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-H₂O₂-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-chlorocholestan-3β,5α-diol), cholesterol β-chlorohydrin (5α-chlorocholestan-3β,6β-diol), and a structurally related cholesterol chlorohydrin. Oxidation of LDL cholesterol by myeloperoxidase required H₂O₂ 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 (Cl₂) through a reaction which requires Cl⁻ and H⁺, this raised the possibility that Cl₂ 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, Cl₂ generated the same family of chlorinated sterols as the myeloperoxidase-H₂O₂-Cl⁻ system. Finally, direct addition of Cl₂ to the double bond of cholesterol accounts for dichlorinated sterol formation by myeloperoxidase. Collectively, these results indicate that Cl₂ derived from HOCl is the chlorinating intermediate in the oxidation of cholesterol by myeloperoxidase. Our observations suggest that Cl₂ 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) 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 H₂O₂ 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 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).

\[ \text{Cl}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O} \] (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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¶¶ 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-\(\text{H}_2\text{O}_2-\text{Cl}^-\) 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 \(\alpha\)- and \(\beta\)-chlorohydrins, and a structurally related cholesterol chlorohydrin. We demonstrate further that LDL cholesterol oxidation is mediated by \(\text{Cl}_2\), which may arise either directly from HOCl or indirectly from a chloramine intermediate.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sodium hypochlorite, \(\text{H}_2\text{O}_2\), 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, oxidoeductase, EC 1.11.1.7) was purified by lectin affinity and size exclusion chromatographies from human leukocytes obtained by leukopheresis (12, 40). Purified myeloperoxidase (\(\text{A}_{430}\) \(\text{nm}\)/\(\text{A}_{280}\) \(\text{nm}\) ratio of 0.6) was dialyzed against water and stored in 50% glycerol at \(-20^\circ\text{C}\). Enzyme concentration was determined spectrophotometrically (\(\text{ε}_{430} = 170 \text{ mM}^{-1} \text{ cm}^{-1}\); Ref. 41).

Preparation of Chloride-Free Sodium Hydrochlorite—All procedures were carried out in chloride-demand-free and chloride-free glassware (19). Chloride-free sodium hypochlorite (\(\text{NaOCl}\)) was prepared by a modification of previous methods (42). Reagent \(\text{NaOCl}\) (100 \(\text{mM}\)) mixed with 100 \(\mu\text{l}\) of ethyl acetate was protonated by dropwise addition of concentrated phosphoric acid (final \(\text{pH} \leq 5\)). The organic phase containing HOCl was washed twice with \(\text{H}_2\text{O}\), and HOCl was re-extracted into \(\text{H}_2\text{O}\) by the dropwise addition of \(\text{NaOH}\) (final \(\text{pH} \geq 9\)). Residual ethyl acetate in the aqueous solution of chloride-free \(\text{NaOCl}\) was removed by bubbling with \(\text{N}_2\). The concentration of \(\text{NaOCl}\) was determined spectrophotometrically (\(\text{ε}_{232} = 350 \text{ mM}^{-1} \text{ cm}^{-1}\); Ref. 43).

**LDL Oxidation—Human LDL (1.020–1.063 \(\text{g}\)/\(\text{ml}\)) was isolated by sequential extraction with bovine serum albumin as standard (45). LDL was rendered chloride-free by exhaustive dialysis against buffer A (65 \(\text{mM}\) sodium phosphate, 100 \(\mu\text{M}\) diethylenetriamine pentaacetic acid, pH 7.4).**

**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 \(\text{[^1]}\text{C}\) cholesterol-labeled LDL with the complete myeloperoxidase-\(\text{H}_2\text{O}_2-\text{Cl}^-\) system. Analysis of oxidized products by normal phase TLC and gas chromatography revealed that LDL cholesterol was converted to a family of chlorinated steroids. The mass spectrometer was operated at unit mass resolution for all experiments to fully resolve individual chlorine isotopes of the chlorinated sterols.

