesterol oxidation, we next compared the pH (Fig. 7) and concentration dependence (Fig. 8) of each of the reactions. Both reactive intermediates demonstrated an acidic pH preference for chlorinating activity. However, over the pH range of 3–7, the total yield of chlorinated sterols was much greater with HOCl than

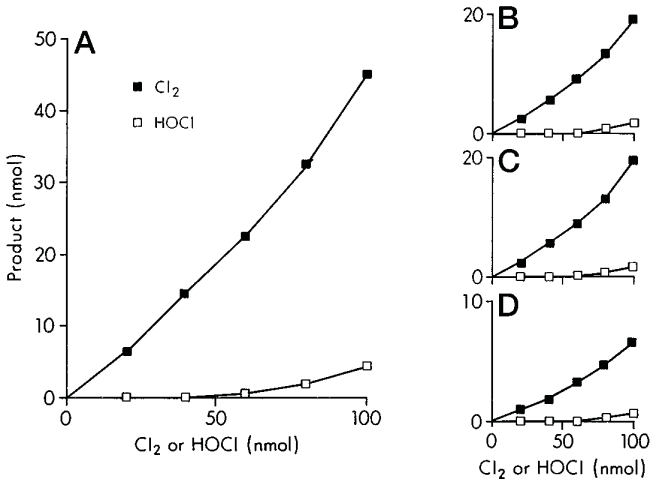


FIG. 5. **Oxidation of LDL cholesterol by molecular chlorine and HOCl.** A gas-tight syringe was used to inject the indicated amount of either Cl_2 or chloride-free HOCl into buffer B (50 mM sodium phosphate; pH 7.0) containing [^{14}C]cholesterol-labeled LDL (512 μg protein/ml) within a sealed reaction vial at 0 °C. The reaction mixture was then incubated at 37 °C for 5 min with constant agitation. Reaction products were extracted with ethyl acetate, resolved by normal phase TLC, and total chlorinated sterol (*A*), α- and β-cholesterol chlorohydrins (*B*), Band 3 cholesterol chlorohydrin (*C*), and the Rapidly Migrating Band (*D*) were quantified by phosphorimaging as described under "Methods."

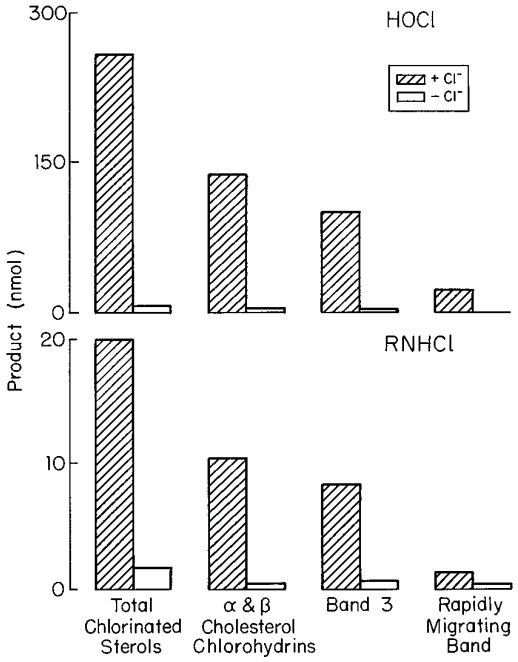


FIG. 6. **Oxidation of LDL cholesterol by HOCl and monochloramine in the presence and absence of chloride.** [^{14}C]Cholesterol-labeled LDL (512 μg of protein/ml) was incubated with either 500 μM chloride-free HOCl (*top panel*) or 500 μM N^{α}-acetyl-L-lysine monochloramine (*bottom panel*) in chloride-free Buffer A for 30 min at 37 °C within sealed reaction vials. Where indicated, NaCl (100 mM) was also included. Reaction products were then analyzed as described in the legend to Fig. 5. *RNHCl*, N^{α}-acetyl-L-lysine monochloramine.