**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, \(\text{v}:\text{v}\)) containing 10 \(\mu\text{M}\) ammonium acetate and were infused into the mass spectrometer at a rate of 4 \(\mu\text{l/min}\). The electrospray interface was maintained at 5.0 \(\text{kV}\) 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 \(\text{[M} + \text{NH}_4^+]^+\) through a 30-V potential difference into argon gas, which was maintained at a target thickness of \(2.0 \times 10^{14} \text{ atoms/cm}^2\). The resulting fragment ions were mass-analyzed by scanning the third quadrupole in 0.1-amu increments from \(m/e\) 1 to 350. The ion intensities were corrected for the mass of the precursor ion. At 30 scans were averaged for each MS/MS spectrum. Precursor ion MS/MS spectra were acquired by scanning for \(\text{[M} + \text{NH}_4^+]^+\) precursors that fragmented to \(m/e\) 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.

**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 \(\text{[^1]}\text{C}\) cholesterol-labeled LDL with the complete myeloperoxidase-\(\text{H}_2\text{O}_2-\text{Cl}^-\) system. Analysis of oxidized products by normal phase TLC and gas chromatography 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 \(\alpha\)-
Myeloperoxidase-generated Cl₂ Oxidizes LDL Cholesterol

Myeloperoxidase-generated Cl₂ Oxidizes LDL Cholesterol

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⁻, H₂O₂, 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 [¹⁴C]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 HOCI 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 + NH₄⁺). The mass spectrum (Fig. 2) also demonstrated the isotopic cluster expected for a dichlorinated sterol, with ions at m/z 474 (M + NH₄⁺) for [³⁵Cl₂], 476 (M + NH₄⁺) for [³⁵Cl³⁷Cl] and 478 (M + NH₄⁺) for [³⁷Cl₂].

Product ion scans of both the [³⁵Cl₂] and [³⁷Cl₂]-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 + NH₄⁺) for [³⁵Cl₂] included m/z 456 ([M + NH₄⁺]⁻ - H₂O), 420 ([M + NH₄⁺]⁻ - H₂O - H²⁵Cl - NH₃), 403 ([M + NH₄⁺]⁻ - H₂O - H²⁵Cl - NH₃) and 367 ([M + NH₄⁺]⁻ - H₂O - 2H²⁵Cl - NH₃). Product ions observed in the mass spectrum of the precursor ion of m/z 478 ([M + NH₄⁺] for [³⁷Cl₂]) included m/z 460 ([M + NH₄⁺]⁻ - H₂O), 422 ([M + NH₄⁺]⁻ - H₂O - H²⁵Cl - NH₃), 405 ([M + NH₄⁺]⁻ - H₂O - H²⁵Cl - NH₃) and 367 ([M + NH₄⁺]⁻ - H₂O - 2H²⁵Cl - NH₃). These results indicate that the Rapidly Migrating Band is a novel dichlorinated sterol, and raise the possibility that the formation of the compound involves Cl₂, 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-H₂O₂-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 H₂O₂ 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 Cl₂ via a reaction that requires Cl⁻ and H⁺ (54) (Equation 2).

\[
\text{HOCl} + \text{H}^+ + \text{Cl}^- \rightarrow \text{Cl}_2 + \text{H}_2\text{O} \quad \text{(Eq. 2)}
\]

This observation, coupled with identification of the novel dichlorinated sterol, suggested that Cl₂ might serve as the oxidizing intermediate in LDL cholesterol oxidation by the myeloperoxidase-H₂O₂-Cl⁻ system. To determine whether HOCl or Cl₂ was the chlorinating intermediate in cholesterol oxida-
tion, we first examined the Cl\textsuperscript{−} and H\textsuperscript{+} dependence of the reaction using HOCl and \[^{14}\text{C}\]cholesterol-labeled LDL. According to Equation 2, no Cl\textsubscript{2} should be generated from HOCl in the absence of Cl\textsuperscript{−}. Remarkably, \[^{14}\text{C}\]cholesterol failed to undergo oxidation when LDL was incubated with HOCl in the absence of Cl\textsuperscript{−} (Fig. 4, right panel). In striking contrast, \[^{14}\text{C}\]cholesterol-labeled LDL exposed to HOCl in the presence of Cl\textsuperscript{−} 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\textsuperscript{−} dependence of LDL cholesterol oxidation by HOCl is consistent with a requirement for the equilibrium-driven formation of Cl\textsubscript{2} as the halogenating intermediate (Equation 2).

To investigate further whether Cl\textsubscript{2} or HOCl served as the reactive intermediate in cholesterol oxidation, we compared the ability of equimolar amounts of Cl\textsubscript{2} and chloride-free HOCl to chlorinate LDL cholesterol (Fig. 5). At neutral pH and under chloride-free conditions, incubation with Cl\textsubscript{2}, but not an equimolar amount of HOCl, resulted in oxidation and chlorination of LDL cholesterol. Collectively, these results suggest that Cl\textsubscript{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\textsubscript{2}—**Cl\textsubscript{2} may be generated by Cl\textsuperscript{−}-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\textsuperscript{\text{-}}-acetyl-l-lysine monochloramine (a model for protein-bound chloramines) were incubated with \[^{14}\text{C}\]cholesterol-labeled LDL under acidic conditions (Fig. 6). Both HOCl and the monochloramine required Cl\textsuperscript{−} to chlorinate LDL cholesterol, indicating that Cl\textsubscript{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\textsuperscript{\text{-}}-acetyl-l-lysine monochloramine.

To investigate further the relative contributions of HOCl and N\textsuperscript{\text{-}}-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...
with the monochloramine.

Two observations indicated that sterol chlorination was possible in the absence of a monochloramine intermediate. First, [14C]cholesterol incorporated into dipalmitoyl phosphatidylcholine liposomes, which lack free amino groups, was readily converted to the sterol oxidation products by reagent HOCl. Second, when [14C]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\(^{2-}\). 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\(_2\)O\(_2\)-Cl\(^{2-}\) system under acidic conditions, yielding a family of chlorinated sterols (Scheme I). Cholesterol oxidation by myeloperoxidase exhibited an absolute requirement for Cl\(^{-}\) and H\(_2\)O\(_2\), 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\(_2\)O\(_2\)-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 cholesterol. Analogous dichlorinated compounds are generated in reactions employing Cl\(_2\) (57, 58). Finally, we have recently demonstrated that the myeloperoxidase-H\(_2\)O\(_2\)-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, 53, 56), which then generate Cl\(_2\) (Equations 3 and 4).
HOCl (Eq. 3)

R₂NCl

H₂O

R₂NCl + H⁺ + Cl⁻ = Cl₂ + R₂NH

(Eq. 4)

The conversion of chloramines to Cl₂ 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 Cl₂ formation (Scheme II). However, several observations suggest that monochloramines are not obligatory intermediates in LDL cholesterol oxidation, and that Cl₂ is likely to be derived predominantly from HOCI. 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 HOCI. Second, cholesterol in LDL treated with acetic acid anhydride, which acetylates reactive amino residues, was still readily oxidized by HOCI in the presence of Cl⁻. Finally, cholesterol incorporated into a synthetic liposome system devoid of all amino groups is rapidly converted by HOCI into the same the same family of chlorinated sterols (23).

One remarkable feature of cholesterol chlorination by myeloperoxidase was the yield of the reaction. Nearly 50% of the H₂O₂ in the reaction mixture was used by myeloperoxidase for chlorination of LDL cholesterol at acidic pH and plasma concentrations of Cl₂. In striking contrast, only trace quantities of lipid oxidation products are formed in LDL exposed to reagent HOCI at neutral pH, where protein amino groups are oxidized preferentially (29, 61). These observations suggest that Cl₂ and HOCI 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 Cl₂.