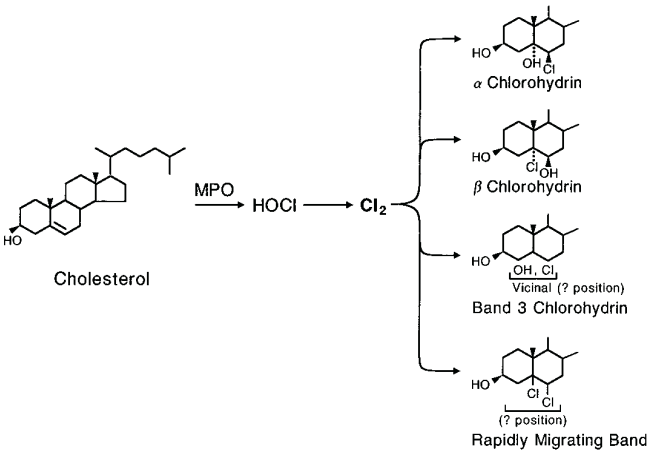
with the monochloramine.

Two observations indicated that sterol chlorination was possible in the absence of a monochloramine intermediate. First, [^{14}C]cholesterol incorporated into dipalmitoyl phosphatidylcholine liposomes, which lack free amino groups, was readily converted to the sterol oxidation products by reagent HOCl. Second, when [^{14}C]cholesterol-labeled LDL was first exhaustively modified with acetic acid anhydride, a potent amino residue modifying reagent, and then exposed to HOCl, cholesterol was rapidly converted into chlorinated sterols. As with the oxidation of LDL cholesterol by reagent HOCl, both reactions demonstrated an absolute requirement for Cl^-. Thus, monochloramines are not an obligatory intermediate in cholesterol oxidation by HOCl.

DISCUSSION

Our results demonstrate that cholesterol is a major target for oxidation in LDL exposed to the myeloperoxidase-H_2O_2-Cl^- system under acidic conditions, yielding a family of chlorinated sterols (Scheme I). Cholesterol oxidation by myeloperoxidase exhibited an absolute requirement for Cl^- and H_2O_2, and was inhibited by NaN_3 and catalase, suggesting that peroxidase-generated HOCl was an intermediate in the chlorination reaction. Consonant with this hypothesis, at acidic pH and in the presence of physiological Cl^- concentrations, reagent HOCl generated a similar spectrum of cholesterol oxidation products.

Multiple lines of evidence implicate Cl_2 derived from HOCl, and not HOCl itself, as the reactive intermediate in cholesterol chlorination by myeloperoxidase (Scheme II). First, oxidation of LDL cholesterol by HOCl possessed an absolute requirement for Cl^-, and was optimal at acidic pH, as expected for the equilibrium-driven formation of Cl_2 from HOCl (Equation 2). Second, Cl_2 converted cholesterol into the same family of sterol oxidation products generated by the myeloperoxidase-H_2O_2-Cl^- system. In contrast to HOCl, oxidation of LDL cholesterol by Cl_2 was independent of Cl^- and was observed at neutral pH. Third, both the myeloperoxidase system and HOCl generated a novel dichlorinated sterol. Formation of this compound is consonant with a Cl_2-mediated reaction involving addition of both chlorine atoms to the double bond in the steroid nucleus of



SCHEME I. **Proposed reaction pathway for the synthesis of cholesterol chlorohydrins and dichlorinated cholesterol by myeloperoxidase (*MPO*).**

cholesterol. Analogous dichlorinated compounds are generated in reactions employing Cl_2 (57, 58). Finally, we have recently demonstrated that the myeloperoxidase-H_2O_2-Cl^- system generates Cl_2, and that activated human neutrophils employ myeloperoxidase to generate a Cl_2-like oxidant within the phagolysosomal compartment (59). Taken together, these results raise the possibility that cholesterol may be a significant target for damage by molecular chlorine generated within acidic compartments.

The generation of Cl_2 by myeloperoxidase may involve at least two different mechanisms. One possible pathway is the direct, equilibrium-driven conversion of HOCl into Cl_2 (Equation 2). Alternatively, HOCl may first react with amino groups to form chloramines (42, 55, 56), which then generate Cl_2 (Equations 3 and 4).

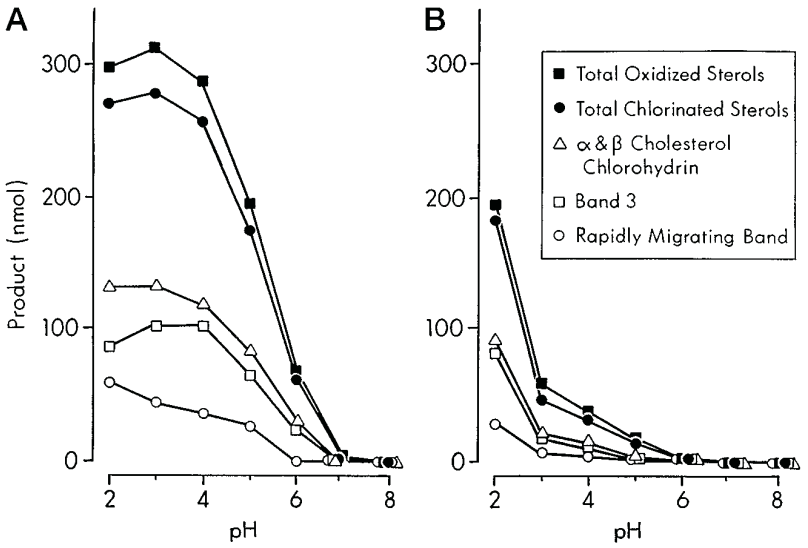


FIG. 7. **pH dependence of LDL cholesterol oxidation by HOCl and monochloramine.** [^{14}C]Cholesterol-labeled LDL (512 μg of protein/ml) was incubated with either 500 μM HOCl (chloride-free) (*A*) or 500 μM N^{α}-acetyl-L-lysine monochloramine (*B*) at the indicated pH in Buffer A supplemented with 100 mM NaCl for 30 min at 37 °C within sealed reaction vials. Reactions products were then analyzed as described in the legend to Fig. 5.

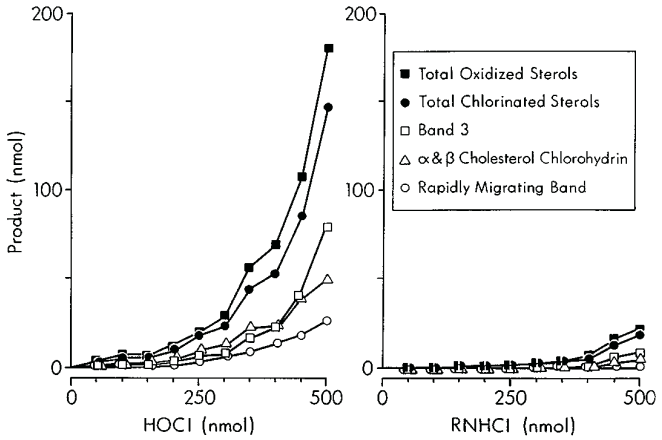
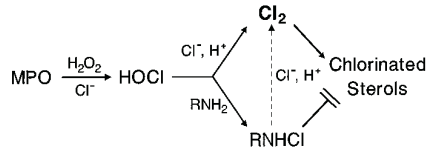


FIG. 8. **Concentration dependence of LDL cholesterol oxidation by HOCl and monochloramine.** [^{14}C]Cholesterol-labeled LDL (512 μg of protein/ml) was incubated within sealed reaction vials with the indicated concentrations of HOCl (*left panel*) or N^{α}-acetyl-L-lysine monochloramine (*right panel*) in Buffer A supplemented with 100 mM NaCl for 30 min at 37 °C. Reaction products were then analyzed as described in the legend to Fig. 5. *RNHCl*, N^{α}-acetyl-L-lysine monochloramine.