Although LDL cholesterol is a major substrate for oxidation by reagent HOCI 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 HOCI-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 Cl₂ may be the oxidizing intermediate in these reactions as well.

One important question is whether acidic conditions that favor Cl₂ 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...
Myeloperoxidase-generated Cl$_2$ Oxidizes LDL Cholesterol

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$_2$O$_2$ 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 imply the presence of Cl$_2$ generation in a pathway for lipid oxidation during inflammation and tissue injury. Oxidized 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$_2$O$_2$-Cl$_2$ 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$_2$O), 491 (M$^+$ - 1 - H$_2$O), 477 (M$^+$ - H$_2$O - CH$_3$), 457 (M$^+$ - Cl - H$_2$O), 403 (M$^+$ + 1 - TMSOH - H$_2$O), 401 (M$^+$ - 1 - TMSOH - H$_2$O), 385 (M$^+$ - TMSOH - Cl), and 367 (M$^+$ - TMSOH - Cl - H$_2$O). 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$_3$F$_7$CO$_2$H - H$_2$O for $^{35}$Cl), 618 (M$^+$ - C$_3$F$_7$CO$_2$H - H$_2$O for $^{37}$Cl) and 581 (M$^+$ - C$_3$F$_7$CO$_2$H - 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$_2$O).

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$_2$O).

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.](image-url)
lesterol (Fig. 9) and contained a molecular ion at m/z 456 (M + NH₄)⁺. The mass spectrum also revealed the characteristic isotopic cluster of ions expected for a monochlorinated sterol, with ions at m/z 456 (M + NH₄)⁺ for 35Cl and 458 (M + NH₄)⁺ for 37Cl, 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 35Cl and 37Cl sterols.

The product ion scan for the precursor ion of m/z 456 (M + NH₄)⁺ for 35Cl) revealed: m/z 438 ([M + NH₄]⁺ - H₂O), 421 ([M + NH₄]⁺ - H₂O - NH₃), 403 ([M + NH₄]⁺ - 2H₂O - NH₃), 367 ([M + NH₄]⁺ - 2H₂O - NH₃ - HCl), and 385 ([M + NH₄]⁺ - H₂O - NH₃ - HCl). The product ion scan for the precursor ion of m/z 458 (M + NH₄)⁺ for 37Cl) demonstrated: m/z 440 ([M + NH₄]⁺ - H₂O), 423 ([M + NH₄]⁺ - H₂O - NH₃), 405 ([M + NH₄]⁺ - 2H₂O - NH₃), 367 ([M + NH₄]⁺ - 2H₂O - NH₃ - HCl), and 385 ([M + NH₄]⁺ - H₂O - NH₃ - HCl).

REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1986) Science 232, 34–47.
2. Witztum, J. L., and Steinberg, D. (1991) J. Clin. Invest. 88, 1785–1792.
3. Estesbauer, H., Gebicki, J., Puh, H., and Jurgens, G. (1992) Free Radical Biol. Med. 13, 341–390.
4. Zhang, H., Yang, B., and Steinbrecher, U. P. (1993) J. Biol. Chem. 268, 5535–5542.
5. Berliger, J. A., and Heinecke, J. W. (1996) Free Radical Biol. Med. 20, 707–727.
6. Agor, K. (1972) in Structure and Function of Oxidation-Reduction Enzymes (Akeson, A., and Ehrenberg, A., eds) pp. 329–335, Pergamon Press, New York.
7. Klenhoff, S. J., and Clark, R. A. (1978) The Neutrophil: Function and Clinical Disorders, pp. 447–451, Elsevier/North Holland Biomedical Press, Amsterdam.
8. Heinecke, J. W. (1994) Coron. Art. Dis. 5, 205-210.
9. Daugherty, A., Dunn, J. L., Ratner, D. L., and Heinecke, J. W. (1994) J. Clin. Invest. 94, 437-444.
10. Rosenfeld, M. E., Patnoki, W., Yla-Herttuala, S., Butler, S., and Witztum, J. L. (1990) Arterioscler. Thromb. 10, 338–339.
11. Hurst, J. K., and & Barette, W. C., Jr. (1989) Crit. Rev. Biochem. Mol. Biol. 24, 271–328.
12. Heinecke, J. W., Li, W., Daehnke, H. L., III, and Goldstein, J. A. (1993) J. Biol. Chem. 268, 4069–4077.
13. Savenkova, M. I., Mueller, D. M., and Heinecke, J. W. (1994) J. Biol. Chem. 269, 29394–29402.
14. Heinecke, J. W., Li, W., Francis, G. A., and Goldstein, J. A. (1993) J. Clin. Invest. 91, 2866-2872.
15. Francis, G. A., Mender, A. J., Bierman, E. L., and Heinecke, J. W. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 6631–6635.
16. Rasmussen, J., and Heinecke, J. W. (1995) Circulation 92, Suppl. I, I-162.
17. Harrison, J. E., and Schultz, J. (1976) J. Biol. Chem. 251, 1371–1374.
18. Foote, C. S., Goyne, T. E., and Lehrer, R. I. (1983) Nature 304, 715–716.
19. Weil, I., and Morris, J. C. (1949) J. Am. Chem. Soc. 71, 1664–1671.
20. Thomas, E. L., Jefferson, M. M., and Grisham, M. B. (1982) Biochemistry 21, 6299–6306.
21. Weiss, S. J., Klein, R., Slivka, A., and Wei, M. (1982) J. Clin. Invest. 70, 598–607.
22. Winterbourn, C. C., Vanden Berg, J. M., Roitman, E., and Kuppers, F. A. (1992) Arch. Biochem. Biophys. 286, 547–555.
23. Heinecke, J. W., Li, W., Mueller, D. M., Boher, A., and Turk, J. (1994) Biochemistry 33, 10127–10136.
24. Hazen, S. L., Hsu, F. F., and Heinecke, J. W. (1996) J. Biol. Chem. 271, 1861–1867.
25. Knox, W. E., VandenBerg, J. J. M., Roitman, E., and Kuypers, F. A. (1993) Science 261, 6631–6635.
26. Test, S. T., and Weiss, S. J. (1986) Adv. Free Radical Biol. Med. 2, 91–116.
27. Albrich, J. M., McCarthy, C. A., and Harut, J. K. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 210–214.
28. Hazell, L. J., and Stocker, R. (1993) Biochem. J. 290, 165–172.
29. Hazell, L. J., Van den Berg, J. M., and Stocker, R. (1994) Biochem. J. 302, 297–304.
30. Hazell, L. J., and Stocker, R. (1993) Biochem. J. 290, 165–172.
31. Yla-Herttuala, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J. L., and Steinberg, D. (1989) J. Clin. Invest. 94, 1086–1098.
32. Hazell, L. J., Arnold, L. Flowers, D., Malle, E., and Stocker, R. (1996) J. Clin. Invest. 97, 1535–1544.
33. Heinecke, J. W., Li, W., Daehnke, H. L., III, and Goldstein, J. A. (1993) J. Biol. Chem. 268, 4069–4077.
34. Savenkova, M. I., Mueller, D. M., and Heinecke, J. W. (1994) J. Biol. Chem. 269, 29394–29402.
35. Kandel, A. A., Chen, H. W., and Heinecke, H. R. (1978) Science 201, 498–501.
36. Sevanian, A., and Peterson, A. R. (1986) Free Radical Biol. Med. 2, 1103–1110.
37. Hwang, P. L. (1991) Food Chem. Toxicol. 29, 205–206.
38. Hwang, P. L. (1991) Food Chem. Toxicol. 29, 205–206.
39. Weiss, S. J., Test, S. T., Eckmann, C. M., Ross, D., and Regiani, S. (1986) J. Biol. Chem. 261, 1075–1080.
40. Rakita, R. M., Michel, B. R., and Rosen, H. (1990) Biochemistry 29, 1057–1060.
41. Morita, Y., Iwamoto, H., Aibara, S., Hasegawa, E. (1986) J. Biochem. (Tokyo) 99, 761–770.
Myeloperoxidase-generated Cl₂ Oxidizes LDL Cholesterol