$$R_2NH + HOCl \rightarrow R_2NCl + H_2O \quad \text{(Eq. 3)}$$

$$R_2NCl + H^+ + Cl^- = Cl_2 + R_2NH \quad \text{(Eq. 4)}$$

The conversion of chloramines to Cl_2 is Cl^--dependent and takes place rapidly under acidic conditions (54–56). Indeed, *N*-chlorinated reagents are employed to halogenate aromatic compounds (55, 56, 60), suggesting that chloramines derived from LDL lipids and proteins could be intermediates in Cl_2 formation (Scheme II). However, several observations suggest that monochloramines are not obligatory intermediates in LDL cholesterol oxidation, and that Cl_2 is likely to be derived predominantly from HOCl. First, under our experimental conditions, the yield of chlorinated LDL sterols from chloramines was over an order of magnitude lower than that observed for an equimolar quantity of HOCl. Second, cholesterol in LDL treated with acetic acid anhydride, which acetylates reactive amino residues, was still readily oxidized by HOCl in the presence of Cl^-. Finally, cholesterol incorporated into a synthetic liposome system devoid of all amino groups is rapidly converted by HOCl into the same the same family of chlorinated sterols (23).



SCHEME II. **Chloride-dependent pathways for cholesterol chlorination by myeloperoxidase (*MPO*).** *RNHCl*, monochloramine.

One remarkable feature of cholesterol chlorination by myeloperoxidase was the yield of the reaction. Nearly 50% of the H_2O_2 in the reaction mixture was used by myeloperoxidase for chlorination of LDL cholesterol at acidic pH and plasma concentrations of Cl^-. In striking contrast, only trace quantities of lipid oxidation products are formed in LDL exposed to reagent HOCl at neutral pH, where protein amino groups are oxidized preferentially (29, 61). These observations suggest that Cl_2 and HOCl react with different biological targets. Another factor influencing the overall yield of oxidation products may be the location of oxidizable substrates. The hydroxyl group of free cholesterol localizes the unsaturated moiety of the sterol at the interface between the aqueous phase and the hydrophobic cholesteryl ester- and triglyceride-rich core of LDL (3). Since myeloperoxidase is presumably active in the aqueous phase, the high yield of LDL chlorinated sterols at acidic pH suggests that cholesterol is a principal target encountered by Cl_2.

Although LDL cholesterol is a major substrate for oxidation by reagent HOCl at plasma concentrations of Cl^- and acidic pH, the curvilinear shape of chlorinated sterol yield (Fig. 8, *left*) suggests that alternative substrates in LDL are oxidized more readily (21, 28, 61). One important scavenger may be α-tocopherol, which is present at high concentrations in LDL (3, 5), and is a potent inhibitor of sterol chlorination by myeloperoxidase in a synthetic phospholipid-cholesterol system (23). The HOCl-dependent oxidation of other compounds that possess extensive π orbital electrons, including hemes, porphyrins, carotenoids, and purines, is optimal at acidic pH (27). Moreover, oxidation of many of these compounds is augmented by Cl^- (27), suggesting that Cl_2 may be the oxidizing intermediate in these reactions as well.

One important question is whether acidic conditions that favor Cl_2 generation exist *in vivo*. Surfaces coated with ligands that promote phagocytosis cause macrophages to generate a tight seal with the underlying matrix (62), and a pH of <4 has been measured in this protected compartment (63). Atherosclerotic tissue itself may be relatively acidic because of impaired

oxygen diffusion and hypoxia (64). One cellular compartment that may become acidified physiologically is the phagolysosome (52, 53). It is noteworthy that oxidation specific epitopes are present in lysosomal-like structures in the macrophages of atherosclerotic lesions (10), and that phagocytosis is a potent stimulus for the secretion of myeloperoxidase and H_2O_2 into the phagolysosome (7, 18). Moreover, aggregated lipoproteins are present in the subintimal space of hypercholesterolemic animals (65, 66), and aggregated LDL is rapidly phagocytosed by macrophages (28, 67). Depletion of antioxidants within protected acidic environments may also favor the halogenation of sterols and other substrates.

Oxysterols are present in plasma and atherosclerotic lesions (33, 34), but the pathways that promote cholesterol oxidation *in vivo* have not been identified. Chlorinated compounds represent attractive candidates for monitoring phagocyte-mediated tissue damage because myeloperoxidase is the only known human enzyme capable of producing HOCl at physiological concentrations of halide ions (17, 39). The detection of chlorinated sterols *in vivo* would implicate Cl_2 generation as a pathway for lipid oxidation during inflammation and tissue injury. Oxygenated sterols are cytotoxic, mutagenic, and powerful regulators of cellular cholesterol homeostasis (34–38). Chlorinated and oxygenated sterols generated by myeloperoxidase may similarly exert potent biological effects in vascular lesions or other sites of inflammation.