42. Thomas, E. L., Grisham, M. B., Jefferson, M. M. (1986) Methods Enzymol. 132, 569–585
43. Morris, J. C. (1966) J. Phys. Chem. 70, 3798–3805
44. Heinecke, J. W., Rosen, H., Suzuki, L. A., and Chait, A. (1987) J. Biol. Chem. 262, 10098–10103
45. Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) Anal. Biochem. 87, 206–210
46. Sevanian, A., Seraglia, R., Traldi, P., Rossato, P., Ursini, F., and Hodis, H. (1994) Free Radical Biol. Med. 17, 397–409
47. Mark, G., Nungenster, E. H., and Bunick, F. J. (1988) Lipids 23, 761–765
48. Sepe, S. M., and Clark, R. A. (1985) J. Immunol. 134, 1888–1895
49. Turk, J., Henderson, W. R., Riebanoff, S. J., and Hubbard, W. C. (1983) Biochem. Biophys. Acta 751, 189–200
50. El-Saadani, M., Estebauer, H., El-Sayed, M., Goher, M., Nassar, A. Y., and Jurgens, G. (1980) J. Lipid Res. 21, 627–630
51. Nelson, D. P., and Kiesow, L. A. (1972) Anal. Biochem. 49, 474–478
52. Jensen, M. S., and Bainton, D. F. (1973) J. Cell Biol. 56, 379–388
53. Lehrer, R. I., and Cech, P. (1984) Blood 63, 88–95
54. White, G. C. (ed) (1972) Handbook of Chlorination, pp. 182–227, Van Nostrand Reinhold, New York
55. Orton, K. J. P., and Bradfield, A. E. (1927) J. Chem. Soc. 986
56. de la Mare, P. B. D., Kesley, A. D., and Vernon, C. A. (1954) J. Chem. Soc. 1290
57. Rivett, D. E. A., and Wallis, E. S. (1950) J. Org. Chem. 15, 55–41
58. Barton, D. H. R., and Miller, E. (1956) J. Am. Chem. Soc. 72, 370–374
59. Hazen, S. L., Hsu, F. F., Mueller, D. M., Crowley, J. R., and Heinecke, J. W. (1996) J. Clin. Invest., in press
60. de la Mare, P. B. D., and Ridd, J. H. (1959) Aromatic Substitution: Nitration and Halogenation, pp. 105–129, Academic Press, New York
61. Arnhold, J., Wiegel, D., Richter, O., Hammerschmidt, S., Arnold, K., and Krumbiegel, M. (1991) Biomed. Biochim. Acta 50, 967–973
62. Wright, S. D., and Silverstein, S. C. (1984) Nature 309, 359–361
63. Silver, I. A., Murillo, R. J., and Etherington, D. J. (1988) Exp. Cell Res. 175, 266–276
64. Hajjar, D. P., Farber, I. C., and Smith, S. C. (1988) Arch. Biochem. Biophys. 262, 375–380
65. Frank, J. S., and Fogelman, A. M. (1989) J. Lipid Res. 30, 967–978
66. Steinbrecher, U. P., and Lougheed, M. (1992) Arterioscler. Thromb. 12, 608–625
67. Suits, A. G., Chait, A., Aviram, M., and Heinecke, J. W. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2713–2717
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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