The demonstrated links between myeloperoxidase and oxidative damage to proteins and lipids implicate the enzyme in tissue injury at sites of inflammation. Catalytically active myeloperoxidase is present in human vascular tissues, and oxidized LDL triggers the pathological events of atherosclerosis. The detection of chlorinated sterols in atherosclerotic tissue would strongly support the hypothesis that myeloperoxidase, with its ability to promote LDL oxidation by pathways involving Cl_2, HOCl, and tyrosyl radical, is of central importance in the development of vascular disease.

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APPENDIX

Formation of Cholesterol α- and β-Chlorohydrins and Cholesterol α- and β-Epoxides in LDL Oxidized by Myeloperoxidase—LDL containing radiolabeled cholesterol was oxidized with the complete myeloperoxidase-H_2O_2-Cl^- system, and the individual sterol oxidation products were isolated by normal phase TLC. The mono-TMS derivative of the compound designated "α-chlorohydrin" (Fig. 1) was analyzed in the positive-ion chemical ionization (PCI) mode. Comparisons with the mono-TMS derivative of authentic cholesterol α-chlorohydrin revealed identical GC retention times and mass spectra. The PCI-mass spectrum of the derivative included ions at m/z 511 ($M^+ + 1$), 509 ($M^+ - 1$), 495 ($M^+ - CH_3$), 493 ($M^+ + 1 - H_2O$), 491 ($M^+ - 1 - H_2O$), 477 ($M^+ - H_2O - CH_3$), 457 ($M^+ - Cl - H_2O$), 403 ($M^+ + 1 - TMSOH - H_2O$), 401 ($M^+ - 1 - TMSOH - H_2O$), 385 ($M^+ - TMSOH - Cl$), and 367 ($M^+ - TMSOH - Cl - H_2O$). Ions in the mass spectrum of the mono-TMS ether derivative of the LDL cholesterol oxidation product designated "β-chlorohydrin" were likewise identical to synthetically prepared and derivatized cholesterol β-chlorohydrin. The mass spectrum of the derivative was similar to that of mono-TMS derivatized cholesterol α-chlorohydrin, with the addition of ions at m/z 459 ($M^+ - HCl - CH_3$). Heptafluorobutyric anhydride derivatization of these LDL cholesterol oxidation products confirmed their structural assignment as the α- and β- chlorohydrins, revealing identical GC retention times and negative-ion chemical ionization (NCI) mass spectra to their corresponding authentic chlorohydrin isomers. Ions consistent with a chlorohydrin structure were observed at m/z 830 (M^- for ^{35}Cl), 832 (M^- for ^{37}Cl), 810 ($M^- - HF$ for ^{35}Cl), 812 ($M^- - HF$ for ^{37}Cl), 616 ($M^- - C_3F_7CO_2H - H_2O$ for ^{35}Cl), 618 ($M^- - C_3F_7CO_2H - H_2O$ for ^{37}Cl) and 581 ($M^- - C_3F_7CO_2H - Cl$).

Structural assignments of the myeloperoxidase-generated LDL cholesterol oxidation products designated "α- and β-epoxides" were confirmed by PCI-GC-MS of their mono-TMS derivatives. Comparisons with the mono-TMS derivatives of authentic cholesterol α- and β-epoxides revealed identical GC retention times and mass spectra. The PCI mass spectra of LDL cholesterol α- and β-epoxides included ions at m/z 475 ($M^+ + 1$), 473 ($M^+ - 1$), 459 ($M^+ - CH_3$), 385 ($M^+ - TMSOH$) and 367 ($M^+ - TMSOH - H_2O$).

Formation of a Cholesterol Chlorohydrin of Unknown Structure in LDL Oxidized by Myeloperoxidase—Initial structural characterization of the major LDL cholesterol oxidation product designated Band 3 was performed by NCI-GC-MS analysis of the heptafluorobutyric anhydride derivative. Ions were apparent at m/z 634 (M^- for ^{35}Cl), 636 (M^- for ^{37}Cl), 614 ($M^- - HF$ for ^{35}Cl) and 616 ($M^- - HF$ for ^{37}Cl). These results are consistent with that previously described for a cholesterol chlorohydrin generated in a dipalmitoyl phosphatidylcholine liposome system which was structurally related to cholesterol α- and β-chlorohydrins but of unclear stereochemical structure (23).

The presence of a chlorohydrin moiety in Band 3 was further suggested by performing base catalyzed dehydrohalogenation (see "Methods"); the presence of vicinal hydroxyl and chlorine groups should yield an epoxide. A product was generated by base treatment whose mono-TMS derivative possessed a retention time and PCI mass spectrum compatible with a cholesterol epoxide; ions were observed at m/z 475 ($M^+ + 1$), 473 ($M^+ - 1$), 459 ($M^+ - CH_3$), 385 ($M^+ - TMSOH$) and 367 ($M^+ - TMSOH - H_2O$).

Further structural analysis of the LDL cholesterol oxidation product Band 3 was performed by electrospray mass spectrometry. The positive ion mass spectrum of the ammoniated compound was that expected for a chlorohydrin derivative of cho-

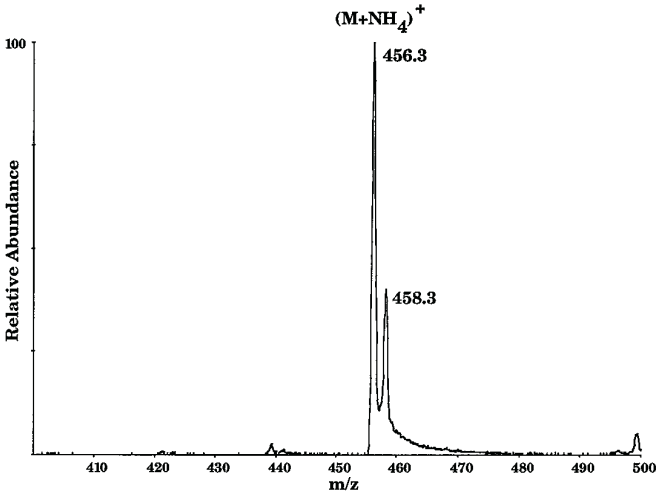

FIG. 9. **Positive-ion electrospray mass spectrum of Band 3.** Band 3 generated by the myeloperoxidase-H_2O_2-Cl^- system was isolated by TLC, extracted into ethyl acetate, protonated with ammonium acetate, and analyzed by electrospray MS/MS as described under "Methods." Note the isotopic cluster of ions with m/z 456 and 458 (relative integrated intensities of 3:1, respectively) characteristic of a chlorinated sterol possessing either a ^{35}Cl or ^{37}Cl atom.

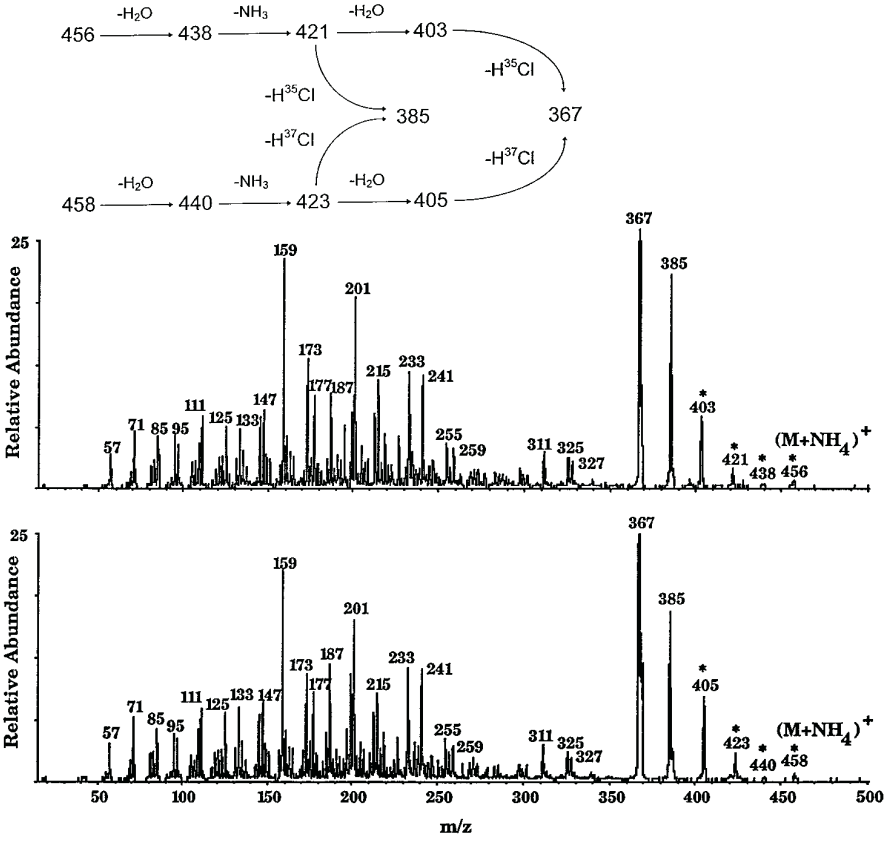

FIG. 10. **Positive-ion electrospray MS/MS spectrum of Band 3.** Band 3 isolated by TLC (see Fig. 1) was extracted into ethyl acetate and analyzed by electrospray MS/MS following addition of ammonium acetate as described under "Methods." Product ion scans of the isotopically distinguishable ions, m/z 456 ($[M + NH_4]^+$ for ^{35}Cl) and 458 ($[M + NH_4^+]$ for ^{37}Cl), are shown in the *upper* and *lower panels*, respectively. *Upper panel*, the product ion scan mass spectrum of m/z 456 ($[M + NH_4]^+$ for ^{35}Cl). *Lower panel*, the product ion scan mass spectrum of m/z 458 ($[M + NH_4]^+$ for ^{37}Cl). *Top inset*, scheme for the proposed fragmentation patterns of the isotopically distinct chlorinated sterols. Low mass ions in each of the spectra arise from sequential fragmentation of the sterol side chain and nucleus. *Asterisk* denotes ions that exhibit the m/z expected for a chlorinated compound (^{35}Cl or ^{37}Cl).

lesterol (Fig. 9) and contained a molecular ion at m/z 456 $(M + NH_4)^+$. The mass spectrum also revealed the characteristic isotopic cluster of ions expected for a monochlorinated sterol, with ions at m/z 456 ($[M + NH_4]^+$ for ^{35}Cl) and 458 ($[M + NH_4^+]$for ^{37}Cl), confirming that this major LDL cholesterol oxidation product is chlorinated (Fig. 9). Subsequent product ion scans (Fig. 10) were consistent with a chlorinated sterol, revealing fragmentation patterns (each with loss of one molecule of HCl) for the isotopically labeled ^{35}Cl and ^{37}Cl sterols. The product ion scan for the precursor ion of m/z 456 ($[M + NH_4]^+$ for ^{35}Cl) revealed: m/z 438 ($[M + NH_4]^+ - H_2O$), 421 ($[M + NH_4]^+ - H_2O - NH_3$), 403 ($[M + NH_4]^+ - 2H_2O - NH_3$), 367 ($[M + NH_4]^+ - 2H_2O - NH_3 - H^{35}Cl$) and 385 ($[M + NH_4]^+ - H_2O - NH_3 - H^{35}Cl$). The product ion scan for the precursor ion of m/z 458 ($[M + NH_4]^+$ for ^{37}Cl) demonstrated: m/z 440 ($[M + NH_4]^+ - H_2O$), 423 ($[M + NH_4]^+ - H_2O - NH_3$), 405 ($[M + NH_4]^+ - 2H_2O - NH_3$), 367 ($[M + NH_4]^+ - 2H_2O - NH_3 - H^{37}Cl$) and 385 ($[M + NH_4]^+ - H_2O - NH_3 - H^{37}Cl$).

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